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| Original Article |

Enhanced Neovascularization by Simultaneous Transplantation of Peripheral Blood CD34$^+$ Hematopoietic Stem Cells and CD14$^+$ Monocytes

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Abstract : Formation of new blood vessels is required for normal embryonic development and healing of damaged tissues, but also is essential for tumor growth. Although CD34$^+$VEGF-R2(KDR)$^+$ endothelial progenitor cells and CD14$^+$ monocytes in human peripheral blood are known to actively participate in angiogenesis and vasculogenesis, the clear role of monocytes in the process of neovascularization is matter of debate. Here, we investigated whether a combination of two types of cells shows synergism in tumor-induced neovascularization. Fluorescently labeled purified CD34$^+$ HSCs, CD14$^+$ monocytes or combination of CD34$^+$ HSCs and CD14$^+$ monocytes were intratumorally injected into nude mice bearing human tumor of pancreatic adenocarcinoma. CD14$^+$ monocytes or combination of CD34$^+$ HSCs and CD14$^+$ monocytes. Injection of a mixture of the 2 subsets resulted in improved neovascularization in vivo to any single-cell-type transplantation. These data demonstrate that human CD14$^+$ monocytes as well as CD34$^+$ HSCs can differentiate along the endothelial lineage in a specific permissive environment and thus this combination represent an autologous transplantable cell source for therapeutic neovasculogenesis.

Key words: *hematopoietic stem/progenitor cells, monocytes, neovascularization*

1. Introduction

New blood vessel formation, or neovascularization, is required for normal embryonic development and promotes healing process of damaged or injured tissues and organs. In addition, the finding that neovascularization also promotes tumor growth and inflammatory diseases[1] makes endothelial progenitor cells (EPCs) of great clinical interest. Through the investigation of participating components and development of model system, the elucidation of the underlying cellular and molecular mechanisms of neovasularization made a noticeable progress. However, controversies over the identity of circulating EPCs have not completely resolved which cells give rise to endothelial cells during in vivo angiogenesis.

The first EPCs were described as CD34$^+$enriched mononuclear cells that acquired endothelial surface marker expression in culture.[2] CD34 is a marker of hematopoietic stem cells (HSCs) and is expressed by less than 0.1 % of circulating peripheral blood mononuclear cells or 1% of cord blood mononuclear cells.[3] Subsequent studies revealed that a subpopulation of circulating CD34$^+$ cells expressing VEGF-R2$^+$ could form endothelial colonies in vitro.[4-5] Only a fraction of these cultured cells incorporated acetylated DiI, expressed several markers in common with endothelial cells such as CD34, CD31 and VEGF-R2.[4] Studies have shown that purified human CD34$^+$ cells can integrate into the vasculature of murine models.[6-7] On the contrary, Fernandez-Pujol et al.[8] demonstrated that CD14$^+$ cells differentiate into endothelial cell-like cells exhibiting characteristics of both endothelial cells and monocytes under appropriate in vitro conditions. Monocytes, circulating and non-proliferating cells in a steady state, play an important role in immune defense, inflammation, and tissue remodeling and they do so by phagocytosis, antigen processing and presentation, and by cytokine production.[9] They can differentiate into dendritic cells during inflammation and thereby playing key roles in linking innate immunity to adaptive immunity.[10] Apart from these immunological roles, CD14$^+$ Monocytes coexpress endothelial and myeloid lineage markers and form vessel-like structure *in vitro*.[11-12] Furthermore, these cells have capacity to integrate into the vasculature of ischemic tissue in non-diabetic mice.[13-14] Myeloid to endothelial plasticity in vivo was shown

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recently through adoptive transfer of immature myeloid progenitors in mice and subsequently confirmed by vessels with donor origin myeloid cells in the liver.[15] In addition, recent reports have also identified a myeloid/endothelial biphenotypic leukocyte population within mouse and human tumors. Thus, it appears that there are two classes of human circulating endothelial progenitor cells, CD34$^+$ and CD34$^-$CD14$^+$ cells. While attention has focused on CD34$^+$ cells, CD34$^-$CD14$^+$ monocytes are far more abundant[16] and may represent the most common class of circulating EPCs

Studies have shown that vascular network in tumor is associated with recruitment of hematopoietic and circulating endothelial precursor cells.[11] However, little is known about the combined effect of freshly isolated monocytes and CD34$^+$ HSC on tumor angiogenesis in vivo. This study examined whether CD14$^+$ cells actually differentiate and integrate into the vasculature in vivo and whether they synergize with CD34$^+$ HSC in neovascularization.

