miR126-5p repression of ALCAM and SetD5 in endothelial cells regulates leucocyte adhesion and transmigration

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Aims

miR126-5p is processed from the miR126-3p/-5p duplex, which is expressed in endothelial cells and gives rise to the guide strand miR126-3p and the passenger strand miR126-5p. miR126-3p has prominent roles in vascular development and diseases, whereas the expression and physiological functions of miR126-5p are unknown. The purpose of this study was to evaluate the expression and role of miR126-5p in blood vessel endothelial cells.

Methods and results

miR126-5p is mostly expressed in blood vessel endothelial cells in vivo and in vitro. Gain- and loss-of-function approaches revealed that miR126-5p promotes leucocyte adhesion and represses leucocyte transendothelial migration. Two distinct target genes of miR126-5p in endothelial cells were identified: the activated leucocyte cell adhesion molecule (ALCAM) gene which codes for an adhesion molecule involved in leucocyte transendothelial migration and SetD5, a gene with previously unknown functions. Using either a blocking antibody or target protectors which specifically disrupt the miRNA/mRNA target pairing, we showed that miR126-5p promotes leucocyte adhesion by controlling the expression of SetD5 and represses transendothelial migration via the regulation of ALCAM. miR126-5p controls ALCAM and SetD5 expression in vivo in separate tissues and regulates leucocyte infiltration into inflamed lungs by repressing ALCAM expression.

Conclusion

miR126-5p is a functional, endothelial-enriched microRNA that participates in the control of leucocyte trafficking by regulating the expression of ALCAM and SetD5.

Keywords

miRNA • miR126-5p • Endothelium • Leucocyte trafficking

1. Introduction

The endothelium forms the interface between blood and tissues and dynamically controls the local extravasation of immune cells from the circulation into inflamed tissues. In response to inflammatory cytokines, the resting endothelium becomes activated and expresses adhesion molecules, such as E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1), which favour the rolling and firm adhesion of immune cells. Transendothelial migration (diapedesis) is then mediated by specific molecules located at the endothelial cell junction, such as JAM, ESAM, PECAM-1, VE-cadherin, and activated leucocyte cell adhesion molecule (ALCAM). ALCAM is a cell adhesion molecule that mediates homo- and heterotypic interactions with the T-cell antigen CD6. ALCAM is expressed by various cell types including microvascular endothelial cells, where it participates in endothelial tube formation, monocyte transendothelial migration, and leucocyte trafficking across the blood–brain barrier.

MicroRNAs (miRNAs) are 21–23 nucleotide long non-coding RNAs that repress protein expression by binding to a complementary sequence of their target mRNAs, leading to mRNA degradation or translation inhibition. MiRNAs are major regulators of gene expression in most biological and pathological processes such as vascular biology, either promoting or repressing angiogenesis. miR21 and miR126-3p were identified as the most highly expressed miRNA in endothelial cells. miR126-3p is a key regulator of embryonic angiogenesis, as both its knockdown in zebrafish and its knockout in mice lead to significant vascular defects. In addition to its involvement in physiological angiogenesis in ischaemia-induced angiogenesis and in erythropoiesis, miR126-3p is also associated with several pathological processes. miR126-3p and its complementary strand miR126-5p derive from the miR126-3p/-5p duplex, which is processed
from intron 7 of the egfl7 gene.\textsuperscript{11,12} It was initially assumed that among miRNAs generated from a miRNA duplex, only the strand with weaker 5′-terminal thermostability (guide strand, miR-3p or miR) would accumulate in cells and be functional, whereas the passenger strand (miR-5p or miR*) would be degraded and supports no function.\textsuperscript{28} This initial model for miRNA maturation contributed to the very limited number of studies performed on miR126-5p\textsuperscript{27,29} and led to underestimate the physiological functions of this miRNA.

Here, the goals were to evaluate whether miR126-5p is a functional endothelial miRNA, to characterize its target genes and its physiological functions.

2. Methods

2.1 Reagents

Anti-mouse ALCAM antibody, recombinant control-Fc, and human ALCAM-Fc were from R&D. Mouse monoclonal anti-human ALCAM (clone MOG/07) was from Novocastra. Locked nucleic acid (LNA) control, miR126-5p inhibitors, LNA SetD5, and ALCAM target protectors were from Exiqon (see Supplementary material online, Table S1). Mimic miRNA control, 126-3p, and 126-5p were from Dharmacon.

