

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/23252> holds various files of this Leiden University dissertation.

**Author:** Bijkerk, Roel

**Title:** MicroRNAs in kidney health and disease

**Issue Date:** 2014-01-29

## Chapter

# 3

*Cardiovasc Res. 2011 Dec 1;92(3):449-55.*

### **MicroRNA-126 modulates endothelial SDF-1 expression and mobilization of Sca-1<sup>+</sup>/Lin<sup>-</sup> progenitor cells in ischemia**

Coen van Solingen<sup>1,2</sup>, Hetty C. de Boer<sup>1,2</sup>, Roel Bijkerk<sup>1,2</sup>, Matthieu Monge<sup>1</sup>, Annemarie M. van Oeveren-Rietdijk<sup>1,2</sup>, Leonard Seghers<sup>2,3</sup>, Margreet R. de Vries<sup>2,3</sup>, Eric P. van der Veer<sup>1,2</sup>, Paul H.A. Quax<sup>2,3</sup>, Ton J. Rabelink<sup>1,2</sup> and Anton Jan van Zonneveld<sup>1,2</sup>

<sup>1</sup>Department of Nephrology and the <sup>2</sup>Eindhoven Laboratory for Experimental Vascular Medicine, <sup>3</sup>Department of Surgery, LUMC, Leiden, the Netherlands.

## Abstract

MicroRNA-126 (miR-126), which is enriched in endothelial cells, plays a role in angiogenesis. Based on the seed sequence, miR-126 can also be predicted to regulate vasculogenesis by modulating the endothelial expression of stromal cell-derived factor-1 (SDF-1). Using miR-reporter constructs, we first validated that miR-126 inhibits SDF-1 expression in endothelial cells *in vitro*. Next, we investigated the potential relevance of this observation with respect to the mobilization of progenitor cells. For this, we studied the migration of human CD34<sup>+</sup> progenitor cells towards chemotactic factors present in endothelial cell-conditioned medium. Antagomir-induced silencing of miR-126 elevated SDF-1 expression by human umbilical vein endothelial cells and enhanced migration of the CD34<sup>+</sup> cells. In a murine model of hind limb ischemia, a striking increase in the number of circulating Sca-1<sup>+</sup>/Lin<sup>-</sup> progenitor cells in antagomir-126-treated mice was observed as compared to scramble-mir-treated controls. Immunohistochemical staining of capillaries in the post-ischemic gastrocnemius muscle of miR-126-silenced mice revealed elevated SDF-1 expressing CD31-positive capillaries, whereas a mobilizing effect of miR-126 inhibition was not detected in healthy control animals. MiR-126 can regulate the expression of SDF-1 in endothelial cells. In the context of an ischemic event, systemic silencing of miR-126 leads to the mobilization of Sca-1<sup>+</sup>/Lin<sup>-</sup> progenitor cells into the peripheral circulation, potentially in response to elevated SDF-1 expression by endothelial cells present in the ischemic tissue.

## Introduction

Integrity of the vascular endothelium is central to vascular homeostasis and is determined by the balance between endothelial injury and repair<sup>1</sup>. In the context of adverse hemodynamic and metabolic risk factors, endothelial cells (ECs) are damaged and constantly replaced by the proliferation of adjacent mature ECs. In the rat aorta, ECs in areas resistant to atherosclerosis have a low rate of cellular replication and have been estimated to have a lifespan of approximately 12 months. In contrast, EC turnover in lesion-prone sites is accelerated to weeks or less as animals age<sup>2</sup>. To maintain the integrity of the endothelium throughout life, endothelial cells in the arterial wall would therefore have to replicate well over a thousand times. However, primary endothelial cell cultures already become senescent after a limited number of passages due to a progressive shortening of telomeres that takes place upon each cell division. Moreover, telomere erosion is accelerated by chronic exposure of endothelial cells to oxidative stress<sup>3</sup>. Consequently, it has been suggested that mature ECs are insufficiently capable of repairing the chronically damaged endothelium throughout life. Support for this notion has been provided by several studies identifying a significant contribution by endothelial progenitor cells as an alternative cellular source for re-endothelialization of the damaged artery wall<sup>4-6</sup>.

Stromal cell derived factor-1 (SDF-1 or CXCL12) has been demonstrated to facilitate the homing of progenitor cells from the peripheral circulation to sites of vascular injury or tissue ischemia. SDF-1 can be actively secreted by injured endothelial cells<sup>7</sup> or activated platelets<sup>8</sup>, leading to the homing of bone marrow-mobilized progenitor cells to the site of injury<sup>9,10</sup>. The beneficial role of SDF-1 in recruiting bone marrow cells to ischemic tissue has been established in several animal models to improve recovery after an ischemic event<sup>7,11-13</sup>. Therefore, SDF-1 is believed to play a central role in regulating both vascular integrity and homeostasis, implicating that its expression should be tightly controlled. Indeed SDF-1 expression is reported to be regulated at multiple levels including transcription and post-translation<sup>7</sup>.

At the post-transcriptional level, SDF-1 expression could be regulated by microRNAs (miRNAs). MiRNAs constitute a class of highly conserved non-coding RNAs that control gene expression by inhibiting the translation of mRNA<sup>14</sup>. The ability of miRNAs to regulate multiple targets provides a means for the coordinated control of gene expression and make these molecules especially attractive candidates for regulating cell type-specific differentiation



and modulating cell function<sup>15</sup>. In recent years, miRNA expression-profiles of ECs have been analyzed in detail and recent studies demonstrated both pro-angiogenic<sup>16-19</sup> as well as anti-angiogenic functions for endothelial miRNAs<sup>20,21</sup>.

*In silico* analyses (<http://www.microrna.org>) for miRNAs targeting the 3' untranslated region (3' UTR) of the SDF-1 mRNA identified miR-126 as a potential post transcriptional regulator of SDF-1 expression. MiR-126 is highly enriched in ECs and has been demonstrated to regulate (ischemia-induced) angiogenesis by blocking the expression of SPRED-1 and PI3KR2<sup>22-24</sup>. Here, we validated the inhibitory actions of miR-126 on expression and function of SDF-1 in ECs *in vitro*. Furthermore, we demonstrate that antagomir-induced silencing of miR-126 prior to acute ischemia leads to the augmentation of SDF-1 levels in both the circulation and ischemic tissue. This is associated with increased mobilization of bone marrow-derived progenitor cells *in vivo*. Our data demonstrate that endothelial miR-126 can regulate both features of angiogenesis as well as vasculogenesis and suggest a role for miR-126 in the maintenance of endothelial homeostasis.