2. Materials and Methods

2.1 Purification of Mononuclear Cells and Isolation of CD34$^+$ HSCs and CD14$^+$ Monocytes

The study protocol was approved by the Institutional Review Board of Severance Hospital (Severance Hospital, Yonsei University Health System, Seoul, Korea). Peripheral blood (50 mL) was obtained from healthy donors following informed consent. Mononuclear cells were fractionated from other components of peripheral blood by centrifugation on Ficol-Hypaq density gradient centrifugation (Pharmacia Biotech, Uppsala, Sweden). CD34$^+$ cells were isolated using standard immunomagnetic techniques (CD34 isolation Kit, MACS; Miltenyi Biotech, Auburn, CA) as described previously.[17-18] From CD34$^-$ fraction, CD14$^+$ monocytes were separated using an anti-CD14 monoclonal antibody (mAb) coupled to magnetic beads (CD14 MicroBeads) followed by magnetic cell sorting (MACS) column separation.

Purified CD34$^+$ HSCs and CD14$^+$ monocytes were cultured with RPMI-1640 supplemented with 10% FBS, antibiotics and L-glutamine (all from Gibco, Grand Island, NY) in the presence of various combinations of 3 hematopoietic cytokines, flt-3 ligand (FL), stem cell factor (SCF) and thrombopoietin (TPO) (10 ng/ml each, all from Peprotech, Rocky Hill, NJ) for 6 days. After culture cells were harvested and counted for cell proliferation.

2.2 Flow Cytometric Analysis

For flow cytometry, cells were stained with various monoclonal antibodies (mAbs) or isotype control antibodies, for 15 min at 4^{o}C in the dark. FITC- or PE-conjugated monoclonal antibodies with the following specificities were used: IgG1 and IgG2a isotype controls, anti-CD34, -CD38, -CD45 and anti-CD14 (all from BD Biosciences, San Jose, CA). Cells were washed in PBS and then fixed in PBS containing 1% paraformaldehyde. For data analysis, a Cytomics™ flow cytometer (Beckman Coulter, Fullerton, CA) was used. The data were analyzed by WinMDI 2.8 (Scripps Institute, La Jolla, CA) or CXP and FCS 3.0 software for the FC500 (Beckman Coulter).

2.3 Cell Labeling

Freshly isolated CD14$^+$ monocytes were stained with either 20 μM of with Cell Tracker Red CMTPX and CD34$^+$ cells were stained with 0.15 μM of Cell Tracker Green CMFDA (Molecular Probes, Eugene, OR), respectively, for 1 hr at 37^{o}C according to the manufacturer's instructions. The cells were then washed 3 times with PBS.

2.4 Mice and Tumor Model

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Yonsei University Severance Hospital, Seoul, Korea. HPac cells, a human pancreatic adenocarcinoma cell line (ATCC, 8×10^6 cells in a volume of 100 μl), were transplanted subcutaneously into the back of female athymic nude mice (Orient, Seongnam, Korea) 6 to 8 weeks old and 17 to 20 g in weight. Seven days after implantation, the fluorescent dye tagged cells (5×10^5 cells/mouse, n=3) were injected to the tumor site. Animals with tumors implanted but injected with PBS instead of human cells served as control. Ten days later, the animals were sacrificed, and tumor were removed and fixed in 10% phosphate-buffered formalin and embedded in paraffin or immediately frozen in isopentane in liquid N_2 for later inclusion in OCT compound.

