Running title **miR-126-3p mediated PVC-matrix interaction**

miR-126-3p promotes matrix-dependent perivascular cell attachment, migration and intercellular interaction

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Abstract

MicroRNAs (miRNAs) can regulate the interplay between perivascular cells (PVC) and endothelial cells (EC) during angiogenesis, but the relevant PVC-specific miRNAs are not yet defined. Here, we identified miR-126-3p and miR-146a to be exclusively upregulated in PVC upon interaction with EC, determined their influence on the PVC phenotype and elucidate their molecular mechanisms of action. Specifically the increase of miR-126-3p strongly promoted the motility of PVC on the basement membrane-like composite and stabilized networks of endothelial cells. Subsequent miRNA target analysis showed that miR-126-3p inhibits SPRED1 and PLK2 expression, induces ERK1/2 phosphorylation and stimulates TLR3 expression to modulate cell-cell and cell-matrix contacts of PVC. Gain of expression experiments in vivo demonstrated that miR-126-3p stimulates PVC coverage of newly formed vessels and transform immature into mature, less permeable vessels. In conclusion we showed that miR-126-3p regulates matrix-dependent PVC migration and intercellular interaction to modulate vascular integrity.
Introduction

Formation of new blood vessels is normally suppressed in adult tissues. Changes in the local environment upon injury or in tumor formation can induce a neoangiogenic response to provide a blood supply that meets the increased metabolic needs of the injured tissue. Lining endothelial cells (EC) and perivascular cells (PVC) are activated and transform into proliferative, migrating cells to form a new vascular network.

PVC can be distinguished according to their abluminal location within the vasculature. Vascular smooth muscle cells are found within arteries, separated by a basement membrane (BM) from the adjacent EC, whereas pericytes are located in the microvasculature enveloped in a BM that they share with EC of small arterioles and capillaries. Such PVC are non-proliferative cells that transform into a migrating and extracellular matrix (ECM)-producing “synthetic” phenotype during neoangiogenesis.

In recent years it became clear that microRNAs (miRNAs) can play a critical role in vascular cell activation. miRNAs can regulate the expression of multiple genes within cells. Most often a single ~22 nucleotide miRNA binds to complementary sequences within the 3'-untranslated regions (3'-UTR) of target mRNAs and inhibits their translation or promote their degradation. Recent findings indicate that the dysregulation of a single miRNA can impair vascular integrity. In vivo downregulation of miR-126 in zebrafish caused vascular leakage and hemorrhage, whereas loss of this miRNA in mice inhibited neointimal lesion formation. Moreover, miR-126 can suppress metastatic angiogenesis and expression of miR-126 in human was linked to the outcome of chronic heart failure. However, only limited data are available on miRNAs that regulate the interaction of PVC with the extracellular environment and EC.

Previously, we isolated PVC from murine brain meninges and showed that the cells can differentiate into multiple mesenchymal lineages, but still maintain their perivascular-like phenotype in culture. PVC express pericyte-specific markers, stimulate the expression of platelet/endothelial cell adhesion molecule 1 (PECAM1) in human umbilical vein endothelial cells (HUVEC) and promote the deposition of BM proteins. Cocultures of PVC and HUVEC mimic initial steps of angiogenesis and were used in this study to analyze the contribution of miRNAs to PVC interaction with HUVEC. Applying microarray-based miRNA profiling miR-126-3p (corresponding to human miR-126) was identified to be upregulated in cocultured PVC. Transfection of PVC with miR-126-3p mimics strongly promoted integrinβ1-dependent vascular cell contacts on a BM-like composite. Subsequent target analysis revealed that miR-126-3p suppresses the expression of sprouty-related protein with EHV-1 domain 1 (SPRED1), polo-like kinase 2 (PLK2) and insulin receptor 1 substrate (IRS1). Suppression of SPRED1 expression stimulated extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation to increase toll-like receptor 3 (TLR3) and chemokine expression and elicited cellular interactions of PVC on matrigel substrate. In vivo gain of expression experiments demonstrated that miR-126-3p stimulates neoangiogenesis and vessel stabilization. Hence, miR-126-3p regulates vascular cell recruitment by modulating cell migration on ECM substrates.
Material and Methods

Cell culture

PVC9, MC3T3 and b.End5 cells were cultured in DMEM (Gibco) supplemented with 10% FCS, penicillin (100units/ml, Biochrom) and streptomycin (100µg/ml, Biochrom). HUVEC were cultured in VascuLife VEGF-Mv medium (Cell Systems). All experiments were performed at 37°C and 5% CO2.

