Nonbone Marrow-Derived Circulating Progenitor Cells Contribute to Postnatal Neovascularization Following Tissue Ischemia

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Abstract—Circulating progenitor cells home to sites of postnatal neovascularization and differentiate into endothelial cells but questions remain regarding the source of these cells. Indeed, a recent study suggests that nonbone marrow-derived cells may be even more important than bone marrow-derived cells in the setting of transplant arteriosclerosis. Thus, we aimed to thoroughly investigate the contribution of nonbone marrow-derived progenitor cells for neovascularization. We exclusively identified nonbone marrow-derived progenitor cells by combining a parabiosis model with reverse bone marrow transplantation followed by hindlimb ischemia. In this model, nonbone marrow-derived circulating progenitor cells attributed for 74±13% of the circulating progenitor cells that incorporated into the ischemic hindlimb. Increasing evidence suggests that organs such as small intestine and liver contain a considerable number of tissue resident progenitor cells and, thus, represent putative sources for nonbone marrow-derived progenitors. To track organ-derived progenitors, we transplanted sex-mismatched small intestine or liver, respectively, into rats followed by induction of hindlimb ischemia. These experiments show that organ-derived progenitor cells are contributing to postnatal vasculogenesis (intestine: 4.7±3.7%; liver: 6.3±2.2%). Based on the subsequent observation that liver-derived nonhematopoietic c-kit+/CD45− progenitors are mobilized on induction of hindlimb ischemia, we prospectively isolated and intravenously infused these progenitors from murine livers. The isolated cells demonstrated a marked capacity for enhancing neovascularization and restoring blood flow to the ischemic hindlimb (no cells: 26.4±8.0% of normal blood flow; c-kit+/CD45− cells: 67.0±8.0% of normal flow; P<0.01). In conclusion, we find that nonbone marrow-derived c-kit+/CD45− progenitors contribute to postnatal neovascularization to an extent that is similar to that of bone marrow-derived progenitor cells. Intestine and liver represent a rich source for mobilized tissue-residing progenitor cells. (Circ Res. 2007;100:581-589.)

Key Words: angiogenesis • vasculogenesis • progenitor cells • stem cells • hindlimb ischemia • parabiosis

Neovascularization in the adult was initially thought to result exclusively from the migration and proliferation of preexisting, fully differentiated endothelial cells (a process referred to as angiogenesis). Recent studies, however, demonstrated that circulating progenitor cells home to sites of postnatal neovascularization and differentiate into endothelial cells in situ in a manner consistent with a process termed vasculogenesis. However, there remain questions regarding the source of these progenitor cells. Although it has conclusively been shown that bone marrow-derived endothelial progenitor cells contribute to corneal and limb neovascularization by using a bone marrow transplantation model, this does not rule out other sources. Using a model of transplant atherosclerosis, one group has suggested that recipient-derived endothelial-cell regeneration may be mediated by circulating cells that are not derived from the bone marrow. Accordingly, we investigated the contribution of circulating cells from bone marrow and nonbone marrow sources to the vasculature by using a parabiosis model with or without reverse bone marrow transplantation, together with a murine hindlimb ischemia model of angiogenesis (see Figure 1a). We provide evidence for the mobilization of tissue resident c-kit+/CD45− progenitor cells (TPC) that have the capacity to form CFU-EC, incorporate into the vasculature of ischemic tissue, and subsequently contribute to blood flow recovery. Furthermore, we find that a surprising percentage of TPC appear to be derived from sites outside of the bone marrow. Finally, we demonstrate that liver and small intestine may

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Figure 1. Murine model of parabiosis to assess the contribution of nonbone marrow derived progenitor cells. a, Experimental setup. In the classic parabiosis model, a transgenic mouse is surgically joined with a wild-type animal. This model allows tracking all TPC that incorporate into the area of neovascularization (upper panel). Using the reverse transplantation model we were able to selectively identify TPC that were derived from nonbone marrow sources (lower panel). Abbreviations: LacZ = Tie2 LacZ transgenic FVB mice; LacZ– FVB wild-type mice; BM = bone marrow. b, Incorporation of tissue progenitor cells into nonischemic and ischemic tissue. Capillary density was assessed after 3 weeks of hindlimb ischemia (black bars; scale on the left-hand side) in nonischemic and ischemic tissue. Capillary density is expressed as the ratio of CD31+ capillaries per myocytes relative to nonischemic control limbs. The percentage of vessels that incorporated TPC (white bars; scale on the right-hand side) was calculated as the number of vessels double-staining for CD31 and β-galactosidase relative to the total number of vessels. n = 6 per group; *P < 0.01 compared with nonischemic mice. c–e, Representative images showing incorporated TPC. Cells that crossover from the transgenic animal to the wild-type animal were identified by double-staining (yellow) for CD31 (green) and the reporter gene β-galactosidase (red). Low- and high power confocal microscopic image of the ischemic adductor muscle (c and d). High-power confocal image of a larger vessel containing TPC (e). f, Contribution of nonbone marrow-derived TPC. Nonbone marrow-derived TPC were identified using a reverse transplantation model (hatched bars; see Figure 1a; lower row). Results are related to the findings obtained with the classic model of parabiosis identifying incorporation of circulating Tie2-LacZ tg cells from any source (black bars). n = 6 animals per group. Data are given as mean ± SEM.
each serve as rich sources of TPC. These findings have physiological implications and possible therapeutic applications for ischemic disorders.