2.5 Confocal Fluorescence Microscopic Analysis

Sections of 5 μm were examined under a fluorescent microscope to visualize the incorporation of the fluorescent-labeled cells into the capillary networks. Frozen sections (10 μm thick) of the tumor were subjected to immunohistochemistry, in which the slides were incubated with mAb to human-specific anti-factor VIII (BD Biosciences) followed by incubation with PE-conjugated secondary antibody (BD Biosciences). Nuclei were counterstained with DAPI (Sigma, St. Louis, MO). These slides were examined with a confocal laser fluorescence microscope. The proportions of CD34$^+$ HSC-derived (CMFDA-stained green fluorescent cells) cells and blood vessels containing human factor VIII-expressing endothelial cells (PE-

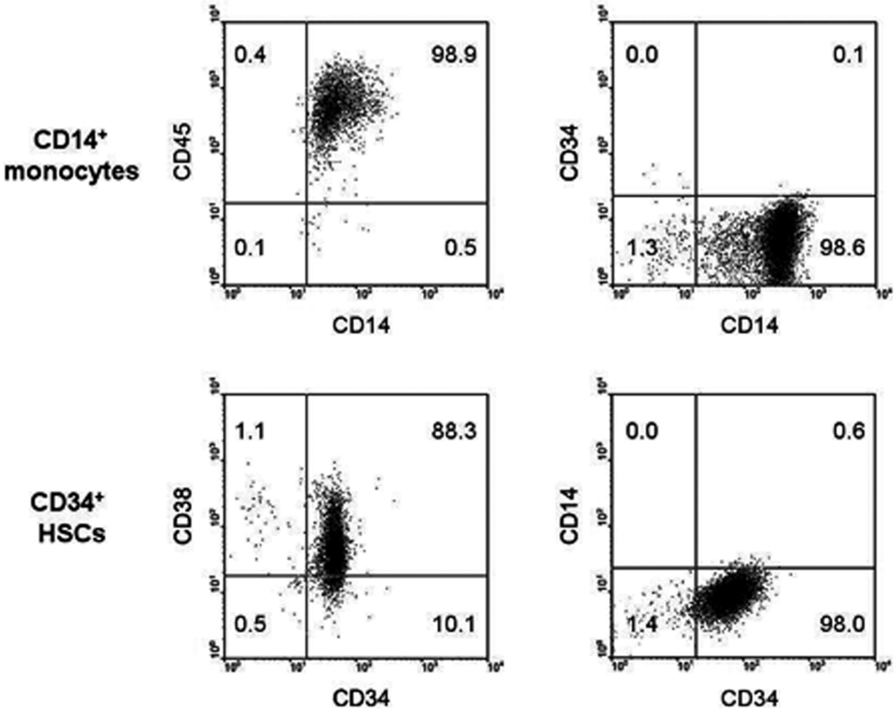

Figure 1. Isolation of CD14$^+$ monocytes and CD34$^+$ hematopoietic stem cells from human peripheral blood. Cells isolated by MACS were stained with the indicated antibodies and analyzed by flow cytometer. CD34$^+$ cell content of MACS-purified CD14$^+$ cells were determined by FACS analysis. Less than 0.1% of freshly isolated CD14$^+$ cells had the progenitor marker CD34 and about 0.6% of isolated CD34$^+$ cells expressed the monocyte marker CD14. Numbers represent % of cells in each quadrant.

stained) were measured by optical density at 492 nm and 540 nm, respectively.

2.6 Statistical Analysis

Data were analyzed using Student's t-test and unpaired t-test with Welch's correction. P-value < 0.05 was considered to be statistically significant.

3. Results

3.1 Phenotypes of Isolated CD14$^+$ Monocytes and CD34$^+$ Hematopoietic Stem Cells

CD34$^+$ cells typically represent less than 0.1% of human PBMCs and flow cytometry analysis of purified cells showed that more than 95% of the selected cells were positive for CD34. CD14$^+$ cells were selected from total CD34$^-$ PBMCs to obtain an essentially pure population of CD14$^+$ cells (Fig 1). In parallel, analysis of expression of CD34 on CD14$^+$ monocytes and CD14 on CD34$^+$ cells revealed that these markers are mutually exclusive. MACS purified CD14$^+$ cells (> 98 % purity) contained little or no CD34$^+$ cells (average 0.41±0.28, mean±SD, n=5).

Next, we evaluated the growth promoting activities of hematopoietic growth factors on the purified monocytes and HSCs to exclude a possibility of HSC contamination in the purified CD14$^+$ cells that may participate neoangiogenesis in vivo (Fig 2). These growth factors, alone or in combinations, had little effect on monocyte proliferation. On the contrary, CD34$^+$ HSCs displayed different responses to the growth factors. Little or no donor-to-donor variability was observed in these assays.

