Molecular pathways regulating mobilization of marrow-derived stem cells for tissue revascularization

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Adult bone marrow is a rich reservoir of hematopoietic and vascular stem and progenitor cells. Mobilization and recruitment of these cells is essential for tissue revascularization. Physiological stress, secondary to tissue injury or tumor growth, results in the release of angiogenic factors, including vascular endothelial growth factor (VEGF), which promotes mobilization of stem cells to the circulation, contributing to the formation of functional vasculature. VEGF interacts with its receptors, VEGFR2 and VEGFR1, expressed on endothelial and hematopoietic stem cells, and thereby promotes recruitment of these cells to neo-angiogenic sites, accelerating the revascularization process. The mobilization of stem cells from marrow is a dynamic process, regulated by shear stress imparted by blood flow, and the activation of metalloproteinases that induce the release of ‘Kit ligand’. Identification of the molecular pathways that support the proliferation and differentiation of vascular stem and progenitor cells will open up new avenues for the design of clinical trials to accelerate tissue vascularization and organogenesis.

Bone-marrow-derived stem cells play a major role in the regulation of several postnatal processes, including wound healing, organ regeneration and tumor growth. Tissue-specific stem cells reside in specific niches within the bone-marrow microenvironment, where they are maintained in an undifferentiated and quiescent state [1]. These niches are crucial for regulating self-renewal and cell fate decisions, as well as providing a dispensable source of stem cells for tissue vascularization and organogenesis [2,3].

During embryonic development, hematopoietic stem cells (HSCs) migrate from the yolk sac to the fetal liver and spleen, and then settle within the bone marrow. Like all other hematopoietic cells, HSCs are motile and, hence, are able to leave the marrow microenvironment in a multistage process known as ‘mobilization’. Physiological stress induces the release of angiogenic factors with chemotactic potential, which promote stem-cell motility. This facilitates the entry of stem cells into a permissive niche where they proliferate, differentiate and are mobilized into the circulation [4–7]. Mobilized stem cells are incorporated either into a specific organ or, as is the case with HSCs, expand in extramedullary sites and recirculate back to the marrow to replenish the stem-cell pool [8].

Bone-marrow-derived circulating endothelial progenitor cells (CEPs) are also recruited from the marrow and contribute to neo-angiogenesis during wound healing [9–12], vascularization post-myocardial ischemia [13–16], limb ischemia [17–19], endothelialization of vascular grafts [20,21], atherosclerosis [22] and retinal neovascularization [23,24]. In addition, the growth of certain tumors is dependent upon the recruitment of CEPs and subsets of hematopoietic cells to the tumor vasculature. The mobilization of CEPs and hematopoietic cells for tumor angiogenesis and growth is induced by the release of angiogenic factors from tumor cells [10,25–30], and inhibition of this mobilization results in retardation of tumor growth. This highlights the importance of bone-marrow-derived cells in supporting tumor angiogenesis.

Collectively, these data suggest that selective expression of organ-specific chemokines promotes the mobilization of bone-marrow-derived pluripotent cells, a process that is essential for tissue vascularization and organ regeneration. Recently, several studies have begun to elucidate the mechanisms by which stem cells are mobilized from bone marrow to particular organs, and the molecular mediators that orchestrate this process. The release of angiogenic factors and hematopoietic cytokines seems to have a major role in the mobilization of endothelial progenitors and subsets of hematopoietic cells [4,31–33]. Shear stress might also activate molecular pathways that increase the response to chemokines and enhance mobilization of HSCs into the circulation [34–36].

These studies have laid the foundations for the design of murine models in which the mechanisms of organ-specific-stem-cell mobilization can be studied. The identification of molecular pathways that are essential in this process, and in the proliferation and differentiation of vascular stem cells, will open up new avenues for accelerating neo-angiogenesis after vascular injury or ischemia.