2.2 Whole-mount in situ hybridization

Pregnant mice were sacrificed by cervical dislocation; embryos were collected, fixed, and treated with proteinase K. After a pre-hybridization step, embryos were incubated overnight in hybridization buffer containing miR126-3p- or miR126-5p-5′- and -3′- digoxigenin-labelled probes. After washes, embryos were incubated with the alkaline phosphatase-conjugated anti-digoxigenin antibody, washed, and stained with 4-Nitro blue tetrazo- lium chloride and 5-Bromo-4-chloro-3-indoly-phosphate, 4-toluidine salt. Embryos were photographed under binoculars.

2.3 Isolation of CD31-positive cells

Mouse lungs and hearts were collected from mouse pups previously sacrificed by decapitation. Tissues were washed in PBS and minced before digestion with Type I collagenase and DNAse I. Cells were then incubated with magnetic beads previously coated with anti-CD31 antibody. CD31\textsuperscript{+} cells were isolated following the manufacturer’s instructions. Isolated cells were centrifuged and suspended in Trizol for total RNA isolation and Relative quantifications (RT-qPCR) analysis.

2.4 Cell culture and transfection

HUVEC endothelial (Lonza), COS-7 (Cell line service), and THP-1 cells were cultured following manufacturers’ instructions. Primelact siRNA (Lonza) and Dharmatext DUO (Dharmacon) were used to transfect HUVEC and COS-7 cells, respectively.

HUVEC were treated with pro-inflammatory cytokines before lysis in Trizol and RNA extraction.

2.5 RNA quantification

Relative quantifications (RT-qPCR) were performed using Taqman gene expression master mix and specific Taqman probes using a StepOne system. miRNA and mRNA expression levels were normalized to U6 or to β-Actin levels, respectively, using the 2\textsuperscript{−ΔΔCT} method. For absolute quantification, standard curves were generated using known amounts of miRNA.

2.6 Adhesion or transendothelial migration of monocytes to endothelial cells

Endothelial cells were seeded in opaque 96-well plates or on 3 μm pore size HTS Fluoroblock inserts (Falcon), transfected with miRNA inhibitors or mimics, and grown to confluence. THP-1 cells were labelled with calcein-AM, plated onto transfected HUVEC cell monolayers, and allowed to adhere or transmigrate. Adherent or transmigrated monocytes were detected using a bottom-reading fluorescent plate reader. Experiments were carried out in triplicate. Four wells were analysed per condition in each experiment.

2.7 Inhibition of miR126-5p in vivo

Cholesterol-conjugated LNA miRNA inhibitors (10 mg/kg) were injected intraperitoneally daily in new born pups from OF1 mice (Charles River). Pups were sacrificed 2 days later by decapitation, organs collected, and lysed in Trizol or RIPA buffer. Experiments were performed three times, and four to eight pups were injected per experiment and per condition. Sub-retinal injections were performed by injecting 0.5 μL of cholesterol-conjugated LNA miRNA inhibitor (1 mg/mL) as described in.\textsuperscript{30} Six retinas were injected per experiment and per condition. V.M. has a level I Animal Experiments diploma and an authorization to perform animal experiments (#59-35066) delivered by the Prefecture de la Region Nord/Pas de Calais.

2.8 LPS and αALCAM antibody injection in vivo

Lipopolysaccharide from Escherichia coli (LPS) (Sigma, 20 mg/kg) were injected intraperitoneally in pups 4 h before sacrifice. Lungs were collected and treated for RNA extraction, protein analysis, or paraffin embedding. When indicated, pups were daily injected with αALCAM antibody (R&D, 25 μg/pup/injection) or control IgG from birth until sacrifice. Lungs were collected and treated as described previously. Experiments were performed at least twice, and four to six pups were injected per experiment and per condition.

2.9 CD45 immunostaining of lung sections

Lungs were fixed in 4% PFA overnight at 4°C before embedding in paraffin. Lung sections were deparaffinized and treated with trypsin for 15 min. Slides were then treated with H2O2 for 20 min and incubated in TNB blocking buffer (PerkinElmer) for 30 min. Slides were incubated with rat anti-mouse CD45 antibody (BD Pharmingen) diluted in TNB at 4°C overnight. Slides were washed before incubation with biotinylated anti-rat antibody (Vector), diluted in TNB for 1 h. After washes, slides were incubated with HRP-labelled streptavidin (Perkin Elmer) and diluted in TNB for 30 min. After washes, staining was finally revealed with DAB (Vector). Slides were counterstained with haematoxylin before mounting in Vectamount (Vector). Slides were analysed under an Axioplan2 Zeiss microscope.