For transfection 3x10⁴ cells were resuspended in 550µl of medium and transferred to a 24-well plate. 10nM mimic, 25nM siRNA, 50nM inhibitor or corresponding concentrations of AllStarsNegativeControl in 50µl HiPerFect transfection solution (Qiagen) and DMEM were added. Cells were incubated for 48hours, detached by trypsinization, resuspended in medium additionally supplemented with PDGF (10ng/ml, Biomol) and VEGF (5ng/ml, Biomol) before used in experiments. Downregulation of target genes were validated by qPCR or immunoblotting (see supplemental figure 7).

Cell morphology and migration assays were performed on glass chamber slides (VWR) precoated with 200µl of collagen I (25µg/ml, Gibco), collagen IV (25µg/ml), laminin 511 (20µg/ml, BioLamina), nidogen-1 (25µg/ml), 16µl matrigel (9.1mg/ml, Becton Dickinson) or 16µl of collagen I gel (0.666mg/ml, Gibco) for 1hour at 37°C. 6.5x10³ transfected cells were added and analyzed by microscopy (Nikon TE2000-U, Olympus IX81).

For two-dimensional cocultures transfected PVC were labeled with CFDA-SE and HUVEC were labeled with SNARF-1 according to the suppliers protocol (Invitrogen). Briefly, 1x10⁵ cells were resuspended in 3% FCS/PBS containing 10µM dye and incubated for 15 minutes at 37°C. After washing cells were resuspended in VascuLifeVEGF-Mv medium. 8x10¹² b.End5 cells were seeded to matrigel-coated wells and after 16 hours 4x10⁵ PVC were added. 4x10⁵ PVC and 1.2x10⁴ HUVEC were also added to matrigel-coated wells and cultured for 16hours. The distribution of cells was determined by microscopy.

Three-dimensional cocultures of 5x10⁵ PVC and 2.5x10⁹ HUVEC were established in 400µl of a 2mg/ml rat tail collagen type I gel containing DMEM, 2% FCS, 22.5mM NaHCO₃ and 1mM sodium pyruvate in a 24-well plate. After gelation (37°C, 20 minutes), 400µl EGM-2 medium, 10ng/ml VEGF, 10ng/ml PDGF and 250µg/ml ascorbic acid phosphate were added. Cultures were maintained for five days. Cells were released by digestion with collagenase E (0.4%), collected by centrifugation and washed with PBS. HUVEC were separated from mouse PVC by anti-human PECAM1+ Dynabeads (Dynal) selection. Total RNA was isolated from both cell types by RNAeasy Micro kit (Qiagen). Purity of cells was confirmed by immunofluorescence analysis.

Proliferation and apoptosis assays

Proliferation was analyzed using the CellTiter 96® AQquous One Solution (Promega) according to the manufacturer’s specification. 5x10³ cells were transfected with oligonucleotides in 96-well plates and cultured for two, 24, 48 and 72hours. Substrate was added and production of colored formazan product detected at 492nm using a 96-well plate reader (Sunrise, Tecan). Cell death was determined as described12. Briefly, PVC were trypsinized 24, 48 and 72hours after transfection, washed, stained with annexin A5 (AnxA5)-Alexa488 and propidium iodide (PI) and analyzed by flow cytometry (FACSCanto II, Becton Dickinson).

RNA isolation and quantification by microarray and quantitative PCR (qPCR)

Total RNA of cells was isolated by phenol-chloroform extraction13 and quality was assessed by micro-capillary electrophoresis (2100 Bioanalyzer, Agilent) according to the manufacturer’s specifications. For microarray analysis 100ng of purified RNA was labeled and hybridized to mouse or human 8x15K-miRBaseV14 microarrays and to Sureprint-G3 mouse 8x60K whole genome mRNA microarrays using the labeling and hybridization protocol of Agilent. The arrays were scanned (Agilent G2595C scanner), data extracted and processed using the GeneSpringXII software (Agilent). miRNA or mRNA were selected according to their differential expression, relative expression levels and the statistical significance14. RNA was also reversely transcribed and used for each prevalidated pri-
miRNA (lifetechnologies) or miRNA-specific SYBR-Green based qPCR assays (Qiagen). The relative expression levels were calculated using the delta-delta C(T) method\textsuperscript{15}. Data were normalized to the expression of Mapk7 or miR-31.