## Materials and Methods

### Cells and Cell Culture

Immortalized human umbilical vein endothelial cells (EC-RF24)<sup>25</sup> were cultured in M199 medium (Gibco, Breda, the Netherlands) supplemented with penicillin/streptomycin (Gibco/Invitrogen), 20% fetal calf serum (FCS, Bio Whittaker/Cambrex, Verviers, Belgium), 10 IU/mL heparin (Leo Pharma, Breda, the Netherlands), 2.5% HEPES buffer (Gibco) and 12.5 µg/mL endothelial cell growth supplement (Sigma-Aldrich, St. Louis, MO). Human umbilical vein endothelial cells (HUVEC) were isolated, cultured and characterized as described previously<sup>26</sup>. In short, ECs were isolated from freshly obtained human umbilical cords by trypsin/EDTA digestion of the interior of the umbilical vein. The cells were cultured in M199 medium supplemented with penicillin/streptomycin, 20% FCS, 10 IU/mL heparin, and 5% bovine pituitary extract (Gibco). CD34<sup>+</sup> cells were isolated from umbilical cord blood using Ficoll (Amersham, 's-Hertogenbosch, the Netherlands) density gradient centrifugation and positive selection using CD34-MACS microbeads (Miltenyi Biotech, Bergish Gladbach, Germany). Umbilical cord blood and umbilical cords were collected non-identifiable and with informed consent. Since the human tissues were unidentifiable, approval from the university ethics review board was not necessary.

### SDF-1 3'UTR Reporter Assays

Synthetic, double-stranded oligonucleotides spanning a 60 base-pair region of the murine 3'UTR of SDF-1 mRNA containing the putative miR-126 binding site with (pSDFmm) or without (pSDF) a C to G mismatch at position 3 of the seed-sequence were cloned into the pMIR-report<sup>TM</sup> Expression Reporter Vector System (Applied Biosystems, Amsterdam, the Netherlands; see online publication for oligonucleotide sequences). All plasmids were sequenced to confirm their structure and exclude cloning artefacts. A renilla luciferase expression plasmid (pRL-SV40, Promega, Leiden, the Netherlands) and a plasmid containing a single, perfect match miR-126 binding site served as controls<sup>23</sup>.

### Design of Antagomirs

Antagomir-126 and a control scramblemir (Dharmacon RNA technologies, Lafayette, CO) were synthesized as previously described<sup>23</sup>.



### **Luciferase Assay**

Antagomir-126 or control scramblemir (5 µg/mL) was added to a near confluent layer of fibronectin-adherent EC-RF24 cells. Twenty four hours after antagomir treatment, the EC-RF24 cells were detached by trypsinization and resuspended in 500 µL serum-free Optimem culture medium (Gibco) and 1.5 µg specific pMIR-report and 150 ng pRL-SV40 were added. The cell suspension was chilled for 10 minutes at 4°C and electroporated using the Gene Pulser II (Bio-Rad Laboratories, Veenendaal, the Netherlands). After 10 minutes recovery time at room temperature, 1.5x10E5 cells were plated in a 24-wells plate coated with fibronectin in triplicate. After 24 hours, the firefly-luciferase and renilla-luciferase signals were measured using a Dual-Luciferase® Assay Reporter System (Promega) in a Lumat LB9507 luminometer (EG&G Berthold, Bundoora, Australia).

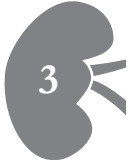
### **Western Blot**

HUVEC were incubated for 48 hours with 5 µg/mL antagomir-126 or scramblemir. Culture medium was aspirated and cells were washed two times with PBS, cellular lysates were harvested using lysis buffer (50 mM Tris-HCl (pH=7.5), 150 mM NaCl, 1% SDS, 0.5% sodiumdeoxycholate and 0.5% Triton X-100 (Sigma-Aldrich)). Protein levels for SDF-1 and β-actin were assessed by Western blot analysis with chemiluminescence detection. Equal amounts of protein were resolved on 15% SDS-polyacrylamide gels and transferred to PVDF membranes (Millipore, Billerica, MA). SDF-1 was detected using a polyclonal rabbit antibody against human SDF-1 (0.5 ug/mL, Abcam, Cambridge, UK) and for β-actin a polyclonal rabbit antibody against human β-actin (0.1 ug/mL, Abcam) was used. Horseradish peroxidase conjugated goat-anti-rabbit IgG (0.05 ug/mL, DakoCytomation, Enschede, the Netherlands) was used as secondary antibody. Bound fragments were detected with chemiluminescent reagents (Supersignal West Dura Extended Duration Substrate, ThermoScientific) and exposed on Hyperfilm ECL (Amersham). Quantitative analysis of the SDF-1 band intensity on Western blot was performed using imageJ software and normalized for β-actin. The ratio of the SDF-1 band intensity over the β-actin band intensity of the antagomir-126 treated HUVEC was arbitrarily set at 100%.

### **Migration Assay**

HUVEC were incubated in serum-free, cell-specific medium with 0.1% insulin-transferrin-sodium selenite media supplement (ITS, Sigma-Aldrich) containing antagomir-126 or scramblemir (5 µg/mL) at 37°C. After 48 hours, cell supernatants were harvested and placed into the lower compartment of

a transwell system with a pore size of 5  $\mu\text{m}$  (Corning B.V. Life Sciences, Amsterdam, the Netherlands). Human CD34<sup>+</sup> cells were added to the upper chamber and migration was followed over a period of 4 hours at 37°C. As a positive control 50 ng/mL recombinant human SDF-1(R&D Systems) was added in the lower compartment. The SDF-1-receptor, CXCR4, was neutralized by incubation of CD34<sup>+</sup> cells with a blocking antibody against CXCR4 (8  $\mu\text{g/mL}$ , R&D Systems) for 30 minutes at 4°C. After 4h, the cell-suspensions in the lower compartment were harvested, spun down and incubated for 30 minutes at 4°C in FACS buffer (PBS + 1% bovine serum albumin (BSA, Sigma-Aldrich)) with directly conjugated antibodies against human CD34 (PerCP-Cy5, BD Biosciences, Breda, the Netherlands) and human CD133 (APC, Miltenyi Biotec). A fixed number of CD34<sup>+</sup> cells was fluorescently labeled by incubation with calcein-AM (R&D Systems, Minneapolis, MD) and added prior to FACS analysis.