Materials and Methods

All animal experiments were approved by the Administrative Panel on Laboratory Animal Care (A-PLAC) at Stanford University School of Medicine, California, or by the Regierungspraesidium Darmstadt, Germany.

Classical Murine Model of Parabiosis

Parabiotic partners share all major histocompatibility antigens and, thus, are free of immunological barriers to cell migration and neovascularization.2 We surgically joined transgenic animals constitutively expressing β-galactosidase (β-gal) under transcriptional regulation of the Tie2 promoter (LacZ⁺; Jackson Laboratory, Bar Harbor, Maine),8 and wild-type animals (LacZ⁻), both of the FVB/N strain (Figure 1a, upper panel). Briefly, mice were joined by a modification of the technique of Bunster and Meyer.9 Matching skin incisions were made from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create about ½ cm of free skin. The olecranon and knee joints were sutured together and the dorsal and ventral skins were approximated by staples.8 Hindlimb ischemia was induced in the wild-type animal 4 weeks after generation of the parabiotic pairs.

Reverse Bone Marrow Transplantation Model

For the modified model of mouse parabiosis (Figure 1a, lower panel), LacZ⁺ mice were transplanted with bone marrow from LacZ⁻ mice (identifying TPC derived from other sources than the bone marrow) before the generation of parabiotic pairs (9.5 Gy; 2 × 10⁶ donor bone marrow cells). Transplantation efficacy was 95.2 ± 1.7% at 6 weeks when parabiotic pairs were generated.

Rat Model of Heterotopic Small Intestine Transplantation

We used a rat model of heterotopic small intestine transplantation as previously described.10 The aorta of the donor organ was connected by end-to-side anastomosis to the aorta of the recipient whereas the portal vein of the donor organ was connected by end-to-side anastomosis to the caval vein of the recipient. The distal part of the donor small intestine and the caecum of the recipient were joined by end-to-side anastomosis whereas the proximal part of the small intestine was ligated.

Rat Model of Orthotopic Liver Transplantation

We used a rat model of physiological liver transplantation as previously described.11 Transplantations were performed either sex-mismatched in Lewis rats or in an allogeneic fashion using DA rats as donors and PVG rats as recipients characterized by spontaneous tolerance.12 For implantation of the donor liver, the portal vein and the caval vein of the donor organ were connected by end-to-end anastomosis to the portal vein and the caval vein of the recipient, respectively, followed by connection of the common biliary duct of the donor organ to the intestine of the recipient (cuff technique; 20G catheter).

Isolation of Murine Liver Progenitor Cells

Dissected livers from C57BL/6/N mice or in some experiments from GFP tg C57BL/6 mice were digested with collagenase (Sigma, St Louis, Mont) for 1 hour under continuous shaking at 37°C. For Fluorescence Activated Cell Sorting (FACS) cells were stained with PE-labeled CD45 antibodies and APC-labeled c-kit antibodies (both ebiosciences). Cells were sorted with a modular MoFlo device using Summit software (Cell Sorting Facility of the Institute of Molecular Immunology at the Helmholtz Center for Environment and Health, Munich, Germany [J.W.E.]). A total of 10⁵ cells was intravenously injected into each NMRI nude mouse (Charles River, Germany) 24 hour after induction of hindlimb ischemia. In addition, single cells were seeded in methylcellulose plates (Methocult GF H3434, Cell-Systems) with 100 ng/mL human recombinant VEGF. On day 7, colony formation was studied by differential interference contrast microscopy.

Hindlimb Ischemia Model

The proximal portion of the right femoral artery including the superficial and the deep branch as well as the distal portion of the saphenous artery were occluded as previously described.13 The overlying skin was closed using surgical staples. After 14 days, relative blood flow was determined by laser Doppler analysis (O2C; Lea Medizin Technik, Giessen, Germany). Calculated perfusion is expressed as the ratio of ischemic to nonischemic hindlimb perfusion.

Tracking of Circulating TEPs

Organ-derived cells in the peripheral blood were characterized by flow cytometry. In the allogeneic rat liver transplantation model, liver-derived progenitors were identified by expression of DA rat specific MHC class I molecules (APC-labeled mouse anti-rat RT1Aa class 1 monoclonal antibody, clone MN4–91 to 6; GeneTex, San Antonio; Tex), lack of CD45 expression (PE-labeled antibody; Pharmingen) and positive staining for c-kit (FITC-labeled antibodies; Santa Cruz Biotech, Santa Cruz, Calif.). In the reverse GFP bone marrow transplantation model, nonbone marrow-derived progenitors were identified by GFP expression, lack of CD45 expression and positive staining for c-kit (both from ebioscience). The endothelial phenotype of the cells was determined by staining for CD45, c-kit, and CD146 (mature endothelial cell marker; all from ebioscience).

