

Differential mobilization of subsets of progenitor cells from the bone marrow.

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Summary

G-CSF stimulates mobilization of hematopoietic progenitor cells (HPCs) from bone marrow by disrupting the CXCR4/SDF-1 α retention axis. We show here that distinct factors and mechanisms regulate the mobilization of endothelial (EPCs) and stromal progenitor cells (SPCs). Pre-treatment of mice with VEGF did not disrupt the CXCR4/SDF-1 α chemokine axis but stimulated entry of HPCs into the cell cycle via VEGFR1, reducing their migratory capacity *in vitro* and suppressing their mobilization *in vivo*. In contrast, VEGF pre-treatment enhanced EPC mobilization via VEGFR2 in response to CXCR4 antagonism. Furthermore, SPC mobilization was detected when the CXCR4 antagonist was administered to mice pre-treated with VEGF, but not G-CSF. Thus, differential mobilization of progenitor cell subsets is dependent upon the cytokine milieu that regulates cell retention and proliferation. These findings may inform studies investigating mechanisms that regulate progenitor cell recruitment in disease and can be exploited to provide efficacious stem cell therapy for tissue regeneration.

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Introduction

The bone marrow is a reservoir of progenitor cells, including haematopoietic progenitor cells (HPCs), fibrocytes, mesenchymal stem cells (MSCs) and endothelial progenitor cells (EPCs). In response to disease or tissue injury these cells are mobilized from the bone marrow and recruited into tissues where they contribute either to disease progression or tissue repair (Takahashi et al., 1999; Orlic et al., 2001; Rankin 2008). Thus, for example, fibrocyte recruitment detrimentally contributes to fibrosis and tissue remodelling in diseases such as idiopathic lung fibrosis and ischemic cardiomyopathy (Philips et al., 2004; Haudek et al., 2006). In contrast, EPCs recruited into ischemic tissues promote angiogenesis and thereby contribute to tissue regeneration (Asahara et al., 1997; 1999; Takahashi et al., 1999; Nolan et al., 2008). Further, the identity of an EPC type, which is distinct from a HPC phenotype has now been reported (Hur et al., 2004; Yoder et al., 2007; Nolan et al., 2007) Indeed, EPCs have been demonstrated to repair damaged myocardium and improve cardiac function (Kocker et al., 2001). Mesenchymal stem cells have the capacity to differentiate into adipocytes, chondrocytes and osteocytes and potentially other cell types including epithelial, myocardial or neuronal cells (Pittenger et al., 1999). It is thought, therefore, that MSCs may be used to promote tissue regeneration in the treatment of diseases such as *osteogenesis imperfecta* and Parkinsons (Hess & Borlongan 2008; Bielby et al., 2007). Additionally MSCs have been reported to have immunosuppressive properties and as such they may be therapeutically useful for the treatment of autoimmune diseases (Le Blanc & Ringden 2007). The therapeutic application of these distinct sub-populations of stem cells in the context of specific diseases is therefore widely anticipated. Currently however, there are both practical and technical complications associated with harvesting, isolation, *ex vivo* expansion and delivery of

these cells. An alternative strategy for stem cell therapy is to stimulate the mobilization of stem cells from the bone marrow into the circulation, thereby circumventing these issues. This approach has been established clinically with respect to the treatment of donors over 3-5 days with the cytokine G-CSF to mobilize of HPCs for bone marrow transplants (BMT) (Cashen et al., 2004).

Mobilization of progenitor cells is a multi-stage process; with initial release from their bone marrow niche followed by active migration across the bone marrow sinusoidal endothelium. The chemokine axis SDF-1 α /CXCR4 is critically involved in the retention of haematopoietic stem cells within the bone marrow (Levesque et al., 2003). At a molecular level, G-CSF has been shown to act by disrupting the SDF-1 α /CXCR4 retention axis, both by reducing CXCR4 expression on HPCs and levels of SDF-1 α in the bone marrow (Levesque et al., 2003; Semerad et al., 2002). This knowledge has led to the development of CXCR4 antagonists as HPC mobilizing agents. In contrast to G-CSF, such antagonists work acutely, mobilizing HPCs within an hour. Moreover, we and others have shown that chronic G-CSF therapy combined with acute administration of a CXCR4 antagonist synergistically enhances HPC mobilization from the bone marrow (Broxmeyer et al., 2005; Martin et al., 2006). This combination therapy has recently shown greater efficacy compared to G-CSF alone in phase III clinical trials for BMT (Calandra et al., 2008). While it has been shown that administration of a CXCR4 antagonist alone increases the circulating numbers of EPCs and improves tissue perfusion following ischemia in animals (Capoccia et al., 2006; Shepherd et al., 2006) it is not known whether G-CSF therapy in combination with acute administration of the CXCR4 antagonist acts synergistically to mobilize EPCs.

In addition to factors that disrupt the retention of EPCs in the bone marrow, EPCs may also be mobilized by factors, such as VEGF, that stimulate their migration

(Asahara et al., 1997). Indeed treatment of mice with VEGF has been shown to increase the circulating numbers of EPCs (Asahara et al., 1999), however, the relative efficacy of VEGF versus G-CSF alone or in combination with other mobilizing reagents has not been specifically assessed with respect to EPC mobilization. There is evidence that bone marrow-derived MSCs contribute to tissue regeneration, suggesting that these cells are also mobilized in response to tissue injury, however the factors and mechanisms regulating the mobilization of MSCs are currently unknown (Rochefort et al., 2006; Au et al., 2008).

We have previously shown that different sub-populations of leukocytes are selectively mobilized from the bone marrow in response to specific blood-borne mediators via distinct mechanisms (Palframan et al., 1998; 1998a; Martin et al., 2003; Wengner et al., 2008). In this study we have identified pathways regulating the differential mobilisation of discrete populations of progenitor cells.

Results

The CXCR4 antagonist mobilizes discrete populations of progenitor cells in mice pre-treated with G-CSF or VEGF.

It has previously been established that G-CSF and the CXCR4 antagonist act synergistically to mobilize HPCs from the bone marrow (Broxmeyer et al., 2005; Martin et al 2006). In the first experiment we examined whether this treatment protocol similarly mobilized maximal numbers of EPCs and stromal progenitor cells (SPCs). Furthermore we investigated whether the profile and numbers of progenitors in the blood changed if mice were pre-treated with VEGF instead of G-CSF. We determined circulating numbers of HPCs, EPCs and SPCs one hour after the administration of the CXCR4 antagonist, AMD3100, to mice pre-treated over 4 days with either G-CSF, VEGF or vehicle control. Administration of the CXCR4 antagonist alone increased circulating numbers of HPCs and EPCs within 1 hour (Figures 1b and 1c, and Shepherd et al., 2006). Interestingly, SPCs were not detected in the blood. G-CSF pre-treatment of mice also increased circulating numbers of HPCs, and to a lesser extent EPCs. (Figures 1b and 1c). Intriguingly, no SPCs were detected (Figure 1d). Whilst G-CSF pre-treatment followed by acute administration of the CXCR4 antagonist synergistically enhanced circulating numbers of HPCs (Figure 1b and Broxmeyer et al., 2005; Martin et al 2006), no such synergism (or even additive effect) was apparent for EPC mobilization (Figure 1c). Furthermore, SPCs could not be detected. Of note, EPCs were scored after 21 days in culture and were shown to be CD34, VEGFR2, VE-Cadherin, and vWF positive (Supplementary Figures 1, 2 and 3). Furthermore these EPCs stained positively with GS-lectin, took up acetyl-LDL and formed tubules *in vitro*. These EPCs are therefore equivalent to the so-called late outgrowth EPCs that have the capacity to form vessels *in vivo* (Hur et al., 2004; Yoder et al., 2007;

Nolan et al., 2007; Hirschi et al., 2008). Importantly the mobilized EPCs did not express CD115, CD14, or CD45 and are thus distinct from both HPCs and the EPCs that are of monocytic origin (Supplementary Figures 1, 2 and 3) (Hur et al., 2004; Yoder et al., 2007; Nolan et al., 2007; Hirschi et al., 2008)

VEGF administration can increase circulating number of cells with an EPC phenotype (Asahara et al., 1997). Similarly we observed an increase in EPC numbers following VEGF treatment of mice over 4 days (Figure 1c). Moreover, a more than additive increase in EPC numbers was observed when the CXCR4 antagonist was administered acutely to mice pre-treated with VEGF as compared to mice treated with VEGF alone or administered a CXCR4 antagonist alone ($P<0.05$ Figure 1c). In contrast to G-CSF, VEGF-treatment did not increase circulating numbers of HPCs (Figure 1b). Further, we found unexpectedly that VEGF pre-treatment profoundly suppressed circulating numbers of HPCs (77% reduction) mobilized by the CXCR4 antagonist ($P<0.01$, Figure 1b). Uniquely, VEGF-treatment combined with the CXCR4 antagonist led to a significant increase in circulating SPCs (CFU-F $P<0.001$, Figure 1d). Importantly, mobilized SPCs displayed the same antigen expression phenotype as bone marrow-derived SPCs (Supplementary figure 4a) which on expansion exhibited tri-lineage differentiation potential (Supplementary Figure 4c-e). Mobilized SPCs were plastic adherent cells, shown to be CD29 and CD105, positive; yet were CD34 and CD45 negative (Supplementary Figure 4b). Mobilized SPCs had the same characteristics as murine mesenchymal stem cells (Phinney et al., 1999; Pittenger et al., 1999; Mereilles & Nardi 2003). Mobilized SPCs were also negative for VE-Cadherin and vWF and were therefore distinct from mobilized EPCs (Supplementary Figure 4b).—These results suggest that fundamentally different mechanisms regulate the mobilization of phenotypically distinct HPCs, EPCs and SPCs.

G-CSF and VEGF pre-treatment differentially regulate the rate of egress of progenitor cell sub-populations from the bone marrow in response to a CXCR4 antagonist

To determine whether the differences in circulating numbers of progenitors cells was due specifically to differences in their rate of egress out of the bone marrow we used an *in situ* perfusion model of the murine hind-limb developed by our group to directly assess mobilization. In this system, the femoral artery and vein are cannulated *in situ* such that the femur and tibia bone marrow are perfused in isolation. Mobilized cells are then collected via the femoral vein (Martin et al., 2003; 2006; Wengner et al., 2008). This technique allows direct comparisons of the number of cells mobilized over a defined period of time, without the complications of progenitor cell trafficking into other tissues including their return to the bone marrow. Mice were pre-treated with vehicle, G-CSF or VEGF for 4 days. On day 5, the femoral artery was infused for 10 minutes with either vehicle or the CXCR4 antagonist and cells mobilized were collected over a period of 1 hour (Figure 2a: experimental protocol). When the CXCR4 antagonist was infused into vehicle pre-treated mice, HPCs and EPCs, but not SPCs were mobilized from the bone marrow ($P<0.001$, Figure 2b-2d). Pre-treatment of mice with G-CSF over 4 days enhanced the basal rate of HPC and EPC egress from the bone marrow ($P<0.001$, Figure 2b and 2c). However, G-CSF pre-treatment did not lead to the mobilization of SPCs (Figure 2d). As indicated above, G-CSF pre-treatment followed by the CXCR4 antagonist led to a synergistic mobilization of HPCs ($P<0.001$, Figure 2b), but not EPCs (Figure 2c). Furthermore, no SPCs were mobilized with the combination of G-CSF pre-treatment followed by the CXCR4 antagonist (Figure 2d). VEGF pre-treatment resulted in significant EPC mobilization ($P<0.001$, Figure 2c),

however, HPCs and SPCs were not mobilized (Figures 2b and 2d). Furthermore, VEGF pre-treatment almost completely suppressed HPC mobilization induced by infusion of the CXCR4 antagonist ($P<0.001$ Figure 1e, 75% reduction), whilst an additive effect was observed for EPC mobilization ($P<0.01$ Figure 2c). Uniquely with this treatment regime, SPCs were mobilized ($P<0.001$, Figure 2d). These experiments definitively show that changes in circulating numbers of progenitors are due to changes in their rate of egress from the bone marrow.

Acute administration of cytokines does not result in the differential mobilization of HPCs, EPCs and SPCs in response to the CXCR4 antagonist.