3.2 In Vivo Vasculogenic Properties of CD34$^+$ HSCs and CD14$^+$ Monocytes

To determine whether CD34$^+$ HSCs and CD34$^-$CD14$^+$ monocytes contribute to the tumor vasculature, human pancreatic adenocarcinoma cell line HPac was implanted subcutaneously into nude mice, and 7 days later freshly isolated fluorescein-labeled CD34$^+$ HSCs or CD14$^+$ monocytes were injected intratumorally at a dose of 5×10^5 cells/mouse. At day 10 after tumor inoculation, the mice were sacrificed. No fluorescent cells were detected in tumors, liver or spleen from mice injected with PBS (data not shown). On the other hand, tumor sections obtained 10 days after transplantation from the green fluorescent CD34$^+$ cell-transplanted mice showed many blood vessels carrying erythrocytes (Fig 3A). Transplanted

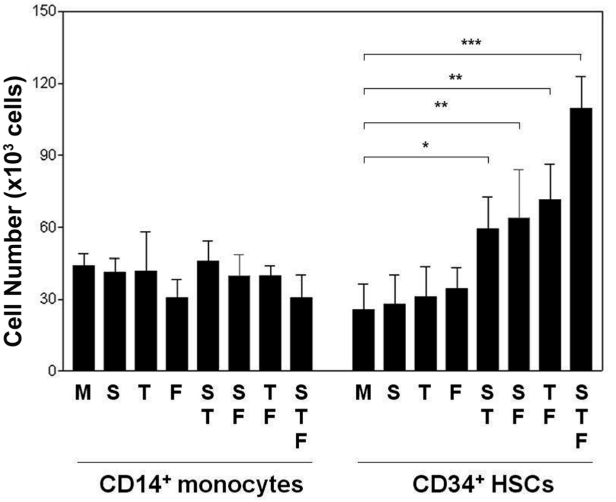

Figure 2. ell proliferation of CD14$^+$ monocytes and CD34+ HSCs by hematopoietic stem cell factors. Proliferation of cells in the absence (M for media) or in the presence of three hematopoietic growth factors, flt-3 ligand (F), thrombopoietin (T) and stem cell factor (S) alone or in combinations, were measured. The cytokine concentrations were fixed at 10 ng/ml each. Cells (1× 10^5 cells/ well) were cultured with the indicated cytokine combinations for 6 days and enumerated with hemocytometer. The results are expressed as mean±SD of triplicates. *p < 0.05, **p < 0.01 and ***p<0.005.

cells were detected in abundance and had organized to form vascular structures. In contrast, only a few vessels were seen in the tumor sections from the red fluorescent CD14$^+$ monocytes cell transplanted mice implying that vasculogenic potential of CD34$^+$ HSCs in vivo was superior to that of CD14$^+$ monocytes. To test whether the combination of CD34$^+$ HSCs and CD14$^+$ monocytes could synergize in neovascularization, equal numbers of CD34$^+$ admixed with CD14$^+$ monocytes were injected intratumorally. Mixed EPC transplantation improved vessel formation compared with any single type of EPC transplantation. The mixed transplantation group had greater capillary density than any group receiving single cell transplantation.

All the tumors were then stained with human-specific Factor VIII. Tumors obtained from the mice that received CD34$^+$ HSCs had blood vessels that included cells strongly expressing human-specific factor VIII (Fig 3B). Many of the factor VIII-positive vessels co-localized with green fluorescent cells (i.e., CD34$^+$ HSC-derived). In contrast, tumors obtained from the mice that received CD14$^+$ monocytes had blood vessels that included cells only weakly expressing factor VIII. Tumors obtained from the mice that received the combination of CD34$^+$ HSCs and CD14$^+$ monocytes had blood vessels that included cells strongly expressing factor VIII with higher density compared to that of tumor received CD34$^+$ HSCs or CD14$^+$ monocytes. These findings indicate that human monocytes contributed to tumor vasculogenesis in vivo by being incorporated and differentiating into the endothelium or to pericytes.

To quantify dye-labeled cells in the tumor, we assessed semiquantitatively the number of human cells in the tumor blood vascular networks by spectrophotometer (Fig 4). Intratumoral injection of any single type of cells led to comparable neovascularization, both of which were better than that of control. However, tumors in mice receiving CD14$^+$ and CD34$^+$ cells had significantly more incorporated human cells in the examined vessels than did tumors from mice receiving CD34$^+$ HSCs or CD14$^+$ monocytes alone. These results demonstrated that both a fraction of human CD34$^+$ HSCs and CD14$^+$ monocytes participate in the formation of human pancreatic cancer vasculartur e and the combination of CD34$^+$ HSC and CD14$^+$ monocytes significantly enhanced the formation of new blood vessels.

