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Analysis of tumor-associated stromal cells using SCID GFP transgenic mice: contribution of local and bone marrow-derived host cells

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ABSTRACT **The green fluorescence protein (GFP) from the UBI-GFP/BL6 transgenic line was bred into C57BL/6J-*scid* and C.B-17-*scid* mice for investigating host-tumor cell interactions. These mice express high levels of GFP under the control of the ubiquitin promoter in virtually all cells examined. In tumor tissue generated by implanting tumor cells in the GFP transgenic SCID mice, the tumor cells and tumor-associated murine host cells were clearly distinguished by GFP expression. A population of cells expressing the endothelial cell marker VEGFR-2/Flk-1, and the progenitor markers c-Kit and Sca-1, were incorporated into tumor tissue. The majority of the Flk-1-positive cells were hematopoietic-derived cells that coexpressed CD45. To investigate the contribution of bone marrow-derived cells to the formation of tumor vessels and stroma, tumor cells were implanted in nontransgenic SCID mice that received a bone marrow transplant from GFP-expressing SCID mice. Although GFP-positive cells were readily detected by histology in tumors taken from bone marrow transplanted animals, they were spatially isolated and lacked organization. In contrast, if tumors were implanted in nontransgenic SCID mice adjacent to a patch of transplanted GFP-expressing skin, these tumors recruited GFP-positive cells that organized into tumor vessels. The results demonstrate that hematopoietic-derived cells, including Flk-1$^+$/CD45$^+$ cells, readily colonized the tumor stroma but were minimally incorporated in the tumor vasculature. The majority of the tumor vessels were instead recruited from tissue adjacent to the tumor. The expression of Flk-1 on nonendothelial, tumor-associated host cells raises the possibility that VEGF antagonists, such as Avastin, could inhibit tumor growth by a mechanism involving hematopoietic-derived CD45$^+$/Flk-1$^+$ cells, in addition to direct suppression of endothelial cell function.—Udagawa, T., Puder, M., Wood, M., Schaefer, B. C., D'Amato, R. J. Analysis of tumor-associated stromal cells using SCID GFP transgenic mice: contribution of local and bone marrow-derived host cells. *FASEB J.* 20, 95–102 (2006)**

Key Words: tumor · stroma · cancer · Flk-1 · VEGFR2 · angiogenesis · endothelial cells · GFP

THE COMPLEX INTERACTIONS between malignant and host cells play a fundamental role in tumor growth. To grow beyond a minimal size, for example, a tumor must undergo vascularization by recruiting either pre-existing endothelial cells (1) or bone marrow-derived circulating endothelial precursors (2). Tumor vascularization is promoted by angiogenesis factors that are elaborated by the tumor cells and tumor-associated host cells such as inflammatory cells (3) and fibroblasts (4). Suppression of angiogenesis using inhibitors (5) or failure to induce angiogenesis can result in a state of tumor dormancy. Conversely, an "angiogenic switch" promoted by changes in critical host-tumor cell interactions can lead to loss of tumor dormancy and to relapse. The magnitude of neovascular responses can be modulated by genetic factors that are strain dependent (6), which suggests that, in turn, tumor growth is controlled in part by polymorphic genes expressed by tumor-associated host cells.

To facilitate the isolation and characterization of tumor-associated stromal cells, we bred the GFP transgene from the UBI-GFP/BL6 line (7) into SCID mice. A β-actin promoter-driven GFP transgenic nude mouse has been described (8). Mice homozygous for the β-actin-driven transgene (9) were abnormal, however, and died shortly after birth. In contrast, UBI-GFP/BL6 mice, which express high levels of GFP under the control of the ubiquitin promoter in all cells examined, are healthy and viable when maintained as a homozygous line. Mice derived from UBI-GFP/BL6 are ideal for cell transplantation studies due to high, uniform expression levels that are distinct for different cell