The selective mobilization of EPCs and SPCs achieved by treating mice over 4 days with VEGF followed by administration of the CXCR4 antagonist was not reproduced when mice were treated acutely with a combination of VEGF and the CXCR4 antagonist. Infusion of either G-CSF or VEGF directly into the femoral artery for 10 minutes and collection up to 60 minutes mobilized both HPCs and EPCs ($P<0.001$ Supplementary Figures 5a and 5b). However, acute administration of VEGF (in contrast to VEGF pre-treatment over 4 days) did not inhibit the mobilization of HPCs with a CXCR4 antagonist and acute administration of G-CSF did not act synergistically with the CXCR4 antagonist to mobilize HPCs (Supplementary Figures 5a and 5b). Further, neither growth factor, when administered acutely or in combination with the CXCR4 antagonist led to mobilization of SPCs (Supplementary Figure 5c). This suggested that the ability of G-CSF and VEGF to promote the differential mobilization of progenitor cell subsets is dependent on changes to the bone marrow environment or the progenitor cell phenotype that occurs over a number of days.

VEGF-treatment does not alter bone marrow morphology or progenitor cell frequency

We next determined whether treatment of mice over 4 days with VEGF or G-CSF affected the number of progenitor cells in the bone marrow. The total number of cells in the femurs of mice treated with VEGF ($1.07 \pm 0.11 \times 10^7$ cells/femur) or G-CSF ($1.41 \pm 0.28 \times 10^7$ cells/femur) was not significantly different compared to control mice ($1.19 \pm 0.07 \times 10^7$ cells/femur). Furthermore, no difference was observed in the frequency of HPCs (Figure 3a), EPCs (Figure 3b) or SPCs (Figure 3c) in the bone marrow of VEGF-treated mice compared to controls. However, G-CSF-treatment did cause a significant reduction in the number of bone marrow EPCs ($P < 0.001$ Figure 3b), and an increase in the number of SPCs ($P < 0.05$ Figure 3c).

To determine whether VEGF-treatment of mice affected the gross morphology of the bone marrow, we performed histological analysis of bone marrow in mice treated over 4 days with PBS or VEGF. As shown in Figure 3d we observed no differences in the gross morphology, the number of sinusoidal blood vessels, or endothelial integrity (Figure 3d). Thus, with no gross morphological changes occurring to the bone marrow of mice treated with VEGF which might affect cellular egress, we next examined whether the molecular retention mechanisms had been disturbed.

VEGF- treatment does not alter the expression of CXCR4 on HPCs or EPCs.

The CXCR4/SDF-1 α chemokine axis is critical for the retention of HPCs in the bone marrow (Levesque et al., 2003). Indeed, G-CSF mobilizes HPCs by reducing their expression of CXCR4 and decreasing levels of SDF-1 α in the bone marrow. To determine whether VEGF similarly mobilized progenitor cells by disrupting this

chemokine axis, we analyzed CXCR4 expression on progenitor cells and SDF-1 α levels in the bone marrow of VEGF and G-CSF-treated mice. In mice treated with G-CSF we observed a significant reduction in levels of SDF-1 α ($P<0.001$, Figure 4a) and CXCR4 expression on HPCs ($P<0.05$, Figure 4b), however, the expression of CXCR4 on EPCs was unchanged (Figure 4c), suggesting that G-CSF does not directly effect EPC retention. This explains why G-CSF is far more effective in mobilizing HPCs than EPCs (Figures 1b-1c and 2b-2c). In contrast, VEGF-treatment did not alter the expression of CXCR4 on either HPCs or EPCs (Figures 4b, and 4c). Moreover, SDF-1 α levels in bone marrow supernatant following VEGF-treatment were not altered (Figure 4a). Whilst the anti-CXCR4 antibody used here is targeted to the epitope matching the first 63 amino acids starting from the N-terminus of a GST-NCXCR4 fusion protein (Forster et al., 1998), it is noted that G-CSF will cleave part of this epitope, and therefore inhibiting the function of CXCR4 (Levesque et al., 2003). Thus reduced antibody binding will therefore indicate either a reduction in the expression of the functional receptor, or an absolute loss of the receptor. Thus we can conclude that VEGF-treatment does not inhibit the function of CXCR4 or affect it's cell-surface expression.

These data indicate that VEGF-treatment does not disrupt the CXCR4/SDF-1 α retention pathway and therefore explains why VEGF alone does not mobilize HPCs from the bone marrow.

VEGF- treatment inhibits HPC but not EPC chemotaxis towards SDF-1 α .

Mobilization is dependent on cellular migration across the bone marrow sinusoidal endothelium. Given that VEGF did not alter CXCR4 expression on HPCs or EPCs

(Figure 4b and 4c) we examined the ability of bone marrow derived HPCs and EPCs from G-CSF or VEGF-treated mice to undergo chemotaxis towards SDF-1 α *in vitro* since in PBS-treated mice, both HPCs and EPCs migrated to SDF-1 α ($P<0.001$ Figure 4d-4g). G-CSF-treatment lead to further increased migration of HPCs and EPCs to SDF-1 α ($P<0.001$ Figure 4d and 4e). In contrast, VEGF-treatment abolished the ability of HPCs to migrate to SDF-1 α but had no effect on EPC migration (Figures 4f and 4g). The suppression of HPC chemotaxis towards SDF-1 α suggested that HPCs from VEGF-treated mice were refractory to migratory stimuli despite expressing CXCR4.

VEGF-treatment does not affect the ability of neutrophils to undergo chemotaxis

Neutrophils, like progenitor cells, are also retained within the bone marrow by the CXCR4/SDF-1 α axis, and as such can be mobilized by G-CSF-treatment or CXCR4 antagonism ($P<0.001$, Figure 5a and Martin et al., 2003). We therefore investigated whether VEGF pre-treatment of mice suppressed neutrophil mobilization as we observed for HPC mobilization (Figure 1b and 2b). As shown in Figure 5a, VEGF pre-treatment profoundly suppressed neutrophil mobilization in response to the CXCR4 antagonist ($P<0.05$, 92% reduction). This effect was not seen when VEGF was administered acutely to mice (Figure 5b). We therefore investigated whether the migratory capacity of neutrophils after VEGF-treatment was impaired in a similar manner to HPCs. During inflammatory reactions, neutrophils are rapidly mobilized from the bone marrow by ELR⁺ CXC chemokines (e.g. KC) which stimulate neutrophil migration across the bone marrow sinusoidal endothelium (Martin et al., 2003; Wengner et al., 2008). Surprisingly, KC-induced mobilization of neutrophils *in vivo* was completely abrogated in mice pre-treated with VEGF ($P<0.001$, Figure 5c). However, VEGF-treatment did not affect either the proportion of Gr-1⁺ bone marrow neutrophils expressing CXCR2 (Figure 5d), or the ability of bone marrow neutrophils to migrate

towards KC *in vitro* (Figure 5e). Thus, whilst VEGF pre-treatment suppressed both HPC and neutrophil mobilization *in vivo*; neutrophils, but not HPCs, retained the ability to undergo chemotaxis *in vitro*, suggesting that the suppression of HPC chemotaxis was particular to their character as progenitor cells compared to neutrophils which are terminally differentiated cells. Analysis of bone marrow revealed a significant reduction in absolute numbers of mature neutrophils (band and segmented nuclei) following VEGF-treatment compared to controls (Figure 5f). It is unclear, however, whether this reduction in the bone marrow reserve accounts for the dramatic reduction in neutrophil mobilization.

VEGF- treatment stimulates entry of HPCs but not EPCs into the cell cycle

Activation of HPCs via VEGFR1 is required for cell survival and cell-cycling (Hattori et al., 2001; Gerber et al., 2002). Since a refractoriness of HPCs to migrate is a feature of cell-cycling (Bowie et al., 2006; Uchida et al., 1997; Roberts & Metcalf 1995), we next tested whether VEGF-treatment of mice stimulates HPC proliferation. Examination of HPC colonies grown from bone marrow of VEGF-treated mice revealed a significant increase in both their cellular content and size compared to controls (Figures 6a, 6b), suggesting that VEGF promotes proliferation of CFU-HPC *ex vivo*. *In vivo* analysis revealed that VEGF-treatment led to a significant increase in the percentage of BM-HPCs (Lin⁻ Sca-1⁺ cells) in the S/G₂/M proliferative phase of the cell-cycle (Figure 6c). In direct contrast, VEGF-treatment did not alter the percentage of CD34⁺ VEGFR2⁺ EPCs in the S/G₂/M phase of the cell-cycle (Figure 6d). Moreover, whilst VEGF has the capacity, as a primary growth factor, to alter the cell cycle status of HPCs (Gerber et al., 2002), we show, as previously described, that G-CSF does not stimulate entry of either HPCs (Figure 6c) or EPCs (Figure 6d) into the S/G₂/M phase of the cell cycle (McKinstry et al., 1997; Nicola et al., 1985; Colvin et al., 2007),

However, as shown by others G-CSF does promote expansion and differentiation of granulocytes as evident by the increased numbers of neutrophils in the bone marrow (Figure 5f) (McKinstry et al., 1997; Lord et al., 1989; 1991).

HPCs are known to express VEGFR1, therefore we next investigated whether the effect of VEGF in stimulating the entry of HPCs into the cell cycle *in vivo* was mediated via this receptor (Supplementary figure 1). We show here that *in vivo* administration of an antibody directed against VEGFR1 completely suppressed the VEGF-stimulated entry of HPCs into the cell cycle (Figure 6e).

VEGFR1 antagonism reverses VEGF-induced suppression of HPC mobilization in response to a CXCR4 antagonist.

Since activation of VEGFR1 was demonstrated to lead to an accumulation of Lin⁻ Sca-1⁺ HPCs within the proliferative phase of the cell cycle (Figure 6e). We next determined whether blocking of VEGFR1 *in vivo* would reverse VEGF-induced suppression of HPC mobilization in response to a CXCR4 antagonist. We show that administration of a VEGFR1 blocking mAb to VEGF-treated mice, completely restored the ability of the CXCR4 antagonist to mobilize HPCs from the bone marrow (P<0.001, Figure 7a). In contrast, anti-VEGFR1 antibody administration did not affect VEGF-induced mobilization of EPCs (Figure 7b). This data indicate that VEGF acting via VEGFR1 stimulates the entry of HPCs into the cell cycle and blocks their mobilization from the bone marrow, while effects of VEGF on VEGFR2 stimulate the mobilization of EPCs. Hence the differential effects of VEGF in regulating HPC and EPC mobilization are due to actions via distinct receptors.

Discussion

We show here, for the first time, that the mobilization of progenitor cell subsets, are differentially regulated by growth factors affecting their retention and cell cycle status. Specifically we show that whilst maximal mobilization of HPCs occurs when the CXCR4 antagonist is administered to mice pre-treated with G-CSF, under these conditions, EPC mobilization is sub-maximal and SPCs are not mobilized. In contrast, when mice are pre-treated with VEGF, the acute administration of a CXCR4 antagonist stimulates mobilization of EPCs and SPCs while suppressing HPC and neutrophil mobilization. As such, the profile of progenitor cells in the blood changes dramatically dependent on the treatment protocol.

The retention of HPCs and mature neutrophils within the bone marrow is dependent on the SDF-1 α /CXCR4 chemokine axis. Thus, mechanisms that disrupt this axis promote the egress of HPCs and neutrophils (Martin et al., 2003; Levesque et al., 2003). Consistent with previous reports, we show here, that G-CSF mobilizes both neutrophils and HPCs, by reducing their expression of CXCR4 and decreasing levels of SDF-1 α within the BM (Levesque et al., 2003; Wengner et al., 2008). In contrast, G-CSF alone mobilized only modest numbers of EPCs and no additive or synergistic effects were observed when mice pre-treated with G-CSF were administered the CXCR4 antagonist. There are no reports that EPCs express G-CSF receptors. Further, in this study G-CSF had no effect on the expression of CXCR4 by EPCs. This data suggests that G-CSF does not have a direct effect on EPCs, which may explain why mobilization of EPCs with the CXCR4 antagonist could not be enhanced by pre-treatment of mice with G-CSF.

