Aging in the Atherosclerosis Milieu May Accelerate the Consumption of Bone Marrow Endothelial Progenitor Cells

Shoukang Zhu, Xialin Liu, Ying Li, Pascal J. Goldschmidt-Clermont, Chunming Dong

Objective—We have demonstrated that bone marrow cells from young and wild-type (WT), but not old apoE<sup>−/−</sup>, mice are capable of preventing atherosclerosis. This study was performed to elucidate the numerical and functional changes underlying the efficacy difference between young and old bone marrow.

Methods and Results—CD34<sup>+</sup>/VEGFR2<sup>+</sup> conventional endothelial progenitor cells and lin<sup>−</sup>/cKit<sup>−</sup>/Sca-1<sup>−</sup> hematopoietic stem cells did not differ numerically or functionally between young and old apoE<sup>−/−</sup> bone marrow. Fluorescence-activated cell sorter analysis, however, showed that a group of cells (simple little cells or SLCs), characteristically located in the lower left quadrant of forward scatter/side scatter flow cytometric plot, were markedly decreased in old WT and apoE<sup>−/−</sup> marrow, but abundantly present in young WT and apoE<sup>−/−</sup> bone marrow. The SLC fraction was mainly composed of lin<sup>−</sup>/cKit<sup>−</sup>/Sca-1<sup>−</sup> cells. In vitro differentiation assay demonstrated substantially more efficient endothelial differentiation of lin<sup>−</sup>/cKit<sup>−</sup>/Sca-1<sup>−</sup> SLCs than other bone marrow fractions at a single cell level and en masse. Furthermore, old lin<sup>−</sup>/cKit<sup>−</sup>/Sca-1<sup>−</sup> SLCs had a trend of decreased endothelial differentiation capability.

Conclusions—Lin<sup>−</sup>/cKit<sup>−</sup>/Sca-1<sup>−</sup> SLCs may represent a previously unrecognized cell population, enriched for endothelial progenitors. The identification of these cells may help improve the efficacy of cell therapy. (Arterioscler Thromb Vasc Biol. 2007;27:113-119.)

Keywords: endothelial progenitor cells  ■  vascular repair  ■  bone marrow  ■  aging  ■  atherosclerosis

The response-to-injury hypothesis presupposed that atherosclerosis was a chronic inflammatory process following localized injury to the vessel wall, in particular to the endothelial layer lining the lumen of the vessel. Recent evidence, however, indicates that the homeostasis of the arterial wall depends on the balance between vascular injury and repair and that endothelial progenitor cells (EPCs) originating from the bone marrow contribute to the vascular repair process, accelerating reendothelialization, and limiting atherosclerotic lesion formation. Indeed, EPC-mediated vascular repair has been demonstrated in acute vascular injury and atherosclerosis. Using genetically marked mice as donors, EPCs have been shown to engrat the injured vascular sites and differentiate into endothelial cells. These observations have generated excitement about the possible use of bone marrow cells as a novel preventative and/or treatment strategy for atherosclerosis.

On the other hand, atherosclerosis risk factors, such as aging and diabetes, reduce the number and functional activity of EPCs. A strong inverse correlation between the number of circulating EPCs and the combined Framingham risk factor score for atherosclerosis was demonstrated. Measurement of flow-mediated brachial-artery reactivity revealed a significant relation between endothelial function and the number of progenitor cells. The levels of circulating EPCs were a better predictor of vascular reactivity than was the presence or absence of conventional risk factors. Furthermore, factors that reduce cardiovascular risk, such as statins or exercise, elevated EPC levels, contributing to enhanced endothelial repair. Remarkably, we demonstrated that injection of unfractionated bone marrow cells isolated from age-matched wild-type (WT) or young, but not old, apoE<sup>−/−</sup> donor mice substantially retarded the formation of atherosclerotic lesions. These data suggest that aging, particularly in the presence of atherosclerosis risk factors, may result in the exhaustion of supply of competent EPCs in the bone marrow, which may undermine the efficacy of certain cell-based therapeutic approaches, especially when autologous bone marrow cells are isolated and simply injected back into the patients. Importantly, the findings underscore the essentiality of understanding the bone marrow biology to vascular biologists and clinicians.