### **Hind Limb Ischemia Model**

All animal experimental protocols were approved by the animal welfare committee of the Netherlands Organization for Applied Scientific Research (TNO, Leiden, the Netherlands) and conform the Directive 2010/63/EU of the European Parliament. One day prior to surgery, C57BL/6 WT mice (n=6 per group, age=10 weeks, Charles River, Maastricht, the Netherlands) were injected intravenously (200  $\mu\text{L}$ ) with antagomir-126 (1.0 mg/animal) or scramblemir (1.0 mg/animal). Before surgery, mice were anesthetized intraperitoneally with a combination of Midazolam (5 mg/kg, Roche Diagnostics, Almere, the Netherlands), Medetomidine (0.5 mg/kg, Orion Corporation, Turku, Finland) and Fentanyl (0.05 mg/kg, Janssen Pharmaceutica, Tilburg, the Netherlands). Ischemia of the left hind limb was induced by electrocoagulation of the left common femoral artery, proximal to the bifurcation of superficial and deep femoral artery, as described<sup>27</sup>. To sacrifice the mice, blood was withdrawn for FACS-analysis (EDTA-anti-coagulated) by heart puncture and the tibia and femur of both legs were kept for isolation of bone marrow cells. Additionally, the gastrocnemius muscle of both hind limbs was dissected and placed in 4% formaldehyde overnight. After paraffin embedding, 4  $\mu\text{m}$  thick serial cross-sections were made for immunohistochemical analysis.

### **Whole Blood 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), FACS-analysis or ELISA.



Hematological values obtained were white blood cell counts (WBC,  $n \times 10^6$ /mL), red blood cell counts (RBC,  $n \times 10^9$ /mL), platelets (PLT,  $n \times 10^6$ /mL), hematocrit (HCT, %/%) and haemoglobin (HGB, mmol/L). For FACS analysis, we incubated 50  $\mu$ L of whole blood for 60 minutes at 4°C with directly conjugated antibodies directed against Sca-1 (FITC, BD-Biosciences), Flk-1 (PE, BD-Pharmingen, San Diego, CA) and a cocktail against lineage-positive cells (APC, BD-Pharmingen). In a separate tube, 50  $\mu$ L of whole blood was incubated with an appropriate cocktail of isotype controls, to identify the threshold for lineage-positivity. SDF-1 levels in serum were assessed with ELISA (R&D Systems).

### **Immunohistochemistry**

Four  $\mu$ m-thick sections of murine gastrocnemius muscle were re-hydrated and incubated with antigen-retrieval buffer (0.1% trypsin/EDTA in PBS). Next, sections were incubated with specific antibodies against murine SDF-1 (rabbit polyclonal IgG, ThermoScientific, Rockford, IL) and murine CD31 (mouse monoclonal IgG2b, Santa Cruz, Heidelberg, Germany) followed by secondary antibodies against goat-anti-rabbit-IgG labeled with Alexa-488 or goat-anti-mouse IgG labeled with Alexa-568 (Molecular Probes). As a negative control, isotype-matched IgGs were used. Images were made using a confocal microscope (Carl Zeiss, Sliedrecht, the Netherlands).

### **FACS Analysis**

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

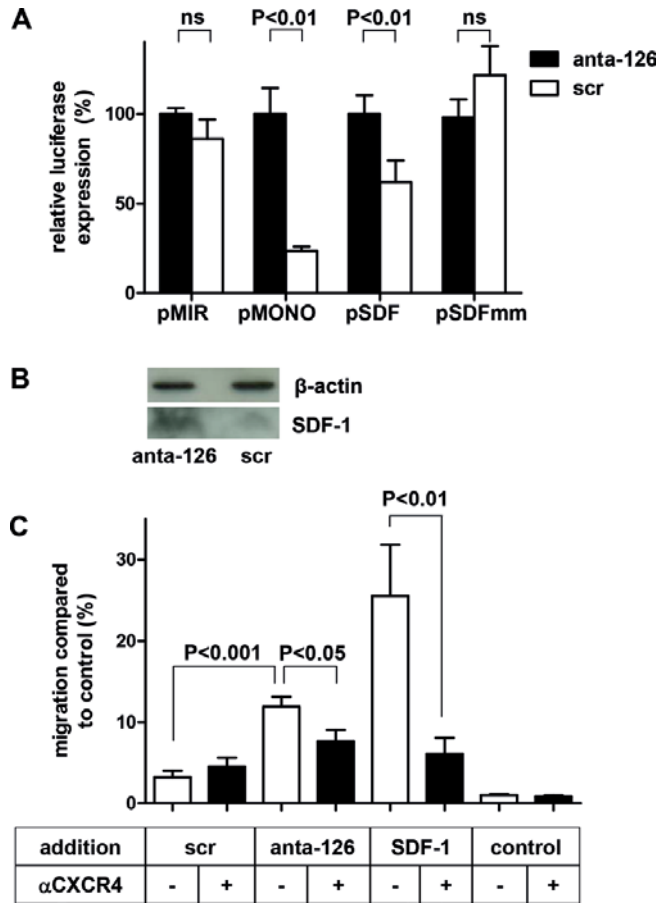
### **Statistical Analysis**

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

## Results

### MiR-126 affects the expression and function of SDF-1 in vitro

Using an on line miRNA target search tool ([http:// www.microrna.org](http://www.microrna.org)) we identified a putative miR-126 binding site in the 3'UTR of the SDF-1 mRNA. To assess its functionality, we cloned this binding site into the 3'UTR of a luciferase reporter gene driven by a constitutive promoter (pSDF) and analyzed luciferase expression and of a control plasmid in the human endothelial cell