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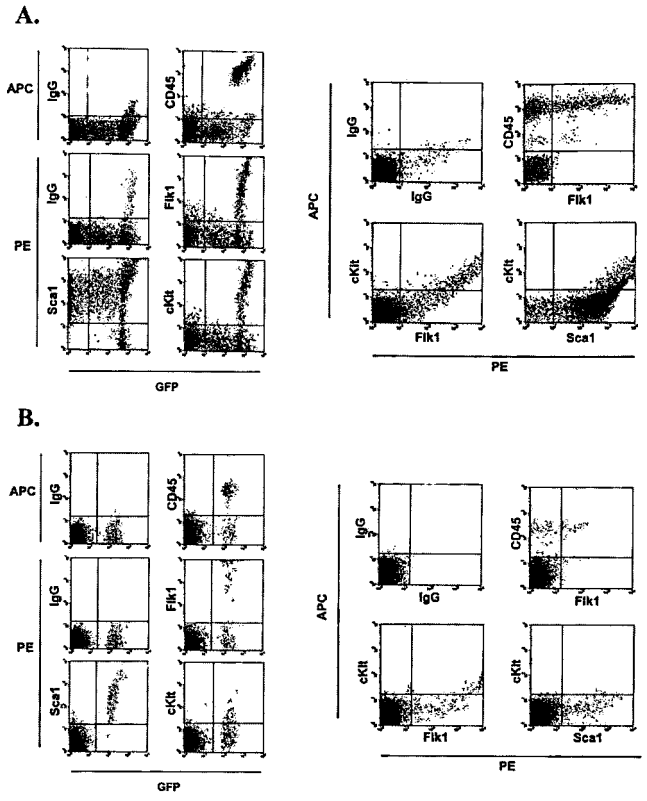

Figure 2. Flow cytometric analysis of tumors growing in GFP transgenic mice. Flow cytometric analysis of a murine LLC tumor tissue implanted in C57BL/6J-GFP-*scid* mice (*A*) and a human MG63Ras osteosarcoma implanted in C.B-17-GFP-*scid* mice (*B*). Tumors ~800–1000 mm^3 in volume were enzymatically digested and analyzed for GFP and the indicated antibodies directly conjugated to either APC or PE as described in Materials and Methods.

gated (Fig. 2*B*). We chose to analyze tumor tissue from C.B-17-GFP-*scid* mice, since the MG63Ras tumors grew ~4-fold faster in this strain than in the C57BL/6J-*scid* strain (data not shown). As shown in Fig. 2, the percentage of GFP-positive host cells in the MG63Ras tumor tissue was lower than in the LLC tumors. The phenotypic profile of the GFP-positive host cells in the MG63Ras xenograft (Fig. 2*B*) and the murine LLC (Fig. 2*A*) were similar, however, and both tumor tissues contained a population of Flk-1 cells that coexpressed the CD45 hematopoietic marker.

Several recent reports have indicated that tumor Flk-1^+ endothelial cells may be bone marrow derived (2). We therefore examined the incorporation of GFP-positive bone marrow-derived cells into tumor vessels. The origin of the tumor vessels was investigated by implanting tumors into C57BL/6J-scid mice that were transplanted with either bone marrow or skin from GFP transgenic SCID donor mice. Since GFP expressed in donor tissue can potentially elicit an immune response, nontransgenic SCID mice were used as recipients. Eight wk after bone marrow transplantation, >90% of the peripheral blood cells in the nontransgenic SCID recipients were replaced by donor GFP-positive cells coexpressing CD45 (**Fig. 3*A***). Specific antibody staining with CD3, CD11b, and Gr1 showed that the donor bone marrow cells from the C57BL/6J-GFP-*scid* mice successfully reconstituted the peripheral blood. The CD3-positive T lymphocyte marker was detectable, but since SCID mice were used, the lymphocyte counts were ~5- to 10-fold lower than in wild-type mice ($0.78\pm0.13\times10^6/\mu L$ for SCIDS vs. $9.20\pm1.91\times10^6/\mu L$ for wild-type mice; not shown). Cells expressing c-Kit and Flk-1 were detected in the peripheral blood of transplanted animals at levels comparable to that of control animals.

