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Regulation of the Hematopoietic Stem Cell Lifecycle by the Endothelial Niche

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Abstract

Purpose of review—Hematopoietic stem cells (HSCs) predominantly reside either in direct contact or in close proximity to the vascular endothelium throughout their lifespan. From the moment of HSC embryonic specification from hemogenic endothelium, endothelial cells (ECs) act as a critical cellular-hub that regulates a vast repertoire of biological processes crucial for HSC maintenance throughout its lifespan. In this review, we will discuss recent findings in endothelial niche-mediated regulation of HSC function during development, aging and regenerative conditions.

Recent findings—Studies employing genetic vascular models have unequivocally confirmed that ECs provide the essential instructive cues for HSC emergence during embryonic development as well as adult HSC maintenance during homeostasis and regeneration. Aging of ECs may impair their ability to maintain HSC function contributing to the development of aging-associated hematopoietic deficiencies. These findings have opened up new avenues to explore the therapeutic application of ECs. ECs can be adapted to serve as an instructive platform to expand *bona fide* HSCs and also utilized as a cellular therapy to promote regeneration of the hematopoietic system following myelosuppressive and myeloablative injuries.

Summary—ECs provide a fertile niche for maintenance of functional HSCs throughout their lifecycle. An improved understanding of the EC-HSC cross-talk will pave the way for development of EC-directed strategies for improving HSC function during aging.

Keywords

endothelial cells; hematopoietic stem cells; bone marrow microenvironment; niche; aging

Introduction

Endothelial cells (ECs) are specialized cells lining blood vessels and that supply tissues with oxygen and nutrients required for homeostasis (1). ECs are an adaptable life-support system essential for promoting organ growth through their ability to extend and remodel the

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Conflicts of interest

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vascular network (2–4). However, ECs are not only passive channels supplying nutrients and fulfilling metabolic demands, but also serve as instructive scaffolds that provide the essential paracrine signaling cues to promote tissue maintenance and regeneration (5–7)*.

From the initial birth of a hematopoietic precursor and subsequent emergence of a definitive hematopoietic stem cell (HSC) during development, to the functional decline of the HSC during physiological aging, the surrounding microenvironment or the niche serve as cellular hubs responsible for the proper balance of HSC self-renewal, expansion, and lineage-directed differentiation. In this review, we will discuss the recent advances that have led to a better understanding of the inherent complexity of HSC niches. The review will focus on the role of ECs in the emergence of HSC activity during development and how ECs support HSC homeostasis and regeneration during adulthood. We will also explore emerging data that suggest that physiological aging of the endothelial niche, and the bone marrow (BM) microenvironment as a whole, can promote functional defects observed in the aged hematopoietic compartment. Lastly, we will discuss the therapeutic application of ECs in the treatment of hematological diseases and how transplantation of ECs can augment hematopoietic regeneration following hematopoietic insults.

Emergence of definitive hematopoietic stem cells

Hematopoietic and vasculogenic precursors originate in the yolk sac as early as day 7 of murine development. At this time, the first primitive hematopoietic cells and ECs are derived from the extraembryonic mesoderm (8). Later during development, definitive hematopoiesis first appears through the production of erythroid and myeloid progenitors followed by the emergence of definitive HSCs that are capable of long-term, multi-lineage engraftment. The emergence of the HSC during embryogenesis occurs in parallel to cardiac output and the onset of blood flow (9–11). The regions of definitive hematopoiesis are known as the para-aortic splanchnopleura (P-Sp) between 8.5 and 10 days of gestation, and as the aorta-gonad-mesonephros (AGM) from day 10.5 to day 12 (12, 13). The P-Sp/AGM contains multipotent progenitors as early as day 8.5 (14–16). Furthermore, transplantation studies have shown that long-term multi-lineage repopulating HSCs (LT-HSCs) initially develop within the P-Sp/AGM, likely serving as a primary source for seeding the fetal liver, where LT-HSC expansion occurs for the remainder of development (17, 18). Notably, ECs that form the ventral surface of the aorta are also derived from the P-Sp/AGM (19). P-Sp/AGM hematopoietic cells are found closely adhering to the ECs on the floor of the dorsal aorta (20). This close temporal and spatial development of hematopoiesis and vasculogenesis suggests not only a potential common cellular origin, but also that constitutive cellular interaction between these two populations promotes the maturation and specification of HSCs. Indeed, a small and unique subset of vascular hemogenic endothelium found in the dorsal aorta, umbilical arteries, and the placenta has been demonstrated to have the ability to acquire hematopoietic potential that allow for the emergence of definitive HSCs during development (21–29). Recent data has also suggested that ECs derived from the somite endotome migrates to the location of the hemogenic endothelium, making up the dorsal aorta and eliciting inductive signals that drive the emergence of the LT-HSC (30)**. Lastly, it has been shown that ECs derived from AGM can recapitulate the developmental *in vivo* endothelial-to-HSC transition in an *in vitro* setting (31)**. These data strongly suggest that