Unexpectedly, pre-treatment of mice with VEGF suppressed the mobilization of both neutrophils and HPCs in response to the CXCR4 antagonist. This was not due to a

change in CXCR4 expression by these cells. HPCs mobilized by G-CSF have been found to be exclusively in the G₀/G₁ phase of cell cycle, whereas HPCs remaining in the bone marrow are actively cycling (Bowie et al., 2006; Uchida et al., 1997; Roberts et al., 1995). This may be explained by the fact that proliferating HPCs cannot migrate, a necessary step in their mobilization from the bone marrow. Here we show, for the first time, that the exogenous administration of VEGF to naïve mice stimulates the entry of HPCs into the S/G₂/M phase of the cell cycle *in vivo* and thereby severely impairs the migratory capacity of these cells, *in vitro*. Furthermore, as a consequence of their inability to migrate, the mobilization of HPCs by the CXCR4 antagonist is completely abrogated in mice treated with VEGF. It has previously been proposed that there are two pools of HPCs, a quiescent dormant reserve of HPCs residing in the endosteal niche that accounts for approximately one third of the HPCs and a 'mobilizable' pool of HPCs, that have the potential to proliferate, residing in the vascular niche adjacent to the sinusoidal endothelium (Wilson et al., 2007). We propose that in stimulating HPCs in the vascular niche to enter the cell cycle VEGF has a profound effect on HPC mobilization.

It has previously been shown that *in vivo* treatment of immuno-suppressed mice with a blocking mAb to VEGFR1 reduces cell cycling of HPCs and the survival of HSCs following engraftment (Gerber et al., 2002 Hattori et al., 2002). This suggests that in this model endogenous VEGF acting via VEGFR1, expressed by HPCs, can regulate the proliferation/survival of HPCs/HSCs (Gerber et al., 2002). In this study we show that that selective blockade of VEGFR1 abolishes the ability of exogenous VEGF to stimulate HPC entry into the cell cycle *in vivo* and restores the ability of the CXCR4 antagonists to mobilize HPCs from the bone marrow.

Interestingly, the mobilization of neutrophils in response to both the CXCR4 antagonist and the chemokine, KC, was dramatically inhibited in mice pretreated with VEGF. This

is not due to an effect on cell proliferation as these are terminally differentiated cells, indeed neutrophils harvested from the bone marrow of VEGF-treated mice migrated normally in response to chemokines. This suggests that VEGF may affect egress of mature cells from the bone marrow by other, as yet identified mechanisms. Tissue specific expression of VEGF has been shown to induce the perivascular expression of SDF-1 α (Grunewald *et al.*, 2006). It is possible, therefore, that neutrophils may be retained within the vascular niche of the bone marrow following chronic VEGF-treatment due to locally increased SDF-1 α expression, acting to re-enforce the retention of neutrophils via the CXCR4/SDF-1 α axis (Martin *et al* 2003).

VEGF stimulates the migration and promotes survival of endothelial cells via signals emanating from VEGFR2 (Gerber *et al.*, 1998). Interestingly, we observed that VEGF-treatment of mice did not stimulate the proliferation of VEGFR2⁺/CD34⁺ EPCs and as such EPCs in the bone marrow of these mice retained their ability to migrate towards chemokines *in vitro* and to be mobilized in response to CXCR4 antagonists *in vivo*. We show here that, anti-VEGFR1 antibody administration did not affect VEGF-induced mobilization of EPCs, indicating that EPC mobilization stimulated by VEGF is indeed mediated via VEGFR2. Hence the differential effects of VEGF in regulating HPC and EPC mobilization are mediated via distinct receptors, VEGFR1 on HPC and VEGFR2 on EPCs. The ability of VEGF to differentially promote the cell cycling of HPCs as compared to EPCs, explains why EPCs can be selectively mobilized by the CXCR4 antagonist in mice treated over 4 days with VEGF. Consistent with this data is the finding that acute administration of VEGF alone or in combination with the CXCR4 antagonist did not selectively mobilize EPCs from the bone marrow. Mechanistic differences between acute and chronic VEGF exposure are unknown, however acute administration of high doses of VEGF, delivered by adenovirus, is associated with

capillary leakiness which may facilitate the escape of progenitor cells from the BM (Moore et al., 2001). Indeed adVEGF has been shown to induce the non-selective mobilization of myeloid cells, HPCs, and EPCs, as observed here with acute VEGF administration (Hattori et al., 2001).

The G-CSF-treatment protocol used here, has been evaluated to promote cardiac regeneration in patients with MI (Orlic et al., 2001; 2001a; Kocker et al., 2001; Powell et al., 2005; Zolnhofer et al., 2006; Ince et al., 2005; Ripa et al., 2006; Ellis et al., 2006; Hill et al., 2005). These trials have been disappointing with only 1 out of 5 trials showing any significant clinical benefit (Zolnhofer et al., 2006; Ince et al., 2005; Ripa et al., 2006; Ellis et al., 2006; Hill et al., 2005). As we reveal here, such limited clinical success might be because G-CSF administration is not a particularly efficacious regime for mobilizing EPCs or SPCs which may be important for cardiac regeneration. Indeed, we have shown that G-CSF treatment actually decreases the number of EPCs in bone marrow, thus depleting the available reservoir for tissue recruitment and revascularization. Further, G-CSF also stimulates neutrophil mobilization, which may exacerbate the inflammatory response and negatively impact cardiac regeneration (Hill et al., 2005; Kang et al., 2004; Zernecke et al., 2008). In this study we have identified a treatment protocol that selectively mobilizes EPCs and SPCs, but not HPCs or neutrophils, from the bone marrow. Future studies will investigate the therapeutic efficacy of this mobilizing regime for cardiac regeneration following myocardial infarction.

Taken together, the results presented here indicate that different factors and molecular mechanisms regulate the mobilization of discrete populations of progenitor cells from the bone marrow. This has far reaching implications for our understanding

of mechanisms regulating the selective recruitment of different populations of progenitor cells in disease and the development of therapeutic strategies to mobilize specific sub-populations for regenerative medicine.

Figure Legends

Figure 1: G-CSF and VEGF pre-treatment differentially regulate circulation of progenitor cells in response to a CXCR4 antagonist.

a, Experimental protocol. Mice were pre-treated with G-CSF, VEGF, or vehicle (PBS) once daily for 4 days (100µg/kg i.p.). 24 hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p, closed bars) or vehicle (PBS, open bars). 60 minutes later, blood was taken for analysis of circulating: **b**, CFU-HPC; **c**, CFU-EPC; and **d**, CFU-SPC. CFU-HPC, CFU-EPC, and CFU-SPC are shown as number of colonies per ml blood. n=5-8 mice per group. Data: means± s.e.m. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$ between selected groups.

Figure 2: G-CSF and VEGF pre-treatment differentially regulate mobilization of progenitor cells in response to a CXCR4 antagonist.

a, overview of procedures set up for identifying the direct mobilization of progenitor cells from the femoral bone marrow. Mice were pre-treated with G-CSF, VEGF, or vehicle (PBS) once daily for 4 days (100µg/kg i.p.). 24 hours after the last injection, mice underwent perfusion of the right hind limb. CXCR4 antagonist (AMD3100 0.1mM, closed bars) or vehicle (PBS, open bars) were then infused directly into the femoral artery for 10 minutes. The hind limb was then perfused for a further 50 minutes to allow collection of **b**, CFU-HPC; **c**, CFU-EPC; or **d**, CFU-SPC; via the femoral vein. CFU-HPC, CFU-EPC, and CFU-SPC are shown as total number of colonies collected in the perfusate. n=5-8 mice per group. Data: means± s.e.m. *: $P<0.05$, **: $P<0.01$, ***: $P<0.001$ between selected groups.

Figure 3: VEGF-treatment does not alter the number of progenitor cells residing in the bone marrow, or the morphology of the bone marrow.

Mice were treated with G-CSF, VEGF, or vehicle (PBS), administered once daily for 4 days (100µg/kg i.p.). 24 hours after the last injection, bone marrow was aspirated for the enumeration of **a**, CFU-HPC bone marrow frequency; **b**, CFU-EPC bone marrow frequency; and **c**, CFU-SPC bone marrow frequency. Femurs from other mice were fixed *in situ* and processed for: **d**, bone marrow morphology of vehicle and VEGF-treated mice, at x20, and x60 objective. n= 4-8 per group. Data: mean± s.e.m. *: $P<0.05$, ***: $P<0.001$ between selected groups.

Figure 4: VEGF-treatment does not alter the expression of CXCR4 on HPCs or EPCs. However, VEGF suppress bone marrow-derived HPC chemotaxis towards SDF-1α

Mice were treated with G-CSF, VEGF, or vehicle (PBS), administered once daily for 4 days (100µg/kg i.p.). Bone marrow was aspirated on day 5 and harvested cells were used to determine **a**, SDF-1α levels in bone marrow supernatant as determined by

ELISA; **b**, % of bone marrow Lin⁻ Sca-1⁺ cells expressing CXCR4; and **c**, % of bone marrow CD34⁺ VEGFR2⁺ cells expressing CXCR4. Harvested cells were also utilized in the following chemotaxis assays: **d**, number of HPCs; and **e**, EPCs undergoing chemotaxis towards 30nM SDF-1 α . Cells taken from bone marrow of mice treated with G-CSF (closed bars) or vehicle (PBS, open bars). **f**, number of HPCs; and **g**, EPCs undergoing chemotaxis towards 30nM SDF-1 α . Cells taken from bone marrow of mice treated with VEGF(closed bars) or vehicle (PBS, open bars). n= 4-8 per group. Data: mean $\pm$ s.e.m. *: $P<0.05$, ***: $P<0.001$ between selected groups.

Figure 5: VEGF pre-treatment suppresses neutrophil mobilization, but does not affect their ability to undergo chemotaxis.

Mice were pre-treated with G-CSF, VEGF, or vehicle (PBS), administered once daily for 4 days (100 μ g/kg i.p.). 24 hours later these mice underwent perfusion of the right hind limb. CXCR4 antagonist (AMD3100 0.1mM, closed bars) or vehicle (PBS, open bars) were then infused directly into the femoral artery for 10 minutes. The hind limb was then perfused for a further 50 minutes to allow collection of **a**, neutrophils via the femoral vein. In other perfusion experiments, CXCR4 antagonist or PBS were infused in addition to VEGF (50nM), G-CSF (50nM), or vehicle (PBS) into the femoral artery of naïve mice for 10 minutes. The hind limb was again perfused for a further 50 minutes to allow **b**, collection of neutrophils. In some groups of mice pre-treated with VEGF over 4 days, KC (30 μ g/kg i.v.) was administered 24 hours after the last injection of VEGF and **c**, circulating neutrophil numbers (determined by blood smears) were counted 60 minutes after administration of KC (closed bars) or vehicle (PBS, open bars). **d**, % of Gr-1^{high} neutrophils expressing CXCR2; and **e**, Neutrophil chemotaxis towards KC (30nM) of neutrophils taken from bone marrow of mice pre-treated with VEGF, or vehicle (PBS). **f**, leukocyte counts of bone marrow taken from mice pre-treated with VEGF, G-CSF or vehicle (PBS). n=4-8 mice per group. Data: mean $\pm$ s.e.m. *: $P<0.05$, ***: $P<0.001$ between selected groups.

Figure 6: VEGF pre-treatment, acting via VEGFR1 enhances HPC cell cycling compared to EPCs.

a, number of cells per colony; and **b**, mean colony area; of CFU-HPC grown for 12 days in methocult. Cells were taken from bone marrow of mice treated (once daily for 4 days) with G-CSF, VEGF, or vehicle (PBS) (100 μ g/kg i.p.). Representative histograms of PI stain of DNA content for Lin⁻ Sca-1⁺ HPCs, **c.i**, PBS-treated, **c.ii** VEGF-treated, **c.iii** G-CSF-treated, and **c.iv**, % of Lin⁻ Sca-1⁺ HPCs residing in S/G₂/M phase of the cell-cycle. Representative histograms of PI stain of DNA content for CD34⁺ VEGFR2⁺ EPCs **d.i**, PBS-treated, **d.ii** VEGF-treated, **d.iii** G-CSF-treated, and **d.iv**, % of CD34⁺ VEGFR2⁺ EPCs residing in S/G₂/M phase of the cell-cycle. In another experiment, mice were treated with anti-VEGFR1 antibody (2.5mg/kg) or control IgG on days 1 and 3, 30 minutes before VEGF administration. Representative histograms of PI stain of DNA content for Lin⁻ Sca-1⁺ HPCs, **e.i**, PBS-treated, **e.ii** VEGF-treated + anti-IgG, **e.iii** VEGF-treated + anti-VEGFR1 antibody, and **e.iv**, % of Lin⁻ Sca-1⁺ HPCs residing in S/G₂/M phase of the cell-cycle. n=4- 7 per group. Data: mean $\pm$ s.e.m. *: $P<0.05$, **: $P<0.01$ between selected groups.