After confirming successful engraftment, LLC tumor cells were implanted in the subcutaneous space of the GFP-bone marrow-reconstituted animals. After 12 days, the animals were killed and the tumor-bearing animals were examined under a fluorescence dissecting microscope. GFP-positive bone marrow-derived cells were disseminated to normal peripheral tissue including skin, bone, and intestine. An intense, GFP fluorescent signal was also observed in tumor tissue (Fig. 3*B*). Immunohistochemical staining of the tumor sections using GFP antibodies revealed numerous solitary single cells (**Fig. 4*A*, *B***), but in contrast to the tumors implanted in GFP transgenic animals (Fig. 1*A*), tumor

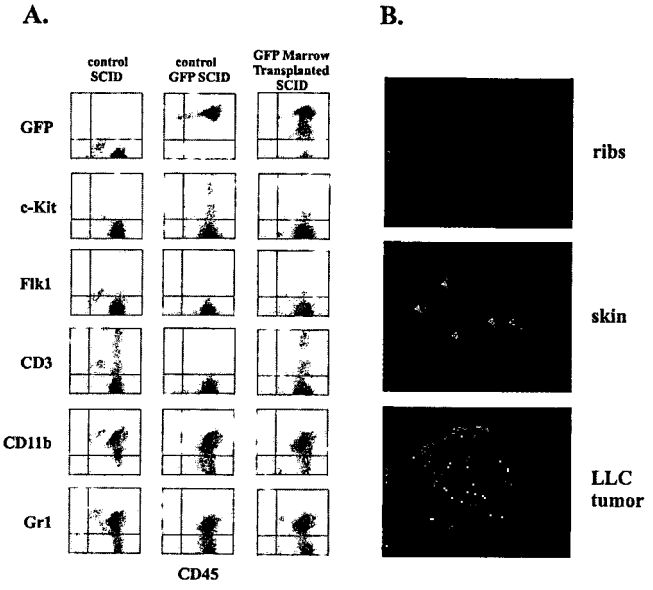

Figure 3. Colonization of GFP-positive cells in normal and tumor tissue in nontransgenic mice transplanted with bone marrow from GFP transgenic mice. *A)* Flow cytometric analysis of peripheral blood mononuclear cells taken from control C57BL/6J-GFP-*scid* mice (control SCID), nontransgenic C57BL/6J-*scid* mice (control GFP SCID), or C57BL/6J-*scid* mice transplanted with bone marrow from C57BL/6J-GFP-*scid* mice (GFP marrow transplanted SCID). Peripheral blood from the bone marrow reconstituted and control mice were analyzed 8 wk after transplantation. *B)* 10 wk after bone marrow transplantation, the mice were inoculated with LLC tumor cells. After 12 days, the tumor-bearing mice were killed and examined with a fluorescence dissecting microscope. GFP-expressing cells were observed in the bone marrow (ribs) and peripheral tissue. Foci of brightly fluorescent, GFP-positive cells in the skin around the mouth are indicated by arrowheads. An intense, GFP fluorescent signal was observed in the tumor tissue.

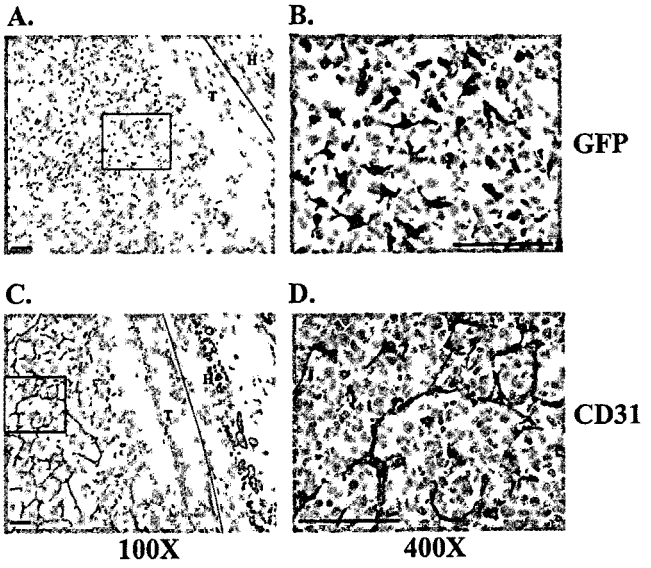

Figure 4. GFP immunohistochemical staining of a LLC tumor implanted in nontransgenic mice reconstituted with GFP-positive donor bone marrow. Tumor sections were stained with antibodies to either GFP (*A, B*) or CD31 (*C, D*). The dotted lines in panels *A, C* indicate the border between tumor tissue (T) and the mouse host tissue (*H*). The images were taken at 100× (*A, C*) and 400× (*B, D*). Boxes in panels *A, C* indicate the area magnified in panels *B* and *D*.