Angiogenesis and hematopoiesis are regulated by lineage-specific and stem-cell-active chemokotkines.
Both hematopoiesis and angiogenesis are dependent upon the recruitment of quiescent stem and progenitor cells to support the large demand for cell proliferation. The release and bio-availability of specific cytokines is the key event that facilitates this recruitment.

The survival and proliferation of hematopoietic progenitor cells is regulated by the expression of lineage-specific cytokines. For example, granulocyte-macrophage colony-stimulating factor (GM-CSF) and granulocyte-CSF (G-CSF) sustain and induce the proliferation of myeloid lineages, and thrombopoietin promotes megakaryocyteopoiesis and thrombopoiesis. Erythropoietin primarily affects expansion and differentiation of the erythroid lineage. Stem-cell-active cytokines, such as ‘Kit ligand’ (KitL; also known as stem-cell factor), support the survival of subsets of stem cells. However, the identity of cytokines that promote the self-renewal of hematopoietic stem cells is not known. Virtually all of the known hematopoietic cytokines, including GM-CSF, G-CSF and KitL, can modulate adhesive properties and promote chemotaxis and chemokinesis of their target cells. Mechanical factors, such as shear stress, might also promote adhesion to the bone-marrow stroma [36].

Among the known angiogenic factors, vascular endothelial growth factor (VEGF) is the most potent and specific of the growth factors that regulate angiogenesis. Revascularization of injured tissue requires angiogenesis – rapid proliferation, migration and stabilization of new blood vessels [37–42] – and an angiogenic switch [40,43] is dependent upon the upregulation of endothelial-specific growth factors, particularly VEGF.

VEGF is a homodimeric, heparin-binding glycoprotein that is produced by almost all cell types [44]. It stimulates angiogenesis by inducing proliferation, differentiation and chemotaxis of endothelial cells [44,45]. Remarkably, mice with only a single allele of the VEGF gene (VEGF+/−) die in utero between days 11 and 12, as a result of impaired hematopoiesis and angiogenesis [46,47].

VEGF isoforms exert their biological effects through interaction with two tyrosine-kinase receptors, VEGF receptor 2 (VEGFR2; also known as KDR human homologue or FLK-1 murine homologue) [48] and VEGFR1 (also known as Flt-1) [49–51]. Whereas VEGFR2 is mostly expressed on endothelial cells, VEGFR1 is expressed on various cell types, including myelomonocytic [52–55] and smooth muscle cells [55,56]. Several studies have suggested that VEGFR2 is the crucial receptor for transmitting cellular signals for the proliferation, differentiation and migration of endothelial cells [49–51], whereas VEGFR1 might be more important for vascular remodeling [57]. Mice deficient in VEGFR2 show dramatic defects in angiogenesis and hematopoiesis. By contrast, VEGFR1-knockout mice develop abnormal vascular channels, suggesting a role for this receptor in the regulation of endothelial cell migration and matrix interactions [57]. Conditional removal of VEGF results in the rapid ablation of newly formed immature tumor endothelial cells [58,59]. These data underscore the significance of VEGF and its receptors, VEGFR2 and VEGFR1, in regulating angiogenesis during wound healing, tissue revascularization and tumor growth.

The majority of cytokines described above exert their effect not only by promoting cell survival and proliferation but also by enhancing cell motility. The physiological significance for the motility of stem and progenitor cells is not well understood, but motility of stem cells might be required when they are forced to translocate from a nonpermissive environment that inhibits their differentiation in a microenvironment that is conducive to proliferation. Enhanced motility of stem cells also facilitates their mobilization to the peripheral circulation. The exact mechanism by which various cytokines or chemokines promote mobilization of stem and progenitor cells has recently been elucidated and is the subject of this review.