Indeed, the lack of comprehensive insights in the bone marrow biology represents a major challenge to clinicians and vascular biologists before performing clinical trials to evaluate cell therapy. Identification of effector EPCs and necessary supporting cells will help improve the efficacy of cell-based treatments and reduce the recruitment of mature...
inflammatory cells or precursors destined for hematopoietic lineages. In the present study, we found that a group of cells characterizedly located in the lower left quadrant of forward scatter (FSC)/side scatter (SSC) flow cytometry plot was markedly decreased in marrows from old WT and apoE−/− mice, but abundantly present in young WT and apoE−/− bone marrow. We term these cells “simple little cells” or “SLCs” because of their modest sideward and forward light scattering properties in flow cytometry, indicative of limited granular content, and small size, respectively. Remarkably, further analysis of SLCs revealed that the majority of these cells fell into the lineage negative, cKit negative and Sca-1 negative (lin−/cKit−/Sca-1−) population and the lin−/cKit+/Sca-1− SLCs differentiated into mature endothelial cells (ECs) more efficiently than other bone marrow fractions, including lin−/cKit+/Sca-1− hematopoietic stem cells (HSCs) and CD34+/VEGFR2+ conventional EPCs (cEPCs). These data underline the notion that after a lifetime of repairing atherosclerotic arteries, the supply of the specific type(s) of EPC(s) because of their modest sideward and forward light scattering properties in flow cytometry, indicative of limited granular content, and small size, respectively. Remarkably, further analysis of SLCs revealed that the majority of these cells fell into the lineage negative, cKit negative and Sca-1 negative (lin−/cKit−/Sca-1−) population and the lin−/cKit+/Sca-1− SLCs differentiated into mature endothelial cells (ECs) more efficiently than other bone marrow fractions, including lin−/cKit+/Sca-1− hematopoietic stem cells (HSCs) and CD34+/VEGFR2+ conventional EPCs (cEPCs). These data underline the notion that after a lifetime of repairing atherosclerotic arteries, the supply of the specific type(s) of EPC(s), probably encompassed in the lin−/cKit+/Sca-1− SLC fraction, needed to maintain the homeostasis of the cardiovascular system, becomes exhausted. Furthermore, lin−/cKit+/Sca-1− SLCs may provide a platform for further study that aims to identify the effector EPCs.

**Methods**

Due to space limits, detailed description of the Methods is presented in the supplemental material (available online at [http://atvb.ahajournals.org](http://atvb.ahajournals.org)).

**Animals**

ApoE−/−, WT and EGFP-expressing mice, all in C57BL/6 background, were used.

**Cell Characterization and Fluorescence Activated Cell Sorting (FACS) Analysis**

Bone marrow was isolated from WT, apoE−/−, and EGFP-positive mouse tibiae and femora and subjected to FACS analysis and sorting.

**En Mass Cell Culture**

Lin−/cKit+/Sca-1− SLCs, lin− whole bone marrow fraction minus SLCs (WB-SLCs), CD34+/VEGFR2+ cEPCs and lin−/cKit+/Sca-1− HSCs were plated on 24-well plate coated with fibronectin at the density of 10⁴/well in 500 μL expansion medium.

**Single-Cell Culture**

Single HSC, cEPC, or lin−/cKit+/Sca-1− SLC was deposited per well in 96-well plates (Corn) and cultured at a single-cell level.

**Capillary-Like Tube Formation on Matrigel**

Capillary tube formation on Matrigel (BD Bioscience) basement membrane matrix was assessed via phase contrast microscopy.

**Immunofluorescence Staining**

Double or triple immunostaining for EC-specific markers or Dil-acetylated low-density lipoprotein (Dil-Ac-LDL) uptake was performed to examine the EC differentiation capability of different bone marrow fractions.

**TaqMan Real-Time RT-PCR**

TaqMan real-time RT-PCR was performed using a sequence detector (ABI Prism 7700, PE Applied Biosystems).

**Statistical Methods**

Group means were compared using either Students t test or ANOVA with Fisher post hoc test as appropriate. Data are presented as mean±SD with P<0.05 accepted as significant.

**Results**

**Aging Results in Exhaustion of Simple Little Cells**

We have demonstrated previously that administration of unfractonated bone marrow from age-matched WT or young, but not old, apoE−/− mice significantly reduced atherosclerotic lesion formation in apoE−/− recipient mice. Thus, aging in the proatherogenic milieu of apoE deficiency eliminated bone marrow cell efficacy in reducing atherosclerosis. Consequently, we reasoned that bone marrow from old apoE−/− mice would be deficient, either functionally or numerically, in vascular repair-competent EPCs and perhaps the needed supporting cells. Identification of these cells, therefore, should be achievable by comparing the bone marrow from old versus young mice. We first focused FACS analysis of the young (3-week-old, weaning) versus old (6-month-old, fed high-fat, high-cholesterol diet) apoE−/− bone marrow on two types of progenitor/stem cells—cEPCs and HSCs—both of which have been shown to differentiate into mature ECs, although the evidence has been conflicting for HSCs,12,13 cEPCs, as identified by the expression of CD34 and flk-1/KDR/VEGFR2, were present in the bone marrow of both young and old mice (accounting for 0.034±0.021% and 0.029±0.017% of the total mononuclear cells in the young and old apoE−/− marrow, respectively), and there was no significant difference between these two groups (P>0.05). Similarly, lin−/cKit+/Sca-1− HSCs constituted 0.067±0.035% and 0.08±0.027% of the mononuclear cells in the young and old apoE−/− marrow, respectively, (P>0.05). These data indicate that atherosclerosis risk factors do not affect the numerical composition of cEPCs and HSCs in the bone marrow.

