CHAPTER 7

MicroRNA-126 overexpression in lineage depleted bone marrow cells leads to increased neovascularization

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Abstract

It has been demonstrated that bone marrow (BM) cells play an important role in the formation of new capillaries. Whether there is a direct differentiation of these cells in to endothelial cells or whether there is only have a supporting role is still unclear. Therefore, the molecular mechanisms that underlie the contribution of BM cells are of high interest. It is well established that microRNAs (miRs) play a key role in cell differentiation as well as cell fate. The endothelial cell enriched miR-126 has been described to be a key factor for angiogenesis, furthermore it has been demonstrated that bone marrow cells also express relevant levels of miR-126. Here, we investigate whether over-expression of the miR-126 in bone marrow cells can have a potential positive effect on neo-vascularization.

Using a lentiviral construct we successfully over-expressed miR-126 in lineage depleted bone marrow cells. Subsequently these cells were intravenously injected into lethally irradiated mice. Seven weeks after reconstitution of the bone marrow an angiogenic matrigel plug containing VEGF and SDF-1 was inserted into the flanks of the animal. After seven days vascularization of these plugs was visualized and a significant increased vessel length was observed in the plugs of the animals that were transplanted with bone marrow cells that over-expressed miR-126 Furthermore, FACS analysis revealed an up-regulation of total white blood cells in the periphery of the mice transplanted with the BM cells that over-expressed miR-126. This enhanced migratory capacity of white blood cells was observed for almost all subpopulation of leukocytes that were tested. Both enhanced neovascularization as well as the increased levels of white blood cells where highly correlated to the expression levels of miR-126 in the bone marrow.

Our data demonstrates that miR-126 has an angiogenic potential that reaches further then its presence in EC. Although the exact mechanisms leading to increased neovascularization and elevated levels of leukocytes is unknown, it is likely that these two effects are linked.

Introduction

It has been demonstrated that the formation of novel capillaries may not only be restricted to the sprouting capability of endothelial cells (ECs) but that bone marrow (BM)-derived progenitor cells also play a facilitary role in neovascularization [1]. Whether these precursor cells can differentiate into mature ECs is extensively studied, but remains unclear [2]. Nevertheless it has been reported that BM-derived endothelial progenitor cells (EPC) can form independent functional vascular structures after migration towards a hypoxic region in the skin [3]. Furthermore, others claim a capability of BM-derived cells to promote vascular growth in (tumor)-ischemia, however this support is not generated by

differentiation of these EPC in to mature endothelium but involves perivascular stabilization of the neovasculature and paracrine effects of these cells [4, 5]. Based on these observations, the contribution of BM-derived progenitor cells might depend on depth of ischemia, release of cytokines and sort of injury.

The molecular mechanisms underlying these processes of vascular homeostasis involve the tight regulation of genes that control of cell proliferation, migration and differentiation [6]. MicroRNAs (miRs) are small regulatory RNAs that function as negative regulators of gene expression [7]. The discovery that miRs are expressed in a tissue- and cell-specific manner during development suggest that miRs could play a role in specifying and maintaining tissue identity [8]. Furthermore, miRs possess the capacity to regulate multiple targets, and can thereby influence the activity of diverse signalling pathways. To this end, ECs, that play a key role in the initiation of neovascularization, have been thoroughly analyzed for the presence of miR. Indeed, several miRs were found to have both pro-angiogenic [9-12]. as well as anti-angiogenic functions in ECs [13, 14].

Of particular interest, with respect to a controlling role in neovascularization, is the endothelial-enriched miR-126. It has been demonstrated that miR-126 targets proteins that play key roles in angiogenesis, vasculogenesis and inflammation [5, 15-19]. MiR-126 is highly expressed in EC and targets two potent repressors of pro-angiogenic signaling sprouty-related EVH1 domain containing 1 (SPRED-1) and phosphoinositide-3-kinase regulatory subunit 2 (PI3KR2) [19, 20]. Therefore, it is likely that ECs require constitutive levels of miRNA-126 to maintain the integrity of the vasculature both during vascular development as well as in adult life. In addition, in experimental ischemia, miRNA-126 can also directly modulate CXCL12/SDF-1 expression and drive the mobilization of vasculogenic Sca-1+/Lin- stem cells into the circulation [18].

Next to its abundant presence in EC, miRNA-126 also circulates in plasma in a complex with Ago2, but also in vesicles or exosomes [21]. Exosomes are vesicles that are secreted as a consequence of the fusion of multivesicular bodies with the plasma membrane and are loaded with distinct sets of miRNAs. Following their release, exosomes can fuse with target cells thereby facilitate functional repression of target cell mRNAs by the exosomal miRNAs [22]. Recently, it was shown that circulating levels of miR-126 decreased during transcoronary passage in patients with evidence of myocardial injury suggesting a role for circulating miR-126 in vascular homeostasis [23].