the surrounding supportive endothelium of AGM can provide the essential educational cues necessary to induce the emergence, maintenance, and amplification of functional HSCs from hematopoietic precursors. Taken together, the current data demonstrates that vascular endothelium is not only the source of the first definitive LT-HSC, but also suggests that neighboring ECs can serve as an instructional guide mediating endothelial-to-hematopoietic transition during embryonic development.

Regulation of HSC maintenance by the endothelial niche

The body must produce about a trillion new blood cells each day to replenish daily losses. This massive production is tightly regulated via coordinated cell-fate decisions made by the HSC. The postnatal HSC is a remarkable somatic cell that is defined by its ability to undergo self-renewal and maintain the capacity to generate all of the mature hematopoietic cell types within the blood and immune system for the life of the organism (32). These unique qualities make the HSC clinically useful in bone marrow (BM) transplantation settings for the treatment of a wide variety of hematological diseases.

Maintenance of HSC function is dependent upon their cell-intrinsic properties, as well as the extrinsic cues from the BM microenvironment (33). To date, most studies have primarily focused on the intrinsic regulation of the HSC. However, research has emerged over the past two decades demonstrating that the BM microenvironment is critical in maintaining the HSC pool and its functional output, with one of the first experiments suggesting that the HSC is dependent on microenvironmental cues from non-hematopoietic BM stromal cells and that these niche cells were capable of maintaining the HSC *in vitro* (34, 35). In 1978 Raymond Schofield was the first to formally suggest that a specialized microenvironment was responsible to maintain stem cell function (36), with his “niche” concept laying the ground work for all subsequent studies related to the microenvironmental control of the HSC.

Within the BM microenvironment there exists a vast array of diverse cellular components that comprise the HSC niche (6, 7, 37–41), including vascular endothelial cells, perivascular stromal cells, osteoblasts, sympathetic nerves, and cells part of the hematopoietic hierarchy such as macrophages and megakaryocytes. Although most of these niche components elicit a positive effect on HSC maintenance, some cell-types such as adipocytes have been shown to impose a negative effect by interfering with homeostatic and regenerative hematopoiesis (42–81)*, **. The BM is a densely vascularized tissue in which the blood vessels encompass a vast surface area, making vascular endothelial cells one of the most abundant niche cells within the BM microenvironment. The endothelial niche is made up of vascular ECs that form monolayers lining the lumen of blood vessels and consists of arteries, veins, and a large network of capillaries that connect the arterial and venous systems (82). The BM endothelial network is comprised of two main endothelial niches, the arteriolar niche identified with a VEcadherin⁺CD31⁺Endomucin^{+/-}SCA1^{high}VEGFR3⁻ cell surface phenotype and the sinusoidal niche which is identified by a VEcadherin⁺CD31⁺Endomucin⁺SCA1^{low}VEGFR3⁺ cell surface phenotype (Figure 1) (61, 63). Although recent studies have suggested another endothelial subset known as Type-H endothelium defined as CD31^{high}Endomucin^{high} (83, 84), when processing ECs from the BM of adult mice with an intravital stain with VEcadherin and excluding Lineage⁺, CD45⁺, and Ter119⁺ cells, both

arterioles and sinusoids are CD31^{high} and all VEGFR3⁺ sinusoids and a subset of arterioles are Endomucin^{high}, suggesting that Type-H endothelium may be more widespread and long-lived than previously thought (Figure 1). Nonetheless, Type-H ECs are suggested to play a major role in driving growth of the bone vasculature and in regulating the metabolic state of the BM microenvironment (83–85)*.