Vascular trauma induces mobilization of bone-marrow-derived endothelial progenitors

One of the most potent physiological effects of VEGF is the induction of endothelial cell motility. Under physiological conditions, only a fraction of the bio-available soluble VEGF is detected in the peripheral circulation, but vascular trauma results in an elevation of plasma VEGF, thereby promoting the recruitment of endothelial cells to the site of active angiogenesis. The vascular trauma (e.g. from surgical intervention or tissue injury) induces the release of specific chemokcytines. Rapid recruitment of endothelial cells accelerates vascular healing and inhibits potential vascular complications secondary to thrombosis or hypoxia. Similarly, tissue ischemia induced by myocardial infarction or cerebrovascular occlusion results in upregulation of angiogenic factors, including VEGF. VEGF and other released chemokines, including platelet-derived growth factors, fibroblast growth factor (FGF) and interleukin-8, promote the migration of endothelial cells to the site of injury.

There are two possible sources of cells for endothelialization: (1) endothelial migration or co-option [40,43,60] from adjacent pre-existing blood vessels and/or (2) recruitment CEPs from the peripheral circulation [20,25,61,62]. CEPs can be considered to be marrow-localized embryonic angioblasts that migrate and proliferate and have the capacity to differentiate into mature endothelial cells [63–65]. The physiological significance of CEPs in the regulation of postnatal processes has recently been the subject of intense scrutiny. Accumulating evidence suggests that bone-marrow-derived CEPs and other types of organ-specific precursors play an essential role in promoting tissue vascularization.

Several studies have implied that vascular healing might be mediated in part by the recruitment of CEPs [5,9]. In these experiments genetically marked bone-marrow-derived endothelial cells were shown to be recruited to ischemic mouse limbs, thereby accelerating the revascularization process. The introduction of cytokines, such as G-CSF or GM-CSF, enhanced the
mobilization of endothelial progenitors to the ischemic limbs, thus augmenting the re-endothelialization process.

The physiological significance of the mobilization of bone-marrow-derived endothelial cells was underscored in studies in which thoracic aortae from adult dogs were replaced with Dacron grafts months after transplantation of genetically haplo-identical bone marrow. The Dacron grafts were made impervious by silicone coating to prevent ingrowth of endothelial cells [20,21]. After three months, the re-endothelialized grafts were removed, and the origin of endothelial cells colonizing the grafts was determined by microsatellite PCR assays. Remarkably, endothelialization was shown to arise exclusively from the mobilized transplanted CD34+ bone-marrow cells.

These data demonstrate that the release of certain chemokines as a result of vascular injury results in the mobilization of endothelial progenitors to the peripheral circulation. Denuded vascular bed, coated with activated platelets and inflammatory cells, provides a permissive environment for the recruitment of these angiocompetent progenitors, accelerating the revascularization process.

In humans, evidence for the contribution of circulating endothelial cells to wound healing originates from patients implanted with a left-ventricular assist device (LVAD). LVADs are peristaltic pumps that connect a failing left ventricle to the aorta, and are implanted in patients with end-stage heart disease that are awaiting a heart transplant. The implantation of an LVAD results in the rapid recruitment of endothelial progenitors to its blood-contacting face, which is composed of a textured metallic material that facilitates the formation of a nonthrombogenic cellular surface. LVADs removed six months after placement were shown to be colonized by CD34+VEGFR2+ endothelial and hematopoietic precursor cells [66], suggesting that co-recruitment of endothelial and hematopoietic progenitors might be essential for the formation of nonthrombogenic vascular surfaces. Taken together, these studies intimate that a specialized population of VEGFR2+ endothelial cells residing in bone marrow have the capacity to mobilize to the peripheral circulation, and might contribute to rapid endothelialization and prevent thrombotic complications.

**Mobilization of CEPs is regulated by a VEGF–VEGFR signaling pathway and is essential for revascularization**

An angiogenic switch requires the upregulation of angiogenic factors, such as VEGF. Under steady-state physiological conditions, VEGFR2+ CEPs represent only a small percentage of circulating mononuclear cells in the peripheral circulation. However, plasma elevation of VEGF in intact adult mice has been shown to result in the mobilization of VEGFR2+ CEPs [4,61,62]. In adult humans, VEGFR2+ CEPs also express another angiogenic stem-cell marker, AC133, but the expression of this protein diminishes rapidly upon maturation of stem cells [28,62].