2.10 Statistics

For each experiment, data were collected at least in triplicates. The ANOVA or Student’s t-test was used to calculate the P-value (\*P < 0.05, \*\*P < 0.01).

3. Results

3.1 miR126-5p is expressed in the endothelium of mouse and human organs

The expression pattern of miR126-5p in normal tissues is not characterized. Since miR126-3p and miR126-5p derive from a duplex embedded in the endothelial-specific egfl7 gene,\textsuperscript{11,12} we assessed whether miR126-5p was expressed in blood vessels in vivo. Using whole-mount in situ hybridization, miR126-5p was detected in the major embryonic cranial vessels of 10.5-day-old mouse embryos, at the same location as miR126-3p (Figure 1A). Expression levels of miR126-5p were higher in lungs and the heart when compared with other organs of mouse pups (Figure 1B) and in the adults (not shown). Absolute quantification showed that miR126-5p was approximately twice as less abundant as miR126-3p in these tissues (see Supplementary material online, Figure S1A and B). To specifically measure the levels of expression of miR126-5p in endothelial cells, CD31\textsuperscript{+} cells were purified from mouse
miR126-5p is expressed in endothelial cells of mouse and human tissues. (A) miR126-5p and miR126-3p detected in the main cranial blood vessels of 10.5-day-old mouse embryos (E10.5) using in situ hybridization (blue staining). No signal was observed when using a control probe (Ctrl probe). (B) Relative quantification of miR126-5p in P8 mouse pup organs using RT-qPCR. Levels were normalized to those in livers, arbitrarily set to 1. (C) Relative quantification of miR126-5p in CD31− and CD31+ cells isolated from P3 mouse hearts and lungs. (D) Relative quantification of miR126-5p in various human organs, using RT-qPCR. (E) Relative quantification of miR126-5p levels in human primary fibroblasts (NHDF), mammary epithelial cells (MCF10A), microglial cells (microglial), and primary endothelial cells (HUVEC) using RT-qPCR. (F) Relative quantification using RT-qPCR of miR126-3p and miR126-5p in HUVEC transfected with the LNA control inhibitor (αmiRCtrl) or with the LNA-miR126-5p inhibitor (αmiR126-5p), or (G) with a control (mimic Ctrl), or a miR126-5p (mimic 126-5p) mature miRNA.
3.2 miR126-5p is involved in leucocyte adhesion and transendothelial migration

High expression levels of miR126-5p were maintained in endothelial cells cultured in vitro when compared with other cell types (Figure 1E). In addition, miR126-5p was three times less expressed than miR126-3p in endothelial cells (see Supplementary material online, Figure S1C), in good correlation with the ratio of expression of these miRNAs measured in vivo (see Supplementary material online, Figure S1A and B).

To study the role of miR126-5p in endothelial cells, we used gain- and loss-of-function approaches using either miRNA mimics or LNA-modified miRNA inhibitors. The efficiency and specificity of these tools were verified by RT-qPCR after transfection in HUVEC (Figure 1F and G). miR126-5p gain- or loss of function did not affect the proliferation of HUVEC, neither their mobility, nor ability to form pseudocapillaries on Matrigel in vitro (see Supplementary material online, Figure S2), indicating that miR126-5p does not play a major role in these endothelial functions, on the contrary to miR126-3p.11–13 Since Egfl731 and miR126-3p19 are both involved in leucocyte trafficking, we then investigated whether miR126-5p played a role in this process. Fluorescently labelled human THP-1 monocytes were used as a model of leucocyte adhesion and transendothelial migration through HUVEC monolayers. Interestingly, when miR126-5p was repressed in endothelial cells, the adhesion of monocytes decreased 38% when compared with control cells. Accordingly, miR126-5p overexpression in endothelial cells increased monocyte adhesion by 40% (Figure 2A). On the other hand, the ability of monocytes to migrate through endothelial monolayers increased 31% when miR126-5p was repressed in endothelial cells, while this effect was reversed when miR126-5p was overexpressed in these cells (Figure 2B). In addition, as a support of miR126-5p involvement in leucocyte trafficking, miR126-5p expression decreased in HUVECs treated with pro-inflammatory cytokines, such as TNF-α or IL-1 (see Supplementary material online, Figure S3).