Figure 7: VEGFR1 blockade reverses VEGF-induced suppression of HPC mobilization in response to a CXCR4 antagonist.

Mice were pre-treated with VEGF, or vehicle (PBS) once daily for 4 days (100µg/kg i.p.). Mice pre-treated with VEGF were additionally administered control IgG or anti-VEGFR1 antibody (2.5mg/kg) on days 1 and 3, 30 minutes before VEGF administration. 24 hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100 5mg/kg i.p, closed bars) or vehicle (PBS, open bars). 60 minutes later, blood was taken for analysis of circulating: **a**, CFU-HPC; **b**, CFU-EPC. **c**, Representation of the divergent roles of G-CSF and VEGF treatment on HPC and EPC status in bone marrow. n= 4 per group. Data: mean± s.e.m. *: $P<0.05$, ***: $P<0.001$ between selected groups.

Experimental Procedures.

Reagents

Recombinant murine chemokines and growth factors KC/CXCL1, SDF-1α/CXCL12, G-CSF, and VEGF were from PeproTech (London, U.K.). For *in vivo* blocking experiments, anti-murine polyclonal VEGFR1 antibody (AF471) was purchased from RnD Systems (Abingdon, UK). Control rat IgG was purchased from Jackson ImmunoResearch (Newmarket U.K.) The CXCR4 antagonist AMD3100, propidium iodide (PI), Triton X, and RNase were purchased from Sigma Aldrich (Poole, U.K.). Dulbecco's Modified Eagles medium (DMEM) was purchased from Life Sciences (Paisley, U.K.). Methocult and supplements was obtained from StemCell Technologies (Vancouver, Canada). EGM-2 basal media and supplements were purchased from Lonza (Wokingham, U.K.). 3µm pore size transwell chemotaxis plates were purchased from Neuroprobe (Gaithersburg U.S.A.). 5µm pore size transwell chemotaxis inserts were purchased from Corning (New York U.S.A). Anti-human/mouse CXCL12/SDF-1 antibody, biotinylated anti-human/mouse CXCL12/SDF-1 antibody, and streptavidin-HRP for ELISA were purchased from RnD Systems (Abingdon, U.K.). Rat anti-mouse CD34-FITC antibody (IgG2a) was purchased from AbD Serotec (Nottingham U.K.). Rat anti-mouse Sca-1-APC (Ly-6A/E) antibody (IgG2a) and rat anti-mouse VEGFR2-APC (Flk-1) antibody (IgG2a) were purchased from eBioscience (Wembley U.K.). Rat

anti-mouse CD11b-FITC antibody (IgG2b), Rat anti-mouse Gr-1-FITC (Ly-6G and Ly-6C) antibody (IgG2b), rat anti-mouse CD45R/B220-FITC antibody (IgG2a), rat anti-mouse CD3e-FITC antibody (IgG2a), rat anti-mouse TER119-FITC antibody (IgG2b), rat anti-mouse CXCR4-PE antibody (IgG2b) were purchased from BD Pharmingen (Oxford U.K.). Rat anti-mouse CXCR2-APC antibody (IgG2a) was purchased from RnD Systems (Abingdon, U.K.). Rat anti-mouse CD16/CD32 Fc-block (IgG2b) was purchased from BD Pharmingen (Oxford U.K.). The following isotype control antibodies were purchased: rat IgG2a-FITC, IgG2b-FITC, and rat IgG2b-PE (BD Pharmingen, Oxford U.K.). Rat IgG2a-APC was purchased from RnD Systems (Abingdon, U.K.). For immunohistochemistry, the following antibodies were used: goat anti-mouse VEGFR1 antibody, rabbit anti-mouse VEGFR2 antibody, rat antimouse CD34 antibody, goat anti-mouse VE-Cadherin antibody were purchased from Santa-Cruz Biotech (Santa Cruz, U.S.A.). Rat anti-mouse CD45 antibody, rat anti-mouse CD29 antibody, rat anti-mouse CD105 antibody were purchased from BD Pharmingen (Oxford, U.K.). rat anti-mouse CD115 antibody was purchased from eBioscience (Wembley, U.K.). Rabbit anti-human vWF antibody was purchased from DAKO (Glostrup, Denmark). The following isotype controls were used: Rat IgG1 from BD Pharmingen (Oxford, U.K.), goat IgG and rabbit IgG from Santa-Cruz Biotech (Santa Cruz, U.S.A.). The following fluorescently labeled secondary antibodies were used: donkey anti-goat Alexafluor 568 antibody, goat anti-rat Alexafluor 594 antibody, goat anti-rabbit Alexafluor 488 antibody were purchased from Molecular Probes (Paisley, U.K.)

Pre-treatment with G-CSF or VEGF

Female BALB/c mice were purchased from Harlan (Oxford, United Kingdom). Mice, at the age of 8-10 weeks were administered VEGF (2.5 µg/mouse i.p.), G-CSF (2.5

µg/mouse i.p.), or vehicle on 4 consecutive days. 24 hours after the last injection, mice were administered a CXCR4 antagonist (AMD3100, 5mg/kg i.p), KC (30µg/kg i.v.) or vehicle and blood was collected via cardiac puncture 60 minutes later for enumeration of circulating leukocyte, HPC, EPC, and stromal progenitor cell levels. In other experiments, mice pre-treated with G-CSF or VEGF were used for *in situ* perfusion of the mouse hind limb as explained below. All studies were carried out under the United Kingdom's Animals (Scientific Procedures) Act of 1986 and local ethical approval from Imperial College, London.

Administration of anti-VEGFR1 antibody

Mice were administered anti-VEGFR1 or control IgG (2.5mg/kg i.p. Ohki *et al.*, 2005) 30 minutes before VEGF administration on days 1 and 3 of the 4 day VEGF-treatment protocol.

In situ perfusion of mouse hind limb

On day 5 following pre-treatment of growth factors, mice were anaesthetized and the femoral artery and vein exposed. The hind limb was isolated by occlusion of the external iliac artery, superficial epigastric and muscular branch. Polyethylene cannulae (Portex, London, UK) were immediately inserted into the femoral artery and vein as previously described (Martin *et al.*, 2003; 2006; Wengner *et al.*, 2008). Perfusion buffer was infused via the arterial cannula and removed from the venous cannula using a Minipuls peristaltic pump (Anachem, Luton, UK). The hind limb was perfused for an initial 2 minutes to remove remaining blood from the vasculature and then perfused for a further 60 min with vehicle alone or the CXCR4 antagonist (AMD3100, 0.1mM), infused over the first 10 minutes using an infusion/withdrawal pump (Harvard Instruments, U.K.).

In some studies the effects of acute administration of G-CSF or VEGF on HPC, EPC, and stromal progenitor cell mobilization were examined. G-CSF or VEGF (50nM) were added to the perfusion buffer for an initial 10 minutes.

Analysis of mobilized cells from the bone marrow in blood and perfusate.

The perfusate was centrifuged and re-suspended in DMEM +20%FBS. In some experiments citrated blood was obtained via cardiac bleed and lysed for red blood cells. Bone marrow and perfusate cytopins, and blood smears were stained using DiffQuik for the enumeration of mononuclear cells, eosinophils, and neutrophils. Bone marrow, perfusate and lysed blood were then used for assays outlined below to enumerate HPCs (CFU-HPC), EPCs (CFU-EPC), SPCs (CFU-F).

CFU-HPC assay

5×10^4 cells were added to Methocult medium (Stem cell technologies) supplemented with specific cytokines and growth factors to enable formation of CFU-HPC. Mobilized cells were incubated for 11 days before quantification. Further characteristics of CFU-HPC colonies were obtained on day 12, namely the mean number of cells in each colony, and the mean colony area. The mean colony area (mm^2) was determined by image analysis using Scion Image analysis program (NIH). The cell number was determined by diluting colonies grown in Methocult into DMEM (to disperse the methylcellulose) and aspirating the colonies into a single cell suspension before obtaining a cell count. Cells taken from HPC colonies and immunostained showed CD115, CD34, CD45, VEGFR1 positive cells, but were negative for VEGFR2, VE-Cadherin, and von Willebrand Factor (vWF) (Supplementary Figure 1)

CFU-EPC assay

5×10^5 cells were added to EPC colony media (EGM-2 basal media + supplements, and additional VEGF: 60 μ g/L, and FBS: 16% final concentration) on fibronectin-coated plates. Dishes were incubated for 7 days before media was changed, and then incubated for a further 14 days before the enumeration of EPC colonies (CFU-EPC). These “late outgrowth” EPC colonies exhibited a cobblestone morphology and by immunohistochemical analysis (Protocol: Supplementary Methods section) expressed CD34, VEGFR2, VE-Cadherin, and vWF (Supplementary Figure 1) stained positively with GS-lectin and were able to uptake acetyl-LDL (Supplementary Figure 2a), as reported by others (Nolan et al., 2007; Yoder et al., 2007; Hur et al., 2004). Importantly, ‘late outgrowth’ CFU-EPC did not express CD115, CD14 or CD45 (Supplementary Figure 1), and are therefore not of a monocyte-macrophage lineage (Hirschi et al., 2008). Moreover, CFU-EPC were able to form tubules in an *in vitro* EC Matrix angiogenesis assay (See Supplementary Methods section) whereby complete mesh-like structures formed after 18 hours in culture (Supplementary Figure 2b-d).

CFU-SPC (CFU-F) assay

5×10^5 cells were added to Mesencult media including supplements (Stemcell Technologies). Dishes were incubated for 7 days before media was changed, and then incubated for a further 14 days before the enumeration of SPC colonies (CFU-F). Plastic adherent bone marrow derived SPCs were shown to express CD29 and CD105 and were negative for CD45 and CD34 (Supplementary Figure 4a) as demonstrated in previous studies on stromal progenitor cells (Phinney et al., 1999; Pittenger et al., 1999). Furthermore, expanded SPC colonies were assessed for their ability to differentiate into other cell types (Supplementary Methods Section), and were capable of mesenchymal tri-lineage differentiation into adipocytes (Supplementary

Figure 3c), osteocytes (Supplementary Figure 3d) and chondrocytes (Supplementary Figure 3e) These progenitor cells exhibit all the characteristics of murine mesenchymal stem cells (Pittenger et al., 1999)

The total number of colony forming units mobilized was calculated according to the number of colonies per plate x by the total number of leukocytes mobilized/ number of cells seeded.

Flow cytometry analysis of chemokine receptor expression on cells residing in bone marrow.

For flow cytometric analysis, cells were re-suspended in FACS buffer. To identify neutrophils in the bone marrow, surface expression of the granulocyte marker Ly-6G/Ly6C (Gr-1) was determined. Neutrophils were identified as Gr-1^{high} positive cells with a characteristic high side scatter profile. HPCs residing in the BM were selected as being negative for lineage (Lin⁻) markers: CD3e, CD11b, CD45R, Ly-76, and Gr-1 and positive for Sca-1 (Lin⁻ Sca-1⁺ cells) (Gerber et al., 2002; Uchida et al., 1997). EPCs residing in the BM were selected as being positive for both CD34 and VEGFR2 (CD34⁺ VEGFR2⁺ cells) (Asahara et al., 1997; Yoon et al., 2005). Furthermore, neutrophils were stained for the surface expression of CXCR2, whilst Lin⁻ Sca-1⁺ cells and CD34⁺ VEGFR2⁺ cells were stained for the cell surface expression of CXCR4. Samples were then washed and then quantified on a FACS Aria cell sorter (BD Biosciences, Oxford, United Kingdom) and analysed using FACSDiva software.

PI staining to determine cell cycle status

Lin⁻ Sca-1⁺ cells and CD34⁺ VEGFR2⁺ cells were analysed to determine their cell cycling status using a propidium iodide (PI) stain. Unfixed cells were initially stained as

explained above to identify their cell type before the addition of 50µL PI (100µg/ ml), and 50µL RNase (100µg/ ml) was added to minimise PI binding to RNA. 5 minutes before flow cytometric analysis, 50µL of 0.1% Triton X (in saline) was added to sample to permeabilize the cell membrane and allow DNA binding. Analysis of flow cytometry data of PI-stained cells was performed using FACSDiva software. A dotplot of PI-Width against PI-Area was recorded. Aggregates of cells were detected as cells having a larger PI-W profile and were not included in the gate. A PI-A histogram of the gated cells showed the first peak to represent cells in G₀/G₁, the second peak cells in G₂/M, and the in between area S phase cells. To calculate the percentage of cells undergoing active cell cycling, a marker was set to exclude the G₀/G₁ peak and the percentage of cells in S/ G₂/ M was calculated.

