Bone Marrow Subsets Differentiate into Endothelial Cells and Pericytes Contributing to Ewing’s Tumor Vessels

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Abstract
Hematopoietic progenitor cells arising from bone marrow (BM) are known to contribute to the formation and expansion of tumor vasculature. However, whether different subsets of these cells have different roles in this process is unclear. To investigate the roles of BM-derived progenitor cell subpopulations in the formation of tumor vasculature in an Ewing’s sarcoma model, we used a functional assay based on endothelial cell and pericyte differentiation in vivo. Fluorescence-activated cell sorting of human cord blood/BM or mouse BM from green fluorescent protein transgenic mice was used to isolate human CD34+/CD38+/CD45+ and mouse Sca1+/Gr1+ cells and mouse Sca1+/Gr1+, CD31+ cells and mouse Sca1+/Gr1+, VEGFR1+, and VEGFR2+ cells. Each of these progenitor subpopulations was separately injected intravenously into nude mice bearing Ewing’s sarcoma tumors. Tumors were resected 1 week later and analyzed using immunohistochemistry and confocal microscopy for the presence of migrated progenitor cells expressing endothelial, pericyte, or inflammatory cell surface markers. We showed two distinct patterns of stem cell infiltration. Human CD34+/CD45 and CD34+/CD38− and murine VEGFR2+ and Sca1+/Gr1+ cells migrated to Ewing’s tumors, colocalized with the tumor vascular network, and differentiated into cells expressing either endothelial markers (mouse CD31 or human vascular endothelial cadherin) or the pericyte markers desmin and α-smooth muscle actin. By contrast, human CD34+/CD45− and mouse Sca1+/Gr1+ cells migrated predominantly to sites outside of the tumor vasculature and differentiated into monocytes/macrophages expressing F4/80 or CD14. Our data indicate that only specific BM stem/progenitor subpopulations participate in Ewing’s sarcoma tumor vasculogenesis. (Mol Cancer Res 2008;6(6):929–36)

Introduction
The creation and expansion of a functional vascular network is essential for tumor growth. Aggressive malignancies, such as Ewing’s sarcoma, depend on angiogenesis and vasculogenesis to support the rapid rate of tumor cell proliferation (1). Therefore, understanding how tumor vessels expand to support the nutritional and oxygen needs of the tumor may identify novel therapeutic targets to interfere with this process. Endothelial progenitor cells (EPCs) have been shown to participate in postnatal vasculogenesis (2). However, defining a set of markers that uniquely identifies these cells has generated much controversy. No clear designation exists for when an EPC becomes a mature endothelial cell, and a universally accepted functional assay for these cells is also lacking. Consequently, several types of assays have been used to show that diverse cell types possess EPC-like capacity. Traditionally, EPCs were believed to express CD34, AC133, and vascular endothelial growth factor receptor 2 (VEGFR2; refs. 2-4). However, it has also been argued that the majority of EPCs are derived from monocytes/macrophages, which are CD34− (5). A specific subset of CD14+ monocytes, those that express VEGFR2, was described to have endothelial differentiation capacity (6). CD34+/VEGFR3+ progenitor cells were reported to play a role in lymphangiogenesis (7). Even mesenchymal stem cells have been shown to form endothelial cells in the presence of VEGF and serum in vitro (8).

Direct stem/progenitor cell incorporation into the endothelial lining of functional tumor neovessels has been reported (9). In addition, recent investigations have shown that bone marrow (BM) cells may have other functions within the tumor microenvironment. Platelet-derived growth factor receptor-β—expressing pericyte progenitor cells were shown to differentiate into NG2+, desmin+, and α-smooth muscle actin–positive (SMA+) pericytes within the tumor vasculature (10). De Palma et al. (11, 12) reported that Tie-2—expressing monocytes homed to tumors in mice and promoted angiogenesis in a paracrine fashion. Similarly, Gr1+/CD11b+ myeloid immune suppressor cells homing to tumors were found not only to directly incorporate into tumor endothelium but also to promote tumor angiogenesis and growth indirectly via release of matrix metalloproteinase-9 (13).