Using adenoviral vectors to increase the plasma levels of various VEGF isoforms, VEGF165, an isoform of VEGF-A, was shown to induce the mobilization of CEPs to the peripheral blood on days 2, 5 and 7 after administration, with a return to control levels by day 14 (Fig. 1a) [4]. In the same experiment, another angiogenic factor, angiopoietin-1 (Ang-1) was shown to induce the same effect. Mobilized CEPs can be distinguished from mature endothelial cells by their capacity to form late-outgrowth colony-forming units of endothelial cells (CFU-EC) (Fig. 1b,c) [67]. By contrast, mature endothelial cells form early-outgrowth colonies. Incubation of mobilized VEGFR2+ peripheral blood mononuclear cells (PBMCs) with VEGF and FGF-2 (basic FGF) resulted in the generation of early- as well as late-outgrowth CFU-EC [62,67], but the majority of the colonies formed were composed of late-outgrowth endothelial cells, suggesting that VEGF and Ang-1 promote the mobilization of bone-marrow-derived CEPs.

The rapid mobilization of AC133+VEGFR2+ CEPs can also be induced by vascular trauma induced by burn injury or surgical manipulation [6]. Six hours after vascular trauma, CEPs were detected in the peripheral circulation and, remarkably, 24 h after vascular injury, 12% of the total circulating PBMCs were AC133+VEGFR2+ cells. Mobilization of CEPs was accompanied by plasma elevation of VEGF, suggesting that VEGF released as a result of vascular injury might be the main factor that promotes mobilization of CEPs. These data are consistent with the notion of chemokine-driven recruitment of bone-marrow-derived precursor cells that restore angiogenesis in ischemic limbs [5].

**Angiogenic factors also promote the mobilization of hematopoietic cells with stem-cell potential**

Owing to the common prenatal origin of CEPs and hematopoietic stem cells from hemangioblasts, both hematopoietic and vasculogenic cells share several survival and chemotactic signaling pathways. For example, the endothelial-specific receptor VEGFR2, and Tie-2 [68] (whose expression was originally shown to be restricted to vascular cells), are also expressed by subsets of human nonobese-diabetic–severe-combined-immunodeficient repopulating cells [69]. Furthermore, VEGFR1 is expressed on subsets of human and murine HSCs [32]; placental growth factor (PIGF), another member of the VEGF family, which selectively signals through VEGFR1 but not VEGFR2, promotes the recruitment and mobilization of HSCs from marrow to the circulation [32]. PIGF is also effective in reconstituting hematopoiesis after myelosuppression, by enhancing the motility and cell cycling of HSCs [32].

The potential of VEGF to induce mobilization of hematopoietic stem cells was examined via elevation plasma VEGF levels by injecting adenoviral vectors expressing VEGF165 into mice. These experiments showed that VEGF165 can induce mobilization to the peripheral circulation of a significant number of hematopoietic progenitors, precursors and stem cells with bone-marrow-repopulating capacity (Fig. 2).

The number of mobilized pluripotent hematopoietic cells with bone-marrow-repopulating capacity was also determined by allogeneic transplantation of VEGF165- and/or Ang-1-mobilized PBMC into lethally irradiated mice. Injection of adenoviral-vector-administered
VEGF165 (AdVEGF165) and/or AdAng-1 (but not AdNull) resulted in the mobilization of hematopoietic stem cells with repopulating potential and these were able to engage and rescue lethally irradiated mice. All mice injected with 10^6 PBMCs from AdNull-treated mice died within 18 hours. However, 56% of mice injected with PBMCs from mice treated with AdVEGF and AdAng-1, 45% of mice injected with PBMCs from AdVEGF-treated mice, and 22% of mice injected with PBMCs from AdAng-1-treated mice survived beyond 150 days. These data suggest that plasma elevation of VEGF165 and/or Ang-1 leads to the mobilization of pluripotent hematopoietic cells with stem-cell potential from the bone marrow to the peripheral circulation.