**Scanning electron microscope analysis**

Silicon wafers were precoated with 16µl matrigel for one hour at 37°C. 6.5x10\textsuperscript{3} transfected PVC were added and incubated for four hours. Cells were fixed in 1% glutaraldehyde in PBS for 30minutes at RT and processed for coating with 2nm of platinum. Attachment of cells to the substrate was analyzed by scanning electron microscopy (Leo1530VPGemini, Zeiss).

**Luciferase reporter assays**

Luciferase reporter assays were performed as described \textsuperscript{16}. Briefly, HEK 293 cells were first transfected with 10nM miR-126-3p mimic or AllStars Negative Control and then cotransfected with 2µg of a psiCHECK\textsuperscript{TM}-2 vector (Promega) which contains the miR-126-3p binding sites. An overview of the oligonucleotides used for the generation of the luciferase reporter constructs is given (supplemental table 1). Luciferase activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

**Immunoblotting**

48hours after transfection cells were starved for 45minutes in VascuLife medium without FCS or supplements in the absence or presence of UO126 (10µM)\textsuperscript{17} or Wortmannin (2µM, Cell Signaling)\textsuperscript{18}. Cells were activated with complete VascuLife VEGF-Mv medium. After cell lysis in RIPA buffer equal amounts of protein (20µg) were resolved on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose (Whatman). Immunostainings were performed with primary antibodies detecting pERK1/2, ERK1/2, pAKT, AKT (Cell Signaling), IRS1, SPRED1, GAPDH (Merck Millipore) and PLK2 (Biorbyt) and with the corresponding secondary antibodies labeled with horseradish peroxidase (DAKO). Band intensities were quantified by ImageJ software\textsuperscript{19}.

**In vivo angiogenesis assay**

**In vivo** angiogenesis experiments were performed as described\textsuperscript{20}. Briefly, miR-126-3p mimic- or AllStarsNegative control-transfected cells were resuspended in a mixture of matrigel, methocoel (Sigma) and fibrinogen (Merck Millipore) containing 500ng/µl VEGF and FGF2 to a final concentration of 500cells/µl. 1µl of thrombin (0.1U/ml, Merck Millipore) was added to 15µl cell suspension, cells were transferred into silicone tubes (Instech) and implanted subcutaneously into the flank of 8 weeks old immunodeficient C.129S6(B6)-Rag2tm1FwaN12 mice. ~16days after implantation 50µl of a FITC-Dextran-70 solution (50µg/ml in H\textsubscript{2}O, Sigma) was administered retroorbitally by intravenous injection. Silicone tubes were isolated 25minutes post injection and assessed by microscopy. Matrigel-fibrin plugs were digested in 100µl dispasel (5mg/ml in PBS, Roche) for 45minutes at 37°C, centrifuged for 5minutes at 4°C and 16.000g and the fluorescence was determined by fluorescence spectroscopy (Infinite M1000, Tecom).

**Statistical analysis**

The statistical analyses were performed with Student's t-test using the unpaired two-tails method. p-values<0.05(*) were considered to be statistically significant, p-values<0.005(**) highly significant. Standard deviations are indicated.
Results

miR-126-3p is upregulated in PVC upon interaction with HUVEC

To identify miRNAs that are upregulated in PVC upon interaction with EC three-dimensional cocultures of PVC and HUVEC were established. In monocultures of HUVEC or PVC (supplemental Figure 1) only few tubes were formed after five days in three-dimensional collagen I gels, whereas the number and length of tubes was markedly increased in cocultures (Figure 1A). Immunodetection of PECAM1 demonstrated that the tubes contain a significant amount of HUVEC (Figure 1B). Total RNA was isolated from PVC or HUVEC monocultures and cocultures of immunomagnetically separated PVC with HUVEC. The purity of the isolated cell population was confirmed by immunofluorescence analysis (supplemental Figure 1). RNA was subjected to microarray analysis and the transcriptome was screened for differentially and significantly expressed miRNAs (Figure 1C). Several miRNAs were significantly downregulated in cocultured PVC. Of note is that only two of 690 analyzed miRNAs were highly abundant in cocultured but absent in monocultured PVC, namely miR-126-3p and miR-146a (Figure 1D, F). Both miRNAs were also expressed in HUVEC but not regulated during coculture (Figure 1E). Therefore, the two miRNAs were specifically upregulated upon PVC-HUVEC interaction only in PVC. Their regulation was then validated by qPCR analysis. A strong upregulation for miR-126-3p and a less pronounced upregulation for miR-146a was detected in this assay (Figure 1G). Characterization of the pri-miRNA expression by qPCR also indicated that the increase in miR-126-3p was accompanied by an upregulation of its pri-miRNA in cocultured PVC (Figure 1H). Hence, we hypothesized that miRNA-126-3p and miR-146a are needed to promote PVC interaction with EC to maintain vascular integrity.