Although the EC is a likely source for circulating vesicles containing miR-126 [24, 25], significant levels of miR-126 are also expressed in circulating cells hematopoietic cells and platelets [26-29]. Moreover, these cells have been demonstrated to be highly capable of exosome/vesicle secretion allowing them to exert transcellular regulation of gene expression [30]. In addition, hematopoietic cells, in particular myeloid cells have been demonstrated to play a rate limiting role on neovascularization facilitating angiogenesis [31] as well as arteriogenesis

[32]. To investigate the role of hematopoietic cell expressed miR-126 in vascular homeostasis we over expressed miR-126 in the hematopoietic compartment of mice. Seven weeks after transplantation, a VEGF- and FGF-rich matrigel plug was inserted to assess the effect of hematopoietic overexpression of miRNA-126 on leukocyte mobilization and neovascularization. Our findings underline a potential role for miR-126 in neovascularization an indicates that this regulatory capacity is not limited to the EC, but may also involve a role for hematopoietic cells via transcellular or direct effects.

Material and methods

Mice

C57BL/6J wild type (WT) and B6.SJL-Ptprca Pepcb/BoyCrl (Ly5.1) were purchased from Charles River Nederland (Maastricht, the Netherlands). Mice were housed under a 12-h light/dark cycle, standard chow diet and drinking water were provided *ad libitum*. All animal experimental protocols were approved by the animal welfare committee of the veterinary authorities of the Leiden University Medical Center.

Lentiviral constructs

The vesicular stomatitis virus G protein-pseudotyped self-inactivating (SIN) HIV type 1 (HIV-1)-based vectors LV-miR126 and LV-control were generated in 293T cells with the aid of the packaging plasmids psPAX2 (Addgene, Cambridge, MA) and pLP/VSVG (Invitrogen, Breda, the Netherlands) as specified before [33]. To concentrate and purify lentivirus vector particles, producer cell supernatants were layered onto 5 mL cushions of 20% (wt/vol) sucrose (Merck, Whitehouse Station, NJ) in phosphate-buffered saline and centrifuged at 15,000 rotations per minute for 2 hours at 10°C in an SW28 rotor (Beckman Coulter, Woerden, the Netherlands). Prior to ultracentifugation, producer cell supernatants were subjected to filtration through 0.45-mm pore-sized cellulose acetate filters (Pall, Port Washington, NY).

Cells and cell culture

Bone marrow (BM) cells were isolated from the femora and tibia of euthanized WT mice. Following isolation, cells were cultured in StemSpan-SFEM (Stemcell Technologies Inc, Vancouver, BC, Canada) supplemented with 50 ng/mL recombinant mouse stem cell factor (rmSCF), 10 ng/mL recombinant mouse thrombopoietin (rmTPO) and 50 ng/mL recombinant mouse fms-related tyrosine kinase 3 ligand (rmFLT3-L) (all R&D Systems, Minneapolis, MI) at 37°C and 5% CO2. After 24 hours the BM cells were transduced by spin occulation in the presence of 4 µg/mL proteamine sulphate (Sigma Aldirch, St Louis, MO) at 800g and at 32°C for 1 hour. Cells were transduced at a multiplicity of infection

(MOI) of 5 with either LV-control or LV-miR126 and kept in culture. Microscopic images were made daily and after five days cells were harvested for FACS analysis and RNA analysis.

Transduction and transplantation of bone marrow cells

BM cells were isolated from the femora and tibia of euthanized CD45.1+ mice (Charles River, aged 8–10 weeks). Upon isolation, lineage negative cells were negatively selected (Lineage Cell Depletion Kit (mouse), Miltenyi Biotec, Bergish Gladbach, Germany) and cultured for 1 day as described before and subsequently transduced at a MOI of 5 with either LV-control or LV-miR126. After transduction, cells were maintained for another 24 hours in the presence of cytokines. Transduced cells (300 000/mouse) were mixed with supportive spleen cells (500 000/mouse) and injected into the tail vein of lethally irradiated (8 Gy) male C57BL/6J recipient mice (n=12 per group, Charles River, aged 8–10 weeks).

Matrigel plug assay

Seven weeks after irradiation, mice (n=10 per group) were anesthetized with isoflurane and injected subcutaneous into the flank with 0.5 mL ice-cold matrigel (BD Biosciences, Breda, the Netherlands). Matrigel was supplemented with 100 ng/mL recombinant mouse SDF-1 (Invitrogen) and 50 ng/mL recombinant mouse VEGF (Invitrogen). After 7 days, mice were anesthetized by an intraperitoneal injection of a mixture of ketamine (100 mg/kg), xylazine (12.5 mg/kg), and atropine (125 µg/kg) and the skin was opened to visualize the vasculature of the implant with a Sidestream Dark Field (SDF)-camera. Thereafter, implants were extracted, imaged with a Leica microscope (DMI6000, Nieuw Vennep, the Netherlands) and subsequently fixed in 4% paraformaldehyde and snap frozen at -80°C. From all microscopic images of the matrigel implants the number of visual vessels was established on both sides of the implants. To obtain total length of the vessels pictures were digitalized and the total pixel area of the vessels was calculated using ImageJ software.