The extent of BM cell incorporation into tumors may be highly variable and depend on the specific tumor model or the quantity/type of BM cells injected (9). Few studies have investigated more than one stem/progenitor cell subpopulation. To clarify the roles of various types of EPCs in tumor vasculogenesis, we investigated the participation of different BM-derived progenitor cell subpopulations in a murine model of Ewing’s sarcoma tumors. We chose this model because we previously showed that Ewing’s tumors recruit BM-derived...
progenitor cells, which participate in tumor vessel formation (1, 14). Here, we show that CD34+/CD38−, CD34+/CD45−, CD34+/CD45+, VEGFR2− (Flk1−), VEGFR1+ (Flt1+), Sca1+/Gr1−, and Sca1+/Gr1+ stem/progenitor cells all migrate to Ewing’s sarcoma tumors. Of these progenitor cell types, CD34+/CD45−, CD34+/CD38−, VEGFR2−, and Sca1+/Gr1+ cells establish residence within the expanding tumor vascular network and differentiate into endothelial cells and pericytes. By contrast, CD34+/CD45+, Sca1+/Gr1+, and VEGFR1+ cells predominantly localize to sites outside the Ewing’s tumor vasculature. CD34+/CD45− and Sca1+/Gr1+ cells differentiate into monocytes/macrophages.

Results

Migration and Differentiation of Human Stem/Progenitor Subpopulations in vivo

Fluorescence-activated cell sorting (BD FACSaria) was used to isolate CD34+/CD45−, CD34+/CD45+, and CD34+/ CD38− cells (Fig. 1). To assess the in vitro endothelial differentiation capacity of human CD34+/CD45− and CD34+/ CD45+, these subpopulations were cultured in endothelial differentiation medium, examined by light microscopy, and immunostained for the endothelial marker human CD31. Cultured CD34+/CD45− cells expressed CD31, with the formation of CD31+ colonies (data not shown). By contrast, no such colony formation was observed for CD34+ cells. We next analyzed the capacity of CD34+/CD45−, CD34+/CD38−, and CD34+/CD45+ cells to migrate into TC71 tumors and participate in tumor neovascularization formation. The three human progenitor populations were green fluorescent protein (gfp) labeled with Ad5/F35-gfp and then separately intravenously injected into nude mice bearing TC71 Ewing’s tumors. One week later, mice were sacrificed and tumors were excised and sectioned. Migrated progenitor cells were detected within tumors by immunostaining with anti-gfp followed by a secondary antibody conjugated to the Alexa Fluor 594 fluorophore. gfp+ cells were therefore red. To determine the location of these human stem/progenitor cells with respect to tumor microvessels, tumor sections were costained for gfp and mouse CD31 (Fig. 2A). The gfp+ migrated human CD34+/CD38− cells (red) were seen both in close association with mouse CD31+ microvessels (green). CD34+−derived cells formed continuous, elongated structures that ran adjacent to and surrounded the mouse CD31+ cells (Fig. 2A, purple arrows). Yellow areas, obtained from merging the gfp and CD31 images, indicated migrated cells that were either on top of or integrated into the tumor vessels. To determine if CD34+ cells were differentiating into endothelial cells, sections were costained for gfp and human vascular endothelial cadherin (hVEC) and analyzed by confocal microscopy. We have previously shown that there is no cross-reactivity between hVEC and mouse endothelial cells; therefore, hVEC+ cells represent migrated human stem cells that differentiated into endothelial cells (14). Colocalization of gfp (red) and hVEC (blue), yielding purple fluorescence in the merged image, indicated that some migrated progenitor subpopulation had differentiated into human endothelial cells. Because the transduction efficiency of Ad5/F35-gfp in human subpopulations was < 50%, migrated CD34+−derived, hVEC+ cells that did not express gfp were also found within the Ewing’s tumor vasculature (Fig. 2B, Merge, blue cells). To determine whether stem cells also contributed to pericyte formation, tumor sections were costained for gfp and the pericyte differentiation markers α-SMA and desmin and then analyzed by confocal microscopy (Fig. 2C). CD34+/CD38− cells expressing α-SMA or desmin were detected in the tumor vascular network, indicating that a fraction of this migrated progenitor subpopulation had differentiated into pericytes/vascular smooth muscle cells (Fig. 2C, arrows). The migration of CD34+/CD38− and CD34+/CD45− cells to TC71 tumors and their contribution to neovascularization formation confirms that vasculogenesis plays a role in the growth of Ewing’s sarcoma.