**Figure 1. MiR-126 affects the expression and function of SDF-1 in vitro.**

A, EC-RF24 were pre-incubated with scrambleir or antagomir-126 and transfected with pMIR as negative control, pMONO as positive control, pSDF with the sequence of the SDF-1 miR-126 binding site or a variant with a single mismatch in the seed sequence (pSDFmmm). Experiments were performed in triplicate and data are expressed as mean values  $\pm$  standard error of the mean. B, HUVEC were incubated for 48 hours with antagomir-126 or scrambleir and conditioned cell supernatants subjected to Western blot analysis for SDF-1. C, HUVEC (n=4) were treated with antagomir-126, scrambleir or vehicle and the conditioned media were used to study the chemotactic activity

towards human hematopoietic CD34<sup>+</sup> cells in vitro. Recombinant SDF-1 was used as positive control and the contribution of the SDF-1 receptor (CXCR4) was studied with the use of a function-blocking antibody ( $\alpha$ CXCR4) (n=2).

line EC-RF24. In these miR-126 expressing cells, luciferase expression driven from a reporter construct lacking a miRNA seed sequence (pMIR) was not affected when miR-126 was silenced using an antagomir approach (anta-126). In contrast, a reporter gene carrying a single perfect miR-126 binding site (pMONO) was fully active when miR-126 was silenced but its expression was



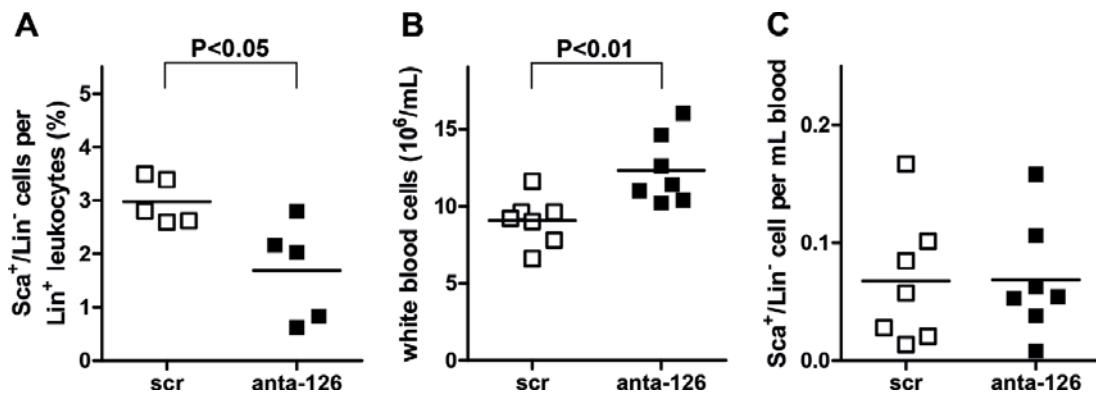
strongly reduced in the presence of a control scramblemir (scr). These studies confirm the functionality of miR-126 in EC-RF24 cells. Likewise, the presence of the putative SDF-1 miR-126 binding site in the reporter construct led to a 40% reduction of luciferase-expression in miR-126 expressing cells (Figure 1A, scramble vs. antagomir-126,  $P < 0.01$ ). A single mismatch in the seed sequence of the putative SDF-1 miR-126 binding site (C to G at position 3 of the seed sequence, pSDFmm) alleviated miR-126-dependent repression of reporter gene expression, confirming the presence and specificity of the miR-126 binding site in the 3'UTR of the SDF-1 mRNA. To validate the regulatory effect of miR-126 on endogenous SDF-1 protein expression in endothelial cells, human umbilical vein endothelial cells (HUVEC) were treated with antagomir-126 or scramblemir, after which SDF-1-protein expression was determined by Western blot analysis. A marked increase of SDF-1 protein expression was observed in HUVEC treated with antagomir-126 as compared to scramblemir (Figure 1B).

Next, we assessed the potential of miR-126 to regulate the extent by which endothelial cell-derived SDF-1 attracts progenitor cells. To this end, conditioned medium derived from HUVEC, incubated with antagomir-126 or scramblemir, was applied to the lower compartment of a transwell-system and umbilical cord blood-derived CD34<sup>+</sup> cells were placed in the upper compartment. As shown in Figure 1C, silencing of miR-126 in HUVEC-enhanced migration of CD34<sup>+</sup> cells compared to the scramblemir-control ( $P < 0.001$ ). Moreover, neutralization of CXCR4 expressed by the CD34<sup>+</sup> cells with a function-blocking antibody ( $\alpha$ CXCR4, 8  $\mu$ g/mL) decreased cell migration towards the conditioned supernatant derived from antagomir-126 treated HUVEC back to the scramble control levels ( $P < 0.05$ ). As a control, maximal cellular migration was observed using medium containing recombinant human SDF-1 (50 ng/mL), which was significantly reduced by  $\alpha$ CXCR4 (Figure 1C,  $P < 0.01$ ). These data suggest that suppression of miR-126 in endothelial cells *in vitro* can augment expression of functional SDF-1, leading to elevated progenitor cell migration.

### **MiR-126 affects progenitor cell mobilization via SDF-1 in vivo**

Endothelial miR-126 expression and functionality is conserved in murine EC, both *in vivo*<sup>23</sup> and *in vitro* (data not shown). To investigate the regulatory role of miR-126 on SDF-1-dependent progenitor cell mobilization *in vivo*, we applied single tail vein injections of antagomir-126 or the control scramblemir into wild type (WT) male C57Bl/6 mice ( $n=6$ ). Ten days after injection, we enumerated the number of circulating Sca-1-positive and lineage-negative

(Sca-1<sup>+</sup>/Lin<sup>-</sup>) cells by FACS analysis. Surprisingly, when we expressed the circulating Sca-1<sup>+</sup>/Lin<sup>-</sup> cell numbers as a percentage of Lin<sup>+</sup> leukocytes, we observed a significant decrease in circulating Sca-1<sup>+</sup>/Lin<sup>-</sup> cells in the mice in which miR-126 had been silenced by antagomir-126 administration as compared to the scramble-mir-treated controls (Figure 2A, P<0.05). This observation could be explained by the fact that antagomir-126 treated mice also displayed a significant increase in total white blood cell (WBC) count (Figure 2B, P<0.01). Such an increase is consistent with reports showing that increased SDF-1 levels in the circulation result in an elevation of WBCs<sup>9</sup>.