vessels were not stained with the GFP antibody. A dramatically different staining pattern was observed when adjacent tumor sections from the GFP bone marrow transplanted animals were stained with CD31 to highlight the tumor vessels (Fig. 4*C, D*). The CD31 antibodies stained some solitary cells, but also revealed the organized architecture of the tumor vasculature. These results suggested that few if any cells contributing to vessels were recruited from the peripheral blood.

To visualize the GFP-positive cells that were recruited to the tumor locally from adjacent tissue, we implanted tumor cells in the subcutaneous space of GFP-positive skin from C57BL/6J-GFP-*scid* mice, which was engrafted onto nontransgenic C57BL/6J-*scid* recipient mice (**Fig. 5*A, B***). Twelve days after tumor cell implantation, the tumors measured ~1 cm in diameter. By epifluorescece illumination, the transplanted GFP-positive skin could be seen overlying a portion of the tumor surface. When the interior of the tumors were examined, GFP-positive cells from the engrafted tissue were observed recruited to the interior of the tumor (Fig. 5*D*). On the histological sections, the transplanted GFP expressing skin and hair follicles were clearly stained using antibodies specific to GFP (Fig. 5*E*). Tumor vessels were stained with the GFP antibody in the tumor tissue adjacent to the transplanted GFP-positive skin (Fig. 5*F*), which is in contrast to the lack of microvascular structures stained with GFP antibodies in tumors implanted in GFP bone marrow reconstituted animals (Fig. 4*A, B*). Control animals injected with saline adjacent to engrafted GFP-positive skin showed no incorporation of GFP-positive vessels into normal adjacent tissue (Fig. 5*G, H*). Together these data indicate that the majority of the vessels were recruited from tissue adjacent to the tumor rather than from the peripheral blood.

DISCUSSION

A tumor is composed not only of transformed cells, but is intimately associated with host cells such as endothe-