**Essential role of mobilization of CEPs and hematopoietic cells for revascularization**

The mobilization of CEPs is a dynamic process that is mediated through adhesion molecules, activation of metalloproteinases and remodeling of the extracellular matrix. VEGF-induced upregulation of certain factors might be essential in providing a permissive environment within the bone marrow to facilitate the mobilization of CEPs and hematopoietic stem and progenitor cells. Interestingly, following tumor inoculation, VEGFR2+ CEPs and VEGFR1+ hematopoietic precursor cells were mobilized and incorporated into tumor vascular bed within 48 h [25]. The necessity for mobilization of VEGFR2+ CEPs in restoring tumor angiogenesis was highlighted by the finding that VEGF-induced mobilization of CEPs was completely impaired in tumor-resistant Id-mutant mice. Furthermore, inoculation with tumor tissue results in the release of chemokines that promote the mobilization of VEGFR2+ CEPs. However, inoculation of the lymphoma cell line, B6RV2, into Id-mutant mice is associated with complete impairment of the mobilization of VEGFR2+ CEPs [25]. The significance of VEGF-mediated mobilization of precursor cells was further underscored by the demonstration that transplantation of Id-mutant bone marrow into wild-type mice resulted in a profound delay in tumor growth [25]. These results suggest that a failure of Id-mutant myeloid cells to express functional metalloproteinases or angiogenic factors might be required for functional integration of CEPs into newly formed vessels. A reduction in the mobilization of VEGFR2+CD11b-CEPs is clearly crucial to the failure of Id-mutant mice to support neo-angiogenesis, but it is also conceivable that Id-mutant myeloid cells fail to express functional metalloproteinases or angiogenic factors that might be required for functional integration of CEPs into neo-vessels. Based on these studies, it is also possible that the rapid revascularization necessary for cardiovascular regeneration requires activation of angiogenic pathways that are required for co-recruitment of endothelial and hematopoietic precursor cells.

**Adhesion molecules involved in the mobilization of stem cells**

CD34+ stem cell homing to the bone marrow might mimic similar pathways involved in leukocyte homing to lymphoid or inflamed tissues [71–73]. Molecular regulation of leukocyte homing and extravasation, and the adhesion molecules involved, have been elucidated by adhesion and targeted-gene-disruption studies. Leukocyte homing is a multistep process, involving the initial selectin-dependent tethering and rolling of leukocytes on the activated endothelial cells, followed by stable adhesion and diapedesis into the organ. The initial phase of tethering of the CD34+ cells might be mediated through selectins, such as E-selectin. This is then followed by firm adhesion mediated by the ligand pairs, vascular cell adhesion molecule 1 (VCAM1)-VLA4 and intercellular adhesion molecule 1 (ICAM1)-VLA4, and then by interaction with interjunctional adhesion molecules, such as platelet endothelial-cell-adhesion molecule (Fig. 3). E-selectin is unique among adhesion molecules in that it is only expressed on activated endothelial cells, whereas other selectins and cell-adhesion molecules are also produced by other cells. Binding of E-selectin to its ligand(s) results in tethering and leukocyte rolling along the luminal surface of blood vessels, slowing the movement enough to allow the
secondary, high-affinity interactions that are involved in the arrest of leukocytes on the endothelium.

Similar to the homing of stem cells, the mobilization of stem cells is a complex process and is dependent upon the activation of a chain of molecular events that increase the motility of quiescent stem cells adherent to stromal cells, facilitating their recruitment to the peripheral circulation (Fig. 3). Using the in vivo mobilization model described above, several molecular pathways that are necessary for the mobilization of subsets of stem cells have been identified.