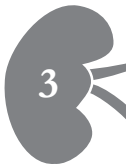


**Figure 2. MiR-126 does not solely affect mobilization of Sca-1<sup>+</sup>/Lin<sup>-</sup> cells in vivo.**

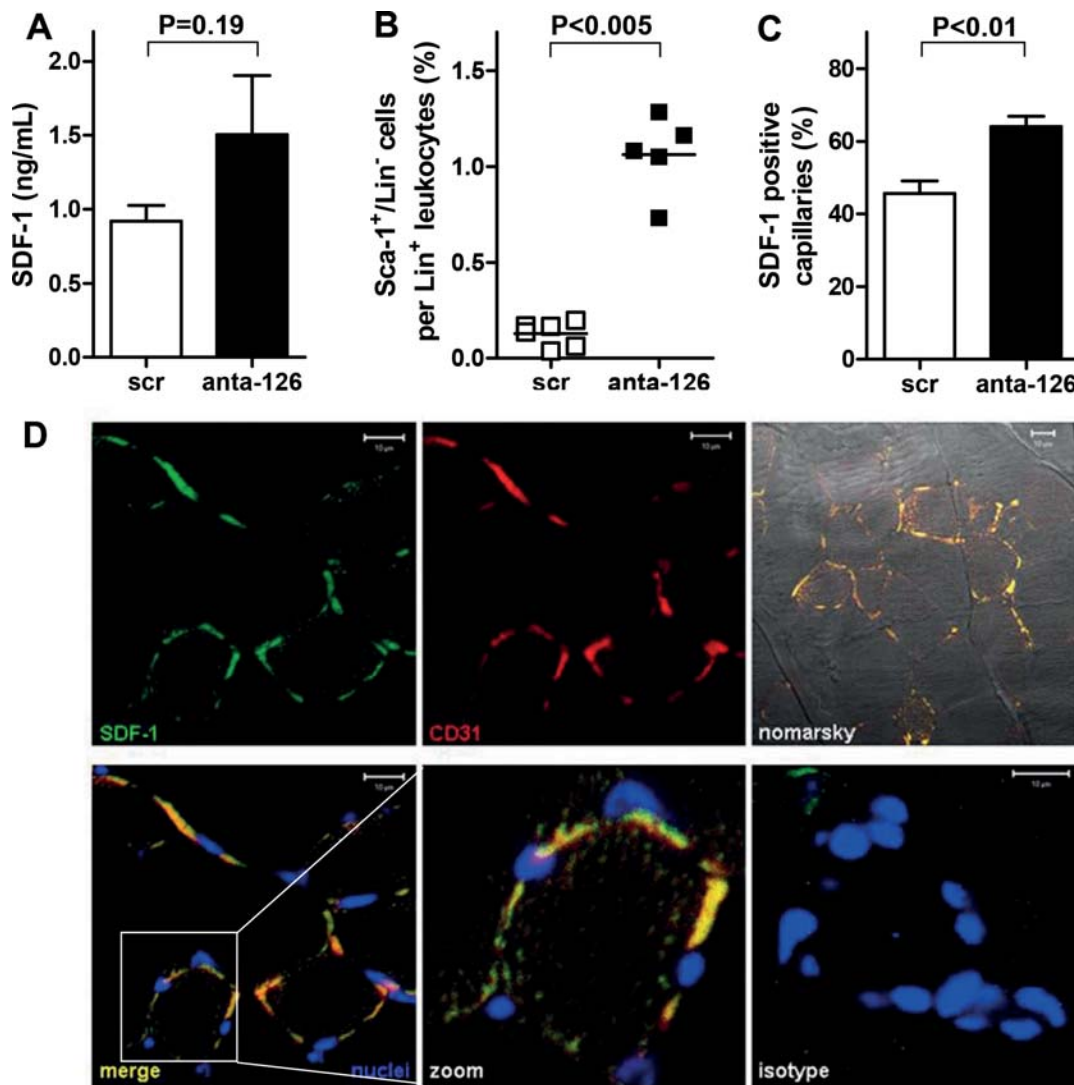
A, Ten days after injection of scramble-mir or antagomir-126, Sca-1<sup>+</sup>/Lin<sup>-</sup> cells in whole blood were analyzed by FACS, expressed as percentage of total Lin<sup>+</sup> leukocytes. B, Ten days after injection of scramble-mir or antagomir-126, total white blood cell counts per mL was determined. C, Ten days after scramble-mir- or antagomir-126-injection Sca-1<sup>+</sup>/Lin<sup>-</sup> cells were analyzed by FACS in whole blood expressed as total number in mL blood.

As such, when we expressed the number of circulating Sca-1<sup>+</sup>/Lin<sup>-</sup> cells per mL blood, the differences between antagomir-126 and scramble-mir-treated animals was lost, indicating that there is no systemic role for miR-126 in Sca-1<sup>+</sup>/Lin<sup>-</sup> progenitor cell mobilization *in vivo* (Figure 2C).

Ischemic endothelial cells have been established to up regulate SDF-1<sup>7</sup>, which we confirmed *in vitro* in HUVEC under hypoxic conditions (data not shown). We investigated whether an ischemic stimulus in combination with attenuation of miR-126 levels would modulate progenitor cell levels in mice. Therefore, we injected male WT mice (n=6) with either antagomir-126 or scramble-mir and, the next day, this procedure was followed by the induction of unilateral hind limb ischemia (HLI). Ten days after induction of HLI, the animals were sacrificed and serum levels of SDF-1 were assessed by ELISA. Although systemic SDF-1 levels were elevated in antagomir-126-treated mice as compared to controls, no significance was reached (Figure 3A, P=0.19).