To determine whether aging and atherosclerosis adversely impacted the function of cEPCs and HSCs, we examined the differentiation capability of these cells isolated from young (3-week-old, weaning) and old (6-month-old, fed high-fat, high-cholesterol diet) apoE−/− bone marrow, both en masse and at the single cell level. Consistent with previous reports,14 both cEPCs and HSCs possessed the ability to convert into a mature EC phenotype, as confirmed by the positive staining for EC surface markers including VEGFR2, von Willebrand factor (vWF), ulex-lectin, and Dil-Ac-LDL uptake, with the plating efficiency of 2.9±1.3% for cEPCs and 2.2±1.7% for HSCs, when old and young bone marrow cells were analyzed together (Figure 1). In addition, comparison of young versus old cEPCs and HSCs revealed that young cells were not superior to their old counterparts in acquiring an EC phenotype (3.3±1.5% versus 2.5±1.3%, P>0.05 for cEPCs, and 1.9±0.8% versus 2.3±2.1%, P>0.05 for HSCs). These findings indicate that aging and hyperlipidemia do not weaken the EC differentiation potential of cEPCs and HSCs.

Because it is controversial regarding whether the CD34+/VEGFR2+ cEPCs represent the authentic EPCs, and there is conflicting evidence for the plasticity of HSCs, in particular in terms of their ability to differentiate into ECs in vivo,13 we chose a more inclusive approach to analyze the bone mar-
Aging Affects the Composition of Simple Little Cells

We have demonstrated that SLCs were markedly decreased with progressive aging, in particular in the presence of hyperlipidemia; next we asked what cells constituted the SLC population. We focused on the young bone marrow, where SLCs were abundant. FACS analysis of the SLC fraction in 3-week-old apoE\(^{-/-}\) mice using lineage cocktails (markers for mature hematopoietic cells) and CD 31 (mature EC marker) showed that most of the cells were lineage low/negative (lin\(^{-/-}\)) and CD31 negative, indicating that the young SLC population is enriched for immature precursors. When the lin\(^{-/-}\)/CD31\(^{-/-}\) fraction was further analyzed for the expression of cKit/Sca-1, surprisingly, most of the cells were cKit and Sca-1 negative (Figure 3A through 3C).

We then determined whether aging would result in exhaustion of selected cell types from the SLC population, in

Aging Affects the Composition of Simple Little Cells

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addition to decreasing the total number of SLCs. Comparison of young (3 weeks) with old (6 months, high-fat, high-cholesterol diet) apoE−/− bone marrow revealed that the percentage of lin− precursors dropped from 58.3±7.4% to 39.6±10.2% (P<0.01), whereas lin+ mature hematopoietic cells increased from 37.5±8.5% to 66.7±3.4% (P<0.01) in the SLC fraction (Figure 3D through 3F). The increase in lin− cells in the SLC population is consistent with an increased

Figure 2. FACS analysis of simple little cells. Simple little cells (SLCs)—cells located in the left lower quadrant of FSC/SSC flow cytometry plot—are abundant in young (3-week-old) apoE−/− bone marrow (A). A marked reduction of SLCs is detected in the marrow of old (6-month old, fed high fat, high cholesterol) apoE−/− mice (B). Similarly, there is a large number of SLCs in young (3-week old) WT bone marrow (C), compared with its old counterpart (2-year-old, D).

Figure 3. Age-dependent selective depletion of precursor cells from the SLC population. FACS analysis of young SLCs reveals that the population (A) is mainly composed of lin− precursors (B), and most of these progenitors cells are cKit and Sca-1 negative (C). In contrast, the old SLC population (D) contains markedly decreased number of lin−/cKit−/Sca-1− cells (E), whereas the relative abundance of CD31+ and lin+/cKit+/Sca-1+ cells remains unaffected by aging (F).
granularity in the bone marrow as a whole (Figures 2B, 2D, and 3D), which may represent increased proportion of neutrophils and decreased fraction of B cells with aging. Instructively, the percentages of CD31+ ECs remained unchanged. Furthermore, the relative abundance of cKit+ Sca-1+ HSCs did not differ between young and old bone marrow within the lin− SLC fraction (Figure 3D through 3F). Thus, lin− cKit−/Sca-1− cells were the subpopulation most affected by aging. These data indicate that aging not only decreases total SLCs, but also selectively depletes immature precursors, in particular the lin− cKit−/Sca-1− subpopulation, within the SLC population.