3.3 miR126-5p controls leucocyte transendothelial migration by regulating ALCAM

To identify the mechanisms by which miR126-5p controls leucocyte trafficking across the endothelium, we searched through computer databases (TargetScan, Diana miRPath, and Diana MicroTV4) for potential mRNA transcripts known to play a role in this process and that could be regulated by miR126-5p. ALCAM was an interesting predicted target for miR126-5p, because it was previously involved in monocyte diapedesis in vitro.9 A perfect pairing between nucleotides 2–8 of miR126-5p (seed site) and the mouse ALCAM 3′UTR was noted (Figure 3A), and this target site is perfectly conserved in mammals (Figure 3B).

To establish whether miR126-5p could directly regulate ALCAM expression, a reporter vector was constructed where the mouse ALCAM 3′UTR was cloned downstream of the luciferase gene. When cells were co-transfected with miR126-5p and this reporter vector, the luciferase activity decreased by 40% when compared with control, whereas miR126-3p had no effects (Figure 3C) and mutation of the identified miR126-5p target site relieved the effect of miR126-5p (Figure 3D) and miR126-5p had no effects (Figure 3E). In addition, inhibition of endogenous miR126-5p induced an accumulation of ALCAM at endothelial cell junctions, whereas miR126-5p overexpression resulted in a strongly reduced detection of ALCAM (Figure 3F), altogether showing that ALCAM is a genuine endogenous target gene of miR126-5p in endothelial cells.

To determine whether ALCAM mediates the effects of miR126-5p on leucocyte trafficking, the adhesion and migration of monocytes through an endothelial monolayer that had been transfected with the miR126-5p
Figure 3  ALCAM is a target gene of miR126-5p. (A) Sequence complementarity between miR126-5p and the predicted miR126-5p-binding site in the 3′ UTR of the mouse ALCAM gene. (B) Sequence alignment of ALCAM 3′ UTR from different species. The red box corresponds to the seed site; the blue box encompasses the additional nucleotides complementary to miR126-5p. (C) Relative luciferase activity measured in COS-7 cells transfected with the pMIR reporter vector containing the mouse 3′ UTR of ALCAM (3′ UTR ALCAM) and with either a control (mimic Ctrl), the miR126-3p (mimic 126-3p), or the miR126-5p (mimic 126-5p) mature miRNA. Values of mimic Ctrl were set to 100, and bars represent SD, **p < 0.01; ns: not significant. (D) Relative luciferase activity measured after COS-7 transfection with mimic Ctrl or mimic 126-5p miRNA and the reporter vector 3′ UTR ALCAM or a miR126-5p site-mutated version of this plasmid (3′ UTR ALCAM mut). Bars indicate SD, *p < 0.05, ns: not significant. (E) Immunoblot of ALCAM protein in HUVEC transfected with the LNA control inhibitor (amiRCtrl) or with the LNA-miR126-5p inhibitor (amiR126-5p, left, upper panel) or with a mimic Ctrl or mimic 126-5p miRNA (left, lower panel). Actin is shown as a loading control; numbers below indicate densitometry measurements of expression relative to Ctrl. Histograms illustrate the means of ALCAM protein levels in HUVEC transfected in similar conditions in independent experiments, Bars indicate SD, *p < 0.05. (F) ALCAM immunofluorescence detection in confluent HUVEC previously transfected with control siRNA (siCtrl), with ALCAM siRNA (siALCAM), or with miRNA inhibitors and miRNA mimics as in E.
inhibitor were analysed after treatment with an ALCAM-Fc blocking antibody. Adhesion was reduced by 30% when miR126-5p was repressed in endothelial cells and this effect was not affected by the presence of the ALCAM-Fc (Figure 4A). On the other hand, the ALCAM-blocking antibody abolished the effects of miR126-5p on leucocyte transmigration (Figure 4B). To confirm these results, a target site protector was designed for ALCAM as an LNA antisense oligonucleotide that binds to the miR126-5p target site identified in the ALCAM transcript and specifically protects ALCAM expression from miR126-5p. Indeed, the ALCAM target protector induced an up-regulation of ALCAM protein in HUVEC (Figure 4C) without modifying the expression of CXCL12, a recently identified target of miR126-5p or miR126-5p itself (see Supplementary material online, Figure S4C and D). As expected, when transfected into HUVEC, this ALCAM target protector favoured transendothelial migration of monocytes and had no effects on their adhesion (Figure 4D and E). In addition, when the ALCAM target protector was co-transfected with the miR126-5p mimic miRNA in HUVEC, it prevented the miR126-5p-mediated inhibition of monocytes transendothelial migration (see Supplementary material online, Figure S5A), whereas it had no effect on the activity of the miR126-5p inhibitor on this process (see Supplementary material online, Figure S5B). All these results show that the endogenous repression of ALCAM by miR126-5p in endothelial cells specifically leads to a repression of leucocyte transendothelial migration.