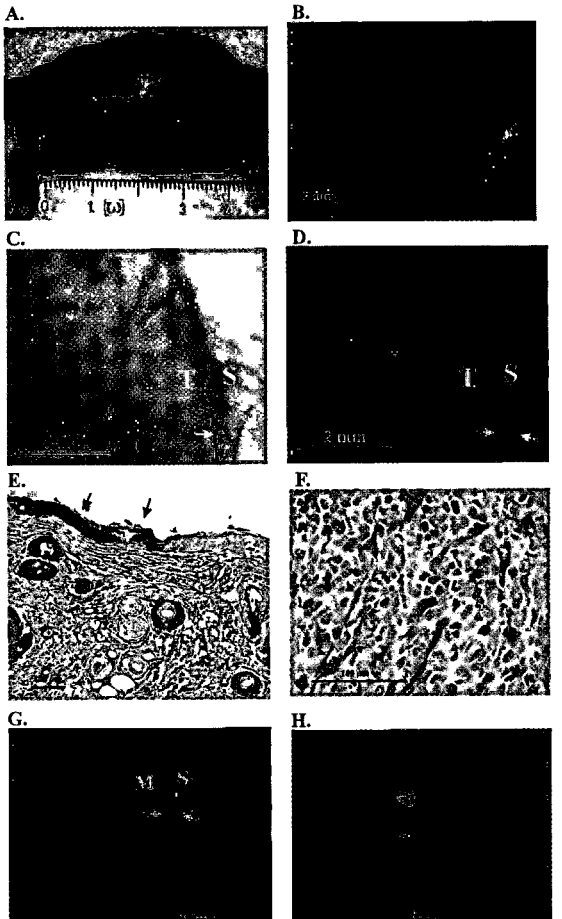

Figure 5. Local recruitment of GFP-positive tumor vessels from the local environment. *A)* Boxed area shows the location of the engrafted skin overlying a Lewis lung tumor. *B)* Epifluorescence illumination of the boxed area in panel *A* reveals the engrafted GFP expressing skin overlying a Lewis lung tumor. *C)* Cross section of a tumor (T) adjacent to skin (S) viewed in phase contrast. A portion of the engrafted GFP-positive skin is indicated by arrows. *D)* Same view as in panel *C* viewed by epifluorescece illumination. A network of GFP-positive cells can be seen extending from the engrafted skin into the tumor. *E)* Anti-GFP immunohistochemical staining of the engrafted skin (arrows) and hair follicles adjacent to a Lewis lung tumor. *F)* Anti-GFP immunohistochemical staining showing GFP-positive tumor vessels (arrowheads) recruited from the engrafted GFP-positive skin. *G)* Cross section of normal mouse tissue (muscle) adjacent to engrafted GFP-positive skin. Saline was injected in the subcutaneous space of the GFP-positive skin 5 days prior to examination. *H)* Higher magnification of panel *G* showing clear delineation between engrafted GFP-positive skin and underlying normal tissue.

lial cells, fibroblasts, and inflammatory cells that can potentially influence tumor growth. Fluorescent proteins are useful markers that have been used for tracking cells in vivo. GFP can be expressed in tumor cells to distinguish tumor cells from host cells. Markers expressed on heterogeneous tumor cell populations often exhibit a wide variability in expression levels, which may be due in part to genomic instability, so GFP may not reliably distinguish between transplanted tumor cells and host tissue in vivo. Here we report the generation of GFP-expressing C.B-17- and C57BL/6J-*scid* mice from the ubiquitin promoter-driven GFP transgenic line UBI-GFP/BL6. UBI-GFP/BL6 mice are ideal for cell transplantation studies due to high uniform expression levels that are distinct for different cell types including RBCs. In cell transfer experiments, leukocytes and dendritic cells from UBI-GFP/BL6 mice were readily identified in secondary lymphoid tissue by flow cytometry and fluorescence microscopy (7).

A transgenic C57/B6 nude mouse expressing GFP under control of the β-actin promoter has been described. However, mice homozygous for the β-actin promoter-driven GFP transgene (9) were abnormal and died shortly after birth. In contrast, homozygous UBI-GFP/BL6 transgenic mice are viable and completely normal despite high levels of GFP in all tissues, including RBCs. Strain differences have been reported with regard to angiogenesis and immune response to transplanted tumors (6, 10–12), which may explain the slow growth rate noted for tumors implanted in the nude C57/B6 transgenic mice (8). We have observed that tumors implanted in the C57BL/6J strain of immunodeficient mice grew more slowly than tumors implanted in the C.B-17 and Balb/cJ strains of immunodeficient mice (data not shown). This suggests that the C.B-17 strain may be more ideally suited for tumor xenograft studies than the C56BL/6J strain.

Alternatively, the differential growth of transplanted tumors in C57BL/6J- and C.B-17-*scid* mice may reflect relevant physiologic host-tumor cell interactions that play an important role in strain-dependent, differential susceptibility to cancer. A recent study has shown that the levels of circulating endothelial progenitor and circulating endothelial cells in the peripheral blood, measured by flow cytometry, correlated with genetically heterogeneous bFGF- or VEGF-induced angiogenesis (19). These cells may therefore serve as useful surrogate markers of angiogenesis, but it remains to be seen whether these surrogate markers participate directly in vessel formation in a strain-dependent manner. The availability of GFP transgenic SCID mice in different strains may prove useful in this regard.

We have used the UBI-GFP/BL6-derived immunedeficient GFP transgenic mice for examining host cells associated with transplanted tumor cells. The LLC tumor line implanted in C57BL/6J-GFP-*scid* mice exhibited an intense, fluorescent signal when examined under a fluorescent dissecting microscope. By flow cytometry, GFP-positive host cells comprised ~30–40% of the tumor tissue. Consistent with the flow cytometric analyses, immunohistochemical staining revealed that a GFP-positive host cell was usually never more than a distance of three cells from a tumor cell. The GFP staining pattern revealed a heterogeneous population of GFP-positive host cells that were dispersed homogeneously throughout the tumor. These observations underscore the notion that tumors are not simply composed of malignant cells, but instead are intimately associated with host cells.