Tracking of Incorporated LacZ or GFP Transgenic Cells

LacZ cells were identified by double-staining for β-galactosidase (antibody labeled with Alexa555; Sigma) and CD31 (FITC-labeled antibody; ebioscience). GFP cells were identified by double staining for GFP (FITC-labeled antibody; Invitrogen; Karlsruhe; Germany) and CD31 (APC-labeled antibody; ebioscience). Nuclei were identified by Topro (for LacZ staining) or Dapi (for GFP staining). TPC frequency was defined as the number of vessels containing transgenic endothelial cells divided by the total vessels examined in ten representative sections. All images were analyzed with a Zeiss LSM 510 Confocal Laser Scanning Microscope.

Statistical Analysis

Continuous variables are expressed as means ± standard deviations if not stated otherwise. Overall comparison of the treatment groups was performed with the Kruskal-Wallis test followed by post-hoc pair wise comparison using the Mann-Whitney test. All analyses were performed with SPSS 11.5 (SPSS Inc, IL). Probability values < 0.05 were considered statistically significant.

Results

Incorporation of TPC From Any Source Into Nonischemic and Ischemic Tissue, Respectively

In nonischemic skeletal muscles (fore limb) of LacZ⁺ animals of the parabiotic pairs, TPC incorporation was a very rare finding. Only 0.14 ± 0.15% of the vessels double-stained for β-gal and CD31, respectively, including 3 animals (out of 7 analyzed animals) without any evidence for incorporated
TPC (Figure 1b). Three weeks after induction of hindlimb ischemia in wild-type animals, capillary density increased to 190 ± 27% of nonischemic tissue (*P* < 0.01). Simultaneously, incorporation of TPC in the vasculature increased to 1.5 ± 1.1% of the capillaries in the ischemic skeletal muscle (n = 6; *P* < 0.01 as compared with nonischemic limbs) (Figure 1b). Representative low- and high-power images of incorporated TPC are depicted in Figure 1c and d. Incorporation of TPC was not restricted to capillaries as they were also found incorporating into the endothelium of larger vessels (Figure 1e).

**Two Distinct Sources of TPC**

In the above described parabiosis model, TPC derived from any circulating cells are tracked. To determine whether the identified TPC also included cells derived from other sources than the bone marrow, we used a parabiosis model combined with reverse bone marrow transplantation in the transgenic animal (Figure 1a, lower panel). In this model, cells that colocalize for β-gal and CD31 must have been mobilized from nonbone marrow sources in the transgenic LacZ- animal. Results were related to incorporation data derived from parabiotic pairs that were created from LacZ- mice that were anastomosed to LacZ+ mice transplanted with LacZ+ bone marrow (LacZ+/LacZ+ group). In these parabiotic pairs, the percentage of vessels containing TPC reached 74 ± 13% of what we observed in the parabiosis model using LacZ+/LacZ- mice (Figure 1f) indicating that nonbone marrow derived cells significantly contributed to neovascularization.

**Mobilization of Nonbone Marrow-Derived Progenitor Cells**

To demonstrate mobilization and subsequent circulation of TPC in response to hind limb ischemia before their incorporation into the vasculature, we used GFP transgenic mice that were reversely transplanted with wild type bone marrow cells. This model allows us to track and characterize nonbone marrow derived cells in the peripheral circulation. Flow cytometry revealed that indeed nonbone marrow derived GFP+ c-kit+/CD45- progenitor cells are mobilized into the peripheral blood following induction of hind limb ischemia. Importantly, these nonbone marrow-derived GFP c-kit+/CD45- cells accounted for ~60% of the mobilized cells (Figure 2). Consistent with the results from the parabiosis model, these data indicate that a significant proportion of the cells must actually have been mobilized from nonbone marrow sources.

**The Liver as a Potential Source for Circulating TPC**

We then aimed to more specifically define putative sources of nonbone marrow-derived TPC. Increasing evidence suggests that organs such as liver15 and small intestine16 contain a considerable number of tissue-residing progenitor cells. Therefore, we investigated the specific mobilization of these organ-derived TPC and determined their original nature before vascular incorporation and subsequent differentiation into mature endothelial cells using a rat model of allogeneic transplantation (liver from DA rat transplanted into PVG rats). We collected serial blood samples on day 3, 5, and 7 following induction of hind limb ischemia. Analysis of the
blood samples by flow cytometry revealed that liver derived c-kit<sup>CD45<sup>−</sup> were mobilized from the donor liver with a maximum on day 5 after induction of limb ischemia (Figure 3a). On average, 0.26±0.05% of the gated cells in the peripheral blood were liver-derived progenitor cells (day 5 post induction of ischemia; n=3).