Bone marrow histology

Femoral bone marrow was fixed by infusion of 2.5% glutaraldehyde in a modified Krebs-Ringers buffer for 15 minutes using the *in situ* perfusion technique previously described (Martin et al., 2003; 2006; Wengner et al., 2008). The femur was removed and placed in fresh glutaraldehyde fixative before being processed and stained with toluidine blue.

In vitro chemotaxis assays of neutrophils, HPCs and EPCs

Bone marrow-derived neutrophils were tested for migration towards KC (30nM) using 3µm pore sized transwell chemotaxis plates as previously described (Wengner et al., 2008). To measure HPC and EPC migration towards SDF-1α, murine bone marrow cells (2x10⁶ cells) were placed in the upper chamber of transwell inserts (5µm pore size Corning). These in turn were placed in individual wells of a 24 well cell culture plate with 30nM SDF-1 α added. Chambers were incubated for 4 hours at 37°c and

migrated cells were then placed in methocult or EPC colony media as described above for the enumeration of CFU-HPC and CFU-EPC respectively.

SDF-1 α ELISA on bone marrow supernatants

Supernatant from bone marrow aspirates was analyzed for SDF-1 α content as per the recommended protocol for the capture and detection antibody (MAB350 and BAF310 respectively . RnD systems).

Statistical Analysis

Data are expressed as mean $\pm$ SEM. *In vivo* mobilization data and *in vitro* chemotaxis data were analysed using two-way analysis of variance (ANOVA), followed by Bonferroni multiple-comparisons test. Flow cytometry data of PI stain were analysed using one-way ANOVA, followed by Bonferroni multiple-comparisons test. Flow cytometry data of chemokine receptor expression were analysed using Student's T-test. All analyses were conducted using the GraphPad Prism statistical package (version 4.0; Graph Pad, San Diego, CA). *P* values less than 0.05 were considered significant.

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Supplementary Methods

Immunohistochemistry of HPC, EPC, and SPC colonies.

EPC and SPC colonies grown in chamber slides, and cytopins of cells from HPC colonies were fixed in ice cold methanol for 10 minutes, before washing in PBS + 1% BSA. Slides were blocked for 30 minutes with PBS+10%BSA. Slides were then stained with the following antibodies: goat anti-mouse VEGFR1, rabbit anti-mouse VEGFR2, rat anti-mouse CD34, goat anti-mouse VE-Cadherin, rat anti-mouse CD45, rat anti-mouse CD29, rat anti-mouse CD105, rat anti-mouse CD115, rabbit anti-human vWF, or goat anti-mouse CD14. The following isotype controls were used: Rat IgG1, goat IgG, and rabbit IgG. After 90 minutes, slides were washed and incubated with the following appropriate fluorescently labeled secondary antibodies: donkey anti-goat Alexafluor 568 antibody, goat anti-rat Alexafluor 594 antibody, goat anti-rabbit Alexafluor 488 antibody. After a further wash, slides were mounted onto coverslips using glycerol based mounting medium containing a 4',6-diamidino-2-phenylindole (DAPI) counterstain. Slides were analyzed using a fluorescent microscope (Leica, Germany).

Staining of EPC colonies with GS-Lectin and uptake of Ac-LDL

DIL-labeled Acetylated-low density lipoprotein (Ac-LDL) was added to EPC media (5µg/ml) and cells were incubated for 4 hours at 37°C. Colonies were then washed in PBS and fixed with 2% PFA for 10 minutes before incubation with fluorescein conjugated *griffonia-simplicifolia* (GS)-lectin (10µg/ml in PBS) for 60 minutes. Colonies were then analyzed using a fluorescent microscope (Leica, Germany).

In vitro angiogenesis assay

The ability of EPCs to form tubules was assessed using an in vitro angiogenesis assay (Madri & Pratt 1986; Salani et al., 2000). EC Matrix Gel solution and EC Matrix diluent buffer (Chemicon) were mixed in a ratio of 9:1 at 4°C. 50 µl was then added wells of a pre-cooled 96 well plate and this was then incubated at 37°C for 1 hour to allow the matrix solution to solidify. EPC colonies were harvested by trypsinization and re-suspended in EPC growth media at a density of 1×10^5 cells/ml and seeded (150 µl) onto the surface of the polymerized matrix. Cells were then incubated for 24 hours, and cellular network structures were fully developed by 12-18 hours.

Tri-lineage differentiation of SPC colonies

To induce differentiation of SPCs into adipogenic and osteogenic lineages appropriate differentiation media was added to SPC colonies. Basic differentiation media consisted of 10% FBS, penicillin/ streptomycin and fungizone. Osteogenic differentiation was induced by adding 50 µg/ml ascorbic acid-2-phosphate, 10nM dexamethasone and 10mM β -glycerol to basic media (Eslaminejad et al., 2006). Adipogenic differentiation was induced by adding 50 µg/ml indomethacine, 100nM dexamethasone and 10ng/ml insulin to basic media (Zuk et al., 2002). Control cultures were incubated in basic media. The differentiation media was replaced three times a week for a period of 20 days. To promote chondrogenic differentiation the micromass culture technique was used (Eslaminejad et al., 2006, Pevsner-Fischer et al., 2006, Pittenger et al., 1999). SPCs were detached by trypsinization and pelleted in a conical polypropylene tube. Chondrogenic differentiation media consisting of basic media supplemented with 10ng/ml TGF- β , 50nM ascorbic acid-2-phosphate and 6.25 µg/ml insulin was added to the cells (Pevsner-Fischer et al., 2006). The chondrogenic differentiation media was changed three times a week for a period of 2-3 weeks.

To confirm differentiation of SPCs to mesenchymal lineages, osteocytes, adipocytes and chondrocytes were stained with cell type specific dyes as reported in previous publications (Pittenger et al., 1999, Zuk et al., 2002). SPCs cultured in osteogenic media were stained with Alizarin red. This dye is used to demonstrate the presence of calcium. Interaction of Alizarin dye with calcium ions results in a bright red stain. SPCs cultured in adipogenic media were stained with Oil red O (Sigma-Aldrich) which is a fat soluble dye that stains triglycerides and lipids of fixed cells with a deep red colour. Cells cultured in chondro-inductive media were stained using Toluidine Blue, which stains the background blue (orthochromatic staining) and the areas of cartilage matrix red-purple (metachromatic staining).

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Supplementary Figure 1: Immunostaining of bone marrow-derived HPCs and EPCs.

Colonies of HPCs **a**, and EPCs **b**, derived from bone marrow cells, were stained for the expression of CD115 (red), CD34 (red), CD45 (red), VEGFR1 (red), VEGFR2 (green), VE-Cadherin (red), vWF (green). Nuclei were counterstained with DAPI (blue).

Supplementary Figure 2: GS-Lectin stain, AC-LDL uptake, and tubule formation of bone marrow derived EPCs.

a, CFU-EPC were stained for GS-Lectin (green) and their ability to uptake Ac-LDL (red). Resuspended EPCs were added to an angiogenesis supporting matrix gel for the evaluation of tubule formation. **b**, EPCs 1 hour after addition to matrix; **c**, tubule formation was apparent after 18 hours in culture, when luminal structures were evident, **d**.

Supplementary Figure 3: Immunostaining of mobilized EPCs.

Colonies of mobilized EPCs were stained for the expression of CD115 (red), CD14 (red), CD45 (red), CD34 (red), VEGFR1 (red), VEGFR2 (green), VE-Cadherin (red), and vWF (green). Nuclei were counterstained with DAPI (blue).

Supplementary Figure 4: Immunostaining of bone-marrow-derived SPCs, mobilized SPCs, and tri-lineage differentiation potential.

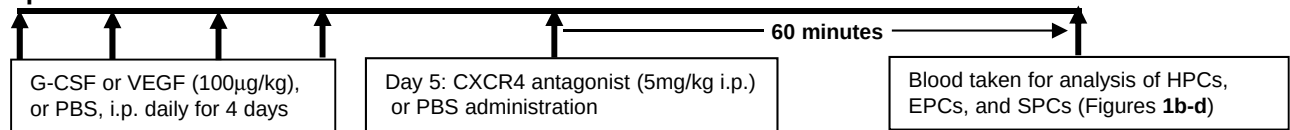
a, colonies of SPCs derived from bone marrow cells were stained for the expression of CD29 (red), Cd105 (red), CD45 (red), CD34 (red), VEGFR1 (red), VEGFR2 (green), and VE-Cadherin (red). **b**, colonies of mobilized SPCs were stained for the expression of CD29 (red), CD105 (red), CD45 (red), CD34 (red), VEGFR1 (red), VEGFR2 (green), VE-Cadherin (red), and vWF (green). Nuclei were counterstained with DAPI (blue). Expanded colonies subjected to specific differentiation media revealed SPCs were able to display a tri-lineage differentiation potential towards **c**, adipocytes (oil red O); **d**, osteocytes (Alizarin red); **e**, chondrocytes (toluidine blue).

Supplementary Figure 5: Acutely administered VEGF or G-CSF do not selectively modulate progenitor cell mobilization either alone, or in combination with a CXCR4 antagonist.

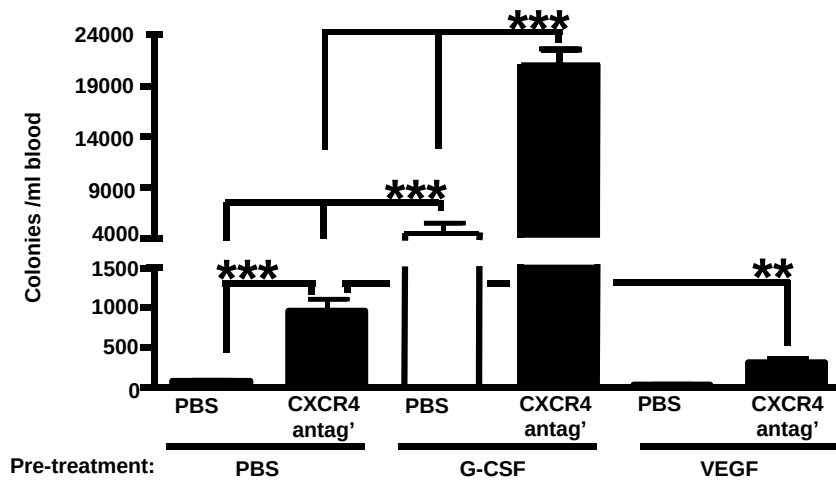
Mice underwent perfusion of the right hind limb. CXCR4 antagonist (AMD3100 0.1mM, closed bars) or vehicle (PBS, open bars) alone, or in combination with G-CSF (50nM), or VEGF (50nM) were then infused directly into the femoral artery for 10 minutes. The hind limb was then perfused for a further 50 minutes to allow collection of **a**, CFU-HPC; **b**, CFU-EPC; and **c**, CFU-SPC via the femoral vein. CFU-HPC, CFU-EPC, and CFU-SPC are shown as total number of colonies per perfusate. n=5-8 mice per group. Data: means $\pm$ s.e.m. **: $P < 0.01$, ***: $P < 0.001$ between selected groups.