4. Discussion

Previous studies showed that there are different types of EPCs present in the human peripheral blood.[19-20] Transplantation of purified CD34$^+$ HSCs or ex vivo endothelial lineage-differentiated cells of derived from human CD34$^+$ hematopoietic stem cells (HSCs) incorporated into newly formed vessels in animal ischemic models or in tumor angiogenesis.[21-23] Although it has been shown that monocytes generate EPCs and exhibit vascularizing properties in vitro[24] as well as in vivo,[22,25] they are considered as terminally differentiated cells and there is still no direct proof of their possible "stemness" or of their relationships with endothelium derived from CD34$^+$ cells. Circulating monocytes may promote the angiogenesis of EPCs indirectly through soluble factor secretion.[26] However, their source, stem cell nature and the possible contribution of monocytes in the HSC-driven angiogenesis was not clearly defined. The proangiogenic effect of myeloid infiltrates[27] and the multipotency of CD14$^+$ monocytes [28] prompt us to investigate the role of monocytes in neovascularization.

Here, we confirmed that freshly isolated human peripheral blood CD34$^+$ HSCs and CD14$^+$ monocytes can participate in tumor neoangiogenesis after intratumoral injection in nude mouse that had received human HPac tumor cells. In addition to endothelial differentiation from CD34$^+$ HSCs, we confirmed that CD14$^+$ fraction can differentiate to factor VIII-expressing endothelial cells in vivo. Integration of freshly isolated and