Prospective Isolation of Identified TPC From Murine Livers

To directly demonstrate the neovascularization capacity of the mobilized progenitor cells, we prospectively isolated murine liver-derived progenitors. Based on our above results, we decided to use c-kit and CD45 for identification of hematopoietic as well as nonhematopoietic cells. We did not use sca-1 as a potential alternative marker as we observed that sca-1<sup>−</sup>CD45<sup>−</sup> contain a marked subpopulation of sca-1 positive mature endothelial cells (Figure 3b). Therefore, we isolated liver-derived c-kit<sup>CD45<sup>−</sup> and, in addition, hematopoietic c-kit<sup>CD45<sup>−</sup> progenitors from C57BL6 wild-type mice and in a second experiment also from GFP transgenic C57BL6 mice to allow tracking of the cell. We then injected the cells intravenously into nude mice 24 hours after induction of hind limb ischemia. Laser Doppler analysis on day 14 demonstrated that c-kit<sup>CD45<sup>−</sup> cells are capable of strongly enhancing neovascularization in ischemic tissue (Figure 3c, left panel and upper right panel). Intriguingly, the neovascularization capacity of c-kit<sup>CD45<sup>−</sup> was significantly greater as compared with c-kit<sup>CD45<sup>−</sup> cells. Moreover, histological evaluation of the ischemic adductor muscles illustrated that isolated GFP<sup>−</sup> c-kit<sup>−</sup>CD45<sup>−</sup> cells actively contributed to postnatal vasculogenesis by incorporating into vascular structures (Figure 3c, right panel). Histological analysis of liver sections revealed the localization of TPCs in perivascular niches (Figure 3d). Isolation of c-kit<sup>CD45<sup>−</sup> and c-kit<sup>CD45<sup>−</sup> single-cells by FACS, respectively, and their subsequent culturing in a CFU assay demonstrated that only c-kit<sup>−</sup>CD45<sup>−</sup> have the capacity to form colonies. Intriguingly, 78% of the single cells formed colonies as illustrated in Figure 3e. This data indicate that the mobilized cells are indeed not mature endothelial cells but rather are progenitor cells.

General Contribution of Organ-Derived TPC to Postnatal Vasculogenesis

Finally, to investigate whether TPC from other organs may also contribute to postnatal vasculogenesis, we used a sex-mismatched rat model of liver or small intestine transplantation to directly demonstrate the mobilization of tissue residing progenitor cells into the ischemic tissue. Immunostaining for the endothelial marker vWF in combination with FISH using a rat Y-chromosomal probe identified incorporated male cells in the limbs of the female rats. In nontransplanted male rats, which served as positive controls, a signal indicating presence of a Y-chromosome could be detected in 75.3±8.8% of vWF-positive cells (Figure 4a and d). In nontransplanted female rats, which served as negative controls, 0.5±0.7% of the vWF positive cells showed background signals. Therefore, the threshold level for the reliable detection of truly Y-chromosome containing cells was conservatively set at 2.6% (corresponding to three standard deviations beyond the mean of the false-positive value). In all nonischemic limbs of female rats that were transplanted with small intestine from a male donor, the number of vWF positive cells also staining for a Y-chromosome was below the threshold level (Figure 4d). In ischemic limbs of 9 of the 10 female rats that were transplanted with either small intestine (n=5) or liver (n=5) from a male donor, the number of vWF-positive cells costaining for the Y-chromosome was above the threshold level. This observation indicated that at least a subset of TPC were mobilized from the male transplanted livers (Figure 4b) and small intestine (Figure 4c).

Discussion

The salient findings of our work are: 1) incorporation of mobilized circulating progenitor cells into the vasculature is rare in nonischemic tissue; 2) incorporation of TPC is more common in the conduit vessels and capillaries of the ischemic limb; 3) a large proportion of circulating progenitor cells are derived from a source other than the bone marrow; 4) in a rat model of intestine and liver transplantation, respectively, we identified these organs as putative, but not necessarily exclusive, sources for circulating TPC; and 5) systemic infusion of progenitor cells derived from a perivascular niche in the liver incorporated into vascular structures and subsequently enhanced neovascularization with improved blood flow recovery in ischemic hindlimbs.

Although the existence and potential of bone marrow-derived progenitor cells has been shown, the actual contribution and biological significance of such cells in physiological and pathological repair processes remains to be definitively established. To demonstrate incorporation of circulating cells we used a model of mouse parabiosis, in which cross-circulation is established between 2 individuals. This is an ideal assay for tracking the migration of any circulating cells with endothelial potential to sites of neovascularization in vivo.® Cells arising from 1 partner can be differentiated from the other by virtue of the presence of a reporter transgene such as LacZ. Our goal was to eliminate biases inherent in models that require pre-selection of a given type or source of the cells, and to avoid manipulations (such as total body irradiation) required to overcome immunological or physiological barriers between the putative precursor cells and the experimental hosts.