miR-126-3p promotes migration and interaction of PVC on matrigel

PVC and EC are connected via a BM and miR-126-3p and miR-146a may modulate the binding of PVC to BM-proteins, which was analyzed by cell-matrix interaction experiments. miR-126-3p or miR-146a levels were increased in PVC using synthetic RNAs that mimic mature endogenous miRNAs (mimics) or nontarget control oligonucleotides. The complex BM-like matrigel was coated on glass chamber slides, transfected PVC were added 48 hours post transfection and cell morphology was determined. Interestingly, miR-126-3p mimic-transfected PVC, but not miR-146a mimic- or control-transfected cells, formed cell aggregates connected by filamentous structures (Figure 1I). Therefore, the analysis was focused on the miR-126-3p in PVC. Quantification of aggregate-associated filaments 16 hours after seeding on matrigel showed that cumulative length of filamentous links between aggregates were fivefold increased in miR-126-3p mimic-transfected PVC compared to control. In contrast, transfection of an anti-miR-126-3p oligonucleotide caused a decrease in length of aggregate-associated links (supplemental Figure 2A). Non-perivascular MC3T3 cells failed to form links after increasing miR-126-3p levels by transfection when compared to PVC in time lapse microscopy (supplemental Figure 2B). The results indicated that the cell-cell interaction on BM-like matrigel is mainly promoted by increased levels of miR-126-3p.

miR-126-3p upregulation in PVC may also promote intercellular interaction with EC, which we analyzed in coculture experiments. Control- or miR-126-3p mimic-transfected, fluorescently labeled PVC were added to preformed networks of mouse brain derived endothelial cells (b.End5 cells) on matrigel. The PVC were rapidly incorporated into endothelial networks 7 hours after seeding (Figure 2A). Networks containing control-transfected PVC partially disassembled after 14 hours, whereas networks containing miR-126-3p mimic-transfected PVC were stabilized. In addition, fluorescently labeled control- or miR-126-3p mimic-transfected PVC (green) and HUVEC (red) were added to matrigel-coated chamber slides and cultured for 16 hours. Network-like structures formed on the matrigel substrate in cocultures of HUVEC and control-transfected PVC (Figure 2B). These were mainly composed of HUVEC (red). In contrast, a large number of PVC was detected in networks of cocultures with miR-126-3p mimic-transfected PVC. Hence, miR-126-3p supported the interaction of PVC and EC on BM-like composites.
Next we analyzed the phenotype of PVC on single BM-components. PVC adapted a spindle-like morphology on glass and on laminin 511, collagen I, collagen IV and nidogen-1 (Figure 3A). No differences in morphology or cell numbers were observed between control- and miR-126-3p mimic-transfected cells and aggregates and filamentous structures were not formed. Hence, miR-126-3p modulated the PVC phenotype on complex BM-like substrates but not on single BM-components. Time lapse microscopy revealed that miR-126-3p mimic-transfected PVC formed multipolar protrusions on matrigel and within four hours cell aggregates appeared, whereas control-transfected PVC still maintained a round morphology (Figure 3B). After seven hours aggregates formed long directional protrusions to contact more distant cells and assembled into larger cell clusters. Control-transfected cells formed small protrusions 12 hours after plating but were unable to form large aggregates and long protrusions. Quantification of cell migration showed that trajectory and the intervals to form cell-cell contacts were reduced in mimic-transfected cells compared to control, while the covered distance and the average speed per cell was unchanged (Figure 3C). 90% of the miR-126-3p mimic-transfected PVC were involved in cell-cell interaction and most cells remained in aggregates throughout the incubation time. In contrast, only 60% of the control-transfected PVC formed aggregates with about half of these remaining in aggregates (supplemental video). Scanning electron microscopy illustrated the effect of miR-126-3p on the morphology of PVC (Figure 3D). After cultivation for four hours on matrigel, miR-126-3p mimic-transfected PVC formed long directional protrusions through cell-cell contacts to assemble thin and long filamentous links on the matrigel surface, while control-transfected cells showed few multipolar protrusions. Therefore, increased abundance of miR-126-3p promoted intercellular contacts of PVC specifically on matrigel.