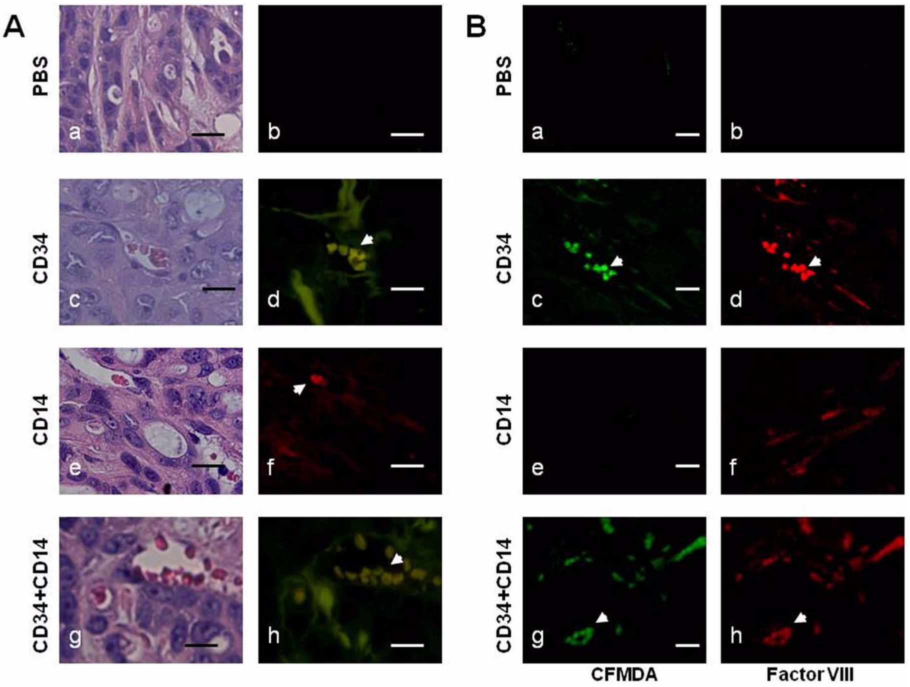

Figure 3. Injected CD34$^+$ HSC and CD14$^+$ monocytes differentiate into endothelial cells in expanding tumor vessel network. (A) Purified CD34$^+$ cells and CD14$^+$ cells were labeled with CMFDA and CMTPX, respectively, and injected intratumorally into nude mice with HPac tumors. Confocal images with hematoxylin and eosin staining of the tumor of PBS-injected control (a,b), CMFDA-labeled CD34$^+$ cell-injected tumor (c,d), CMTPX-labeled CD14$^+$ cells-injected tumor (e,f) and CMFDA-labeled CD34$^+$ cell and CMTPX-labeled CD14$^+$ cells-injected tumor (g,h) reveal vessels with blood cells. Scale bar indicates 20 μm, initial magnification ×20. (B) Injected CD34$^+$ HSC and CD14$^+$ monocytes differentiate into human factor VIII-expressing endothelial cells in tumor vessels. Purified CMFDA-labeled CD34$^+$ cells and unlabeled CD14$^+$ cells were and injected intratumorally, alone or in combination, into nude mice with HPac tumors. Confocal images of PBS-injected tumor (a,b), CD34$^+$ cell-injected tumor (c,d), unlabeled CD14$^+$ cells-injected tumor (e,f) and mixed population of CMFDA-labeled CD34$^+$ cell and unlabeled CD14$^+$ cells-injected tumor (g,h) with human factor VIII-specific cells (PE-conjugated secondary antibody) show vessels of human cell origin. Scale bar indicates 20 μm, initial magnification ×100. The Arrowheads show RBCs associated with blood vessels.

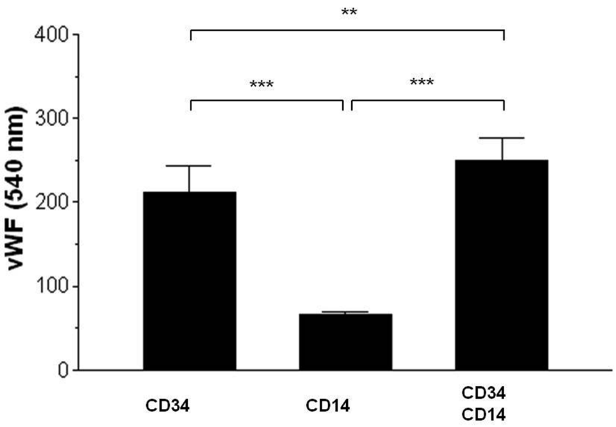

Figure 4. Combination of CD34$^+$ HSC and CD14$^+$ monocytes generates more human factor VIII-expressing cells in HPac tumor of nude mice. The quantitation of human Factor VIII-expressing endothelial cells in the tumor sections was spectrophotometrically analyzed from the fluorescent images. **p < 0.01 and ***p<0.005.

undifferentiated HSCs into blood vessels in vivo implies that differentiation of precursor cells to endothelial cell is driven by the tumor microenvironment. While CD14$^+$ monocytic EPCs promote tumor neovasculogenesis, they do so less well than their CD34$^+$ EPC counterparts. However, combination of CD34$^+$ HSCs and CD14$^+$ monocytes cooperated to enhance neovasculogenesis in the human tumor in the nude mice. It has been shown that circulating monocytes play a key role in neovascularization[29-30] and an infusion of bone marrow-derived CD14$^+$ monocytes contributes to the regeneration of functional endothelium.[31] Our findings are consistent with these reports showing that CD14$^+$ monocytes are not solely immune cells but also potential precursors for endothelium.

Different EPC subsets can influence the function of other subsets, and the combination of these subsets can be beneficial for enhanced neovascularization than using one subset alone.[32] In addition, the function of CD34$^+$ HSCs is severely compromised in certain pathological conditions such as diabetes. In this case, EPCs of CD14$^+$ monocyte origin can be of a therapeutic alternative.[33-34] CD14$^+$ cell-based therapy may be feasible in an acute setting, as large quantity of CD14$^+$ monocytes can be easily isolated with no pre-activation when directly injected into the affected tissue.

In conclusion, the transplantation of mixed EPCs (of CD34$^+$ HSC and CD14$^+$ monocytic) results in synergistic augmentation of angiogenesis in athymic nude mice with human tumor. Such synergistic interactions may also be present among other types of stem or progenitor cells that may shed light on the future direction of stem cell therapy. Our data indicate that local injection of small numbers of freshly isolated circulating EPCs can integrate to the tumor-induced vasculature, but that CD34$^+$ HSCs are more effective than CD14$^+$monocytes in doing so. The different roles played by two types of EPC in vasculogenesis would be an interesting topic for a future study.

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