CD34+/CD45− cells also migrated to the TC71 tumor area. These migrated cells were observed at the tumor periphery and also deeper into the tumor tissue (Fig. 2D, top left). But unlike the CD34− subpopulations, CD34+/CD45+ cells were found

![Image of figure 1: Isolation of human and murine stem/progenitor subpopulations. Fluorescence-activated cell sorting of human umbilical cord blood–derived or BM-derived ex vivo expanded CD34+ cells was used to isolate CD34+/CD45− (1), CD34+/CD45− (2), and CD34+/CD38− cells (3). Similarly, VEGFR2− (4), Sca1+/Gr1− (5), Sca1+/Gr1+ (6), and VEGFR1+ (7) cells were isolated from whole gfp transgenic mouse BM.](image-url)
mostly as single cells (Fig. 2D, top right), as opposed to large clusters/arrangements (Fig. 2A). CD34+/CD38− derived cells did not express either mouse CD31 (Fig. 2D, top right) or hVEC (data not shown). Rather, the CD34+/CD45+ migrated cells expressed F4/80, showing that they had differentiated into macrophages (Fig. 2D, bottom).

**FIGURE 2.** Migrated CD34+ cells differentiate into both endothelial cells and pericytes in the expanding tumor vessel network, whereas CD34− cells differentiate into macrophages. A, Sorted CD34+/CD38− cells were gfp labeled and intravenously injected into nude mice with TC71 tumors. Immunohistochemistry (magnification, ×20) identified migrated gfp+ CD34+/CD38− cells (red) near and around mouse CD31+ vessels (green). Hoechst 33342 identifies cell nuclei. B, Confocal microscopy. Migrated CD34+/CD38− cells differentiated into endothelial cells expressing hVEC. Magnification, ×40. C, Confocal microscopy. CD34+/CD38− cells (arrows) expressing pericyte markers α-SMA (left; magnifications, ×20 (GFP and α-SMA) and ×40 (Merge)) and desmin (right; magnification, ×20) were present within the tumor vascular smooth muscle cell network. The merged images show that α-SMA and desmin pericyte networks were mosaics composed of locally derived (blue) as well as CD34+/CD38− progenitor-derived (magenta) cells. Sytox was used to stain nuclei green. D, Sorted CD34+/CD45+ cells were gfp labeled and intravenously injected into nude mice with TC71 tumors. Top left, immunohistochemistry of tumor sections revealed migrated CD34+ cells (red) throughout the tumor area. Magnification, ×10. Top right, CD34+ progenitors (red) were single cells in the vicinity of green CD31+ tumor vessels. Magnification, ×20. Bottom, CD34+ progenitors differentiated into cells expressing macrophage antigen F4/80. Magnification, ×20.
cells. Similar to the VEGFR2+ cells, Sca1+/Gr1+ progenitor cells (Fig. 3C) migrated into the tumor and were associated with CD31+ microvessels. Using confocal microscopy, we showed that Sca1+/Gr1+ cells differentiated into CD31+ endothelial cells (purple cells) and were directly incorporated into vessels, thereby forming a mosaic pattern of BM progenitor-derived (purple) and locally derived (blue) endothelial cells adjacent to each other within the vascular endothelial lining (Fig. 3C, left). Sca1+/Gr1+-derived cells expressing α-SMA (Fig. 3C, right, arrow) and desmin (data not shown) were also seen, indicating pericyte differentiation.