Immunohistochemistry on matrigel plugs

Twenty µm-thick sections of matrigel plugs were fixed with methanol on a glass side and subsequently blocked with 2% fetal calf serum (FCS, Bio Whittaker/Cambrex, Verviers, Belgium) 3% bovine serum albumin (BSA, Sigma Aldirch) in PBS. Next, sections were incubated with specific antibodies against murine vWF (Dako Netherlands, Heverlee, Belgium) or F4/80 (Abcam, Cambridge, UK) followed by secondary antibodies against goat-anti-rabbit-IgG labeled with Alexa-488 (Molecular Probes). Ly6C (ABD Serotec, Düsseldorf, Germany) was directly labeled with Alexa-488. As a negative control, isotype-matched IgG were used.

Whole blood and bone marrow analysis

Whole blood was collected by incision of the tail vein or heart puncture and analyzed by semi-automatic hematology analyzer F-820 (Sysmex; Sysmex Corporation, Etten-Leur, the Netherlands) microscope (Leica) and flow cytometry (FACS, LSR II, BD Biosciences). Hematological values obtained were white blood cell counts (WBC, n*10^6/mL), red blood cell counts (RBC, n*10^9/mL), platelets (PLT, n*10^6/mL), hematocrit (HCT, %/%) and hemoglobin (HGB, mmol/L). For microscopic images a blood smear was made on a glass cover and images were made using a fluorescence microscope. For BM cells the same procedure was followed For FACS analysis, we incubated 35 µL of whole blood or 106 bone marrow cells for 30 minutes at 4°C with directly conjugated antibodies directed against CD45.1 (PE-Cy7, eBioscience, Vienna, Austria), CD45.2 (FITC, BD Biosciences) to analyze the percentage of chimerism of the mice after BM transplantation, CD11b (PercP, BD Biosciences), CD3e (APC, BD Biosciences) and B220 (APC-eFluor780, eBioscience). A different sample was prepared with the same amount of cells and antibodies directed against Ly6G (PE, BD Biosciences), CD115 (biotin, eBioscience), CD11b (APC, BD Biosciences) and MP20 (FITC, kindled provided by Erasmus University Rotterdam). These cells were subsequently incubated with secondary antibody with streptavidin (PercP-Cy5.5, BD Biosciences) to visualize the biotin labeled antibody. Furthermore, a third mix was prepared with 50 µL of whole blood or 106 bone marrow cells with antibodies directed against Sca-1 (FITC, BD Biosciences), CD117 (PE-Cy7, BD Biosciences) and a cocktail against lineage-positive cells (APC, BD-Biosciences). In a separate tube, 50 µL of whole blood was incubated with an appropriate cocktail of isotype controls.

Quantification of miR levels

Total RNA from BM cells was isolated using Trizol reagent (Invitrogen). Expression levels of miR-126 were validated by quantitative RT-PCR (qRT-PCR). Reverse transcription was performed using a 5 minute 65°C incubation of 250 ng total RNA with specific Taqman® miR probes for miR-126 (Applied Biosystems, Nieuwerkerk a/d IJssel, the Netherlands). cDNA was synthesized using a M-MLV First-Strand Synthesis system (Invitrogen). Validation of miR-126 levels was performed using Taqman® miR assays and qRT-PCR. For normalization, a qRT-PCR on RNU6B was performed on cDNA obtained from the same RNA. The following primers were used for PCR: U6 (sense) CTCGCTTCGGCAGCACA and U6 (antisense) AACGCTTCACGAATTTGCGT. Results were normalized using Gene Expression Analysis for iCycler IQ® RT-PCR Detection System (Bio-Rad Laboratories, Veenendaal, the Netherlands).

FACS analysis of blood and bone marrow

All samples obtained for FACS analysis were either immediately analyzed by

flow cytometry analysis (FACS, LSRII) or were fixed in 1% paraformaldehyde and analyzed within 24 hours after preparation. Data were analyzed using FACSDiVa software (BD Biosciences).

Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical analysis was performed using the Mann-Whitney or a standard T-test. $P<0.05$ was considered statistically significant.

Results

Transduction of BM cells with LV-miR126 leads to overexpression of miR-126 in vitro

The hairpin for miR-126 was cloned into a pLV-U6 construct that also contained the sequence for dsRED behind a PGK promoter. A control was used that lacked the hairpin structure (Figure 1A). Lineage-depleted BM cells were transduced with LV-miR126 and LV-control at a MOI of 5. As compared to non-transduced cells a high number of fluorescent dsRED-positive BM cells were observed 5 days after transduction with either viral construct. Quantification by flow cytometry indicated a transduction efficiency of around 30% for both constructs (Figure 1B). Quantitative real-time PCR analysis (qPCR) revealed a 30 fold increase in miR-126 expression levels in the BM cells transduced with the lentiviral construct harboring the gene for miR-126 (LV-126, n=3, Figure 1C) as compared to non-transduced BM cells (mock) and BM cells transduced with control virus (LV-C).