Cell-matrix and cell-cell interactions are mainly mediated by ß1-integrin cell surface receptors. siRNA-mediated knock down approach was used to demonstrate that ß1-integrin contributes to the miR-126-3p mimic induced phenotype in PVC. Control- or miR-126-3p mimic-transfected PVC were transfected with siRNAs directed against ß1-integrin and 48 hours later added to matrigel coated chamber slides. After four hours network formation was studied (Figure 3E). Cells transfected with siRNA against ß1-integrin lost the ability to form aggregates and network-associated links but showed a spindle-like morphology. This resembled the phenotype of control- or miR-126-3p mimic-transfected PVC cultured on glass or on single BM substrates (Figure 3A). No differences between control- and miR-126-3p mimic-transfected PVC were detected. The results pointed to a role of ß1-integrin-mediated formation of aggregates and intercellular connections.

miR-126-3p targets Spred1, Plk2, and Irs1 and activates the ERK signaling pathway in PVC

Target scan database analysis revealed that 20 conserved target genes are predicted for the miR-126-3p, but only a few may represent true targets of the miRNA. We hypothesized that target genes need to be expressed in the vasculature to modulate the activity of major signaling pathways and induce aggregate and protrusion formation of PVC. Therefore, gene expression atlas analysis (www.genepaint.org) and web-based literature search was used to exclude targets that were not described to be expressed in the vasculature and were not known to be involved in signaling pathway modulation. Among the 20 target genes SPRED1 and phosphatidylinositol 3-kinase regulatory subunit polypeptide 2 (PIK3R2) could regulate the activation of AKT and ERK1/2 signaling pathways in ECs and IRS1 was described to be a target of miR-126-3p in smooth muscle cells. In addition, the signaling proteins regulator of G-protein signaling 3 (RGS3) and PLK2 were expressed in the developing vasculature of the mouse embryo. Hence, we focused our analysis on SPRED1, PIK3R2, IRS1, RGS3 and PLK2. First, we studied the interaction of miR-126-3p with its binding sequences in the 3'UTR of selected target genes using luciferase assays. The luciferase activity was significantly reduced in miR-126-3p mimic-transfected HEK293 cells that contain the miR-126-3p binding sequence of Spred1, Plk2 or Irs1 in the 3'UTR of the luciferase reporter gene (Figure 4A). The inhibitory effect was abolished by two point mutations within the binding sequences of Spred1, Plk2 or Irs1, demonstrating the specificity.
of the miR-126-3p interaction. The luciferase activity was not decreased in cells containing the miR-126-3p 3'-UTR binding sequences of Pik3r2 and Rgs3. Interestingly, the luciferase activity was reduced for the binding sequence of the human PIK3R2 gene, pointing to species-specific differences in target gene recognition. In conclusion, miR-126-3p interacted with the 3'-UTR of mouse Spred1, Irs1 and Plk2.

Next, their expression was studied in an existing mRNA microarray dataset (E. Pöschl, unpublished results) of the three-dimensional coculture system and in immunoblot analysis. The selected target genes were expressed in three-dimensional monocultures of PVC. The mRNA expression of Plk2 and Irs1 was reduced in PVC upon coculture with HUVEC (Figure 4B) and in immunoblot analysis a significant twofold reduction was found for all proteins in miR-126-3p mimic-transfected PVC compared to control (Figure 4C). To study the role of each target gene in PVC migration and interaction PVC were transfected with siRNAs directed against Spred1, Plk2 or Irs1 and added to matrigel-coated chamber slides. 16 hours later network formation was compared to control or miR-126-3p mimic-transfected PVC. siSpred1- and siPlk2- transfected PVC formed network-like structures as seen for miR-126-3p mimic-transfected PVC, whereas control- and siIrs1-transfected PVC formed only few multipolar protrusions (Figure 4D). The cumulative tube length was fivefold increased in miR-126-3p mimic-transfected PVC and fourfold in PVC transfected with siSpred1 and siPlk2 compared to control- or siIrs1-transfected PVC. In contrast, aggregate and network-associated link formation was inhibited when overexpressing SPRED1 in miR-126-3p mimic-transfected PVC (supplemental Figure 3). Taken together, SPRED1 and IRS1 are regulatory targets of miR-126-3p and PLK2 was identified as a novel target of miR-126-3p in PVC. Inhibition of SPRED1 and PLK2 expression could mimic the effect of miR-126-3p in PVC on matrigel and the phenotype of miR-126-3p mimic-transfected PVC could be rescued by SPRED1 overexpression.