**Sca1+ Gr1+ and VEGFR1+ Progenitor Subpopulations Do Not Differentiate into Vascular Endothelial Cells**

Although Sca1+/Gr1+ progenitor cells also migrated to the TC71 tumors, very few were found to reside in the tumor vessel vicinity. Similar to what was seen with CD34+ cells, none of the Sca1+/Gr1+-derived cells expressed CD31 (data not shown), indicating that this BM subpopulation did not differentiate into endothelial cells (Table 1). Similar to the human CD34+ subpopulation, Sca1+/Gr1+-derived cells expressed CD14, a monocyte/macrophage marker (Table 1).

Migrated VEGFR1+-derived cells were found in close proximity to CD31+ tumor vessels as well as away from the vessels (data not shown). However, these VEGFR1+-derived cells did not express CD31, again indicating that this murine BM subpopulation did not differentiate into endothelial cells within Ewing’s sarcoma tumors (Table 1).

**Discussion**

The data presented show that both human and mouse BM-derived stem/progenitor subpopulations fall into two categories: those that differentiate into endothelial cells and pericytes and participate in Ewing’s sarcoma tumor neovascularization and those that differentiate into myeloid cells and do not contribute to tumor vessel development. We showed that human CD34+/CD38–, CD34+/CD45+, and CD34+ /CD45+ stem/progenitor cells migrated into TC71 tumors. However, only the CD34+ cells differentiated into endothelial cells and pericytes. Evidence of endothelial differentiation *in vivo* was established using hVEC staining, a marker of human endothelial cells. We have previously shown that hVEC does not cross-react with mouse endothelial cells, allowing us to separate out the endothelial cells derived from human CD34+
cells. These human CD34\(^+\)–derived endothelial cells may be forming functional anastomoses to tumor vessels composed of murine endothelium. We further showed that many migrated CD34\(^+\) cells were associated with mouse CD31\(^+\) vessels and expressed the pericyte differentiation markers α-SMA and desmin. By contrast, migrated CD34\(^-\) cells did not differentiate into endothelial cells and mostly resided distant from the tumor vessels. Because we showed that CD34\(^+\) but not CD34\(^-\) stem/progenitor subpopulations contributed to the tumor vessel architecture, our findings refute the contention that migration of progenitor cells to human tumors is nonspecific.

CD34\(^+\)–derived human endothelial cells were observed wrapped around the tumor vessels and seemed distinct from the tumor vessel mouse endothelium. For this reason, we were concerned that species mismatch may interfere with the ability of recruited human progenitor cells to interact with the local mouse endothelial cells in the formation of tumor vessels. We therefore also evaluated whether mouse BM-derived stem/progenitor subpopulations showed a similar pattern of migration and differentiation. Sca1 is a marker of hematopoietic stem cells found in the mouse BM, whereas Gr1 is a myeloid differentiation antigen (Table 2). We selected to evaluate Sca1 \(^+/\)/Gr1\(^+\) and Sca1 \(^-\)/Gr1\(^+\) cells because these subpopulations represented the mouse counterparts of human CD34\(^+\)/CD45\(^+\) and CD34\(^-\)/CD45\(^-\) cells, respectively. Sca1 \(^+/\)/Gr1\(^+\) and Sca1 \(^-\)/Gr1\(^+\) cells, as well as VEGFR2\(^+\) and VEGFR1\(^+\) cells, all migrated into Ewing’s tumors. Similar to the human CD34\(^+\) subpopulation, VEGFR2\(^+\) and Sca1\(^+\)/Gr1\(^+\)-derived cells were within the expanding tumor vascular network. It is unclear at this point if these cells proliferated once within the tumor tissue or whether the large clusters of BM-derived cells resulted solely from migration. A portion of the VEGFR2\(^+\)– and Sca1\(^+\)/Gr1\(^+\)-derived cells colocalized with CD31 and incorporated into tumor vessels, indicating that they had differentiated into endothelial cells (Fig. 3A and C). A substantial fraction, however, resided in very close proximity to tumor vessels but did not express CD31. Rather, these recruited Sca1\(^+\)/Gr1\(^+\)– and VEGFR2\(^+\)-derived cells expressed the pericyte markers α-SMA and desmin (Fig. 3B and C). These data indicate that, similar to human CD34\(^+\) cells, these mouse BM subsets also contribute to the tumor vessel pericyte/vascular smooth muscle cell network. More importantly, our confocal studies (Fig. 3B and C) indicate for the first time that the endothelial and smooth muscle cell components of the tumor vasculature are mosaics composed of both BM and locally derived cells. Indeed, the vessel wall is composed of BM-derived and local endothelial cells side by side. This finding suggests that BM cells play an important, perhaps even critical, role in tumor vessel formation and that the BM serves as a source for the needed progenitor cells as the tumor vasculature expands during tumor growth. Interestingly, the incorporation of BM-derived cells in tumor vessels is not a uniform process, and these cells do not distribute homogeneously throughout the tumor tissue. Rather, recruited BM-derived cells form “hotspots” of incorporation at distinct sites within the tumor neovasculature. In our analysis of the Ewing’s tumor vasculature, certain microscopic tumor fields were found to contain numerous BM-derived endothelial cells and pericytes, with nearby microscopic fields often showing a relative absence of BM-derived cells. Previous work in our laboratory has shown that, on average, 10% of Ewing’s tumor vessels contain at least one BM-derived cell (1).