Nevertheless, when we examined the *in vivo* mobilization of Sca-1<sup>+</sup>/Lin<sup>-</sup> cells to the circulation after ischemia, we observed an 8-fold increase of Sca-1<sup>+</sup>/Lin<sup>-</sup> cells expressed as percentage of total Lin<sup>+</sup> leukocytes in the blood of



**Figure 3. MiR-126 can alter SDF-1-expression and induce progenitor mobilization after ischemic injury in vivo.** A, Ten days after HLI antagomir-126-treated mice showed non-significantly elevated levels of SDF-1 in the serum as compared to scramblemir-treated controls (n=6). B, Ten days after HLI and injection of scramblemir or antagomir-126, Sca-1<sup>+</sup>/Lin<sup>-</sup> cells were measured in whole blood, expressed as percentage of total Lin<sup>+</sup> leukocytes C, Immunohistochemical micrographs of immunohistochemistry of the gastrocnemius muscle after HLI showed colocalization of SDF-1 and CD31. SDF-1 was visualized with alexa-488 (green) and CD31 with alexa-568 (red). Nuclei were stained with DAPI and shown in blue, Nomarsky contrast images show muscle tissue. Scale bars represent 10  $\mu$ m. D, Quantification of microscopic images displayed that antagomir-126-treated mice have increased levels of SDF-1 positive capillaries as percentage of total number of capillaries (n=6).

antagomir-126-treated animals as compared to scramblemir-treated control animals (Figure 3B,  $P < 0.005$ ). The absolute number of Sca-1<sup>+</sup>/Lin<sup>-</sup> cells per mL blood was raised from  $0.24 \pm 1.4$  in the control group to  $2.1 \pm 1.4$  in the antagomir-126 treated animals. As the observed effects suggested an ischemia-dependent elevation of SDF-1, we next investigated whether the ischemic gastrocnemius muscle could be a potential contributor to the elevated levels of circulating SDF-1. Therefore, we performed both detailed qualitative and quantitative immunohistochemical analysis of SDF-1 expression in relation to CD31-positive capillaries. Indeed, we observed a clear co-localization of SDF-1 and CD31 expression in sections of the ischemic muscle (Figure 3D). Furthermore, the ischemic muscle of antagomir-126-treated animals showed a higher percentage of double-positive capillaries (Figure 3C,  $P < 0.01$ ) as opposed to scramblemir-treated controls. These data imply that the mildly elevated levels of SDF-1 protein in the circulation upon antagomir-126-treatment may be a reflection of the locally enhanced expression of SDF-1 protein in the vessels of ischemic tissue.



## Discussion

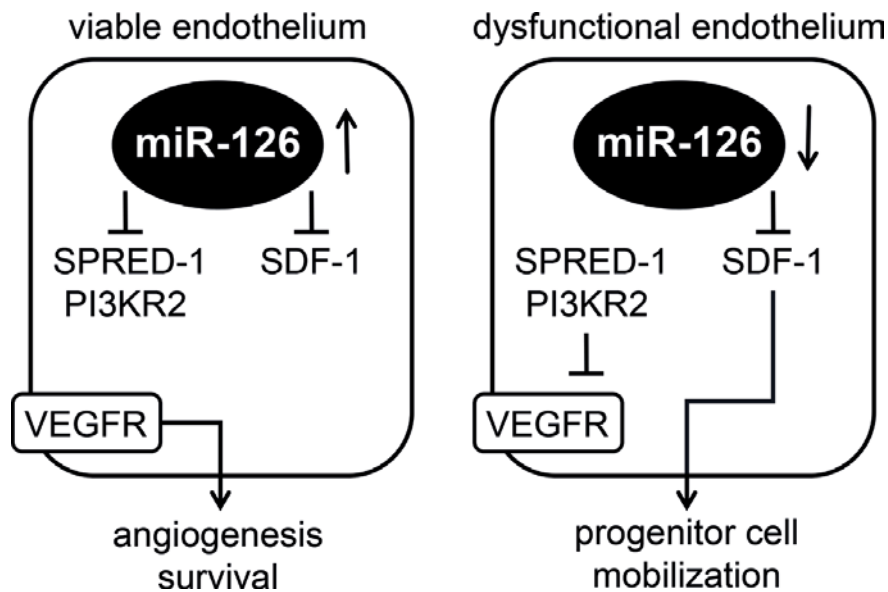
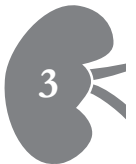
A function for miR-126 in angiogenic processes in vascular maintenance and during development has been shown in a number of publications demonstrating that loss of miR-126, either in knockout models or mediated by treatment with antagomirs, leads to structural impairment of the vascular bed<sup>22-24</sup>. In the current study, we provide evidence for a vasculogenic role for miR-126 in regulating the mobilization of endothelial progenitor cells via the release of chemokine SDF-1 from ischemic endothelial cells. *In vitro*, the increased secretion of SDF-1 upon silencing of miR-126 was sufficient to stimulate the migration of human CD34<sup>+</sup> stem cells. In mice however, systemic silencing with a single tail vein injection of antagomir-126 was not sufficient to raise the levels of circulating murine Sca-1<sup>+</sup>/Lin<sup>-</sup> progenitor cells. However, in combination with the ligation of the femoral artery, we demonstrated an increase in circulating Sca-1<sup>+</sup>/Lin<sup>-</sup> cells following miR-126 silencing, strongly suggesting that tissue ischemia is needed to reveal the regulatory role of miR-126 *in vivo*.

Our data suggest that elevated numbers of circulating Sca-1<sup>+</sup>/Lin<sup>-</sup> cells in the antagomir-treated animals are the result of SDF-1 mediated mobilization of these cells following ischemia. This is supported by the fact that SDF-1-protein expression is also up regulated in the endothelial cells of the ischemic tissue as well as in the peripheral circulation. Nevertheless, definitive proof of a causal role of miR-126 in SDF-1 dependent mobilization of progenitor cells would require an *in vivo* blockade in the SDF-1/CXCR4 axis. For this, inhibitors of the interaction of SDF-1 with CXCR4, such as AMD3100, could be administered to antagomir-126 treated mice and used to alleviate the elevated progenitor cell mobilization following ischemia. This approach is however complicated by the direct effects of these inhibitors on the egress of progenitor cells from the bone marrow which could potentially override the SDF-1 effects elicited by the endothelium in the ischemic tissues in the periphery<sup>28</sup>. Also, impaired progenitor homing to the ischemic tissue or the spleen cannot be fully excluded<sup>29</sup>.