**Lin− cKit−/Sca-1− SLCs Differentiate Into Mature ECs**

Because lin− cKit−/Sca-1− SLCs represent the cell type that is affected most profoundly by aging and atherosclerosis and both of which are associated with decreased bone marrow efficacy, we investigated whether these cells could adopt a mature EC phenotype under conditions in favor of EC differentiation in vitro. When 10⁴ lin− cKit−/Sca-1− SLCs were plated in 24-well plate, a fraction of the cells were capable of unlimited self-replication, which further formed colonies. When counted two weeks after seeding, the colony formation efficiency (CFE) was 9.6±3.1% for young WT cells. We also cultured the lin− fraction of the bone marrow mononuclear cells depleted of SLCs (WB-SLCs). The CFE for these cells was 1.9±2.1% (P<0.01). Immunofluorescence examination of the SLC progeny revealed that these cells expressed markers for mature ECs, including CD31, VEGFR2, VE-Cadherin, and were capable of AcLDL-Dil uptake (data not shown).

To further confirm that the lin− cKit−/Sca-1− SLC population was indeed enriched for progenitors that were capable of converting to mature ECs, we optimized single-cell culture conditions for these cells, which, in contrast to cEPC and HSC single cell culture, required the presence of OP-9 stromal cells as feeders. Thus, we isolated lin− cKit−/Sca-1− SLCs from 3-week-old EGFP mice and cultured these cells at a single-cell level together with WT OP-9 stromal cells. Indeed, a portion of these cells formed colonies (Figure 4A through 4C). The SLC progeny formed vascular tubes when cultured on matrigel (Figure 4D). Furthermore, progeny of single lin− cKit−/Sca-1− SLCs stained positive for VEGFR2 and CD31 (supplemental Figure II). To confirm the immuno-fluorescence data, we performed TaqMan real-time RT-PCR analysis for CD31 and VEGFR2, which revealed substantially increased CD31 and VEGFR2 mRNA expression in the progeny of single lin− cKit−/Sca-1− SLCs compared with uncultured whole bone marrow cells (supplemental Figure III). Consistent with the CFE data obtained in en masse culture, the plating efficiency for these cells were 7.4±2.3% for lin− cKit−/Sca-1− SLCs, and 2.1±1.8% for WB-SLCs. Collectively, these data indicate that lin− cKit−/Sca-1− SLC fraction, the supply of which is exhausted with aging and atherosclerosis, is enriched for progenitors that are capable of adopting a mature EC phenotype in vitro. It is noteworthy that the plating efficiency for the lin− cKit−/Sca-1− SLCs is much higher than that for cEPCs (2.9±1.3%) and HSCs (2.2±1.7%).

We then examined the EC differentiation efficiency of old lin− cKit−/Sca-1− SLCs by using 2-year-old GFP bone marrow whose number of SLCs was equivalent to 6-month-old apoE−/− mice fed high-fat diet, high-cholesterol diet. The plating efficiency for these cells was 6.3±2.7% for en masse culture and 4.1±2.3% for single cell culture. The difference in plating efficiency between young and old lin− cKit−/Sca-1− SLCs was borderline significant (P=0.051 for en masse and P=0.053 for single cell culture). Although we were unable to use lin− cKit−/Sca-1− SLCs isolated from young and old apoE−/− mice to study the combined effects of aging, apoE deficiency and high-fat diet, high-cholesterol diet...
on the differentiation capability of these cells because of the difficulty to distinguish apoE<sup>−/−</sup> lin<sup>−/−</sup> cKit<sup>−/−</sup>/Sca-1<sup>−/−</sup> SLC progeny from OP-9 feeder cells, the findings indicate that additional work, particularly in vivo experiments, is warranted to fully characterize the impact of aging and atherosclerosis on the function of lin<sup>−/−</sup>cKit<sup>−/−</sup>/Sca-1<sup>−/−</sup> SLCs.

**Discussion**

The identification of EPCs extracted from human peripheral blood in 1997 by Asahara et al<sup>1</sup> inspired substantial efforts to investigate the mechanisms that maintain and restore endothelial integrity and function, the disruption of which represents a critical event in atherogenesis. Clinical studies have shown that traditional risk factors for atherosclerosis are associated with low levels of circulating EPCs,<sup>8,15–17</sup> whereas factors that reduce cardiovascular risk, such as statin therapy or exercise, appear to elevate EPC levels.<sup>9–11</sup> Recently, Werner and colleagues<sup>18</sup> found that higher levels of EPCs were associated with a reduced risk from cardiovascular causes and of the composite end point of major cardiovascular events, after adjustment for traditional risk factors and prognostic variables. Hill et al<sup>18</sup> found that even in healthy subjects, the levels of EPCs were inversely correlated with the combined Framingham risk factor score for atherosclerosis and predicted vascular function better than the Framingham risk score. Numerous animal studies have shown that EPCs participate not only in forming new blood vessels but also in maintaining the integrity and function of vascular endothelium.<sup>1,5,19</sup> We have demonstrated that repeated injection of bone marrow–derived cells into atherosclerosis-prone apoE<sup>−/−</sup> mice reduced the rate of plaque formation without altering serum lipids levels, and that donor EPCs engrafted and differentiated into ECs in the recipient’s blood vessels.<sup>5</sup> These studies have provided insights into the vascular repair mechanisms and basis for the development of new therapeutic approaches involving bone marrow cells.