miR-126 can repress SPRED1 expression to stimulate the activation of the ERK1/2 and AKT signaling pathways in human EC6. We asked whether the pathways were also targeted in murine PVC. Cells were transfected with control or miR-126-3p mimic oligonucleotides or with siRNAs directed against Spred1, Plk2 or Irs1, cultured on plastic and stimulated with serum. ERK1/2 phosphorylation was determined 15 minutes later (Figure 5A). ERK1/2 phosphorylation was significantly increased in miR-126-3p mimic- and siSpred1-transfected PVC compared to control, whereas phosphorylation was not affected in siPlk2- or siIrs1-transfected cells. This indicated that increased miR-126-3p levels inhibit Spred1 expression to modulate ERK1/2 phosphorylation in PVC. The phosphorylation status of ERK1/2 of control- or miR-126-3p mimic-transfected PVC was assessed in (Figure 5B) 5, 15 and 30 minutes after stimulation with complete VascuLife VEGF-Mv medium. In addition, AKT phosphorylation was analyzed. The amounts of pERK1/2 were significantly increased in miR-126-3p mimic-transfected PVC, whereas pAKT was not significantly increased compared to control. Hence, miR-126-3p mainly stimulated ERK1/2 phosphorylation in PVC.

To demonstrate that cell-cell and cell-matrix interactions were induced by ERK1/2 but not by AKT signaling pathway activation we cultured control- and miR-126-3p mimic-transfected PVC for 16 hours on matrigel in the presence of 10μM U0126 inhibitor to suppress the phosphorylation of ERK1/2 or 2μM Wortmannin to inhibit AKT phosphorylation. The addition of U0126 blocks aggregate-associated link formation in miR-126-3p mimic-transfected PVC, but not the addition of Wortmannin (Figure 5C). The inhibition of ERK1/2 or AKT phosphorylation was confirmed by immunoblot analysis indicating that both inhibitors acted specifically on their individual kinases (Figure 5D). Therefore, only the inhibition of ERK1/2 phosphorylation blocked aggregate-associated link formation in miR-126-3p mimic-transfected PVC.

miR-126-3p stimulates chemokine expression in PVC
The miR-126-3p-mediated activation of the ERK1/2 signaling pathway could induce downstream changes to stimulate cell-matrix and cell-cell contacts. These changes were studied by global transcriptome analysis using a 60k whole genome array of control- and miR-126-3p mimic-transfected cells after four hours culture on matrigel, when multipolar
protrusions and cell aggregates start to form in miR-126-3p mimic but not in control-transfected PVC. To determine the influence of miR-126-3p on genes involved in cell-matrix interaction we initially studied the expression of vascular basement membrane proteins and integrin receptors. Collagens, fibronectin, perlecan, laminins and nidogens and the corresponding integrin receptors were not differentially expressed in miR-126-3p mimic-transfected PVC compared to control (supplemental Figure 4A). We then focused on significantly upregulated and annotated genes in miR-126-3p mimic-transfected PVC (Figure 6A). 47 upregulated genes were imported into the String 9.1 database and functional partnerships between the imported entities were determined. 38 genes were clustered and seven were linked to cytokine-cytokine receptor interaction (supplemental Figure 4B). Among those chemokine (C-C motif) ligand 5 (CCL5) was reported to promote endothelial cell migration, spreading and neo-vessel formation.\(^{23}\) Chemokine production can be regulated by mitogen activated kinases via the Toll-like receptor 3 (TLR3)\(^{24,25}\) and this was an upregulated cell surface receptor in miR-126-3p transfected PVC. The ECM can interact with Toll-like receptors\(^ {26}\) and TLR3 can mediate endothelial cell migration and vascular sprouting.\(^ {27}\)

Hence, CCL5 and TLR3 may promote PVC migration and interaction upon miR-126-3p mimic transfection in PVC on matrigel. To study their function we performed siRNA-mediated knock down studies and transfected control- or miR-126-3p mimic-transfected PVC with siRNAs directed against CCL5 or TLR3. PVC were added to matrigel and 16 hours later formation of network-like structures was analyzed (Figure 6B). PVC transfected with siCCL5 and miR-126-3p mimic formed network-like structures, whereas PVC transfected with siTLR3 and miR-126-3p mimic showed only few multipolar protrusions. The cumulative tube length was fourfold decreased compared to siCCL5 or control-transfected PVC with elevated levels of the miR-126-3p. Therefore, miR-126-3p induced, presumably through increased ERK1/2 phosphorylation, TLR3 expression in PVC on matrigel to stimulate PVC migration and intercellular interaction, whereas induced CCL5 expression was not needed for cell-matrix and cell-cell contacts.