Figure 1

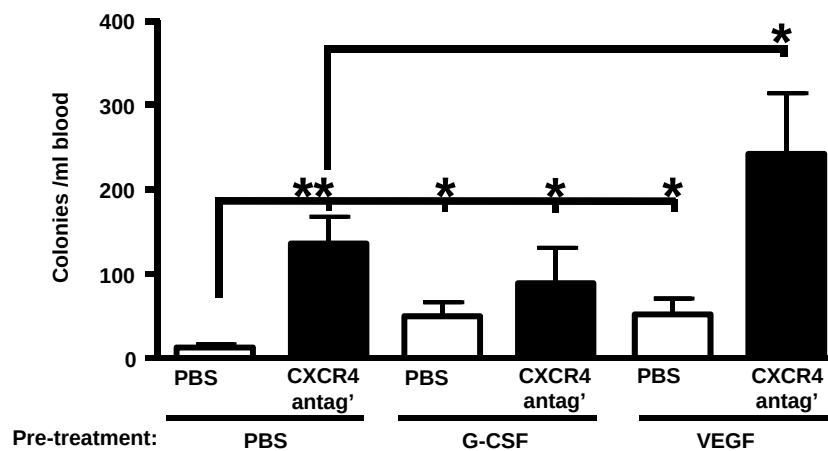
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b. HPCs



c. EPCs



d. SPCs

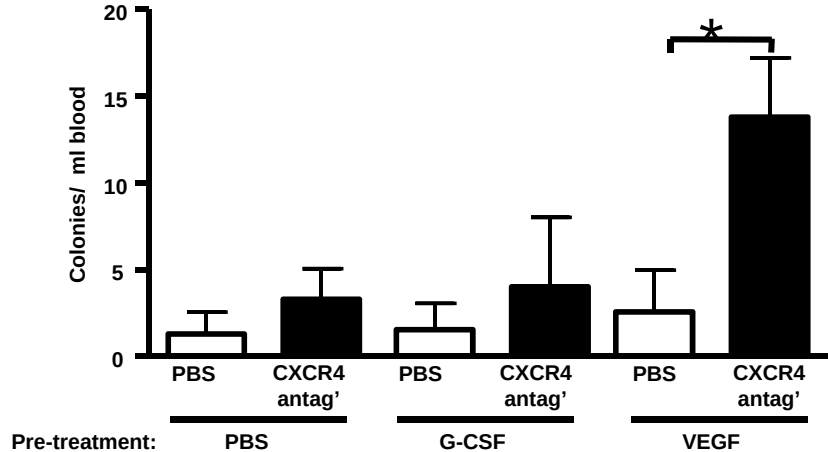
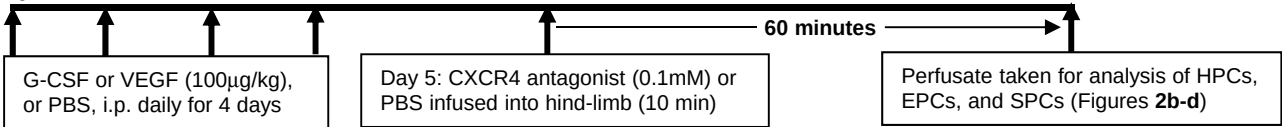
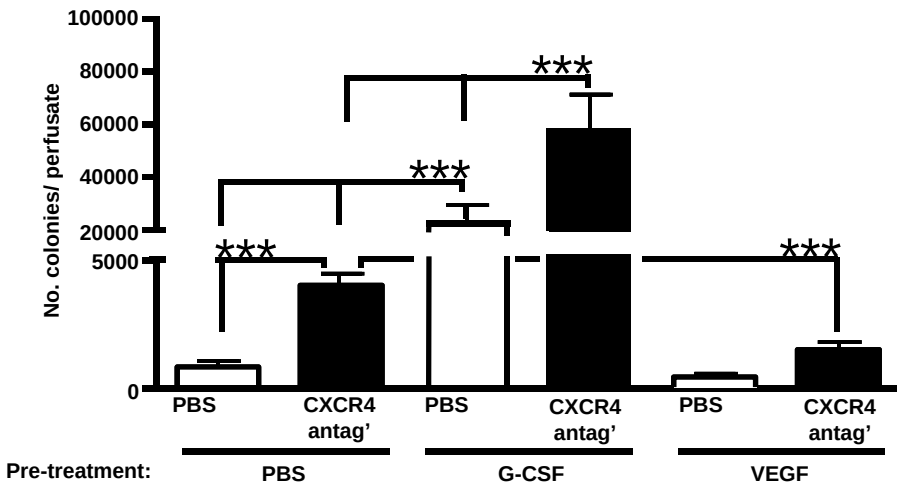


Figure 2

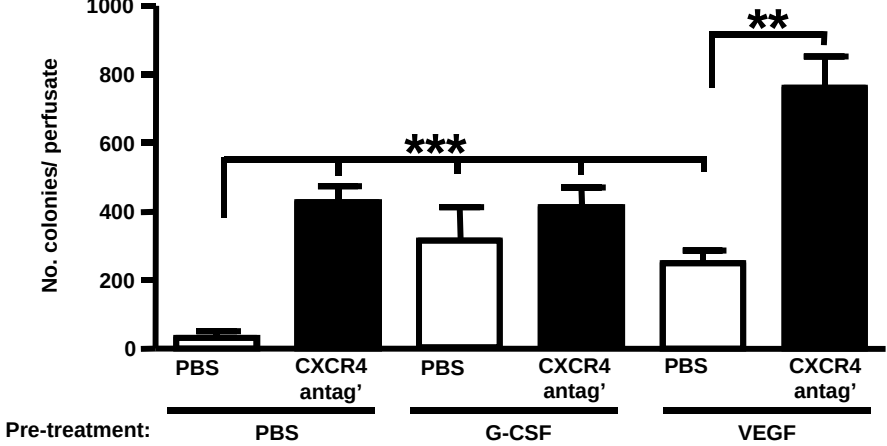
a. Experimental Protocol:



b. HPCs



c. EPCs



d. SPCs

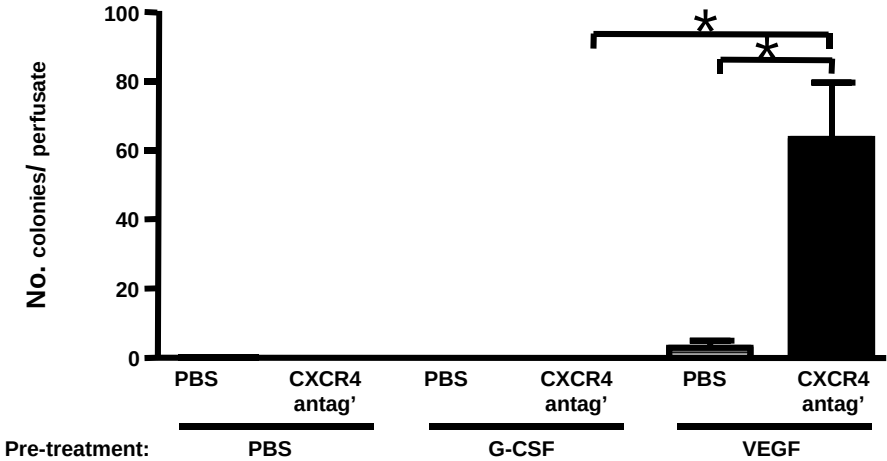
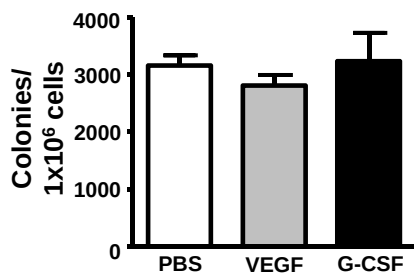
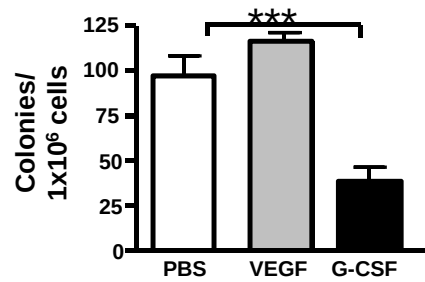


Figure 3.

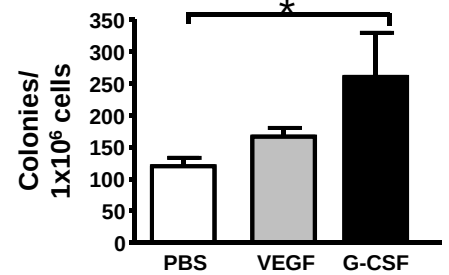
a. CFU-HPC



b. CFU-EPC

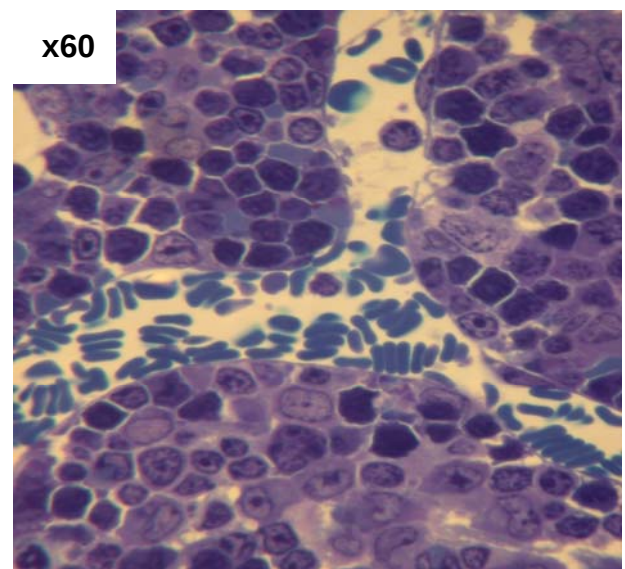
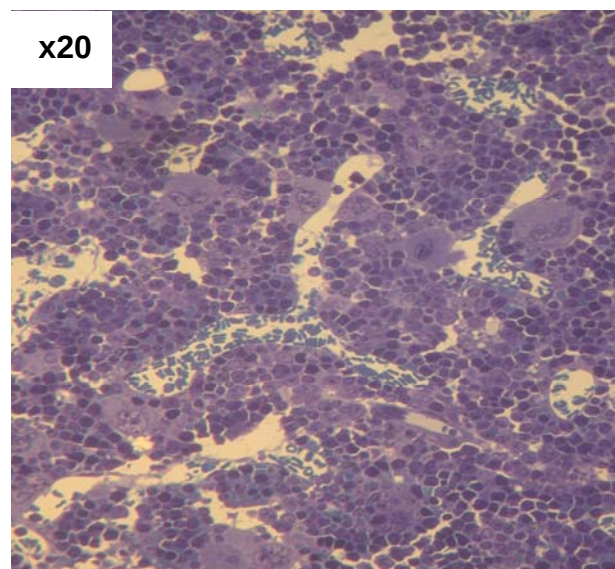


c. CFU-SPC



d.

BM from PBS treated mice:



BM from VEGF treated mice:

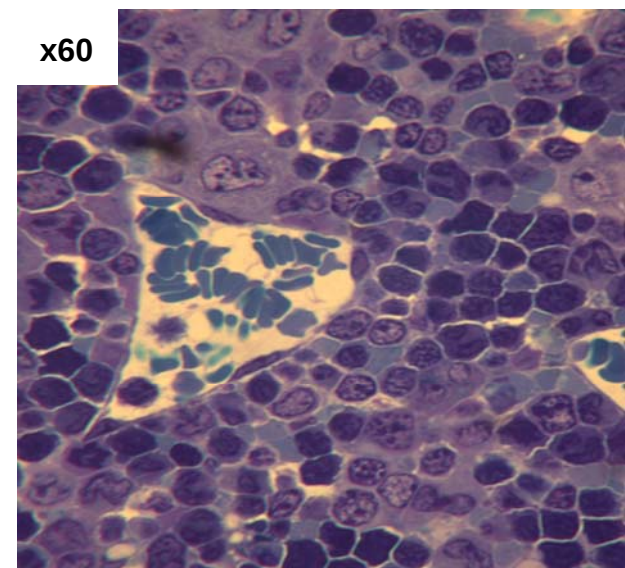
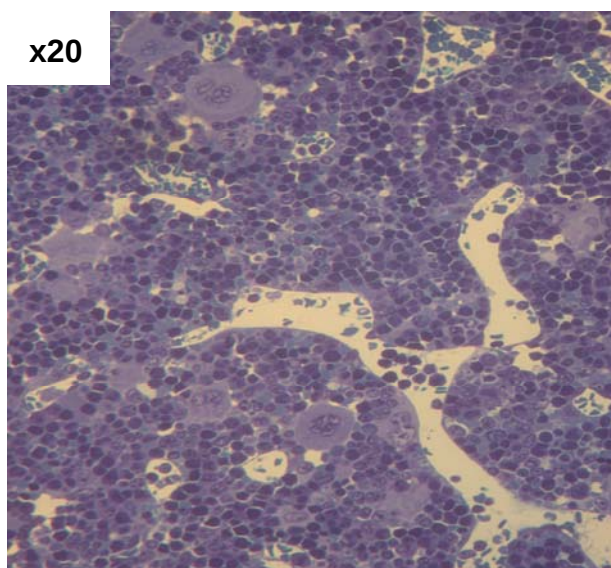
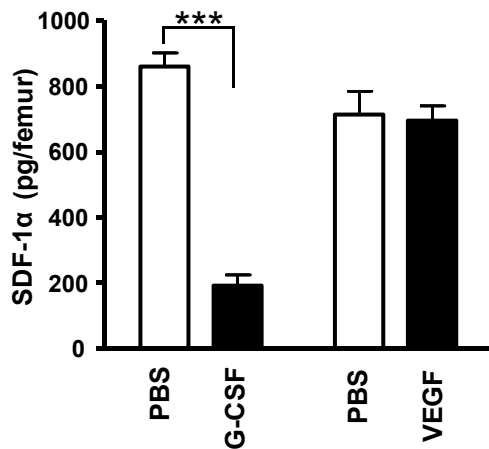
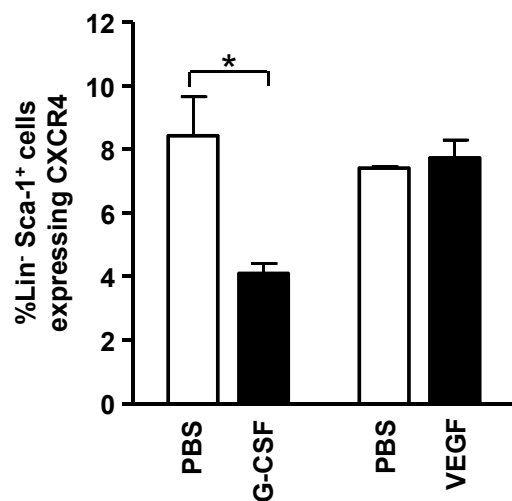


Figure 4.