Prior work from our group has demonstrated that signaling pathways within the endothelial niche can dictate HSC cell fate; with AKT signaling supporting HSC self-renewal and activation of endothelial MAPK signaling driving HSC differentiation into hematopoietic progenitors and terminally differentiated cells (86). AKT-activated ECs promote the self-renewal of HSCs via the expression of HSC supportive paracrine factors, including KITL, CXCL12 and JAGGED-1. Additionally, we have demonstrated that inhibition of canonical NF-κB signaling in endothelium results in a profound increase in HSC self-renewal and regenerative potential (65). Subsequent *in vivo* studies have proven that EC-specific deletion of *Kitl*, *Cxcl12*, and *Jag1* impaired the maintenance of the HSC at homeostasis (53, 54, 64). Additionally, conditional deletion of *Jag1* from ECs resulted in profound defects in hematopoietic regeneration following myelosuppressive injury (64). It is also important to note that many of the EC-derived paracrine factors are also expressed in other BM niche constituents. In particular, there has been extensive data demonstrating that deficiency of factors in multiple niche cells, such as KITL and CXCL12, results in defects in homeostatic hematopoiesis. More importantly, depending on the cellular source of some of these factors, there could be differential regulation of the HSC and its progeny. For instance, it has been shown that deletion of *Cxcl12* in ECs (53, 57) and NG2⁺, LEPR⁺, or PRX1⁺ perivascular cells results in defects in HSC maintenance, as well as localization of the HSC within the BM microenvironment (53, 57, 71). However, osteoblast-specific deletion of *Cxcl12* resulted in defects in lymphopoiesis but not the HSC itself (53, 57). These findings have recently been extended to the cytokine IL-7 and IL-7⁺ mesenchymal cells, where conditional deletion of *Il7* in ECs and mesenchymal progenitors affected B-lymphopoiesis and conditional deletion of *Kitl* and *Cxcl12* in IL-7⁺ cells results in defects in the HSC and in multipotent progenitor cells (52). Taken together, these data suggest that the BM microenvironment is made up of multiple HSC niches that, depending on the type of growth factor and the cellular source producing that growth factor, regulates HSC function and its cell fate decisions. It is also important to take into account the possibility that the structural integrity and function of endothelial and perivascular niches are intimately dependent on one another to provide the instructional framework essential for governing HSC self-renewal and differentiation. It is likely that loss of factors such as KITL or CXCL12 in perivascular cells could have deleterious effects to the functionality of endothelium, and vice versa. Indeed, cKIT (87) and CXCR4 (88) are expressed on endothelium and deletion of their respective ligands, KITL and CXCL12, in perivascular cells could perturb integrity or function of neighboring ECs. Recent work by Zhou *et al.* demonstrated that conditional deletion of *Angpt1* from perivascular and hematopoietic cells resulted in increased vascular leakiness, suggestive of a cross-talk between the EC and perivascular components (89)*. One must also take caution when ablating niche cells to study their function, as the removal of specific niche cells can perturb the structural framework of the compound BM architecture leading to adverse effects on their neighboring niche cells. For instance, our lab has demonstrated that

both sinusoidal and arteriole bone marrow endothelial cells (BMECs) are highly invested with LEPR⁺ perivascular cells (63) (Figure 2A) and when we ablate LEPR⁺ cells using an inducible diphtheria toxin system we find that the VEGFR3⁺ sinusoidal vasculature is more tortuous, dilated, and leaky (Figure 2B–C). Moving forward, it will be important to take into account the notion of a compound HSC niche and determine whether the alterations made on a particular niche sub-type directly influences hematopoietic function or causes indirect effects mediated via compound niche interactions. Currently, the lack of genetic tools and assays limit us from adequately testing these issues; at a minimum, the potential of off-target effects of gene deletion and cell ablation studies should be taken into consideration.