**miR-126-3p modulates PVC-dependent angiogenesis in vivo**

To analyze the role of miR-126-3p in vivo we used a directed in vivo angiogenesis assay.\(^ {20}\) Control- or mimic-transfected PVC were transferred to a silicone tube in a matrigel-fibrin matrix enriched in VEGF and FGF2 and tubes were implanted subcutaneously under the dorsal skin of immunodeficient mice. \(~16\) days after implantation, matrigel-fibrin plugs were isolated and vascular invasion was assessed by microscopy. Matrigel-fibrin plugs containing control-transfected PVC were invaded by few vessels and small blood lacunae were formed (Figure 7A). In contrast the anterior and central area of the plugs containing miR-126-3p mimic-transfected cells were highly vascularized. Newly formed vessels deeply infiltrated the matrix and blood lacunae were detected. Vascularization and permeability of the newly formed vessels was assessed by intravenously injection of a FITC-dextran tracer (70kDa) prior to isolation of the tubes. This tracer can diffuse into the extracellular space of immature vessels but is retained in mature, less permeable vessels.\(^ {28}\) A diffuse staining of the matrigel-fibrin matrix was seen for plugs containing control-transfected cells 30 minutes after injection, whereas a strong vessel associated staining was observed for plugs containing miR-126-3p mimic-transfected cells (Figure 7B). These FITC-dextran positive vessels originate from the anterior end to form a vascular tree branching into the central area of the plug. The matrigel-fibrin matrix and blood lacunae were hardly stained. The accumulation of the FITC-dextran conjugate in plugs was also analyzed by fluorescence spectroscopy. Plugs were digested with dispase, centrifuged and the fluorescence of the supernatant was determined at 510nm (Figure 7C). The fluorescence signal was significantly increased for plugs containing miR-126-3p mimic-transfected cells compared to control. The results show that miR-126-3p mimic-transfected PVC induced a strong angiogenic response in vivo. The lack of FITC-dextran leakage into the perivascular space demonstrated that the newly formed vasculature was stabilized by perivascular cells.
Discussion

Blood vessel formation and stabilization depends on the control of interactions between perivascular and endothelial cells. Our analysis identified murine miR-126-3p (corresponding to human miR-126) to be selectively upregulated in PVC upon interaction with EC to promote BM-dependent migration and mutual interactions.

Originally, miR-126 was described to increase the migration of human EC in scratch assays and reduced levels affected the stability of tubes formed on matrigel substrate⁶. The present study shows for the first time that increased levels of miR-126-3p in murine PVC stimulate formation of network-like assemblies on matrigel, a composite substrate resembling a BM. In contrast, individual components of the vascular BM, e.g., laminin 511, collagen IV and nidogen 1 failed to promote network formation. Knock down of β1-integrin in miR-126-3p mimic-transfected PVC resulted in the loss of aggregate formation and cultures on collagen I gels failed to form any networks (supplemental Figure 5). The results indicate that miR-126-3p promotes integrin-mediated synergistic interaction of PVC with more than one BM component and, most likely, with vascular BM.

miR-126 was described to induce SMC proliferation and apoptosis⁷. We could not observe changes in PVC proliferation or survival (supplemental Figure 6), which could be due to the use of different culture systems in the two studies. Here, PVC were transfected with miR-126-3p mimic to determine the direct effects of the miRNA upregulation on the cellular phenotype, whereas in the earlier study EC were transfected with inhibitors of miR-126-3p and indirect effects on SMC survival were analyzed. This suggests that secondary signals transmitted from EC in response to changes in miR-126-3p expression modulate survival and proliferation of cocultured PVC rather than direct effects of miR-126-3p on PVC.