Transduction of bone marrow cells with LV-miR126 leads to over-expression of miR-126 in vivo after eight weeks

To generate mice overexpression miR-126 in the hematopoietic compartment, ex vivo transduced BM cells were intravenously injected into lethally irradiated mice. Eight weeks after transplantation mice were sacrificed and blood and bone marrow samples were obtained. Smears of blood and BM suspensions isolated from the animals transplanted with transduced BM showed dsRED positivity, validating successful transplantation. Blood and BM smears derived from control donor mice showed no dsRED positivity (Figure 2A and 2B). To determine the degree of chimerism, the ratio between congenic markers CD45.1 (donor) and CD45.2 (acceptor) in blood and BM cells was assessed by FACS analysis. As shown in Figure 2C and D, the percentage of $CD45.1^+$ cells in total $CD11b^+$ leukocytes (top left quadrant of FACS plot) of the animals was between 90% and 100%. The overall percentage of CD45 cells in either blood (~90% $CD45^+$ cells) as well as in BM (~75% $CD45^+$ cells) was comparable in transduced with LV-miR-126 as well as LV-control, indicating that the BM of the trans-

planted animals was successfully reconstituted after whole body irradiation and subsequent bone marrow transplantation. A small trace of CD45.2$^+$ cells was still found in blood as well as bone marrow of the transplanted mice, however the presence of a high percentage of CD45.1$^+$ cells in blood as well as BM indicates that these cells have been responsible for the repopulation of the bone marrow compartment.

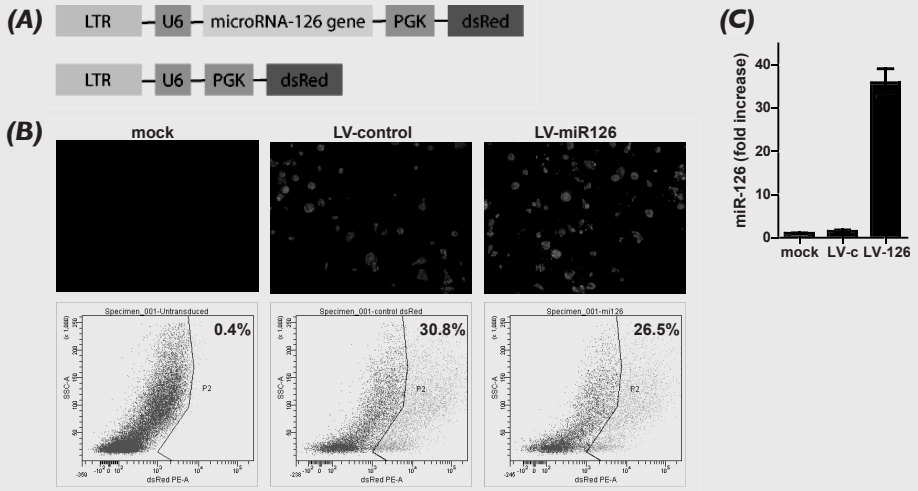

Figure 1. Overexpression of miR-126 in bone marrow in vitro

(A) Schematic representation of either the miR-126 over expression virus (LV-126, upper scheme) or the control virus (LV-C, lower scheme). The DNA encoding the precursor RNA hairpin of miR-126 was cloned behind the constitutive U6 promoter. Both viruses contain the dsRED reporter gene behind a PGK promoter to be able to detect transduced cells with a fluorescence microscope. ***(B)*** Fluorescence microscopic images show dsRED positivity in bone marrow cells indicating successful transduction by LC-V and LV-126. Non-transduced (mock) cells are shown in left panel as a negative control. FACS plots depict the percentage of dsRED positive cells of total number of cells. ***(C)*** qRT-PCR on RNA isolated from total BM cells (n=3) demonstrates up regulation of mir-126 expression in LV-126 transduced cells when compared to cells transduced with LV-C or non-transduced cells (mock).

Quantitative PCR on total RNA isolated from BM suspension cells revealed a 8.2-fold increase in miR-126 expression levels in the BM cells from the animals transplanted with miR-126 over expressing BM (LV-126) as compared to animals transplanted with BM cells transduced cells with control virus (LV-C) and BM from CD45.1$^+$ donor animals $P<0.0001$, n=11, Figure 2E).

Sidestream dark field imaging can be used to show functional vessels in an angiogenic matrigel plug in vivo

Seven weeks after BM transplantation, matrigel plugs containing recombinant

SDF and VEGF were inserted in the flank of mice. After 7 days, mice were anesthetized and the skin was opened to visualize the vasculature in the implant by sidestream dark field camera. Figure 3B shows a still of one of the movies that showed active flow of red blood cells through the vessels that had grown into the angiogenic plugs. The movies confirmed neovascularization of the matrigel SDF-1/VEGF supplemented plugs.