Because of the lack of understanding of bone marrow biology, most preclinical and clinical studies have been based on introducing either whole bone marrow cells or a crude bone marrow cell population potentially containing EPCs, hematopoietic cells, and irrelevant pluripotent cells, with some animal experiments using either purified conventional EPCs, such as CD133+/CD34+ or CD34+/VEGFR2+ cells, or CD34+ hematopoietic stem cells. The use of whole bone marrow or crude cell preparations risks the introduction of nonessential, and at time noxious, cells into the vessel wall, which may be associated with increased toxicity; for example, injection of monocytic precursors may result in accumulation of inflammatory cells within the vessel wall, further aggravating the inflammatory process associated with atherosclerosis. The isolation and characterization of EPCs have been confounded by the lack of specific endothelial markers on angioblast-like progenitors and assays to distinguish EPCs from mature ECs sloughed from the vessel wall, and from hematopoietic cells.<sup>20</sup> For example, both putative EPCs with angioblastic potential and vessel wall-derived mature ECs may express similar endothelial-specific markers, including VEGFR2, Tie-1, Tie-2, vWF, and VEGFR2, are expressed in both hematopoietic cells and ECs.<sup>20</sup> Furthermore, HSCs and even bone marrow–derived macrophages have been shown to transdifferentiate into endothelial-like cells.<sup>21</sup> Recently, tissue-resident stem cells have been isolated from the heart, which are capable of differentiating into the endothelial lineage.<sup>22</sup> These data support the notion that it will be virtually impossible to identify the “true” EPCs based on the available markers. Conversely, highly purified EPCs may not be better suited for vascular repair, because several cell types (endothelial progenitors and supporting cells) may synergize in endothelialization and vascular healing. Hence, identification of the cell population that is enriched for EPCs and also contains necessary supporting cells is a critical step in enhancing therapeutic efficacy and reducing untoward side effects of cell therapeutic approaches. In the present study, we provide evidence indicating that aging, in particular when it is accelerated by the presence of atherosclerosis risks, results in selective reduction/exhaustion of the supply of SLCs. Furthermore, lin<sup>−/−</sup>cKit<sup>−/−</sup>/Sca-1<sup>−/−</sup> cells constitute the bulk of the SLC population and are affected most profoundly by aging and hyperlipidemia. Remarkably, lin<sup>−/−</sup>cKit<sup>−/−</sup>/Sca-1<sup>−/−</sup> SLCs are more efficient in converting to a mature EC phenotype than other bone marrow fractions, including cEPCs and HSCs. These data indicate that lin<sup>−/−</sup>cKit<sup>−/−</sup>/Sca-1<sup>−/−</sup> SLCs may represent a cell population that is enriched for EPCs.

In addition to endothelial progenitors, which, for the most part, are presumably lineage restricted, two other stem/progenitor cell types—mesenchymal stem cells (MSCs) and multipotent adult progenitor cells (MAPCs)—with multipotent differentiation and extensive proliferation potential have been extensively investigated for vascular repair. MSCs are capable of stimulating angiogenesis and arteriogenesis after acute myocardial infarction.<sup>23</sup> MAPCs copurify with MSCs and, when cultured with VEGF, differentiate into CD34<sup>+</sup>, VE-cadherin<sup>+</sup>, Flk1<sup>+</sup> cells—a phenotype consistent with angioblasts—which subsequently differentiate into cells that express endothelial markers, functioning in vitro as mature endothelial cells and contributing to neoangiogenesis in vivo.<sup>24</sup> Because both cell types are lin<sup>−/−</sup>cKit<sup>−/−</sup>/Sca-1<sup>−/−</sup>, it is conceivable that the lin<sup>−/−</sup>cKit<sup>−/−</sup>/Sca-1<sup>−/−</sup> SLC population is enriched for MSCs and MAPCs. Indeed, the discovery of SLCs may provide an efficient alternative to MSCs and MAPCs, whose isolation requires extended culture and may introduce alterations to the cell phenotype.

In conclusion, the depletion of EPC-enriched lin<sup>−/−</sup>cKit<sup>−/−</sup>/ Sca-1<sup>−/−</sup> SLCs in aging and atherosclerotic mice in combination with observations that decreased circulating EPC levels predict atherosclerosis disease outcome<sup>18</sup> and that the function of EPCs are impaired in high-risk patients<sup>9</sup> suggest that the patients most in need of EPCs may be those who least possess them for autologous transplantation. Hence, approaches to enrich EPCs and/or enhance their function may be necessary to increase efficacy of bone marrow transplantation. Furthermore, lin<sup>−/−</sup>cKit<sup>−/−</sup>/Sca-1<sup>−/−</sup> SLCs may serve as a marker to screen candidate patients, in particular those with atherosclerosis, for their suitability for autologous bone marrow transplantation.
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Disclosures
None.