miR126-5p regulates ALCAM and SetD5 in endothelial cells

Figure 4 miR126-5p regulates monocyte transendothelial migration via ALCAM. (A) THP-1 cell adhesion onto confluent HUVEC transfected with the LNA control inhibitor (amiR Ctrl) or with the LNA-miR126-5p inhibitor (amiR126-5p) and pre-incubated with a recombinant human chimeric Fc-control (Ctrl-Fc) or an anti-ALCAM-blocking antibody (ALCAM-Fc) before plating of the labelled THP-1. **P < 0.01. Data are shown as fold of change over Ctrl set to 100%. (B) THP-1 transendothelial migration through confluent HUVEC transfected as in A. *P < 0.05, ns: not significant. Data are shown as fold of change over Ctrl. (C) Upper panel: ALCAM protein expression analysed by the western blot in HUVEC transfected with Target Control or Target ALCAM in independent experiments, Bars indicate SD. *P < 0.05. (D) THP-1 transmigration through HUVEC transfected with Target Ctrl or with Target ALCAM. **P < 0.01. (E) THP-1 cell adhesion measured as described above on confluent HUVEC previously transfected with Target Ctrl or with Target ALCAM. ns: not significant.
3.4 miR126-5p controls leucocyte adhesion to endothelium by regulating SetD5

Except for ALCAM, the computer databases did not predict any other miR126-5p target gene known to be involved in leucocyte trafficking through the endothelium. To identify such targets, a comparative microarray analysis was performed on HUVEC in which miR-126-5p had been knockdown. Of the common transcripts identified in the three cell lots used, 16 transcripts corresponding to 9 LincRNA (not shown) and 7 coding RNA had more than 1.5-fold change of expression after inhibition of miR126-5p (see Supplementary material online, Figure S6A and B). Among these, the gene with unknown functions SetD5 was considered as the most interesting candidate after RT-qPCR validation, as it was the sole transcript among those tested which was consistently up-regulated in all HUVEC lots (see Supplementary material online, Figure S6C). Furthermore, when analysing the SetD5 gene sequence, a perfect match between the seed site of miR126-5p described above and the 3′ UTR sequence of SetD5 was found (Figure 5A). The complementary sequences between miR126-5p and the 3′ UTR of SetD5 are totally conserved from lizard to mammals (Figure 5B), suggesting that this target site could be functional. Indeed, co-transfection of miR126-5p with a reporter vector where the 3′ UTR of SetD5 was cloned downstream of the luciferase gene induced a 35% decrease in luciferase activity when compared with control (Figure 5C). This effect was dependent on the identified miR126-5p target site in the 3′ UTR of SetD5, because the mutation of this site abolished the repression exerted by miR126-5p (Figure 5C). Furthermore, in HUVEC, the inhibition of endogenous miR126-5p induced a significant up-regulation of SetD5 expression (Figure 5D), while over-expression of this miRNA repressed SetD5 expression (Figure 5E), confirming that the expression of endogenous SetD5 transcripts is under the control of miR126-5p in endothelial cells. The repression of SetD5 protein by miR126-5p could not be addressed in this study because of the lack of valid antibodies.