Essential role of the endothelial niche in hematopoietic regeneration

It has long been suggested that hematopoietic recovery is dependent upon the regeneration of the BM vasculature (90, 91). Utilizing *in vivo* genetic models, *Heissig et. al.* was the first to demonstrate that the BM endothelial niche was a supportive cellular hub for functional hematopoiesis (92). In this study, the authors demonstrated that mice lacking the soluble form of the pro-HSC factor KITL were unable to effectively regenerate the hematopoietic system following physiological insults as a result of their inability to properly position HSCs for self-renewal and directed differentiation. Notably, this landmark study was one of the first to coin and recognize the term “vascular niche”. Follow-up studies later determined that phenotypically marked HSCs were in close association with the vascular niche (93) and that conditional deletion of *Vegfr2* in adult ECs (61) or infusion of neutralizing antibodies to VEcadherin (51, 94) interfered with the proper reassembly of the vascular niche and blocked hematopoietic recovery following hematopoietic insult. Conversely, endothelial-specific deletion of intrinsic drivers of apoptosis, *Bak1* and *Bax*, conferred a striking radio-protective effect and subsequently promotes rapid recovery of the hematopoietic system following insult, due to the preserved integrity of the BM endothelial niche (56). Additionally, recent studies have shown that endothelial-specific deletion of paracrine factors such as *Jag1* (64), *Ptn* (60), and *Egf* (55), as well as vascular adhesion molecules such as E-selectin (66) can have deleterious effects to the regenerative capacity of the HSC. Taken together, these data demonstrate that the regeneration of the structural and functional integrity of the endothelial niche is a vital process in ensuring that the hematopoietic system returns back to homeostasis following hematopoietic insult.

The aging endothelial niche and its effect on HSC function

Most reports describing aging-related alterations in the hematopoietic compartment have focused on cell-intrinsic alterations within HSCs. These studies have shown that while the absolute number of immunophenotypically defined HSCs increases with age, aged HSCs exhibit a decrease in their long-term reconstitution abilities (95–99). Furthermore, aged HSCs exhibit a significant myeloid bias at the expense of lymphopoiesis (96, 100–102), leading to a predisposition towards the development of myeloid neoplasms. While these studies show that cell-intrinsic changes that include defects in cellular polarity, altered epigenetic landscape, and impaired genomic integrity and DNA damage largely contributes to the aging of the hematopoietic system, the role of the aged microenvironment is largely unexamined. As previously discussed, there is an ever-increasing body of evidence

demonstrating essential interactions between the HSC and its niche, suggesting that both local and systemic factors regulate HSC function (42–81). Indeed, it has been demonstrated that transplanting young HSCs into aged recipients can, in part, can lead to reduced hematopoietic clonality (103) and can lead to myeloid skewing which is driven by the inflammatory cytokine CCL5 (104). Even more recently, a study by *Guidi et. al.* has demonstrated that stromal-derived osteopontin is decreased with age and that the infusion of thrombin-cleaved osteopontin has therapeutic effects in regards to rejuvenating aged HSC phenotypes (105)**. Our laboratory has determined that physiological aging results in defects in BMEC numbers and function. Analysis of the femurs in aged mice (24 months) revealed a tortuous and dilated vascular network when compared with young (3 month) counterparts (Figure 3A). Moreover, we observed a significant decrease in the frequency of functionally potent CD45⁺Ter119⁺CD31⁺VEcadherin⁺ BMECs (Figure 3B). In line with these findings, it has recently been demonstrated that aged BMECs have impaired niche function in regards to regulating hematopoietic niche cells and that it is possible to rejuvenate their functional readout. However, these studies were unable to restore the full functional capacity of the aged HSC (106)*. Taken together, these data suggest that changes in aged ECs and other BM niche constituents inefficiently sustain HSC homeostasis, potentially leading to aged-related hematopoietic disorders. Therefore, understanding the intimate relationship between the BM microenvironment and its resident HSCs during homeostasis and aging will provide a therapeutic opportunity to mitigate and potentially reverse the age-related functional decline observed in the hematopoietic system, including the development of hematologic malignancies.

Therapeutic application of the endothelial niche

Despite advances in the understanding of HSC biology, the exact mechanisms that regulate the balance between self-renewal and lineage-specific differentiation are still unknown, which has limited the development of new *in vivo* and *ex vivo* HSC-based therapies. The necessity to build upon current HSC expansion strategies may potentiate the therapeutic use of HSCs, as well as ECs, for the treatment of hematological disorders. The use of endothelium to expand hematopoietic stem and progenitor cells (HSPCs) was first demonstrated by co-culturing human BMECs with CD34⁺ umbilical cord blood (107, 108). Subsequent studies showed that ECs isolated from fetal tissues, heart, and brain had the capacity to expand HSPCs (109–113). However, these studies were limited in that they relied on significant supplementation with serum and supraphysiological levels of growth factors to maintain ECs, causing adverse effects on HSPCs (114, 115). To study the role of ECs in the *ex vivo* expansion of HSCs, our group has developed an approach that allows for the maintenance of primary ECs by constitutively activating AKT-signaling, enabling EC survival for weeks under serum- and growth factor-free conditions, in part, by activating the downstream AKT signaling pathway mTOR (116). Additionally, AKT activation maintains the paracrine repertoire of the ECs without inducing immortalization or tumorigenic potential (116). Maintenance of primary mouse and human ECs without exogenous growth factors or serum allowed us to develop an unbiased EC-HSPC co-culture system, in which the co-culture of mouse HSCs (51) and human umbilical cord blood (UCB) hematopoietic stem and progenitor cells (HSPCs) (117) with ECs resulted in the expansion of *bona fide* repopulating