Our findings are consistent with other recent reports showing that BM-derived cells differentiate into tumor pericytes in melanoma, pancreatic carcinoma, and neuroblastoma (10, 15-17). Pericytes envelope the endothelial lining of blood vessels, supporting and regulating blood vessel formation

### TABLE 1. Differentiation of Selected Stem/Progenitor Subpopulations within the Ewing’s Sarcoma Tumor Microenvironment

<table>
<thead>
<tr>
<th>Stem/Progenitor Subpopulation</th>
<th>Differentiation into Endothelial Cells</th>
<th>Differentiation into Pericytes</th>
<th>Differentiation into Inflammatory Cell Types (Do Not Contribute to Neovessel Formation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34(^+)/CD45(^-)</td>
<td>+</td>
<td>+</td>
<td></td>
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<tr>
<td>CD34(^-)/CD45(^+)</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>Sca1(^+)/Gr1(^+)</td>
<td>–</td>
<td>+</td>
<td>(macrophages)</td>
</tr>
<tr>
<td>VEGFR2(^+)/Flk1(^+)</td>
<td>–</td>
<td>+</td>
<td>(macrophages)</td>
</tr>
<tr>
<td>VEGFR1(^+)/Flk1(^+)</td>
<td>+</td>
<td>+</td>
<td></td>
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</tbody>
</table>

### TABLE 2. Significance of Selected Stem/Progenitor Subpopulations

<table>
<thead>
<tr>
<th>Stem/Progenitor Subpopulation</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34(^+)/CD45(^-)</td>
<td>Primitive CD34(^+) cell; important in late engraftment and enriched in cord blood</td>
</tr>
<tr>
<td>CD34(^-)/CD45(^+)</td>
<td>Human hematopoietic stem cell</td>
</tr>
<tr>
<td>Sca1(^+)/Gr1(^+)</td>
<td>Human myeloid progenitor cell</td>
</tr>
<tr>
<td>VEGFR2(^+)/Flk1(^+)</td>
<td>Hematopoietic stem cell of mouse BM (also displaying evidence of myeloid differentiation)</td>
</tr>
<tr>
<td>VEGFR1(^+)/Flk1(^+)</td>
<td>Consensus marker for EPCs (also important in multiple angiogenic processes)</td>
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Pericytes in tumor blood vessels provide an escape mechanism to antiangiogenic therapy by promoting endothelial cell survival/stability (18). Here, we have identified three BM subpopulations (i.e., CD34+, VEGFR2+, and Sca1+/Gr1+ cells), which act as both pericyte precursors as well as endothelial cell precursors in Ewing’s sarcoma. Interfering with the homing of these particular progenitor cell types to Ewing’s tumors might provide a dual insult to the tumor vasculature by intervening with the supply of endothelial cells and pericytes that are needed to form functional tumor vessels. By disrupting vasculature expansion, this strategy could potentially allow better inhibition of tumor growth.