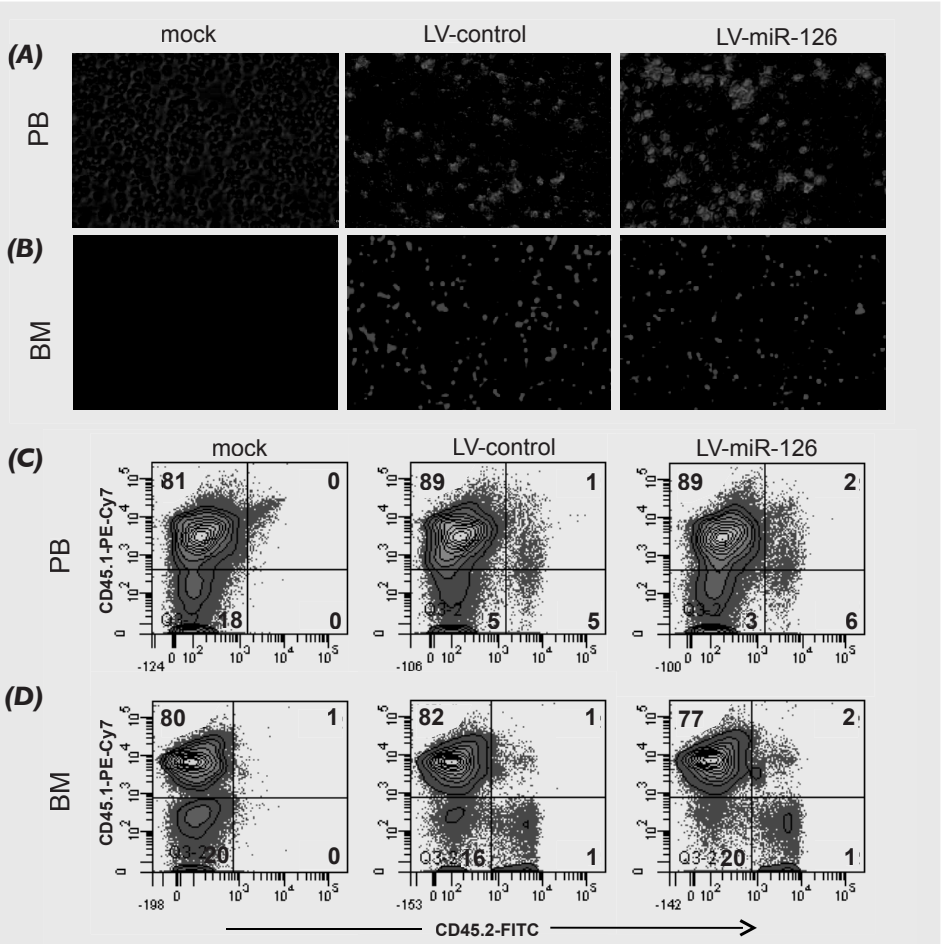

Figure 2A-D. Overexpression of miR-126 in blood and bone marrow cells in vitro

(A) Microscopic images of blood smears show clear dsRED positive blood cells in animals transduced with either LV-C or LC-126 when compared to cells of non-transduced animals. ***(B)*** Microscopic images of bone marrow cells show clear dsRED positive blood cells in animals transduced with either LV-C or LC-126 when compared to cells of non-transduced animals. ***(C,D)*** Representative picture for FACS plots that demonstrate successful chimerism on CD45.1 (donor) and CD45.2 (acceptor) levels in blood and bone marrow cells. All animals were over 95% chimeric.

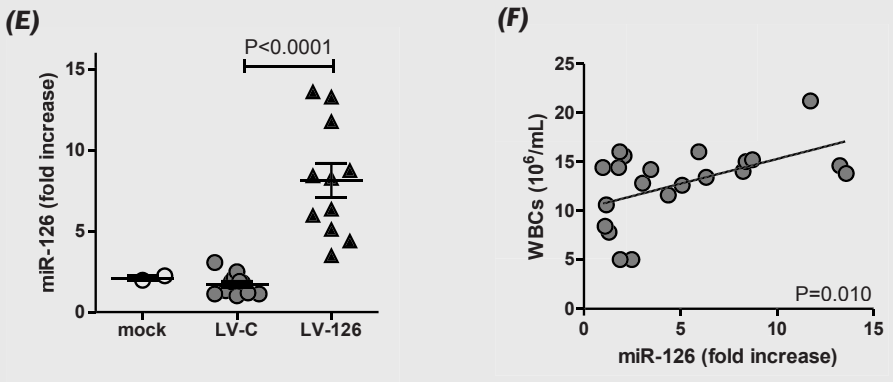

Figure 2E,F. Overexpression of miR-126 in blood and bone marrow cells in vitro

(E) qRT-PCR on bone marrow cells (n=10-12) demonstrates up regulation of mir-126 in LV-126 transduced when compared with cells transduced with LV-C or non-transduced cells (mock). ***(F)*** Overexpression of miR-126 leads to increased levels of white blood cells in the periphery. A correlation was found between the levels of white blood cells in the periphery and the expression levels of miR-126 in the bone marrow (n=10-12).