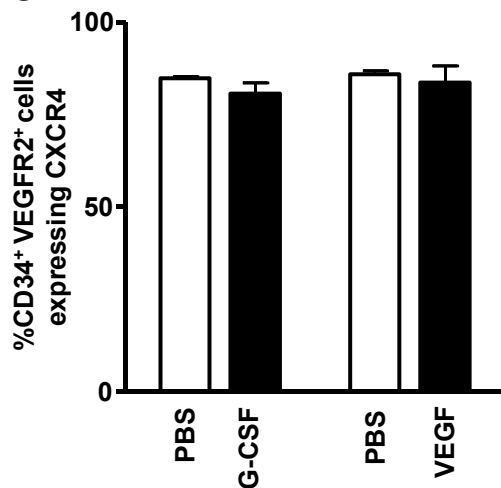
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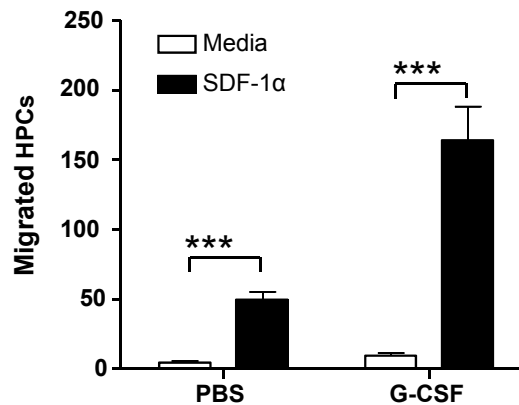
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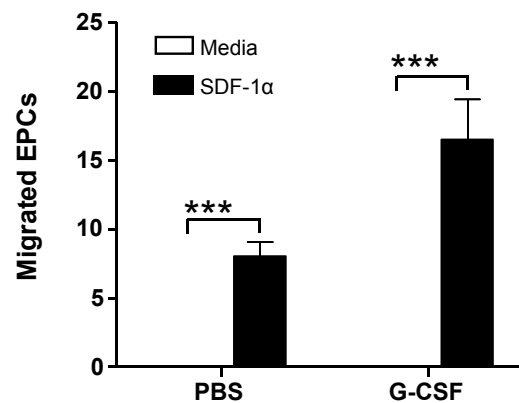
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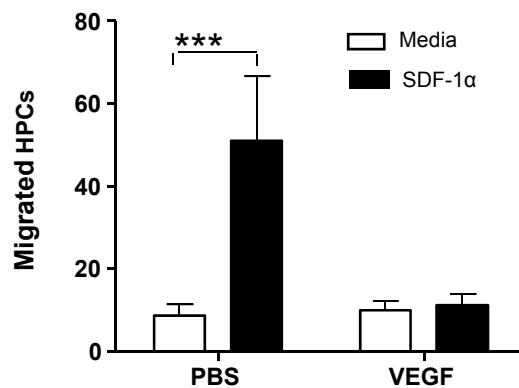
d. HPCs



e. EPCs



f. HPCs



g. EPCs

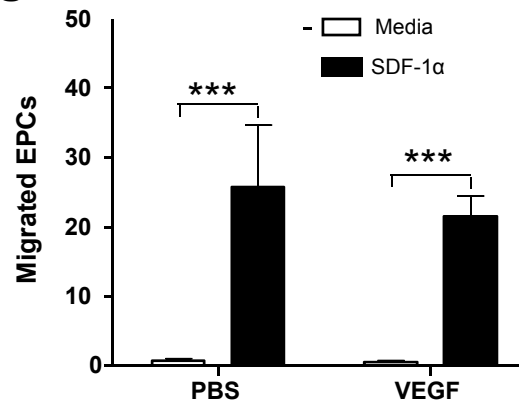


Figure 5.

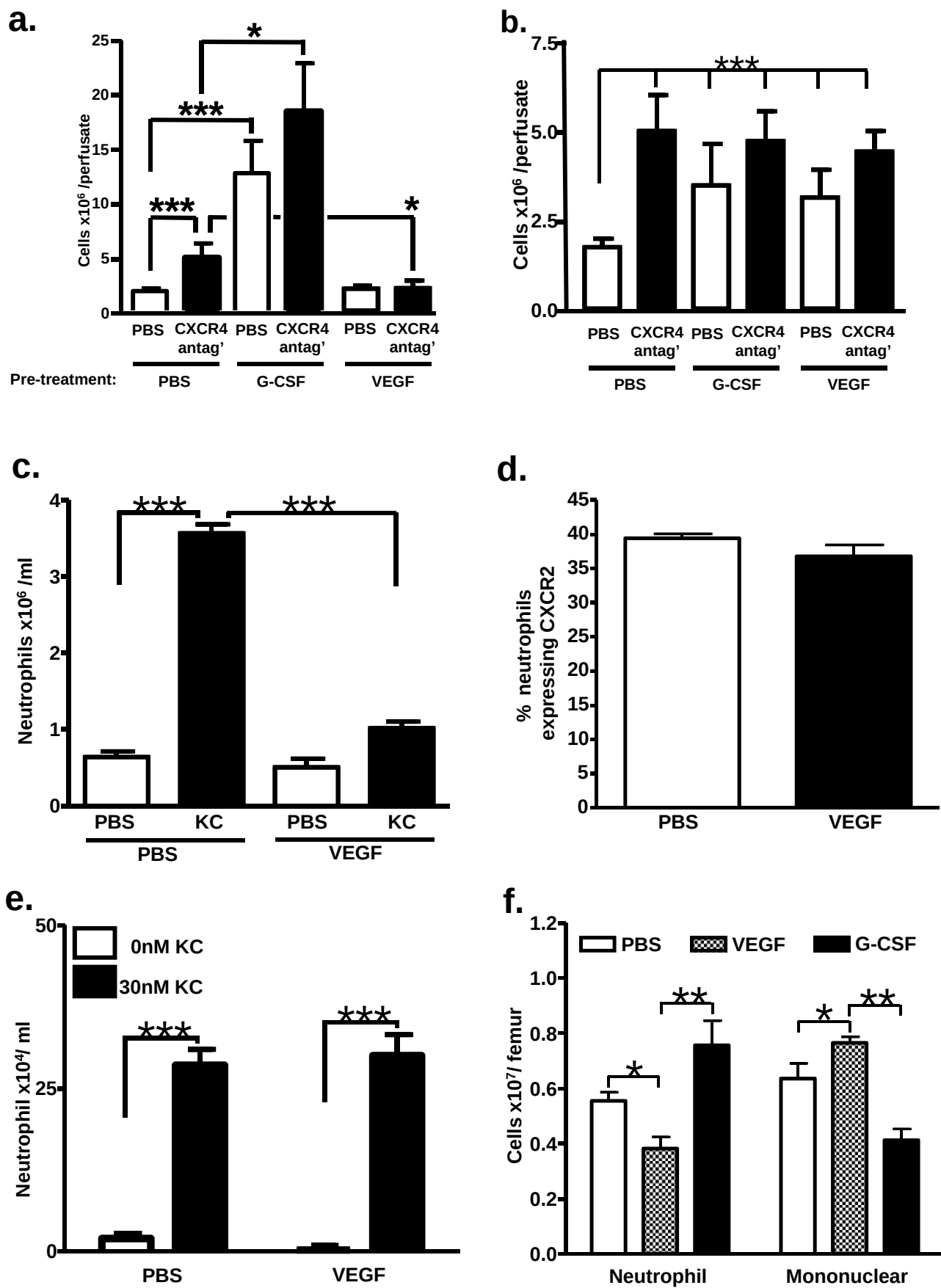
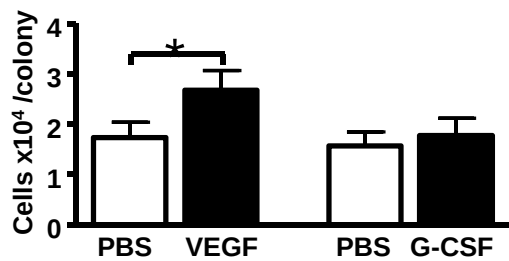
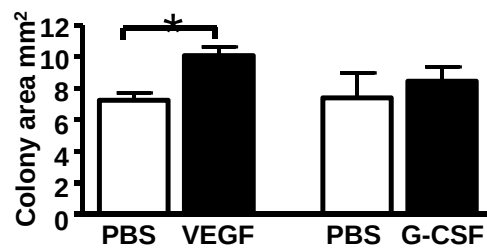