To determine whether miR126-5p controlled leucocyte trafficking through endothelial cells also via SetD5, we designed a target protector aimed at specifically protecting the SetD5 transcripts from the effects of the miR126-5p. As expected, the SetD5 target protector induced a 40% up-regulation of SetD5 expression in HUVEC (Figure 5F), whereas it did not affect the expression of ALCAM, miR126-5p, or CXCL12, as expected (see Supplementary material online, Figure S7A–C). Conversely, the ALCAM target protector used earlier did not alter the expression of SetD5, validating the specificity of each target protector (see Supplementary material online, Figure S7D). Interestingly, THP-1 adhesion to HUVEC transfected with the SetD5 target protector was reduced 40% when compared with control cells, indicating that miR126-5p favours leucocyte adhesion to the endothelium through the down-regulation of SetD5 in endothelial cells (Figure 5G). In contrast, THP-1 transmigration through HUVEC transfected with the SetD5 target protector was similar to that observed through control cells (Figure 5H). In accordance with these observations, the co-transfection of the SetD5 target protector with the miR126-5p mimic inhibited the effects of miR126-5p on monocyte adhesion to transfected HUVEC (see Supplementary material online, Figure S8A). In addition, the SetD5 target protector did not further increase the repressing effect of the miR126-5p inhibitor on monocyte adhesion (see Supplementary material online, Figure S8A). Finally, when a SetD5 siRNA was simultaneously transfected with the miR126-5p inhibitor in endothelial cells, SetD5 expression was rescued to its basal levels (see Supplementary material online, Figure S8C) and leucocyte adhesion was not affected anymore (Figure 5I), confirming the specific involvement of SetD5 in the control of leucocyte adhesion by miR126-5p.

3.5 Tissue-specific control of ALCAM and SetD5 expression by miR126-5p in vivo

To establish whether miR126-5p regulates the expression of these identified target genes in vivo, expression of ALCAM and SetD5 was analysed after inhibition of miR126-5p in mouse pups. When compared with control animals, intraperitoneal injection of the LNA-miR126-5p inhibitor induced a repression of miR-126-5p, especially in the heart (not shown) and lungs (see Supplementary material online, Figure S9A), but did not affect miR126-5p expression in the brain and retina (not shown), probably because of the blood–brain or –retinal barrier. We therefore focused our analysis on lungs where both ALCAM and SetD5 are expressed (Figure 6A), and where miR126-5p is also mainly detected (Figure 1B). The levels of ALCAM protein increased in the lungs of pups injected with the miR126-5p inhibitor when compared with controls, as assessed by ELISA (Figure 6B) and by western blotting (see Supplementary material online, Figure S9B), thus demonstrating that miR126-5p controls ALCAM expression in vivo at least in this organ. Surprisingly, SetD5 expression in the lungs was not significantly affected by the inhibition of miR126-5p (Figure 6C), suggesting that miR126-5p does not control both targets simultaneously in this organ.

As reported earlier, target genes can be protected from miRNA repression depending on the tissue where they are expressed in vivo.34 We thus investigated whether miR126-5p could control the levels of expression of SetD5 in other tissues than the lungs. In mouse pup organs, SetD5 was predominantly detected in the heart and the retina when compared with other organs (Figure 6A). As we found no variation in SetD5 expression in the heart after miR126-5p inhibition (not shown), SetD5 regulation by miR126-5p was investigated in the retina. To bypass the blood–retina barrier, the LNA-miR126-5p inhibitor was directly injected into the subretinal space, leading to an effective silencing of endogenous miR126-5p (see Supplementary material online, Figure S10). This induced a significant up-regulation of SetD5 expression (Figure 6E), indicating that miR126-5p also controls SetD5 expression in vivo, at least in the retina. Interestingly, the inhibition of miR126-5p did not alter ALCAM expression in the retina (Figure 6D), confirming that the expression of ALCAM and SetD5 are actually separately regulated by miR126-5p in different organs in vivo.