Chemokine-induced activation of metalloproteinases is necessary for the mobilization of stem and progenitor cells

The majority of quiescent stem cells reside in close cellular contact with stromal elements and with the complex network of extracellular matrix, where they receive survival signals. Physiological stress results in the release of stem-cell-active cytokines, such as KitL, that promote rapid cycling of the stem cells, setting the stage for mobilization to the peripheral circulation. Remodeling of the extracellular matrix could be an essential step in the mobilization and recruitment of hematopoietic stem cells. Furthermore, inhibition of MMP-9 has been shown to influence the mobilization and release of mature hematopoietic cells, including platelets [74]. Similarly, administration of a matrix-metalloproteinase inhibitor blocked chemokine-induced mobilization of 82 ± 3% of cells with stem-cell potential. Of the matrix metalloproteinases, only MMP-9 has been shown to be produced by hematopoietic stem cells in response to chemokine activation, and it is required for migration through reconstituted extracellular matrix in vitro. To define the physiological role of MMP-9 in vivo, chemokine-induced mobilization was examined in MMP-9-deficient mice: mobilization of hematopoietic progenitors was partially blocked whereas chemokine-driven mobilization of stem cells was significantly impaired. These mice also showed delayed hematopoietic reconstitution following treatment of the bone marrow with the cell-cycle-specific suppressive agent, 5-fluorouracil (5-FU) [33]. In fact, 70% of mice treated with 5-FU succumbed to complications associated with prolonged marrow suppression.

Further investigation demonstrated that activation of MMP-9 is essential for the release of soluble KitL (sKitL). An increase in the bio-availability of sKitL results in increased cycling of quiescent stem and progenitor cells, and enhanced translocation to a vascular niche that is conducive to stem-cell proliferation, differentiation and mobilization to the peripheral circulation. Angiogenic factors released as a result of vascular injury can also activate MMP-9, resulting in a rapid increase in sKitL and enhanced recruitment of CEPs and HSCs from the marrow to the peripheral circulation [32, 33]. These data suggest that angiogenic-factor-mediated MMP-9 activation is the decisive checkpoint regulating the mobilization of CEPs and HSCs.

Concluding remarks

The organ-specific stem cells reside in an adaptable bone-marrow microenvironment in which they can readily sense and respond to the high demand for revascularization. To meet this demand, rapid bio-availability of chemokines and cytokines is essential for proliferation and timely recruitment of quiescent stem cells.

Accumulating evidence suggests that like the homing of stem cells to the marrow, mobilization of stem cells from the marrow to the peripheral circulation is a dynamic multistage process, and requires sequential activation of particular signaling molecules. Murine mobilization models, in which the plasma elevation of stem-cell-active cytokines can increase the motility of stem cells, provide an ideal means of identifying the molecular pathways that are essential for the mobilization of organ-specific stem cells (Fig. 4).

Plasma elevation of angiogenic factors, including VEGF and PIGF, released as a result of tissue injury, results in the rapid mobilization of both VEGF R2 CEPs and VEGFR1 hematopoietic progenitor and stem cells. The mobilization process is dynamic and requires sequential chemokine-mediated activation of MMP-9, followed by release of soluble bio-available stem-cell-active cytokines, including sKitL. An increase in sKitL levels enhances the cycling of VEGF R2 endothelial cells and VEGFR1 hematopoietic stem and progenitor cells, thereby augmenting their motility. This facilitates their translocation to a vascular zone that is permissive to their mobilization to the peripheral circulation. These studies introduce a novel concept in which the activation of a metalloproteinase functions as a molecular switch, activating signaling cascades that are essential for recruiting quiescent stem and progenitor cells from the protected microenvironment of the bone marrow. This sequence of events leads to the mobilization of stem and progenitors to the peripheral circulation where they incorporate into neo-angiogenic sites. Co-mobilization of hematopoietic cells facilitates the incorporation of endothelial cells into functional neo-vessels (Fig. 4).