In contrast to CD34+ and Sca1+/Gr1+ cells, we found that CD34+ and Sca1+/Gr1+ progenitor cells had markedly reduced endothelial differentiation capacity in vitro and in vivo. Human CD34+ and mouse BM-derived Sca1+/Gr1+ cells did not contribute to the TC71 tumor neovasculature. Tumor-infiltrating CD34+ and Sca1+/Gr1+ cells differentiated into macrophages/microcytes within the TC71 microenvironment. As was the case for the BM-derived endothelial cells and pericytes described above, BM-derived macrophages did not seem to distribute uniformly throughout the tumor tissue. Although distinct areas within the Ewing’s tumor showed an abundance of BM-derived macrophages, other areas showed many host-derived (non–graft derived) macrophages. Although some of these BM subpopulations seem to play no role in the formation of tumor vessels, they nevertheless may have a different role that contributes to tumor cell growth and survival. Macrophage/microcyte progenitors may promote tumor growth via the production of angiogenic factors. For example, human stem/progenitor cells expressing monocye/macrophage markers have previously been shown to secrete VEGF, hepatocyte growth factor, granulocyte colony-stimulating factor, and granulocyte-macrophage colony-stimulating factor (4). Furthermore, Bingle et al. (19) used breast cancer spheroids to show that macrophages can initiate tumor angiogenesis in vivo. Not only have tumor-infiltrating macrophages been linked to tumor progression (20) but specifically eliminating these cells suppressed tumor growth and metastasis (21, 22). Targeting the specific BM stem/progenitor cell types that migrate to tumors and promote tumor growth by differentiating into macrophages may represent another potential therapeutic approach.

The objective of these studies was to determine whether specific BM subpopulations contributed to the formation of the tumor neovasculature. We have shown that human CD34+ hematopoietic stem cells and the mouse counterpart Sca1+/Gr1+ cells are the key progenitor cells that contribute to tumor vessel formation and that these cells migrate to the tumor and differentiate into either endothelial cells or pericytes. It is impossible to uniquely label each individual BM subset within a single animal and distinguish their individual contributions to the tumor vasculature. We therefore characterized each BM subpopulation of interest separately in a tumor-bearing animal. As was the case for the BM-derived endothelial cells and pericytes, it is important to note that the surface markers expressed by the BM-derived subpopulations analyzed are certainly not mutually exclusive. Specifically, we showed that 52.5% of VEGFR2+ cells were also Sca1+, whereas 58.2% of Sca1+ cells coexpressed VEGFR2. In addition, 8.5% of Sca1+ cells also expressed VEGFR1, whereas 38.5% of VEGFR1+ cells coexpressed Sca1. Six percent of VEGFR2+ cells were also VEGFR1+, whereas 29.4% of VEGFR1+ cells coexpressed VEGFR2.

Determining the relative importance of each subpopulation is beyond the scope of this work and cannot be ascertained by the methodology described. As an initial step toward comparing various progenitor subpopulations, an equal number of sorted cells of each type could be intravenously injected into tumor-bearing mice and the relative numbers of migrated cells were assessed. However, the detection of a larger number of migrated cells of a particular type within the tumor may not necessarily equate to greater importance. It is likely that the migrated cells divide, and the present detection system cannot identify daughter cells from parental migrated cells. For this reason, determining the percentage of intravenously injected BM cells of a particular type, such as Sca1+Gr1+ cells, that contribute to the vessel network was not possible in this work, as the number of these cells within the final tumor is potentially very different from that introduced initially. Different BM subpopulations are also likely to proliferate at different rates within tumors, making it difficult to meaningfully interpret any quantitative comparison between various BM subsets. In addition, a few BM cells of a specific type may exert great influence on tumor growth via production of cytokines affecting both resident tumor cells and vascular endothelial cells. At the same time, the tumor microenvironment may in part dictate the functional roles assumed by infiltrating BM stem/progenitor cells. A better understanding of the specific types of BM cells that migrate to tumors and how these progenitor cells contribute to the expansion of the tumor vascular network within the tumor microenvironment may aid in the identification of new therapeutic targets. Additionally, understanding this process may help in ascertaining how some tumors circumvent the effect of antiangiogenic therapy.