Overexpression of miR-126 in the hematopoietic compartment leads to increased neovascularization in angiogenic matrigel plugs

To quantify the impact of hematopoietic overexpression of miR-126 on neovascularization microscopic images were taken from both sides of the angiogenic plugs that had been implanted for 7 days. Figure 3A shows a panel of representative microscopic images of the plugs and the arrowheads indicate the blood filled microvascular structures that had grown into the matrigel plugs following implantation. The number of ingrowth vessels per matrigel implant was determined by counting the vessels present on the photographs taken from both sides of the matrigel plug. A nearly significant up regulation was found in the number of vessels of the animals transplanted with miR-126 as compared to the control-transplanted animals (Figure 3C, n=10-12, P=0.053). However, when the images were digitalized with imageJ, quantification of the total microvascular surface revealed a significant 1.4-fold increase in vascularization confirming augmented neovascularisation in the mice overexpressing mir-126 in the hematopoietic compartment (Figure 3D, n=10-12, P<0.02). Finally, we observed a near significant direct correlation between the total length of the vessels per mice and the levels of BM miR-126 expression (Figure 3E, n=22, P=0.054). Our data indicate that over expression of miR-126 in the hematopoietic compartment augments neovascularization of angiogenic plugs that were subcutaneously implanted in mice for 7 days.

Immunohistochemical analyses of the vascularized matrigel plugs demonstrated

a profound infiltration of dsRED positive cells directly adjacent to the infiltrating von Willebrand factor positive microvessels (Figure 4A and 4B), indicating that the BM-derived cells merely act as pericytes to support the ingrowth of endothelial cells into the matrigel plug. Further immunohistochemical analyses shows that there was co-expression of the dsRED positive with macrophage and monocyte markers such as F4/80 and Ly6c (Figure 4C-D, indicated with asterisks in Figure 4E-F). These results may suggest that leukocytes that are derived from the bone marrow may create an angiogenic environment in the plug into which endothelial cells can sprout and be subsequently supported by pericytic function

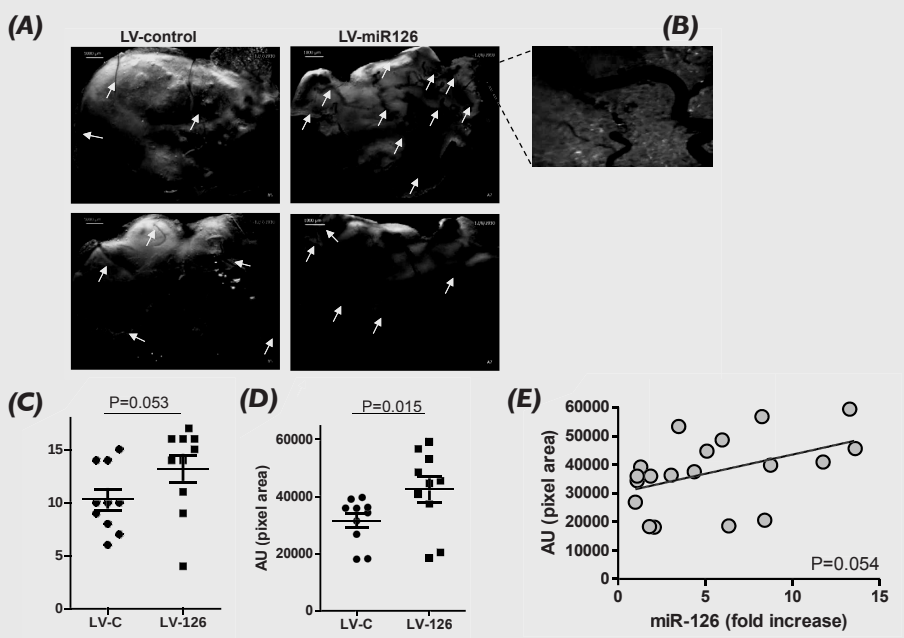

Figure 3. Overexpression of miR-126 in BM cells leads to increased neovascularization

(A) Representative microscopic images of both sides of matrigel plugs seven days after implantation in the flank of mice. Right panels show matrigel plugs obtained from animals transplanted with bone marrow cells transduced with LV-126, left panels demonstrate control animals. Arrowheads show penetrated vessels. ***(B)*** Movies were shot with SDF-camera 7 days after insertion of the matrigel plug in to the flank of the mice. A still of one of the movies is shown that displayed the active flow of red blood cells through the vessels that had grown into the angiogenic plugs. ***(C)*** Number of vessels was counted per plug and plotted. Animals transplanted with bone marrow cells that were transduced with LV-126 show higher number of vessels (n=10-12). ***(D)*** Vessels were 'digitalized' and the total length (in pixel area) was calculated in imageJ. LV-126-animals demonstrated a significant higher total pixel area as compared to LV-C-animals (n=10-12). ***(E)*** A correlation was found between the total length of vessels in the plug and the expression levels of miR-126 in the bone marrow (n=10-12).

of the bone marrow cells.