In the absence of ischemia, we observed a very low but consistent incorporation of TPC. Three weeks after induction of ischemia, however, the percentage of capillaries incorporating TPC increased to 1.5±1.1%. If one takes into account the fact that only half of the TPC are detectable by our approach, TPC contributed to ~3% of the capillary endothelium (Figure 1b). Our data may appear to be in contrast to a very recent report where, in a parabiosis model of myocardial infarction, no evidence for a contribution of circulating cells to neovascularization or cardiac regeneration was found.® Apart from the obvious difference between our study and the study by Balsam and coworkers as we used a model of hind limb ischemia, we also used a different mouse strain and the reporter gene LacZ driven by the Tie2 promoter/enhancer. Indeed, a recent study by Duda demonstrated that the contribution of bone marrow-derived cells to postnatal vasculogen-
Figure 3. Identification and characterization of liver-derived tissue progenitor cells. a, An allogenic, but tolerant model of orthotopic liver transplantation was used to track liver-derived tissue progenitor cells in the circulating blood. Blood samples were drawn on day 3, 5, and 7 and subsequently analyzed for the expression of DA rat MHC class I, CD45, and c-kit with DA rat MHC class I c-kit’ CD45’ cells classified as liver-derived progenitor cells. b, Expression of the mature endothelial marker CD146 on liver-derived progenitor cells identified by staining for c-kit and sca-1, respectively. c, Murine liver-derived c-kit’ CD45’ and c-kit’ CD45’ cells isolated by FACS were infused into NMRI nu/nu mice 24 hours after induction of hindlimb ischemia. Laser Doppler analysis on day 14 is depicted. *P<0.01 vs no cells; **P<0.05 versus c-kit’ CD45’. n=5 animals per group. Vascular incorporation of infused GFP’ c-kit’ CD45’ cells in the ischemic adductor muscles was determined by double staining for GFP (green) and CD31 (red). Nuclei were stained by Dapi (blue). Representative images are shown. d, The localization of liver resident progenitor cells was identified in cryosections from murine livers. Representative images are shown. e, Colony forming capacity of single cells isolated by FACS. Cells were cultured for 7 days in methylcellulose.
esis varies considerably with organ site and mouse strain. Moreover, in the studies by Balsam and coworkers, parabiosis was created simultaneously with the induction of myocardial infarction whereas in our model, parabiosis was created 4 weeks before induction of ischemia. Because it takes 10 to 14 days for the establishment of the cross circulation between the conjoined mice, it is possible that Balsam and coworkers had not achieved full chimerism at the time that they induced ischemia in their model.

Interestingly, 2 recent studies also generated conflicting data on the incorporation of bone marrow-derived cells in tumor vessels despite using similar tumor models. Garcia-Barros and colleagues offered compelling evidence for the critical role of bone marrow-derived cells in solid tumor growth in nonmutant mice. Bone marrow cells transplanted from Rosa-26 mice, which ubiquitously express β-galactosidase, formed about half of the tumor-vascular endothelium. The work of De Palma and colleagues also attests to the critical role played by bone marrow-derived cells in the neovascularization and growth of mouse tumor xenografts, although they found very few if any GFP-positive cells incorporating into the tumor endothelium. The latter study provided evidence for a new subset of bone marrow-derived cells that are involved in postnatal neovascularization. Expression of GFP under the Tie2 promoter/enhancer revealed that bone marrow contribution to the tumor vessels relied on Tie2-expressing mononuclear cells (TEMs). These TEMs home specifically to angiogenic sites and contribute indirectly to neovascularization. The selective elimination of the TEM cells by delivery of a suicide gene resulted in substantial inhibition of angiogenesis and slower tumor growth. It is possible that such cells may represent a subset of our TPC.

It is difficult to distinguish bone marrow-derived endothelial cells from nonbone marrow-derived endothelial cells, as both cell types display nearly the same set of phenotypic markers. Thus, the relative contribution of bone marrow versus nonbone marrow-derived endothelial cells in tissue neovascularization has remained undefined. By combining reverse bone marrow transplantations with the parabiosis model, we were able to discriminate between bone marrow-derived circulating cells and circulating cells from other sources (Figure 1a, lower panel). In this model, we were able to demonstrate that the majority (74 ± 13%) of the TPC incorporating into the endothelium of the ischemic tissue were derived from a source other than the bone marrow (Figure 1f). To provide even stronger evidence for the contribution of nonbone marrow-derived TPC to neovascularization, we used a rat model of liver and intestine transplantation, respectively. After transplantation of male donor organs into female recipients, male cells detected in the vasculature should be derived from the transplanted organ.