Flow cytometric analysis of tumors implanted in GFP mice revealed a significant population tumor-associated host cells that expressed Flk-1, Sca-1, c-Kit, and CD45. Studies by Lyden et al. reported that bone marrow-derived cells contribute to tumor vascularization (2). To investigate the origin of the stromal cells, we implanted tumors in nontransgenic SCID mice transplanted with bone marrow or skin from GFP^+ donor SCID mice. Immunohistochemical staining of tumor tissue from the bone marrow reconstituted mice using a GFP antibody revealed that bone marrow-derived cells incorporated in tumors but were spatially isolated and lacked organization. Similar staining patterns were obtained in tumors that were implanted in immune competent and SCID mice reconstituted with wild-type GFP transgenic mice (UBI-GFP/BL6). In addition, we have not seen a difference in the neovascular response between SCID and wild-type mice in a corneal model of neovascularization (data not shown), which demonstrates that the lack of bone marrow contribution to tumor vessels was not attributable to reduced lymphocyte counts in SCID mice. The use of immune-deficient mice is advantageous in GFP tissue engraftment studies because the potential for an immune response to GFP is minimized, and the engrafted mice could be applied for the study of human tumor xenografts.

In contrast to the bone marrow transplantation experiments, tumors implanted in nontransgenic SCID mice, adjacent to GFP-positive skin from transgenic donors, incorporated GFP^+ tumor vessels. The results indicate that, in our model, most of the tumor vessels were nonhematopoietic, tissue resident cells from the local environment rather than bone marrow-derived cells from the circulation. Our findings support the recent report showing that bone marrow-derived cells comprise precursor for periendothelial vascular mural cells but do not contribute significantly to tumor and cytokine induced angiogenesis (20).

An important issue that remains is the mechanism by which the nonhematopoietic tissue contributes to tumor neovascularization. It is well known that VEGF and its receptor Flk-1 are critical role for vasculogenesis, angiogenesis, and tumor growth (13, 21, 22). A number of other vascular specific molecules such as the angiopoietins and their receptors (23–25), as well as nonspecific molecules such as platelet-derived growth factor (26), TGF-β (27), insulin-like growth factor II (28), and retinoic acid binding protein-4 (29), coordinate with VEGF to promote vascular development (22, 30, 31). The GFP^+ bone marrow transplant experiments indicate that the cellular target(s) that participate in neo-

vascularization in response to the growth factors likely resides predominantly in nonhematopoietic tissue, although the nature of the tissue-resident, nonhematopoietic cellular target that is incorporated into tumor vessels in response to tumor-derived angiogenesis factors is not yet clear.

A population of bone marrow-derived Flk-1$^+$ cells that expressed Sca-1, c-Kit, and CD45 were found in transplanted murine LLC tissue in C57BL/6J-GFP-*scid* mice as well as human MG63Ras osteogenic sarcoma tumor tissue in C.B-17-GFP-*scid* mice. The proportion of Flk1$^+$/CD45$^+$ cells in the Lewis lung tumor tissue in vivo was higher than in normal tissue (not shown). Flk-1 expression on the bone marrow-derived cells suggests they may also be recruited or stimulated by the angiogenic factor VEGF. These cells do not appear to incorporate into vessels, but may indirectly contribute to tumor angiogenesis by elaborating additional factors or enzymes that stimulate the recruitment of endothelial cells from neighboring tissue. Bone marrow-derived Flk1$^+$ cell may potentially stimulate tumor proliferation and invasion.

A relevant issue that might arise from these observations with regard to therapy is whether disrupting the binding of VEGF to nonendothelial, Flk-1$^+$/CD45$^+$ cells in the tumor stroma, using Avastin (32), for example, could inhibit tumor growth by a mechanism other than antagonizing VEGF signaling of endothelial cells. In addition, the presence of both c-Kit and Sca-1 suggests that the Flk-1$^+$ cells may be cells that are capable of reconstituting the hematopoietic system. The GFP SCID transgenic mice will facilitate the isolation and characterization of tumor host stromal elements that participate in the regulation of angiogenesis and tumor growth. FJ

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