Overexpression of miR-126 in bone marrow leads to increased levels of white blood cells in the circulation

To begin to determine whether over expression of miR-126 in the bone marrow augmented matrigel vascularization due to elevated leukocyte infiltration we assessed the distribution of circulating cells in the periphery by sysmex and FACS analyse. Indeed we observed that the total number of white blood cells per mL blood was significantly correlated with the miR-126 expression levels found in the bone marrow (WBC, Figure 2F, n=22, $p<0.01$). FACS analysis of the various circulating hematopoietic subsets was performed to establish whether elevated numbers of circulating WBC was caused by a general mobilization effect of all WBCs or whether overexpression of miR-126 affected the differentiation of specific populations of the white blood cells, such as granulocytes, eosinophils and monocytes. When we plotted the number of cells per mL we observed that mice that overexpressed miR-126 displayed a higher number of circulating WBC and that the mobilizing effect of placing a VEGF/SDF-1 rich plug was also only observed in the mice overexpressing miR-126 in the hematopoietic compartment. In particular, in monocytes this mobilizing effect reached statistical significance (Table 1, n=22). When the number of the circulating subset cells are as percentage of total number of white blood cells we did not observe selective enrichments for any of the subpopulations indicating that, when assessed in the periphery, overexpression of miR-126 did not have a major impact on hematopoietic lineage specification (Table 1, n=22).

Discussion

Various studies have demonstrated a role for endothelial miR-126 in angiogenesis and the mobilization of vasculogenic cells [18-20]. However, significant expression of this miR has been demonstrated in circulating hematopoietic cells and platelets [26-29]. In this study we have set out to determine whether miR-126 expression in the hematopoietic compartment can also affect neovascularisation. As previously used methods to silence miR-126 with, for example, antagomirs [17, 18] act systemically and do not allow to assess the function of hematopoietic miR-126 independently from that of endothelial miR-126 we have chosen to overexpress miR-126 in the hematopoietic compartment specifically. To that end, we transduced lineage-depleted BM from donor mice with a lentiviral vector that drives the expression of miR-126 by a constitutive U6 promoter. Subsequently these BM cells were used to reconstitute the BM of lethally irradiated mice. After eight weeks chimeric mice displayed an over 8-fold elevation of miR-126 expression in the BM and consequently in all hematopoietic cells while we observed no major changes in weight, behavior

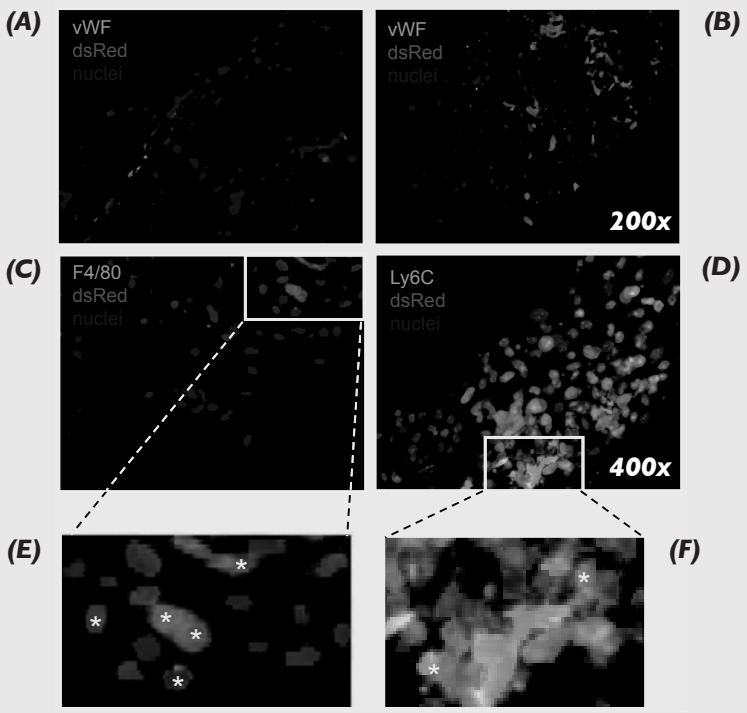

Figure 4. Matrigel plug slides were stained with endothelial marker von Willebrand Factor, macrophage marker F4/80 or monocytic marker Ly6C

(A,B) Microscopic images shows a positive staining for von Willebrand Factor adjacent to dsRED positive cells indicating a percytic role for infiltrated bone marrow. Macroscopic images show double positive staining for dsRED as well as ***(C)*** Ly6C and ***(D)*** F4/80 suggesting that monocytes and macrophages in the plug are derived from the bone marrow. Magnification of images show distinct double positive cells for dsRED and for ***(E)*** Ly6C and ***(F)*** F4/80 indicated with asterisks.

other health indicators. To begin to elucidate the relevance of hematopoietically expressed miR-126 to neovascularization we implanted VEGF and SDF-1 rich matrigel plugs subcutaneously in these mice. Surprisingly, after seven days, both image analysis as well as visual inspection of the matrigel plugs with a sidestream dark field camera revealed an almost two fold increase in vascularization of the plugs in mice overexpressing miR-126 in the hematopoietic cells.