An interaction between miR-126 and SDF-1 has previously been shown to increase miR-126 uptake of endothelial cell-derived apoptotic bodies by endothelial cells. This resulted in increased SDF-1 expression through inhibition of Regulator of G-protein signaling 16 (RGS16)<sup>30</sup>. In contrast, our studies implicate that the abrogation of miR-126 is associated with increased expression of SDF-1, suggesting that miRNAs could serve as a biological

rheostat, with the response magnitude of biological pathways being dependent on the context and source of the external stimulus.

Recently, it has been demonstrated that miRNAs are present in the circulation and that alterations in the profile of plasma or serum miRNAs can be associated with disease states<sup>31,32</sup>. These early reports mainly displayed a link between circulating miRNAs and cancer, while subsequent studies have also revealed a clear association of circulating miRNAs with cardiovascular disease<sup>33-35</sup>. Since endothelial injury is considered one of the hallmarks of patients at risk for cardiovascular disease and, upon injury, endothelial cells can secrete miRNA-containing microvesicles<sup>36-38</sup>, it is of interest to address the value of endothelial cell-derived circulating miRNAs as biomarkers for cardiovascular disease. Indeed, two recent clinical studies revealed a decrease in circulating miR-126 levels in patients with coronary artery disease (CAD)<sup>39</sup> and diabetes mellitus 2 (DM2)<sup>40</sup>. It was suggested that lowered levels of miR-126 in DM2 patients might be explained by the fact that high glucose levels can lead to a decrease of the miR-126 content in the endothelial particles, while cellular miRNA levels remained unaltered<sup>40</sup>. One may speculate that lowering of circulating miR-126 levels might be a specific signal of the injured



**Figure 4. MiR-126 acts as a vasculogenic switch.** In viable endothelium miR-126 inhibits the expression of SPRED-1 and PI3KR2 thereby facilitating VEGF-dependent angiogenesis. Furthermore, the expression of SDF-1 and VCAM-1 are inhibited. When miR-126 is lost, SPRED-1 and PI3KR2 are up regulated thereby blocking angiogenesis, at the same time SDF-1 and VCAM-1 levels are up regulated and this elevation subsequently leads to an increased mobilization and adhesion of bone marrow derived progenitors.



endothelium to increase the expression levels of distinct miR-126 targets that are critical for the integrity of the endothelium.

We suggest here that miR-126 functions as a regulator of endothelial homeostasis (Figure 4). In the healthy, viable endothelium, miR-126 is readily expressed and serves to down regulate SPRED-1 and PI3KR2, both of which are inhibitors of angiogenic and cell survival signals in response to vascular endothelial cell growth factor (VEGF)<sup>22,24</sup>. This condition favors the angiogenic response to injury, as SDF-1 expression is concomitantly repressed by miR-126. On the other hand, under conditions associated with endothelial cell dysfunction or senescence, a decrease in miR-126 levels would inhibit angiogenic and cell survival signals. Furthermore, this condition favors the expression of SDF-1 and VCAM-1<sup>41</sup> of the affected endothelial cells and support re-endothelialization by a vasculogenic response through the recruitment and subsequent adhesion of vascular progenitor cells. As bone marrow-derived CD34<sup>+</sup> cells were recently demonstrated to represent a more functional EPC population than Sca-1<sup>+</sup>/Lin<sup>-</sup> progenitor cells, future studies may include these progenitor cell population as well<sup>42</sup>.

Although speculative, our model is supported by the observed decreased levels of circulating miR-126 in DM2 and CAD patients<sup>39,40</sup> and elevated levels of SDF-1 in patients with acute coronary syndrome (ACS)<sup>43</sup>. Future studies in patient cohorts will provide insight as to whether the down regulation of circulating miR-126 can be correlated with increased levels of SDF-1.

In conclusion, both *in vitro* and *in vivo*, miR-126 can regulate the expression of SDF-1 in endothelial cells, which may lead to the mobilization of Sca-1<sup>+</sup>/Lin<sup>-</sup> progenitor cells into the peripheral circulation following conditions of acute ischemia. As the endothelial miR-126 regulates both features of angiogenesis as well as vasculogenesis, a regulatory role for this miRNA in endothelial homeostasis is proposed.

## Acknowledgements

CvS is supported by the Netherlands Heart Foundation (grant number 2006B145). MM and RB are supported by the Dutch Kidney Foundation (grant number C08.2282 and C07.2227 respectively). EvdV is supported by the Translational Excellence in Regenerative Medicine (TeRM) Smart Mix Program of the Netherlands Ministry of Economic Affairs and the Netherlands Ministry of Education, Culture and Science. AJvZ is supported by a grant from the Genzyme Renal Innovations Program.

## References

1. Rabelink, T.J., de Boer, H.C. & van Zonneveld, A.J. Endothelial activation and circulating markers of endothelial activation in kidney disease. *Nature reviews. Nephrology* 6, 404-414 (2010).
2. Schwartz, S.M. & Benditt, E.P. Clustering of replicating cells in aortic endothelium. *Proceedings of the National Academy of Sciences of the United States of America* 73, 651-653 (1976).
3. Kurz, D.J., et al. Chronic oxidative stress compromises telomere integrity and accelerates the onset of senescence in human endothelial cells. *Journal of cell science* 117, 2417-2426 (2004).
4. Op den Buijs, J., et al. Mathematical modeling of vascular endothelial layer maintenance: the role of endothelial cell division, progenitor cell homing, and telomere shortening. *American journal of physiology. Heart and circulatory physiology* 287, H2651-2658 (2004).
5. Foteinos, G., Hu, Y., Xiao, Q., Metzler, B. & Xu, Q. Rapid endothelial turnover in atherosclerosis-prone areas coincides with stem cell repair in apolipoprotein E-deficient mice. *Circulation* 117, 1856-1863 (2008).
6. Asahara, T., et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *The EMBO journal* 18, 3964-3972 (1999).
7. Yamaguchi, J., et al. Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation* 107, 1322-1328 (2003).
8. Massberg, S., et al. Platelets secrete stromal cell-derived factor 1alpha and recruit bone marrow-derived progenitor cells to arterial thrombi in vivo. *The Journal of experimental medicine* 203, 1221-1233 (2006).
9. Hattori, K., Heissig, B. & Rafii, S. The regulation of hematopoietic stem cell and progenitor mobilization by chemokine SDF-1. *Leukemia & lymphoma* 44, 575-582 (2003).
10. de Boer, H.C., et al. Human CD34+/KDR+ cells are generated from circulating CD34+ cells after immobilization on activated platelets. *Arteriosclerosis, thrombosis, and vascular biology* 31, 408-415 (2011).
11. Hiasa, K., et al. Gene transfer of stromal cell-derived factor-1alpha enhances ischemic vasculogenesis and angiogenesis via vascular endothelial growth factor/endothelial nitric oxide synthase-related pathway: next-generation chemokine therapy for therapeutic neovascularization. *Circulation* 109, 2454-2461 (2004).
12. Askari, A.T., et al. Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet* 362, 697-703 (2003).
13. Tepper, O.M., et al. Adult vasculogenesis occurs through in situ recruitment, proliferation, and tubulization of circulating bone marrow-derived cells. *Blood* 105, 1068-1077 (2005).
14. Kloosterman, W.P. & Plasterk, R.H. The diverse functions of microRNAs in animal development and disease. *Developmental cell* 11, 441-450 (2006).
15. Xie, X., et al. Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals. *Nature* 434, 338-345 (2005).
16. Urbich, C., Kuehbach, A. & Dimmeler, S. Role of microRNAs in vascular diseases, inflammation, and angiogenesis. *Cardiovascular research* 79, 581-588 (2008).
17. Kuehbach, A., Urbich, C., Zeiher, A.M. & Dimmeler, S. Role of Dicer and Drosha for endothelial microRNA expression and angiogenesis. *Circulation research* 101, 59-68 (2007).
18. Fasanaro, P., et al. MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. *The Journal of biological chemistry* 283, 15878-15883 (2008).
19. Chen, Y. & Gorski, D.H. Regulation of angiogenesis through a microRNA (miR-130a) that down-regulates antiangiogenic homeobox genes GAX and HOXA5. *Blood* 111, 1217-1226 (2008).