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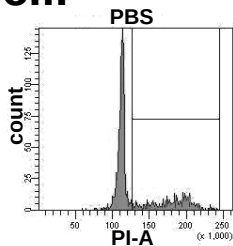
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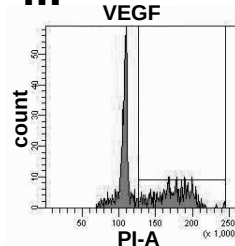
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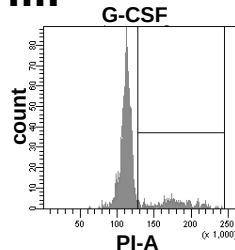
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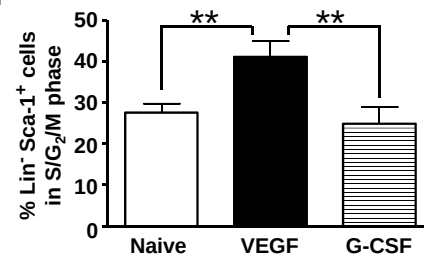
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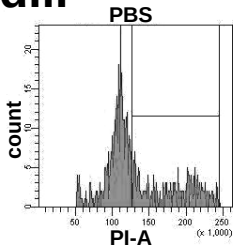
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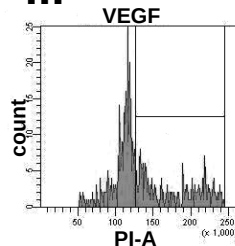
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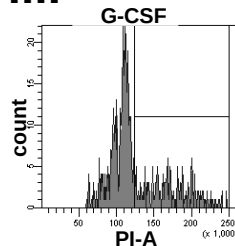
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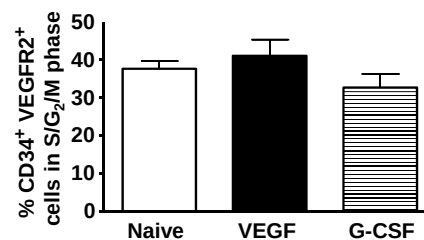
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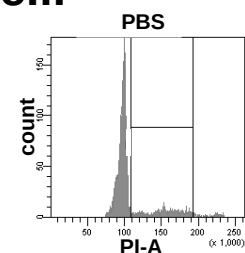
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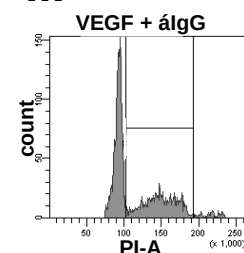
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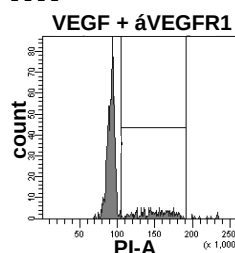
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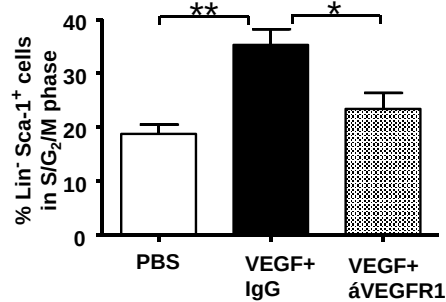
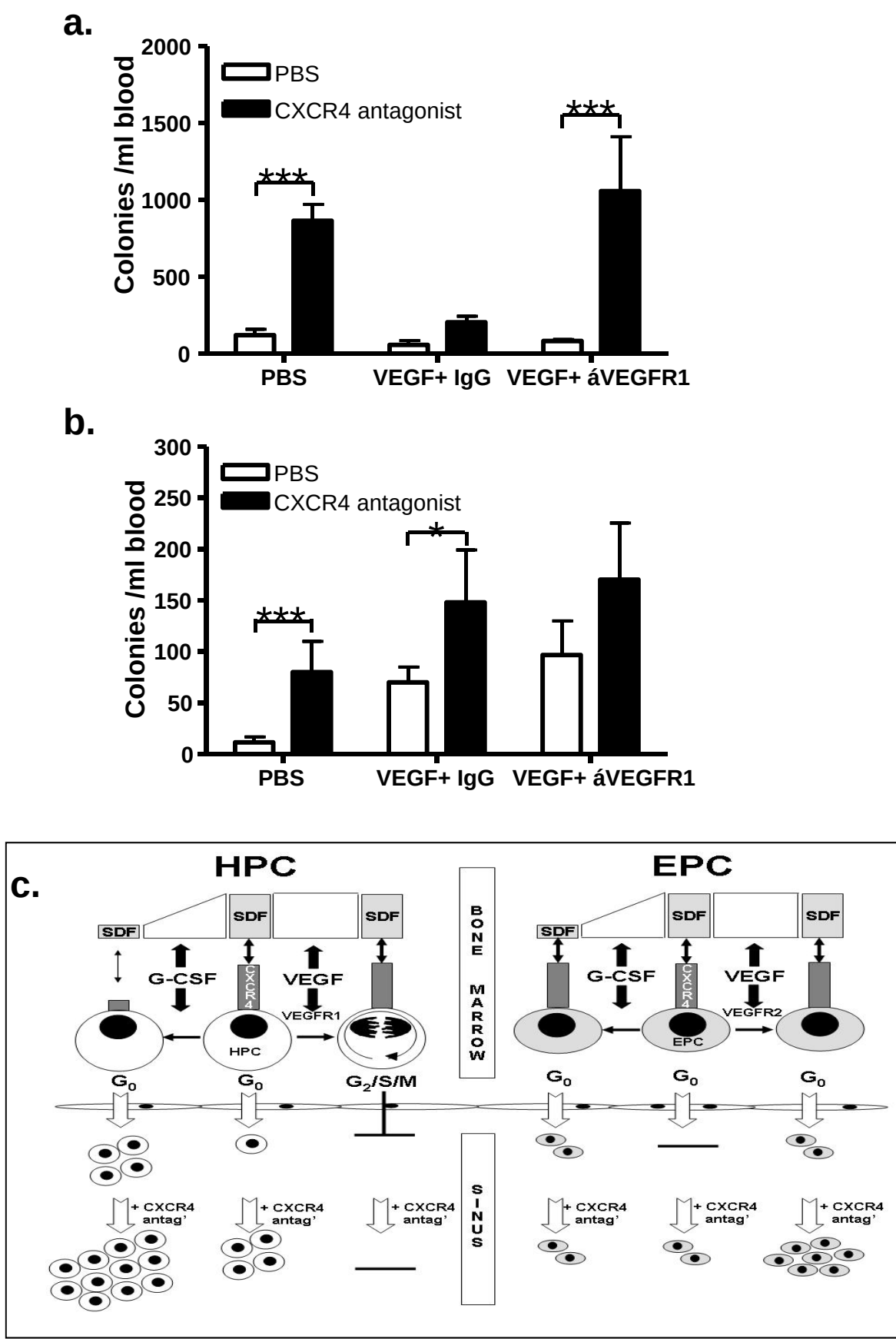
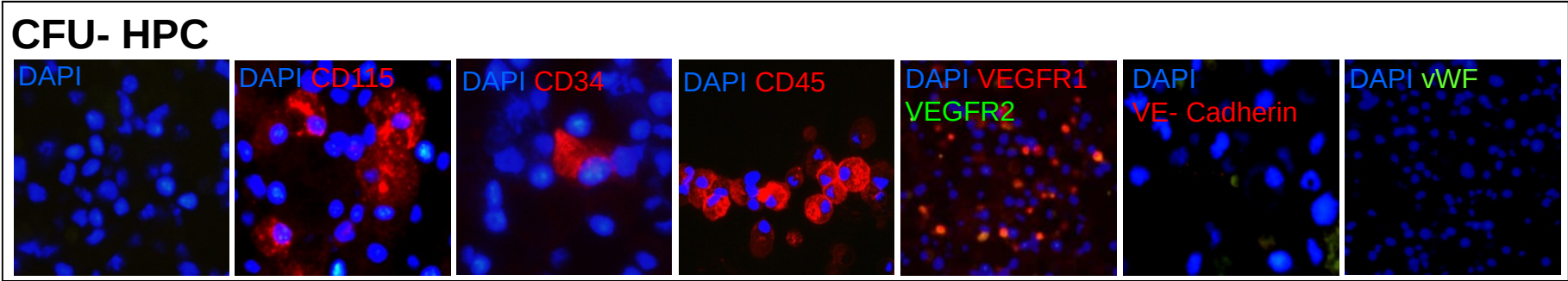


Figure 7.

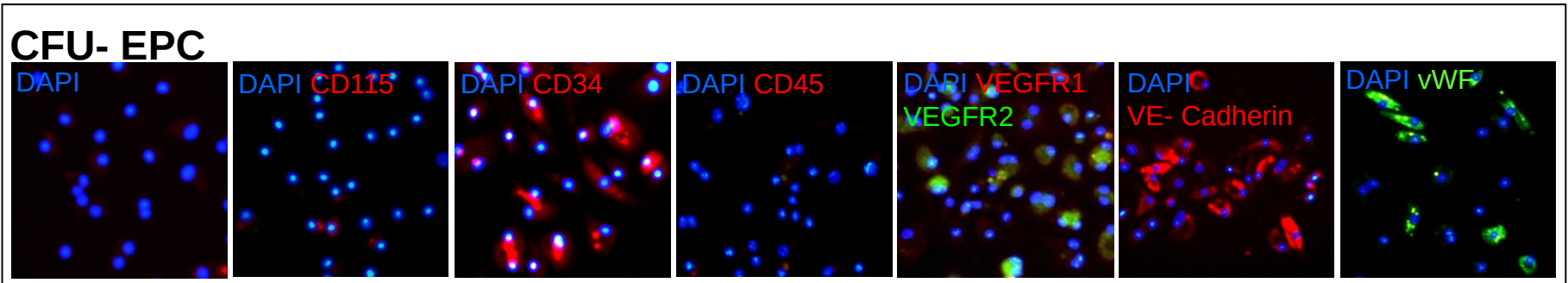


Suppl Figure 1 Stains of BM-derived HPCs and EPCs

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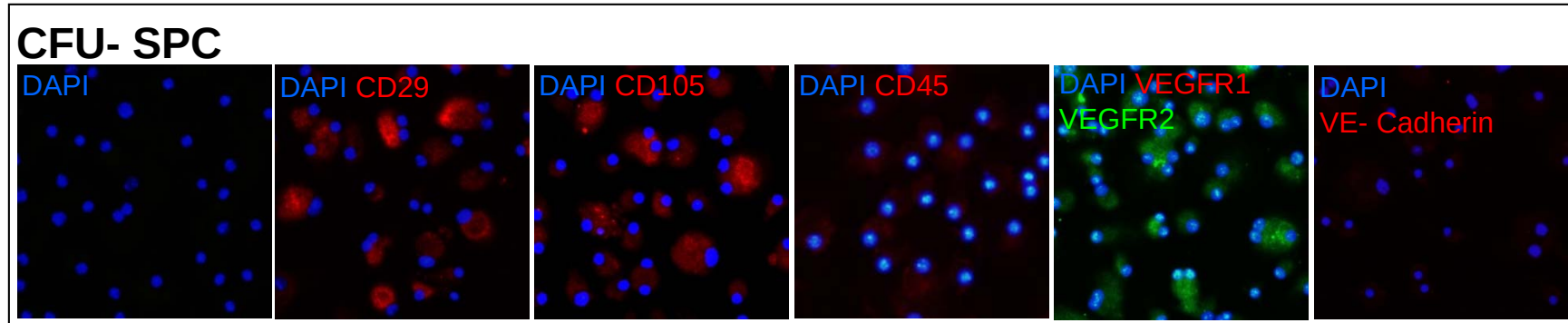


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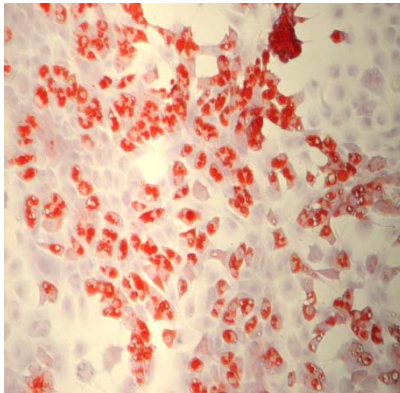


Suppl Figure 3 Stains of BM-derived SPCs

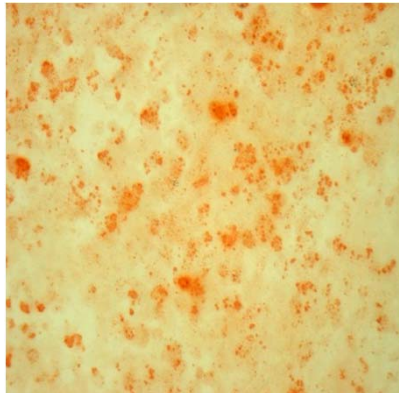
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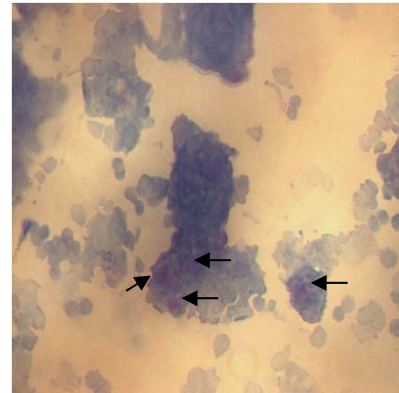
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c.

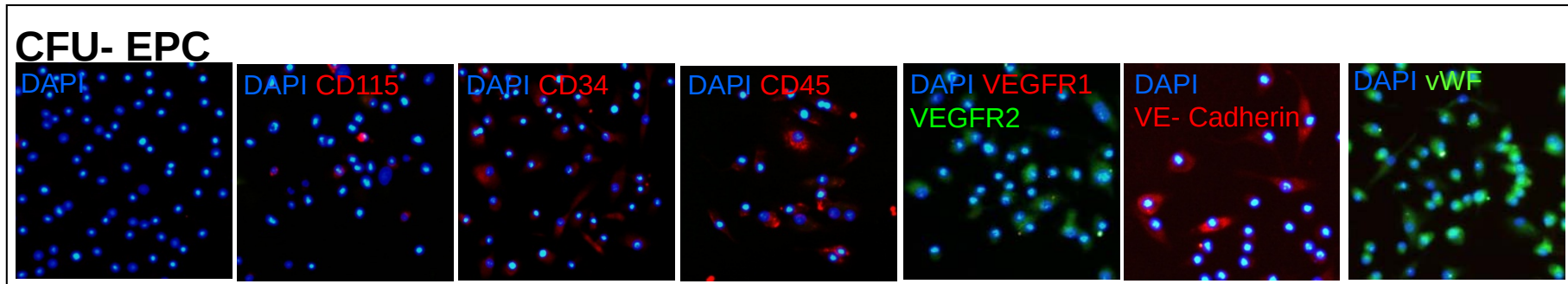


d.



Suppl Figure 4. Stains of mobilised EPCs and SPCs

a.



b.