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METHODS

Animals
ApoE<sup>−/−</sup> C57BL/6J mice and Wild-type (WT) C57BL/6 mice (6–8 weeks of age) were purchased from The Jackson Laboratory (Bar Harbor, Maine). EGFP-expressing C57BL/6 mice were kindly provided by Dr. Nelson J Cao, Division of Cellular Therapy, Duke University Medical Center. Pups were weaned at 3 weeks (wks) of age and fed either regular chow or a Western diet #88137 (Harlan-Teklad; 42% fat, 1.25% cholesterol, Teklad, Madison, WI). Animals were housed in sterile microisolator cages in which they received autoclaved food and autoclaved acidified drinking water in a specific pathogen-free facility throughout the study. Mice were euthanized at the age of 3 wks, 6 months, 1 year or 2 years. At the time of euthanization, bone marrow cells were collected. Studies were performed in accordance with Duke University Institutional Animal Care and Use Committee-approved procedures.

Cell Characterization and Fluorescence Activated Cell Sorting (FACS) Analysis
Bone marrow was isolated from wild type, apoE<sup>−/−</sup>, and EGFP positive mouse tibiae and femora. Single-cell suspensions were obtained in staining medium (HBSS + 3% fetal calf serum, 10 mM Hepes, PH 7.0, and 2 mM EDTA) after ammonium chloride lysis of the red blood cells and straining through a nylon mesh. Bone marrow cells were stained with biotinylated monoclonal antibodies to the following mouse cell surface antigens: CD3 (KT31.1), CD4 (GK1.5), CD5 (53-7.8), CD8 (53-6.7), CD45R/B220 (6B2), erythroid cells (Ter119), Mac-1 (M1/70), and 8C5 (Gr-1) followed by incubation with antibiotin Streptavidin PE-Texas Red. The gating of the lineage negative (lin-) versus lineage positive (lin+) cells was determined using parameter histograms that display the relative fluorescence plotted against the number of events. The line separating lin- from lin+ cells was placed in between the peaks representing the two cell populations. The gating was further aided with the use of negative control (biotinylated IgG isotype control) and positive control (peripheral blood mononuclear cells stained with lineage cocktail). Hematopoietic stem cells (HSCs) were identified using PE–E13-161-7 (anti–Sca-1), allophycocyanin (APC)-2B8 (anti–c-Kit) antibodies in the lin- population. Conventional endothelial progenitor cells (cEPCs) were detected with a PE–conjugated rat anti-mouse VEGFR2 antibody and FITC-conjugated rat anti-mouse CD34 antibody. All rat antibodies were obtained from BD-Pharmingen. Labeled cells were detected with a dual laser FACS Vantage™ (Becton Dickinson) flow cytometry. Dead cells were excluded from analysis using propidium iodide staining. For the identification of lin-/cKit-/Sca-1- SLCs, unfractionated bone marrow mononuclear cells were utilized. SLCs were selected first on the FSC/SSC flow cytometric plot, followed by lineage negative and CD31 negative cells, which were further gated for Sca-1 and c-Kit expression. The
gating of lin- versus lin+ SLCs was performed in the same fashion as described above for the whole bone marrow cells. Flow cytometry data were analyzed using FlowJo software (Treestar Inc.), and two parameter data are presented as 5% probability plots with outliers.

**En Masse Cell Culture**

Lin−/cKit−/Sca-1− SLCs, lin− whole bone marrow fraction minus SLCs (WB-SLCs), CD34+/VEGFR2+ cEPCs and lin-/cKit+/Sca-1+ HSCs were plated on 24-well plate coated with fibronectin at the density of 10⁴/well in 500 µl expansion medium composed of endothelial basal medium-2 (EBM, Clonetics) supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin and mouse cytokines: 50 ng/mL SCF, 50 ng/mL class III receptor tyrosine kinase ligand (Flt3-L), 10 ng/mL IL-3, 100 ng/ml angiopoietin I, 10 ng/ml VEGF and 10 ng/mL IL-6, 10 ng/ml EGF, 10 ng/ml IGF-1 and 10 ng/ml bFGF (Biosource International, Rockville, MD). After two wks, EGF, IL-3 and Flt3-L were withdrawn from the expansion medium to induce EC differentiation. The plating efficiency/colony forming units (CFUs) were counted under a phase-contrast microscope after 14 days of incubation at 37°C in a humidified atmosphere of 5% CO2. The endothelial identity of the cultured cells was determined by fluorescence staining as described below for the expression of EC specific markers.