Figure 4. Rat model of sex-mismatched liver and small intestine transplantation, respectively. a, Fluorescent in situ hybridization analysis. Incorporated male cells were detected by immunostaining for the endothelial marker vWF (FITC; green) in combination with FISH using a Y-chromosomal probe (Spectrum Orange; red). Nuclei were counterstained with DAPI (blue). Adductor muscles from nontransplanted male rats serving as positive controls show red signals indicating presence of the Y-chromosome in the majority of the nuclei. b and c, Transplantation of liver and small intestine. Incorporation of cells derived from the male liver (b) or the small intestine (c) were analyzed three weeks after induction of hindlimb ischemia. Representative images are shown. d, Quantification. The data are depicted as the percentage of vWF positive cells containing a signal indicating the presence of a Y-chromosome relative to the total number of evaluated vWF positive cells. The threshold level for the detection of the Y-chromosome containing cells within the population of vWF positive cells was 2.6% based on the rate of false-positive cells in nontransplanted female rats. *P < 0.05 vs nontransplanted female rats. At least 5 transplanted female rats were analyzed.
However, as a potential limitation of this animal model, it is conceivable that not all of the circulating bone marrow-derived stem and progenitor cells were removed before transplantation. Because bone marrow-derived stem and progenitor cells represent an extremely rare population in the circulating blood during physiological conditions (eg, no remote tissue ischemia), it is very unlikely that these cells have accounted for the observed robust mobilization and subsequent incorporation of the organ-derived cells following hindlimb ischemia in our studies. Indeed, combined immunophenotyping and FISH analysis detected a significant number of vWF-positive cells in the ischemic hindlimb that were derived from the transplanted organ. By contrast, TPC derived from the transplanted organ were not found in the nonischemic limbs. (Figure 4a–d). These results indicate that a significant proportion of TPC are derived from nonbone marrow sources, and that liver and intestine may represent putative sources for these cells.

What is the nature of the cells mobilized from these organs? One possible hypothesis is that circulating mature endothelial cells are shed from the vessel wall and home to areas of neovascularization. Mature endothelial cells would be relatively radiation-resistant, and would survive the irradiation used in this reversed bone marrow transplantation model. Circulating vessel wall-derived endothelial cells have been identified in various physiological and pathological conditions. Although the regenerative capacity of vessel wall-derived endothelial cells is considered to be lower than that of progenitor cells, these cells may still home to sites of neovascularization. However, because the capacity of mature endothelial cells to contribute to postnatal neovascularization is limited, an alternative explanation for our observations is that TPC arise from tissue progenitor cells in organs such as skin, spleen, liver, and, intestine. Indeed, our data demonstrate that c-kit+CD45– tissue-residing progenitors can be detected in their vascular niche of the liver and, following induction of hindlimb ischemia, are mobilized into the circulating blood stream and subsequently incorporated into the vasculature of the ischemic tissue. Many terminally differentiated tissues have been shown to harbor a population of cells with progenitor cell-like activity, which also maintain site-specific functions. In the liver, a potential stem cell compartment located within the intraportal biliary tree can give rise to cords of so-called oval cells within the lobules. Accordingly, we isolated c-kit+CD45– progenitor cells from the murine liver and determined their neovascularization capacity in vivo. Other markers such as sca-1 have previously been used to isolate tissue resident progenitors. However, it was previously shown that sca-1 is also expressed on murine liver endothelial cells from the hepatic sinusoid and we consistently found a higher number of sca-1+CD45+ cells that coexpressed the mature endothelial marker CD146 whereas c-kit+CD45– progenitor were only moderately contaminated by mature endothelial cells. Furthermore, c-kit+CD45+ progenitors show strong colony forming capacity. Subsequent systemic infusion of isolated c-kit+CD45– progenitor cells into a model of hindlimb ischemia demonstrated that the neovascularization capacity of these cells was even greater than that of c-kit+CD45+ cells. Most likely, c-kit+CD45– cells still possess a progenitor phenotype with a marked capacity to undergo further vascular differentiation. Of note, in the present study, we cannot exclude that TPC mobilized from organs such as liver and intestine and, thus, classified as nonbone marrow-derived TPC did originally reside in the bone marrow. However, as a potential limitation of this animal model, it is very unlikely that these cells have accounted for the observed robust mobilization and subsequent incorporation of the organ-derived cells following hindlimb ischemia in our studies. Indeed, combined immunophenotyping and FISH analysis detected a significant number of vWF-positive cells in the ischemic hindlimb that were derived from the transplanted organ. By contrast, TPC derived from the transplanted organ were not found in the nonischemic limbs. (Figure 4a–d). These results indicate that a significant proportion of TPC are derived from nonbone marrow sources, and that liver and intestine may represent putative sources for these cells. In conclusion, we find that a surprising percentage of circulating progenitor cells bearing the capacity to enhance postnatal neovascularization is derived from sites outside of the bone marrow. We provide evidence that liver and small intestine may each serve as sources of TPC. Our data support the notion that these circulating cells have diverse origin and differentiation states.

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Disclosures

None.

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Detailed Material and Methods