**Materials and Methods**

**Ewing’s Sarcoma Cell Lines**

TC71 human Ewing’s sarcoma cells were cultured in Eagle’s MEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 1 mmol/L nonessential amino acids, 1 mmol/L penicillin-streptomycin, and 2-fold vitamin solution (Life Technologies). These cells have the Ewing’s sarcoma t(11; 22) translocation as detected by reverse transcription-PCR.

**Mice and Murine BM Cells**

All experiments were approved by the Institutional Animal Care and Use committee of the University of Texas M. D. Anderson Cancer Center. All animals were housed in a specific pathogen-free facility. Four- to six-week-old athymic (T-cell deficient) nude mice were purchased from the National Cancer Institute. gfp transgenic mice (strain 003115; The Jackson Laboratory) were purchased from M. D. Anderson Cancer Center, Genetic Engineering Mouse Facility. This transgenic strain expresses an enhanced gfp driven by chicken β-actin promoter and cytomegalovirus intermediate early enhancer; intensity of gfp expression varies in a tissue-dependent manner. gfp transgenic mice were used as BM...
donors. BM cells were isolated from donors by flushing hind femurs with PBS. Freshly isolated BM cells were resuspended in PBS and kept on ice until injected into the tail veins of recipient nude mice.

**Isolation of Human and Murine Stem/Progenitor Cell Subpopulations**

Human CD34+ cells were isolated from umbilical cord blood obtained from the St. Louis Cord Blood Bank (St. Louis, MO) as previously described (14). Alternatively, frozen BM-derived CD34+ cells were purchased from the National Disease Research Interchange (Philadelphia, PA). CD34+ cells were cultured at 37°C in serum-free expansion medium (Stemcell Technologies) supplemented with Flt3 ligand, stem cell factor, interleukin-3, and interleukin-6 (StemSpan CC100 cytokine cocktail, Stemcell Technologies). Culture-expanded CD34+ cells were analyzed using the following antibodies: phycoerythrin (PE)-conjugated mouse anti-human CD34 (BD Biosciences), FITC-conjugated mouse anti-human CD45 (BD Biosciences), and FITC-conjugated mouse anti-human CD38 (BD Biosciences). Isotypic mouse IgG1κ antibodies conjugated to either PE or FITC were used as negative controls (BD Biosciences). Fluorescence-activated cell sorting (BD FACSAria) was used to isolate CD34+/CD45+, CD34−/CD45−, and CD34+/CD45− cells (Fig. 1).

Whole BM obtained from gfp transgenic mice was used as the source of all murine progenitor cell types and analyzed using the following antibodies: PE-conjugated anti-mouse Flk1 (VEGFR2, Ly-73; BD Biosciences), PE-conjugated anti-mouse Scal1 (Ly-6A/E; BD Biosciences), allopheocyanin-conjugated anti-mouse Gr1 (Ly-6G; BD Biosciences), and PE-conjugated rat anti-mouse VEGFR1 (Flt1; R&D Systems). Isotypic mouse IgG1κ antibodies conjugated to either PE, FITC (BD Biosciences), or allopheocyanin (R&D Systems) were used as negative controls. Fluorescence-activated cell sorting (BD FACSAria) of freshly isolated whole mouse BM was used to isolate Flk1+ Scal1−Gr1+, Scal1+Gr1+, and Flt1+ cells (Fig. 1). The significance of these human and mouse lineages is described in Table 2.

**Transduction of Human Stem/Progenitor Cell Subpopulations with Ad5/F35-gfp**

CD34+/CD45+, CD34+/CD45−, and CD34−/CD45− cells were cultured for 24 h with the adenovirus vector Ad5/F35-gfp. Ad5/F35 binds cells in a CAR-independent manner, allowing effective gene delivery to human stem/progenitor cells (23).