**Single-Cell Culture**
HSCs and cEPCs: Single Lin\textsuperscript{neg}Sca-1\textsuperscript{pos}c-Kit\textsuperscript{high} HSC or CD34+/VEGFR2+ cEPC derived by two successive sorts was deposited per well in 96-well plates (Corn) using the single cell deposition unit and FACS Diva software on a FACS Vantage SE muti-color high-speed sorter. The wells contained 100 µl of serum-free medium (Xvivo15; BioWhittaker) supplemented with 10% fetal bovine serum, penicillin (100 U/mL), streptomycin (100 µg/mL), 5 x 10\textsuperscript{-5} M β-mercaptoethanol, IL-3 (20 ng/ml), IL-6 (10 ng/ml), SCF (30 ng/ml), Flt3 (100ng/ml), Flt3 ligand (Flt3-L, 30 ng/ml), thrombopoietin (Tpo, 100 ng/ml), Angiopoietin I (100ng/ml), basic fibroblast growth factor (bFGF, 100 ng/ml), epidermal growth factor (EGF, 100 ng/ml), and vascular endothelial growth factor (VEGF10 ng/ml). All factors were purchased from R&D Systems. After two weeks, a colony was formed from a single HSC in the center of well. Cells were then passaged into 8-well chamber slides containing EGM plus medium to induce EC differentiation. After 4 or 6 weeks, cells were identified with VEGF, CD31 staining and Ac-LDL uptake.

SLCs: An OP9 mouse stromal cell (American Type Culture Collection, Manassas, VA) layer was prepared in 96-well microtiter plate in OP9 growth medium consisting of α-modified minimum essential media (α-MEM) supplemented with 20% FBS, 1% PSA and 2-mercaptoethanol (10\textsuperscript{-4} M). A single GFP positive Lin\textsuperscript{-}/cKit\textsuperscript{-}/Sca-1\textsuperscript{-} SLC, or WB-SLC cell, was sorted out and deposed onto each well with pre-cultured OP9 stromal cell layer using Clone Cyt (Becton Dickinson) apparatus of FACS Vantage and subjected to \textit{in vitro} differentiation assay. Recombinant mouse IL-3 (20 ng/ml), SCF (20 ng/ml), IL6
(20 ng/ml) and VEGF (10 ng/ml) were added in the culture medium. For each cell type, at least 192 wells (2x96-well plates) per mouse and from five mice per group were used. The progeny were either stained directly with EC-specific markers as described below to establish their endothelial identity or harvested and stained with APC-conjugated rat anti-mouse CD31 and PE-conjugated rat anti-mouse VEGFR2 antibody. CD31 or VEGFR2 positive cells were sorted out by flow cytometry and analyzed for expression of GFP. GFP+/CD31+/VEGFR2+ cells within the lin~/cKit~/Sca-1 SLCLC fraction were subjected to further culture for induction of capillary-like tube formation and for gene expression study at the RNA level using Real time PCR.

**Capillary-Like Tube Formation on Matrigel**

Matrigel (BD Bioscience, Bedford, MA) basement membrane matrix was added to 96-well culture plates and incubated at 37°C until gelation occurred. Sorted GFP+/CD31+/VEGFR2+ cells (7,000) from cultured lin~/cKit~/Sca-1 SLCs were added to each well in 100 μL EGM-2 media containing 15% FBS, 1% PSA, 10 ng/mL recombinant mouse VEGF and 10 ng/ml mouse IL-6. Twelve hours later, capillary tube formation was assessed via phase contrast microscopy.

**Immunofluorescence Staining**

For the identification of endothelial cells derived from lin~/cKit~/Sca-1 SLCs, WB-SLCs, CD34+/VEGFR2+ cEPCs, or lin-/cKit+/Sca-1+ HSCs, cells cultured either *en masse* or at a single cell level were fixed for 10 minutes in 4% paraformaldehyde in PBS and subjected to double or triple immunostaining for EC-specific markers or Dil-acetylated
low-density lipoprotein (Dil-Ac-LDL) uptake. Briefly, after washing with PBS, slides were blocked for 30 minutes with 2% BSA in PBS, incubated for 1 hour with primary antibodies against CD31 (1:50), VEGFR2 (1:50) or VE-Cadherin (1:50) (R&D Systems). Secondary antibodies and Hoechst 3342 for DNA staining (Molecular Probes) were used at concentrations of 1:250 to 1:500 and incubated for 30 minutes. Between each step, slides were washed with PBS in 0.3% BSA. All procedures were performed at room temperature in dark. For Dil-Ac-LDL uptake, cells were incubated in EGM2 serum-free medium containing 10 μg/ml Dil-Ac-LDL (Molecular Probes) for 4 hours. Cells were then washed and observed by fluorescence microscopy.