Classical murine model of parabiosis. Parabiotic mouse pairs were created to investigate the mobilization and incorporation of progenitors to vessel formation in normal and ischemic conditions using a hindlimb ischemia model of neovascularization (Fig. 1a; upper panel). Parabiotic partners share all major histocompatibility antigens and, thus, are free of immunological barriers to cell migration and neovascularization. Weissman and colleagues have previously shown that parabiosis of syngenic males to females does not result in a detectable anti H-Y immune response. Unambiguous cell tracking between the mice was possible by assaying for genetic markers unique to one animal in the pair. We surgically joined transgenic animals constitutively expressing β-galactosidase (β-gal) under transcriptional regulation of the Tie2 promoter (LacZ⁺; Jackson Laboratory, Bar Harbor, Maine), and wild type animals (LacZ⁻), both of the FVB/N strain. The Tie2 receptor is expressed in endothelial lineage cells that participate in neovascularization. Briefly, mice were anesthetized to full muscle relaxation with xylazine and ketamine HCl (1.67 mg per 10 g of body weight) by intraperitoneal injection and joined by a modification of the technique of Bunster and Meyer. After shaving the corresponding lateral aspects of each mouse, matching skin incisions were made from the olecranon to the knee joint of each mouse, and the subcutaneous fascia was bluntly dissected to create about ½ cm of free skin. The olecranon and knee joints were attached by a single 2-0 silk suture and tie, and the dorsal and ventral skins were approximated by staples. Cross-circulation in these animals was confirmed by tracking the flow of Evans blue dye from mouse A to mouse B following intravenous injection. Consecutive peripheral blood chimerism of parabiotic mice was determined in preliminary tests using CD45 allotype analysis. Following the surgical procedure, blood was drawn after an increasing time interval from the parabiotic partners that differ in their CD45 locus (CD45.1 and CD45.2; Jackson Laboratory). The samples were stained for 30 min at 4°C with anti-CD45.1-FITC and anti-CD45.2-PE (both ebiosciences, San Diego, CA), respectively, lysed with hypotonic FACS lysing solution (BD biosciences) for 5 min, and washed twice with PBS prior to analysis on a FACSCalibur (BD biosciences). For the final
experiments, hindlimb ischemia was induced in the wild type animal 4 weeks after generation of the parabiotic pairs.

**Reverse bone marrow transplantation model.** To investigate the source of TPC, we conducted experiments with a modified model of mouse parabiosis (Fig. 1a; lower panel). In those experiments, LacZ+ mice were transplanted with bone marrow from LacZ– mice (identifying TPC derived from other sources than the bone marrow) prior to the generation of parabiotic pairs. For this purpose, recipient mice were lethally irradiated with 9.5 Gy and received intravenous injection of $2 \times 10^6$ donor bone marrow cells each. To determine the transplant efficiency of our protocol, we transplanted bone marrow from CD45.1 mice into CD45.2 mice revealing a transplantation efficacy of $95.2\pm1.7\%$ after 6 weeks. Therefore, for the final experiments, transplanted LacZ+ animals were used 6 weeks following the reverse bone marrow transplantation to generate the respective parabiotic pairs.

**Rat model of heterotopic small intestine transplantation.** We used a rat model of heterotopic small intestine transplantation as previously described. To harvest the donor small intestine, the colon arteries, the portal vein and the pylorus vein were ligated followed by ligation of the caval vein. After ligation of the gastric and splenic veins, the pancreas was separated from the small intestine. Then, the aorta was dissected free below the mesenteric artery and ligated. Then the small intestine was separated from the caecum, the aorta was perfused with cold sodium chloride solution to remove all blood cells from the donor organ, and the small intestine was washed with cold sodium chloride solution. The donor small intestine was transferred to the recipient rat and the aorta of the donor organ was connected by end-to-side anastomosis to the aorta of the recipient whereas the portal vein of the donor organ was connected by end-to-side anastomosis to the caval vein of the recipient. When appropriate perfusion of the transplanted organ was established, the distal part of the donor small intestine and the caecum of the recipient were joined by end-to-side anastomosis whereas the proximal part of the small intestine was ligated. Hindlimb ischemia was induced three weeks after organ transplantation.

**Rat model of orthotopic liver transplantation.** We used a rat model of physiological liver transplantation as previously described. Transplantations were performed either sex-mismatched in
Lewis rats or in an allogeneic fashion using DA rats as donors and PVG rats as recipients (both from Harlan-Winkelmann, Borchen, Germany). The latter model is characterized by spontaneous tolerance. To harvest the donor liver, the caval vein was sub-diaphragmatically ligated and the gastric vein including side branches were also ligated. Then the portal vein was dissected free and the hepatic artery was ligated. After ligation of the distal caval vein (above the suprarenal vein) and the portal vein, the common biliary duct was prepared using the Cuff technique and the liver was perfused using cold sodium chloride solution. In a similar procedure, the liver of the recipient rat was removed and discarded. For implantation of the donor liver, the portal vein and the caval vein of the donor organ were connected by end-to-end anastomosis to the portal vein and the caval vein of the recipient, respectively, followed by connection of the common biliary duct of the donor organ to the intestine of the recipient (Cuff technique; 20G catheter). Hindlimb ischemia was induced one to two weeks after organ transplantation.

Isolation of murine liver progenitor cells. Murine livers from C57BL6/N mice were dissected, liver cell suspensions prepared by mechanical dissociation. For Fluorescence Activated Cell Sorting (FACS), murine livers from C57BL6/N mice or in some experiments from GFP tg C57BL6/J mice were incubated with Collagenase Type IV (Sigma, St. Louis, Montana) for one hour under continuous shaking at 37°C. Then, cells were stained with PE labeled CD45 antibodies and APC-labeled c-kit antibodies (both ebiosciences) and sorted by using a modular MoFlo device and Summit software (Cell Sorting Facility of the Institute of Molecular Immunology at the Helmholtz Center for Environment and Health, Munich, Germany [J.W.E.]). A total of 10⁵ cells was intravenously injected into each NMRI nude mouse (Charles River, Germany) 24 h after induction of hindlimb ischemia. In addition, single cells were seeded in methylcellulose plates (Methocult GF H3434, CellSystems) with 100 ng/ml human recombinant VEGF. On day 7, colony formation was studied by differential interference contrast microscopy.