**In vitro EPC Culture**

To determine the ability of human CD34+/CD45+ and CD34−/CD45− cells to differentiate into endothelial cells in vitro, these sorted cell populations were cultured in fibronectin-coated chamber slides (BD BioCoat Human Fibronectin Cellware, BD Discovery Labware) in endothelial differentiation medium (EndoCult Liquid Medium kit, Stemcell Technologies). Cells were cultured in either EndoCult basal medium alone, medium + EndoCult supplements (Stemcell Technologies), or medium + supplements + recombinant human VEGF (200 ng/mL; Stemcell Technologies). After 5 d, cell proliferation and endothelial colony formation were assessed by direct observation via light microscopy.

**Assessment of In vivo Migration of Stem/Progenitor Subpopulations to Ewing’s Tumors**

Nude mice were injected subcutaneously with TC71 Ewing’s sarcoma cells plus growth factor–depleted Matrigel basement membrane matrix (BD Matrigel Basement Membrane Matrix, 2 × 106 cells/0.3 mL Matrigel; BD Biosciences). One week following tumor cell inoculation, one of the BM-derived mouse progenitor subpopulations or Ad5/F35-infected human subpopulations was separately injected intravenously into a Ewing’s tumor-bearing mouse. Mice were euthanized 1 wk later, and tumors were resected, placed in OCT compound, snap frozen in liquid nitrogen, and stored at −80°C.

**Immunohistochemistry**

For in vitro analysis of EPC differentiation, chamber slides (see above) were fixed in acetone and incubated with mouse anti-human CD31 (DakoCytomation). For in vivo detection of mouse platelet-endothelial cell adhesion molecule (mCD31), gfp, hVEC, α-SMA, desmin, platelet-derived growth factor receptor-β, CD14, or F4/80, frozen tissue sections were fixed in acetone, blocked using 4% fish gelatin in PBS, and incubated with the appropriate primary antibody. The following antibodies were used: rat anti-mouse CD31/PECAM (BD Biosciences), rabbit anti-gfp (Santa Cruz Biotechnology), mouse anti-hVEC (Chemicon International, Inc.), mouse anti-SMA (Abcam), rabbit anti-desmin (Abcam), rabbit anti-platelet-derived growth factor receptor-β (Santa Cruz Biotechnology), mouse anti-CD14 (Abcam), and rat anti-mouse F4/80 (Serotec). When using mouse monoclonal antibodies, slides were preblocked overnight with F(ab)2 fragment (Jackson ImmunoResearch Laboratories, Inc.) before incubation with primary antibody. The following secondary antibodies were used: goat anti-rat Alexa Fluor 488, goat anti-rabbit Alexa Fluor 594, goat anti-rabbit Alexa Fluor 488, or goat anti-mouse Alexa Fluor 488 (Invitrogen). All antibodies were diluted in PBS containing 4% fish gelatin. Hoechst 33342 dye (Molecular Probes) was used to stain cell nuclei. Fluorescent images were captured at 10× (eyepiece) and 10× or 20× (objective) with Optimas imaging software.

For confocal microscopy, the following secondary antibodies were used: goat anti-mouse Cy-3 [Cy-3−conjugated AffiniPure F(ab)2 fragment goat anti-mouse IgG + IgM, Jackson ImmunoResearch Laboratories], goat anti-mouse Cy-5 (Jackson ImmunoResearch Laboratories), goat anti-rabbit Cy-3 (Jackson ImmunoResearch Laboratories), goat anti-rabbit Cy-5 (Jackson ImmunoResearch Laboratories), goat anti-rat Cy-3 (Jackson ImmunoResearch Laboratories), and goat anti-rat Cy-5 (Jackson ImmunoResearch Laboratories). Sytox Green (Invitrogen) was used to stain cell nuclei. Images were collected using a Zeiss laser confocal microscope (Carl Zeiss MicroImaging, Inc.).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.
References

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