HSCs which were able to engraft lethally irradiated primary and secondary recipients. This platform's utility has also been extended to expand engraftable adult BM HSPCs and HSPCs derived from induced pluripotent stem cells (118, 119)*.

It has been over 3 decades since the first scientific experiments demonstrated that ECs are a critical part of the BM "stroma" (120, 121). These studies confirmed the notion that ECs may have instructive capabilities, sparking a line of seminal papers leading to the discovery that various sources of endothelium have the capacity to support many aspects of hematopoiesis, particularly in the *ex vivo* setting (51, 109, 122–124). The culmination of all of these experiments laid the foundation for all of the recent data confirming the essential role of ECs and the vascular niche in maintaining functional hematopoiesis, both *in vivo* and *ex vivo*. In addition to the expansion of repopulating HSCs, direct injection of various sources of allogeneic or syngeneic endothelium has been shown to enhance hematopoiesis following myelosuppression (62, 63, 65, 125–127). These data suggest that ECs, regardless of source, may be useful for acceleration of hematopoietic recovery. Although it appears that the source of ECs for therapeutic transplantation may not be a barrier in the clinical arena, our group (128) and others (2) have demonstrated that there is significant heterogeneity within the body's vascular network, suggesting that each organ is endowed with a specialized endothelium with a unique paracrine signature that specifically supports their tissue-resident stem cell and their progeny. Notably, it has been shown that tissue specific ECs can enhance organ regeneration with other sources of ECs unable to elicit the same regenerative effect (129, 130). In line with this notion, we have demonstrated that transplantation of niche-specific BMECs can rapidly and efficiently enhance hematopoietic recovery following myeloablation (63, 65). Additionally, transplantation of BMECs resulted in the preservation of the BM vascular niche and the rapid regeneration of VEGFR3⁺ sinusoidal endothelium. It is important to note that we have attempted to transplant ECs isolated from other organs (i.e. BM-derived endothelial progenitor cells, lung ECs, and brain ECs) and found that only the niche-specific BMECs result in this profound protection of the hematopoietic system and the vascular niche following myeloablative insult (63, 65). These data suggest that BMECs are a specialized subtype of endothelium that can produce factors to supportive regenerative hematopoiesis and microenvironmental radioprotection (63). In summary, transplantation of ECs may create a more permissive microenvironment by protecting the structural and functional integrity of the endothelial niche following hematopoietic insults, as well as enhance the engraftment of HSPCs following BM transplantation. These finding have laid a scientific and therapeutic justification for the transplantation of endothelium to accelerate the rate of hematopoietic recovery following radiation or chemotherapeutic regimens and decreasing the morbidity/mortality resulting from associated pancytopenias.

Concluding Remarks

Understanding the intimate relationship between the endothelial niche and HSCs that maintains homeostatic hematopoiesis is critical to the future discovery of therapeutic pro-HSC paracrine factors that modulate self-renewal and expansion of adult HSCs and will aid in the development of strategies to enhance hematopoietic reconstitution in the clinical arena. However, there is still a great need to develop novel approaches to facilitate processes

allowing for the unraveling of the complex BM microenvironment-HSPC interactions that are critical in providing the knowledge for the development of new HSC therapeutic applications. The field will need to be dedicated in understanding the role of tissue-specific ECs in establishing unique instructive vascular niche cells that produce the correct milieu and stoichiometry of paracrine factors to direct organ regeneration, in particular within the BM microenvironment. It is likely that the reconstitution of hematopoiesis along with endothelial transplantation will not only co-operatively augment both the stability and integrity of the newly formed vessels but also promote tissue repair and multi-organ regeneration via an inductive mechanism. At the field's current pace, we will have a significant and positive impact on novel therapeutics aimed at decreasing the morbidity and mortality associated with life-threatening pancytopenias associated with hematopoietic disorders and myelosuppressive regimens.