20. Suarez, Y., Fernandez-Hernando, C., Pober, J.S. & Sessa, W.C. Dicer dependent microRNAs regulate gene expression and functions in human endothelial cells. *Circulation research* 100, 1164-1173 (2007).
21. Poliseno, L., et al. MicroRNAs modulate the angiogenic properties of HUVECs. *Blood* 108, 3068-3071 (2006).
22. Fish, J.E., et al. miR-126 regulates angiogenic signaling and vascular integrity. *Developmental cell* 15, 272-284 (2008).
23. van Solingen, C., et al. Antagomir-mediated silencing of endothelial cell specific microRNA-126 impairs ischemia-induced angiogenesis. *Journal of cellular and molecular medicine* 13, 1577-1585 (2009).
24. Wang, S., et al. The endothelial-specific microRNA miR-126 governs vascular integrity and angiogenesis. *Developmental cell* 15, 261-271 (2008).
25. Fontijn, R., et al. Maintenance of vascular endothelial cell-specific properties after immortalization with an amphotrophic replication-deficient retrovirus containing human papilloma virus 16 E6/E7 DNA. *Experimental cell research* 216, 199-207 (1995).
26. Jaffe, E.A., Nachman, R.L., Becker, C.G. & Minick, C.R. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *The Journal of clinical investigation* 52, 2745-2756 (1973).
27. van Weel, V., et al. Vascular endothelial growth factor overexpression in ischemic skeletal muscle enhances myoglobin expression in vivo. *Circulation research* 95, 58-66 (2004).
28. Devine, S.M., et al. Rapid mobilization of functional donor hematopoietic cells without G-CSF using AMD3100, an antagonist of the CXCR4/SDF-1 interaction. *Blood* 112, 990-998 (2008).
29. Zhao, X., et al. The spleen recruits endothelial progenitor cell via SDF-1/CXCR4 axis in mice. *J Recept Signal Transduct Res* 30, 246-254 (2010).
30. Zernecke, A., et al. Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection. *Sci Signal* 2, ra81 (2009).
31. Mitchell, P.S., et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proceedings of the National Academy of Sciences of the United States of America* 105, 10513-10518 (2008).
32. Lawrie, C.H., et al. Detection of elevated levels of tumour-associated microRNAs in serum of patients with diffuse large B-cell lymphoma. *Br J Haematol* 141, 672-675 (2008).
33. Ji, X., et al. Plasma miR-208 as a biomarker of myocardial injury. *Clin Chem* 55, 1944-1949 (2009).
34. Corsten, M.F., et al. Circulating MicroRNA-208b and MicroRNA-499 reflect myocardial damage in cardiovascular disease. *Circ Cardiovasc Genet* 3, 499-506 (2010).
35. Ai, J., et al. Circulating microRNA-1 as a potential novel biomarker for acute myocardial infarction. *Biochem Biophys Res Commun* 391, 73-77 (2010).
36. Sapet, C., et al. Thrombin-induced endothelial microparticle generation: identification of a novel pathway involving ROCK-II activation by caspase-2. *Blood* 108, 1868-1876 (2006).
37. Combes, V., et al. In vitro generation of endothelial microparticles and possible prothrombotic activity in patients with lupus anticoagulant. *The Journal of clinical investigation* 104, 93-102 (1999).
38. Boulanger, C.M., et al. In vivo shear stress determines circulating levels of endothelial microparticles in end-stage renal disease. *Hypertension* 49, 902-908 (2007).
39. Fichtlscherer, S., et al. Circulating microRNAs in patients with coronary artery disease. *Circulation research* 107, 677-684 (2010).
40. Zampetaki, A., et al. Plasma microRNA profiling reveals loss of endothelial miR-126 and other microRNAs in type 2 diabetes. *Circulation research* 107, 810-817 (2010).

41. Harris, T.A., Yamakuchi, M., Ferlito, M., Mendell, J.T. & Lowenstein, C.J. MicroRNA-126 regulates endothelial expression of vascular cell adhesion molecule 1. *Proceedings of the National Academy of Sciences of the United States of America* 105, 1516-1521 (2008).
42. Yang, J., et al. CD34+ cells represent highly functional endothelial progenitor cells in murine bone marrow. *PLoS One* 6, e20219 (2011).
43. Stellos, K., et al. Expression of stromal-cell-derived factor-1 on circulating platelets is increased in patients with acute coronary syndrome and correlates with the number of CD34+ progenitor cells. *Eur Heart J* 30, 584-593 (2009).