**TaqMan Real-Time RT-PCR (TRT-PCR)**

Sorted GFP+/CD31+/VEGFR2+ cells isolated from lin−/cKit−/Sca-1−/SLC/OP9 stromal cell co-culture were further cultured for 5 days. RNA was isolated from GFP+/CD31+/VEGFR2+ Cells using RNeasy Mini kit (Qiagen). Freshly isolated bone marrow mononuclear cells were used as a baseline control. One μg total RNA was used for the synthesis of first strand cDNA using the SUPERSCRIPT Preamplification System (Life Technologies). PCR was optimized for the quantitation of CD31 and VEGFR2 with specific primers and probes (mCD31-forward promer, 5’-gcccaatcaagttctgttt-3’, mCD31-reverse primer, 5’-tgcttcctctgctttgct-3’, mCD31-probe, 5’-6-FAM-cattccacagcggtgttg-TAMRA-3’; mVEGFR2-forward primer, 5’-atgaattgccctggtgagttg-3’, mVEGFR2-reverse primer, 5’- agegtctgcctaatcact-3’, mVEGFR2-probe, 5’-6-FAM-cgctgtaacgctgctta-TAMRA-3’). House-keeping gene 18S ribosomal RNA was used as an internal control. A sequence detector (ABI Prism 7700, PE Applied Biosystems) was
used to measure the amplified product in direct proportion to the increase in fluorescence emission continuously during the PCR amplification. For each sample, a threshold cycle (Ct) value was calculated from each amplification plot, representing the PCR cycle number at which the fluorescence was detectable above an arbitrary threshold. To normalize Ct of the target gene copies to 18S rRNA, ΔCt was calculated as Ct (target) - Ct (18S rRNA). Data presented were derived from ΔCt and expressed as the mean of 5 independent experiments, with each assayed in duplicate.

**Statistical Methods**

All flow cytometry and *en masse* culture data were derived from five mice per group. Single cell culture data were derived from at least 192 cells per mouse and 5 mice per group. TRT-PCR data were derived from GFP+/CD31+/VEGFR2+ cell population generated from 5 single Lin-/cKit-/Sca-1- SLC cells in duplicate and freshly isolated bone marrow mononuclear cells from four mice. Group means were compared using either Students *t* test or ANOVA with Fisher post hoc test as appropriate. Data are presented as mean ± SD with *p*<0.05 accepted as significant.

**SUPPLEMENTAL FIGURES**

**Supplemental Figure I** Decrease in the number of SLCs in relation to aging, apoE deficiency and atherogenic diet. The number of SLCs is determined in the bone marrow of 3-wk-old WT and apoE−/− mice, WT mice fed regular chow (age: 6 months, 1 year and 2 years), apoE−/− mice fed regular chow (age: 6 months and 1 year), and apoE−/−...
mice fed high-fat, high cholesterol diet (age: 6 months). A graded decrease in the number of SLCs is observed. *: p<0.05 compared with 3-wk-old mice of the same genotype; **: p<0.01 compared with 3-wk-old mice of the same genotype.

**Supplemental Figure II** Phenotypic characterization of lin<sup>-</sup>/cKit<sup>-</sup>/Sca-1<sup>-</sup> SLC progeny. The endothelial identity of cells derived from a single GFP<sup>+</sup>/lin<sup>-</sup>/cKit<sup>-</sup>/Sca-1<sup>-</sup> SLC (A and E, green color) is determined by the expression of endothelial surface markers CD31 and VEGFR2 (B and F, red color); C and G represent Hoechst 33342 staining (blue color) for nuclei. D and H are the overlay of the three colors, revealing the expression of CD31 and VEGFR2 in virtually all GFP<sup>+</sup> progeny. The excess nuclei are due to the presence of GFP negative OP-9 stromal cells. Cultured GFP<sup>+</sup> SLCs incubated with rat anti-mouse IgG instead of rat anti-mouse VEGFR2 primary antibody followed by Alexa Fluor 594 donkey anti-rat IgG (red) staining serves as negative control for VEGFR2 (I to L) and CD31.

**Supplemental Figure III** Enhanced CD31 and VEGFR2 expression in GFP<sup>+</sup>/lin<sup>-</sup>/cKit<sup>-</sup>/Sca-1<sup>-</sup> SLC progeny. RNA was prepared from single GFP<sup>+</sup>/lin<sup>-</sup>/cKit<sup>-</sup>/Sca-1<sup>-</sup> SLC derived CD31+ and VEGFR2+ progenies (n=5) and freshly isolated whole bone marrow cells (n=4) and analyzed for CD31 and VEGFR2 expression by Taqman real-time RT-PCR. The results represent fold changes obtained from duplicate experiments and normalized by 18s rRNA expression. A substantial increase in mouse CD31 (A) and VEGFR2 (B) mRNA expression in GFP<sup>+</sup>/lin<sup>-</sup>/cKit<sup>-</sup>/Sca-1<sup>-</sup> SLC progenies is noted as compared with uncultured whole bone marrow cells.