The identification of molecular pathways that selectively enhance the mobilization of organ-specific stem cells will lay the foundations for designing strategies for recruiting and isolating a large number of stem cells that might ultimately be used to augment vascularization in a specific target organ.

However, despite the discovery of the molecular mechanisms involved in the mobilization of stem cells, harnessing the potential of these cells for tissue vascularization remains a daunting task. In particular, the exact molecular pathways involved in the differentiation of immature marrow-derived stem and progenitor cells into mature endothelial cells are not well defined. Furthermore, it is not clear whether the process of differentiation of the endothelial and hematopoietic progenitors is initiated during the recruitment process (in the bone marrow) or at the neo-angiogenic site. The tissue-specific extracellular matrix, adhesion molecules and chemokines expressed at the neo-angiogenic site are crucial for the selective homing and differentiation of
stem and progenitor cells, thereby facilitating tissue vascularization of that particular target organ. In this regard, the identification of tissue-specific chemokines and adhesion addressins, collectively referred to as ‘molecular zip codes’, will provide for a novel means of selectively directing marrow-derived stem and progenitor cells to a particular organ.

It also remains to be elucidated how mechanical forces imparted on stem and progenitor cells during the mobilization process influence their differentiation and survival. Stem cells are subjected to various mechanical forces as they undergo trans-endothelial migration and exit the marrow microenvironment. In this respect, the bone-marrow endothelial monolayer might act as a signal transduction interface for hemodynamic forces. Shear stress imparted by blood flow might also be among the variables that control the proliferative-differentiative balance of endothelial progenitor cells during the mobilization process (Fig. 4). However, the molecular mechanisms by which shear stress regulates the proliferation and trafficking of hematopoietic and endothelial cells are not fully understood, and are the subject of ongoing studies.

Finally, chemokine-induced mobilization and proliferation of stem and progenitor cells will allow the isolation from the circulation, of an enriched population of these pluripotent cells, which exist in low numbers under normal physiological conditions. Recovery of a large number of marrow-derived stem and progenitor cells should enable the development of strategies for in vitro expansion and genetic modification. Re-infusion of ex vivo expanded endothelial and hematopoietic stem and progenitor cells might then be used to augment tissue vascularization in clinical conditions that require rapid angiogenesis, including myocardial infarction, vascular injury and organ regeneration.