Hindlimb ischemia model. The proximal portion of the right femoral artery including the superficial and the deep branch as well as the distal portion of the saphenous artery were occluded. The overlying skin was closed using surgical staples. After 14 days, relative blood flow was determined by laser Doppler analysis (O2C; Lea Medizin Technik, Giessen, Germany). Prior to analysis, animals were
placed on a heating pad at 37°C to minimize variations in temperature. Calculated perfusion is expressed as the ratio of ischemic to non-ischemic hindlimb perfusion.

**Tracking of mobilized progenitor cells in mice.** In order to more precisely define the phenotype of mobilized tissue resident progenitor cells, we characterized organ-derived cells in the peripheral blood by flow cytometry. In the allogeneic rat liver transplantation model, liver-derived progenitors were identified by expression of DA rat specific MHC class I molecules (APC-labeled mouse anti-rat RT1Aa Class 1 monoclonal antibody, Clone MN4-91-6; GeneTex; San Antonio; Texas), lack of CD45 expression (PE-labeled antibody; Pharmingen) and positive staining for c-kit (FITC-labeled antibodies; Santa Cruz Biotech, Santa Cruz, California). In the reverse GFP bone marrow transplantation model, non-bone marrow-derived progenitors were identified by GFP expression, lack of CD45 expression and positive staining for c-kit (both from ebioscience). The endothelial phenotype of the cells was determined by staining for CD45, c-kit, and CD146 (matue endothelial cells marker; all from ebioscience).

**Tracking of incorporated LacZ or GFP transgenic cells in mice.** For the identification of LacZ cells, sections were permeabilized in 0.3% Triton X-100 (Roche) and double-stained with anti-β-galactosidase monoclonal antibodies (labeled with Alexa555; Sigma) and antibodies against the endothelium-associated antigens CD31 (FITC-labeled antibodies; ebiosciences). GFP cells were identified by double staining for GFP (FITC-labeled antibody; Invitrogen; Karlsruhe; Germany) and CD31 (APC-labeled antibody; ebioscience). Nuclei were identified by Topro (for LacZ staining) or Dapi (for GFP staining). TPC frequency was defined as the number of vessels containing transgenic endothelial cells divided by the total vessels examined in representative sections. Non-ischemic skeletal muscles from wild type animals served as negative controls and non-ischemic tissue from transgenic animals served as positive controls. All images were generated on a Zeiss LSM 510 Confocal Laser Scanning Microscope.

**Tracking of incorporated male cells.** To identify cells that were derived from the transplanted male organs, we applied combined immunostaining for the endothelial marker vWF labeled with FITC (Acris;
Hiddenhausen; Germany) and interphase FISH using the rat Y-chromosome probe 9.1ES8 (a generous gift of Barbara Hoebee; Laboratory of Toxicology, Pathology, and Genetics, National Institute of Public Health and the Environment, Bilthoven, The Netherlands) according to a slightly modified FICTION protocol published by Martin-Subero and colleagues 9. Briefly, for FICTION, the cryosections were thawed and dried for 30 minutes at room temperature, fixed in acetone for 10 minutes, and air-dried. Slides were incubated for 30 minutes at room temperature with the FITC-labeled vWF antibody (1:100) diluted in PNM buffer (5% no-fat milk/PN buffer [0.1 M NaPO4 buffer pH 8.0]). After immunophenotyping, slides were fixed in Carnoy’s fixative (ethanol:acetic acid, 3:1) for 10 minutes and in paraformaldehyde solution (1%) for 1 minute. Then, slides were dehydrated through increasing ethanol concentrations and air-dried. DNA extraction, labeling with Spectrum Orange (Vysis, Downers Grove; Illinois), and preparation of the Y-chromosomal FISH probe were performed as previously described 9. On each section, 1.5 µL of the hybridization solution was applied and both probe and target DNA were simultaneously denatured at 70°C for 12 minutes and incubated for two days at 37°C. After hybridization, slides were washed in 0.1 x standard saline citrate at 60°C followed by a wash step with PNM buffer. Finally, nuclei were stained with DAPI (4’,6-diamidino-2-phenylindole, Roche Diagnostics, Mannheim, Germany) and the slides were mounted in anti-fade solution for analysis. Slides were evaluated using a Zeiss Axioskop 2 microscope equipped with appropriate filter sets and documented using the ISIS software package (MetaSystems, Altluhsheim, Germany). In female rats transplanted with either liver or small intestine from a male donor, at least 200 vWF-positive cells per section and at least three slides per animal were analyzed.

References