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Key points

- Endothelial cells serve as an instructive platform to nurture the emergence, maintenance, and regeneration of the HSC.
- Improving our understanding of the intimate relationship between the HSC and its specialized niche by investigating the role of the BM microenvironment in supporting HSC function during aging may prove to be beneficial in reversing or preventing the age-related functional decline observed in the hematopoietic system.
- Endothelial cells can be utilized as a stand-alone or adjuvant cellular therapy to temporize pancytopenia associated with myelosuppressive or myeloablative treatments.

Figure 1. VEGFR3 and intravital VEcadherin identify distinct immunophenotypic arteriole and sinusoidal BMEC niches

Mice expressing a *Vegfr3::YFPBAC* transgene reporter were intravitaly-labeled using an EC-specific antibody (VEcadherin). **A**) Representative flow cytometry contour plots of enzymatically-dissociated and hematopoietic lineage⁺ cell-depleted whole bone marrow, stained with antibodies raised against CD45, TER119, CD31, VEcadherin, and Endomucin. Gating strategies (dashed line) and percentage of parent populations are indicated. Note: VEGFR3^{high}VEcadherin^{high}CD31⁺ sinusoid bone marrow endothelial cells (BMECs) (green) and VEGFR3^{low/-}VEcadherin^{low}CD31⁺ arteriole BMECs (red) can be distinguished using intravital VEcadherin staining in combination with hematopoietic exclusion (lineage⁻CD45⁻TER119⁻). **B**) Representative histogram of Endomucin stained VEGFR3^{high}VEcadherin^{high}CD31⁺ sinusoid BMECs (green) and VEGFR3^{low/-}VEcadherin^{low}CD31⁺ arteriole BMECs (red). **C**) Representative confocal images of femoral trabecular and diaphysis regions demonstrating VEGFR3⁺VEcadherin⁺CD31⁺Endomucin⁺ (yellow arrow) and VEGFR3^{low/-}VEcadherin⁺CD31⁺Endomucin⁻ (white arrow) arteriole BMECs. Scale bar = 100 μ m.

Figure 2. Perivascular LEPR⁺ cells support arteriole and sinusoidal BMECs

Mice expressing *Vegfr3-YFP; Lepr-cre* transgenes were crossed with mice carrying either a *Rosa26*-expressing *loxP*-STOP-*loxP* *tdTomato* reporter or a *Rosa26*-expressing *loxP*-STOP-*loxP* inducible Diphtheria Toxin Receptor (iDTR) cell ablation cassette and intravitaly-labeled with an Alex Fluor 647-conjugated antibody against VEcadherin. **A**) Representative confocal images of VEGFR3⁺VEcadherin⁺ sinusoid bone marrow endothelial cells (BMECs) (white arrow), VEGFR3⁺VEcadherin⁺ arteriole BMECs (yellow arrow), and sinusoid/arteriole BMEC-invested tdTOMATO⁺ *Lepr*-expressing cells (merged image). Scale bar = 50 μ m. **B–C**) Representative confocal images of **(B)** wild type (*wt*) or **(C)** homozygous *Rosa26-iDTR* (flanked by *loxP*; *fl*) *Vegfr3-YFP; Lepr-cre* mice following diphtheria toxin administration. Dashed box indicates magnification. Note: Vascular BMECs appear dilated following LEPR cell ablation. Scale bar = 500 μ m and 100 μ m, respectively.

Figure 3. Physiological aging alters BMEC function

Endothelium from young and aged mice were intravitaly-labeled using an Alex Fluor 647-conjugated antibody against VEcadherin. **A)** Representative confocal images of femoral trabecular region from young and aged mice. Scale bar = 200 μ m. **B)** Quantification of hematopoietic lineage-depleted, CD45⁻TER119⁻VEcadherin⁺CD31⁺ BMECs from young and aged mice. Note: All quantifications were performed on enzymatically-disassociated and lineage+ cell-depleted femurs and gated on CD45⁻TER119⁻VEcadherin⁺CD31⁺ populations. Error bars represent mean $\pm$ SEM. Pairwise two-tailed comparisons using Student's t-test were performed to determine significance. * P<0.05; ** P<0.01; *** P<0.001.