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Figs 1. Adenoviral-vector-administered vascular-endothelial-growth-factor-165 (AdVEGF165) and angiopoietin-1 (AdAng-1) promote the mobilization of circulating endothelial progenitor cells (CEPs) with late-outgrowth potential. (a) Mobilized peripheral blood mononuclear cells (PBMC) were isolated from AdVEGF165 and/or AdAng-1 or AdNull-treated severe-combined-immunodeficient (SCID) mice, between day 0 and day 28, and stained with fluorescein-isothiocyanate-conjugated anti-VEGF-receptor-2 (VEGFR2) monoclonal antibody and propidium iodide. Ten or more cells were analyzed by flow cytometry. The percentages of PBMCs that were VEGFR2+CD11b− CEPs, from AdVEGF165-, AdAng-1- or AdNull-treated SCID mice (n = 3), are shown. (b) To quantify bone-marrow-derived CEPs with late-outgrowth potential, mobilized PBMCs were obtained from AdVEGF165 and/or AdAng-1- or AdNull-treated SCID mice in the third week following treatment and plated in the presence of endothelial growth medium. Colony-forming units of endothelial cells (CFU-EC) were identified by immunostaining and metabolic labeling with diI-acetylated low-density lipoprotein (Dil-Ac-LDL). Colonies that formed within the first three days (early-outgrowth colonies) and colonies formed 14 days after vector administration (late-outgrowth colonies) were quantified by Dil-Ac-LDL labeling. The majority of CFU-EC proliferated 2–3 weeks after the start of culture, forming confluent Dil-Ac-LDL endothelial monolayers. (c) Endothelial monolayers at 14 days (i), 20 days (ii) and 25 days (iii).
Fig. 2. Increases in plasma levels of adenoviral-vector-administered vascular endothelial growth factor (AdVEGF) and/or angiopoietin-1 (AdAng-1) promote the mobilization of mature and immature hematopoietic cells. Severe-combined-immunodeficient (SCID) mice received AdVEGF165, AdAng-1 or AdNull vector by a single intravenous administration on day 0. Plasma concentrations of VEGF and Ang-1 were measured using a sensitive enzyme-linked immunoabsorbent assay (ELISA). (a) Total white blood cells were counted and stained with crystal violet (n=6). (b) The pluripotency of the mobilized cells was determined by quantification of colony-forming units of stromal cells (CFU-S). Compared with AdVEGF alone, the combination of AdVEGF and AdAng-1 induced significant long-term mobilization of CFU-S up to day 21 (*p<0.01 on days 3, 7 and 14; **p<0.05 on day 21). Ang-1, VEGF, or a combination of VEGF and Ang-1, promoted mobilization of bone-marrow-repopulating cells (n=9). (c) The pluripotency of the mobilized cells was also determined by a bone-marrow-repopulating assay. Peripheral blood mononuclear cells (PBMCs) from SCID mice (H-2Kd) treated with AdNull, AdVEGF165, AdAng-1 or a combination of AdVEGF165 and AdAng-1 were transplanted into irradiated C57BL/6 (H-2Kb) mice by intravenous injection on day 0. The number of engrafted H-2Kd cells was determined by flow cytometry. Compared with the AdNull group, AdAng-1- and AdVEGF-treated mice showed significant mobilization of cells capable of reconstituting hematopoiesis in lethally irradiated mice (**p<0.05). By contrast, all the mice transplanted with PBMCs from the peripheral blood of AdNull treated mice failed to engraft. As a control group, 90% of the mice transplanted with untreated bone marrow were engrafted and survived the effects of lethal irradiation.

Fig. 3. Trafficking of stem cells is regulated through sequential interaction with chemokines and adhesion molecules. Homing of hematopoietic stem cells to the bone marrow is dependent upon a multistep process in which tethering, mediated by E-selectin, is followed by firm adhesion mediated by vascular cell adhesion molecule 1 (VCAM1)–VLA4 and intercellular adhesion molecule 1 (ICAM1)–LFA1 ligand pairs. This process is orchestrated by chemokines, which provide directional cues for the stem cells to home to the bone marrow where, ultimately, they settle within a safe haven of stromal cells. Chemokine-mediated mobilization of stem cells reverses the homing process by increasing the motility of the stem cells, facilitating their release from the extracellular matrix and stromal cells. Shear stress plays a crucial role in altering the adhesion and chemokine profile of motile hematopoietic cells, thereby guiding their homing or mobilization.
Chemokine-mediated mobilization of endothelial and hematopoietic stem and progenitor cells is a dynamic multistep process. Physiological stress, such as vascular trauma, ischemia or tumor growth induces the release of specific chemokines, such as vascular endothelial growth factor (VEGF) and placental growth factor (PlGF). These stimulate the upregulation and activation of matrix-metalloproteinase-9 (MMP-9), resulting in the release of stem-cell-active cytokines, including soluble Kit ligand (sKitL). This promotes cell cycling, thereby increasing the motility of otherwise quiescent sessile VEGF-receptor-2+ (VEGFR2+) endothelial, and VEGF-receptor-1+ (VEGFR1+) hematopoietic, stem and progenitor cells. As a result, these cells are mobilized to the peripheral circulation and are ultimately incorporated into injured tissue or growing malignant tumors, accelerating the revascularization process. Reciprocal interaction between co-mobilized VEGFR1+ hematopoietic and VEGFR2+ endothelial cells is necessary for the functional incorporation of circulating endothelial progenitor cells into newly formed vessels.