Over expression of miR-126 in the transplanted bone marrow also led to an up regulation of total white blood cells in the circulation as compared to animals that were transplanted with control BM cells. As we showed this was a general effect on mobilization and this effect was directly related to the BM miR-126 levels in the tested mice, we concluded that miR-126 in the hematopoietic compartment serves to modulate VEFG and/or SDF-1 driven mobilization of BM cells to the

Table 1. Overview of FACS results and the correlation per population of leukocytes in the periphery

Population	% of cells (miR-126 versus control)	# of cells/mL (miR-126 versus control)	Correlation with miR-126 in BM
Lymphoblasts	Not upregulated	Upregulated (ns)	P<0.10
Lymphocytes	Not upregulated	Upregulated (ns)	P<0.15
Activated T-cells	Not upregulated	Not upregulated	-
Eosinophils	Not upregulated	Upregulated (P<0.05)	P<0.05
Granulocytes	Not upregulated	Upregulated (P<0.05)	P<0.10
Monocytes	Not upregulated	Upregulated (P<0.05)	P<0.02
NK-cells	Not upregulated	Upregulated (P<0.001)	P<0.17

periphery. Interestingly, a recent paper demonstrated that the general mobilizer GCSF promotes the accumulation of miR-126 loaded microvesicles in the BM. Subsequently, these vesicles are proposed to fuse to BM progenitor, stromal and endothelial cells and reduce the expression of vascular cell adhesion molecule-1 (VCAM-1) by these cells in a miR-126 dependent fashion. As VCAM-1 is critical to the retention of hematopoietic stem and progenitor cells in the BM, loss of VCAM-1 expression would drive the mobilization of these cells to the periphery [34]. As granulocytes are the main target of GCSF and express relatively high levels of miR-126 (van Solingen et al, unpublished data) and granulocytes might also be responsive to both VEGF and SDF-1 increasing miR-126 levels in these mobilizing vesicles could provide an explanation for the general mobilizing effect we observed when implanting VEGF/SDF-1 rich matrigel plugs into the miR-126 overexpressing mice. Alternatively, overexpression of miR-126 in BM cells could inhibit the expression of miR-126 target genes PI3KR2 and SPRED-1[19, 20] two major negative mediators of the VEGF receptor and CXCR4 signaling. Therefore, overexpression of miR-126 may render the hematopoietic cells more responsive to VEGF and SDF-1 gradients and lead to an increased migratory capacity.

Increased recruitment of leukocyte subsets to the matrigel plug could also contribute to the observed increase in vascularization. For instance, granulocytic neutrophils are able to infiltrate a foreign body [35] and are major sources of matrix metalloprotease type 9 (MMP-9) a protein that, by breaking down the extracellular matrix, functionally contributes to angiogenesis [36]. Furthermore, the secondary granules of eosinophils contain large amounts of preformed VEGF [37] and when these cells are in direct contact with ECs they enhance EC

proliferation and as such, angiogenesis [38] also, high levels of SDF-1 and VEGF have been demonstrated to be chemotactic for monocytes [39, 40] and upon extravasation into a foreign body these cells can differentiate into macrophages that then can support angiogenesis. Subsequently, macrophage can contribute to angiogenesis by producing angiogenic factors like VEGF and TGFβ [41, 42] or facilitating anastomosis of the forming vessels [43]. Taken together, the elevated levels of any of these cellular populations may affect the number and length of the vessels in the angiogenic plugs.

We also observed that the vascularization of the matrigel implants is closely associated with a profound infiltration of BM derived cells that organize alongside the von Willebrand factor-positive microvasculature suggesting that these cells fulfill a perivascular role supporting the ingrowth of the vessels. A mere higher number of possible pericytes that can invade the plug may also augment the vascular in growth either via stabilizing the growing vessels or in a paracrine way that may involve the secretion of miR-126 containing exosomes that can be taken up by ECs [30]. Whether such pro-angiogenic exosomes are also elevated in the circulation in the miR-126 overexpressing mice is the topic of current investigations.

In conclusion, overexpression of miR-126 in the hematopoietic compartment leads to increased neovascularization and has a general augmenting effect on the mobilization of of BM-derived to the peripheral circulation. While the exact mechanism by which hematopoietic miR-126 increases neovascularization is yet unclear we demonstrates a novel role for hematopoietic miR-126 in vascular homeostasis.

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