3.6 miR126-5p controls leucocyte trafficking in inflamed lungs

To determine whether miR126-5p controls leucocyte trafficking through the regulation of ALCAM or SetD5 expression in vivo, the phenotype induced by the inhibition of miR126-5p was analysed in the retina and lungs. In the retina, the inhibition of miR126-5p led to vascular defects of the developing vasculature (data not shown), which prevented the analysis of leucocyte recruitment by the endothelium. Conversely, in lung sections of pups injected with the miR126-5p inhibitor, leucocyte infiltration was analysed by immunohistochemistry against the CD45 leucocyte marker. Surprisingly, whereas ALCAM expression was up-regulated in the lungs of pups injected with the miR126-5p inhibitor (Figure 6B), the infiltration of leucocyte was not increased after miR126-5p inhibition compared with control conditions (Figure 7A). As ALCAM is exclusively involved in leucocyte transmigration,3 we speculated that leucocyte infiltration via ALCAM into the lungs would not be noticeable without a preliminary induction of leucocyte adhesion
Figure 5  SetD5 is a target gene of miR126-5p that represses monocyte adhesion. (A) Sequence complementarity of the predicted miR126-5p-binding site with the 3′UTR of mouse SetD5 gene. (B) Sequence alignment of SetD5 3′UTR from different species. The red box corresponds to the seed site; the blue box encompasses the additional nucleotides complementary to miR126-5p. (C) Relative luciferase activity measured in COS-7 transfected with the control (mimic Ctrl) or miR126-5p (mimic 126-5p) mature miRNA and the pMIR reporter vector containing the mouse 3′UTR of SetD5 (3′UTR SetD5) or a miR126-5p site-mutated version of this reporter (3′UTR SetD5 mut). Bars indicate SD, *P < 0.05, **P < 0.01, ns: not significant. (D) Relative quantification of SetD5 using RT-qPCR in HUVEC transfected with the LNA control inhibitor (amiRCtrl) or with the LNA-miR126-5p inhibitor (amiR126-5p). *P < 0.05. (E) Relative quantification of SetD5 using RT-qPCR in HUVEC transfected with mimic Ctrl or mimic 126-5p mature miRNA. **P < 0.01. (F) Relative quantification using RT-qPCR of SetD5 in HUVEC transfected with Target Ctrl or with Target SetD5. *P < 0.05. (G) THP-1 cell adhesion onto confluent HUVEC transfected with the Target Ctrl or with Target SetD5, **P < 0.01. (H) THP-1 cell transmigration through confluent HUVEC transfected with Target Ctrl or with Target SetD5. (I) THP-1 cell adhesion measured as described above on confluent HUVEC transfected either with amiRCtrl or with amiR126-5p and a control non-targeted siRNA (siCtrl) or with the siRNA directed against SetD5 (siSetD5). Data are shown as fold of change. **P < 0.01; ns: not significant.
of the lung up-regulated ICAM-1 and VCAM-1 expressions (see Supplementary material online, Figure S11A), but did not dramatically alter the expression of miR126-5p (see Supplementary material online, Figure S11B). The inhibition of miR126-5p was therefore carried out in order to investigate its consequences in inflamed tissues. We first verified that LPS injection in pups did not affect the down-regulation of miR126-5p after inhibitor injection (Figure 7B), nor the up-regulation of ALCAM expression into the lungs (Figure 7C). Interestingly, in inflamed lungs, the inhibition of miR126-5p induced an increase in leucocyte infiltration, as demonstrated by CD45 immunostaining (Figure 7D), indicating that miR126-5p controls leucocyte trafficking in the inflamed lung. This phenotype was not due to adhesion molecule up-regulation by miR126-5p inhibition in inflamed tissues, since the expression levels of ICAM-1 and VCAM-1 were not modulated by inhibitor injection in inflamed lungs (see Supplementary material online, Figure S12). To establish whether this increased leucocyte infiltration was actually due to ALCAM up-regulation after miR126-5p inhibition in the inflamed lung, similar experiments were performed after intraperitoneal injection of an ALCAM-blocking antibody. In these conditions, leucocyte infiltration levels were no longer different than in control lungs (Figure 7E), indicating that miR126-5p controls leucocyte infiltration through a regulation of ALCAM expression in inflamed lungs.

4. Discussion

In this study, we showed that miR126-5p is a functional miRNA which accumulates in several tissues, mainly in endothelial cells. miR126-5p takes part in the control of leucocyte trafficking by modulating the expression of two genes: ALCAM and SetD5. Repression of ALCAM by miR126-5p regulates leucocyte trafficking in inflamed lungs.

Passenger strands of miRNA duplexes were initially considered to be degraded, but no study had clearly addressed this point for miR126-5p. We show here that miR126-5p accumulates in various mouse and human tissues, together with its guide strand. This is consistent with miRNA profiling analyses, which showed that both strands of a miRNA duplex may be simultaneously detected in the same tissues, at various ratios. Previous studies had established the presence of miR126-5p in embryonic bodies, in human prostate cancer, and in human breast cancer cell lines, suggesting that miR126-5p might have both physiological and pathological functions.

Despite a concomitant regulation of ALCAM and SetD5 by miR126-5p in vitro, our results in vivo show that miR126-5p does not control simultaneously the expression of ALCAM and SetD5 in the same tissues. The context-dependent miRNA target selection was also observed for miR126-3p. Indeed, Asgeirsdo´ttir et al. showed that the inhibition of miR126-3p in vivo leads to an up-regulation of VCAM-1 in kidneys and the liver, but not in lungs and the heart. In addition, Zhang et al. demonstrated that miR126-3p differently controls its target genes in mammary tumour cell lines, strengthening the hypothesis that the regulation of miRNA target expression depends on the tissue context. The molecular mechanisms governing this miRNA target selection are not known yet; however, we observed that, in lungs and in the retina, miR126-5p inhibits highly expressed target genes, suggesting that a titration of the miRNA may take place in these tissues to prevent the regulation of all miR126-5p targets at the same place and time. Tissue-specific RNA-binding proteins may also interfere with the binding of miR126-5p to its targets leading to their protection from miRNA control. We cannot exclude that miR126-5p may control the expression of ALCAM and SetD5 in separate locations owing to
miR126-5p regulates ALCAM and SETD5 in endothelial cells

Figure 7 miR126-5p regulates leucocyte trafficking in the inflamed lung by controlling ALCAM expression. (A) CD45 immunostaining (brown) of lungs from mice injected intraperitoneally with the LNA control inhibitor (amiRCtrl) or with the LNA-miR126-5p inhibitor (amiR126-5p). Left panel, images of lung sections immunostained for the CD45 leucocyte marker; right panel, quantification of the surface occupied by CD45-positive cells per field. The surface occupied by CD45-positive cells measured in lung injected with the control inhibitor was arbitrary set to 100. (B) Relative quantification of miR126-5p in lungs from mice injected intraperitoneally with the LNA control inhibitor (amiRCtrl) or with the LNA-miR126-5p inhibitor (amiR126-5p) and treated with LPS 4 h before sacrifice. (C) Quantification of ALCAM protein by ELISA in lungs from mice injected intraperitoneally with the LNA control inhibitor (amiRCtrl) or with the LNA-miR126-5p inhibitor (amiR126-5p) and treated with LPS. (D) CD45 immunostaining of lungs from mice injected intraperitoneally with the LNA control inhibitor (amiRCtrl) or with the LNA-miR126-5p inhibitor (amiR126-5p) and treated with LPS. Left panel, images of lung sections immunostained for the CD45 leucocyte marker; right panel, quantification of the surface occupied by CD45-positive cells per lung section field. P-values are indicated.
the expression of the miRNA and its target genes by different cell popu-
lations in the same organ.

This tissue-specific control of miRNA targets suggests that miR126-5p independently favours leucocyte adhesion and represses diapedesis in dif-
f erent locations. Given that miR126-3p and its host gene Egfl7 control leucocyte adhesion to endothelial cells by inhibiting the expression of VCAM-1,31 and ICAM-1,31 miR126-5p may work in concert with its
breast tumour cells.27 Interestingly, the study of Zhang
highlighting a cooperative role between miR126-5p and miR126-3p in
concept and possibly to identify new targets for anti-inflammatory
player, which preserves the non-activated state of the endothelium in
VCAM-1, or E-selectin. In such case, SetD5 would be another new
trols the expression of endothelial adhesion molecules, such as ICAM-1,
pecially during neuroinflammation where it controls the migration of
leucocytes into the CNS.4 As our cholesterol-conjugated miRNA inhib-
itors do not cross the blood–brain barrier, we were not able to estab-
lish whether ALCAM expression is also controlled by miR126-5p in the
brain. However, the delivery of miR126-5p into the brain is a promising challenge in order to try to negatively regulate ALCAM expression and to prevent leucocyte infiltration into the CNS, notably in patients with active multiple sclerosis lesions.4

Regarding SetD5, the SET domain predicted in the SetD5 protein sug-
ests that it may take part in epigenetic regulation,31 but no data support
this hypothesis yet. It will be interesting to establish whether SetD5 controls the expression of endothelial adhesion molecules, such as ICAM-1, VCAM-1, or E-selectin. In such case, SetD5 would be another new player, which preserves the non-activated state of the endothelium in the presence of inflammatory stimuli, in addition to Egfl7.31 Further studies are now required to improve our knowledge on this concept and possibly to identify new targets for anti-inflammatory therapy.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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