We focused on the consequences of increased miR-126-3p expression in PVC since miR-126-3p is strongly upregulated in PVC upon interaction with EC. Nevertheless, inhibition of miR-126-3p caused a significant reduction in network formation, demonstrating that limited levels of miR-126-3p were present in PVC monocultures and modulated the PVC response to matrigel. In PVC specifically the miR-126 3′-strand modulates the interaction with the ECM environment. The effect is mediated by activating the ERK1/2 signaling pathway as we could demonstrate that miR-126-3p suppresses SPRED1 protein expression in PVC to stimulate ERK1/2 phosphorylation leading to network-like structure formation. Moreover, we could block matrix-dependent intercellular interactions in miR-126-3p mimic-transfected PVC by ERK1/2 pathway inhibition or SPRED1 overexpression. This is in line with previous reports showing that miR-126-3p can inhibit SPRED1 expression to increase the activation of the ERK1/2 pathway in human EC⁵. SPRED1, a member of the sprouty-related protein with EHV-1 domain (SPRED) family of proteins, can translocate to the membrane to down-regulate Ras-GTP levels and inhibit the ERK1/2 signaling pathway²⁹. Knock down of miR-126 in zebrafish stimulated SPRED1 expression and enhanced ERK1/2 signal pathway activation and resulted in the loss of vascular integrity and hemorrhage during embryonic development⁶. This was mainly attributed to altered ERK1/2 signal pathway activation in endothelial cells. Our study now points to an additional role of the miR-126-3p-SPRED1-ERK1/2 axis in PVC and in cell-matrix interactions. We also showed that *Irs1* is downregulated by miR-126-3p in PVC upon coculture with EC. A previous study demonstrated that miR-126-3p represses IRS1 protein expression in cocultured SMC⁷, but the relevance for the cellular phenotype is less clear. PVC attachment and cellular interactions are not affected upon *Irs1* knock down and PLK2 as a novel target for the miR-126-3p in PVC seems to be of greater significance. PLK2 belongs to the polo-like family of serine/threonine kinases, which can modulate dendritic spine remodeling in neurons²⁸,²⁹. Interestingly, the siRNA-mediated knock down of PLK2 protein, which is expressed in the vasculature and is a direct target of miR-126-3p, also promotes PVC protrusion formation on matrigel. The molecular link between reduced PLK2 activity and the stimulation of PVC-EC interactions on BM-like composites is not yet known. The siRNA studies indicated that ERK and AKT signaling pathways are not targeted by PLK2, but recent results linked PLK2 to the TLR signaling response²⁹.
In contrast, SPRED1-dependent suppression of ERK1/2 signaling pathway seems to be an essential molecular link. The inhibitor studies showed that an impaired ERK1/2 phosphorylation blocks network-like tube formation of PVC and we identified that downstream chemokine-receptor targets that are upregulated upon culture of miR-126-3p mimic-transfected PVC on a matrigel substrate. Several of these cytokines are associated with the toll-like-receptor pathway and only recently it was shown that miR-126-3p can control the expression of Toll-like receptor genes in dendritic cells. Interestingly, Tlr3 mRNA expression was also significantly increased upon culturing of miR-126-3p transfected PVC on matrigel substrate. Tlr3 mRNA expression can be stimulated by ERK1/2 signaling and TLR3 signaling can augment the production of chemokines. Although Toll-like receptor signaling is mainly related to the innate immune response and recognition of dsRNA, a recent report showed that Toll-like receptors can also recognize ECM molecules and promote endothelial cell migration and vascular sprouting. We showed that in PVC miR-126-3p can reduce SPRED1 protein levels to induce ERK1/2 phosphorylation, increase Tlr3 mRNA expression and modulate PVC-matrix interaction and network-like structure formation. Hence, miR-126-3p-induced ERK1/2 phosphorylation and Tlr3 upregulation fulfil important functions during vascular homeostasis.

These findings are relevant in the context of new vessel formation in normal and pathological angiogenesis, where deposition of basement membranes is critical for EC-PVC interactions and stabilization of the maturing vessels. The upregulation of miR-126-3p in PVC upon coculture with EC, the increased cell-matrix and cell-cell contacts upon miR-126-3p transfection as well as the proangiogenic and vessel-stabilizing effects in vitro and in vivo suggest that miR-126-3p promotes the maturation of newly formed vessels. The effects are mainly mediated by the inhibition of PLK2 and SPRED1. PLK2 downregulation by miR-126-3p overexpression induces phenotypic changes in PVC irrespective of ERK activation, whereas SPRED1 downregulation by miR-126-3p overexpression induces changes via stimulation of the ERK signaling pathway. The latter is in line with a previous published link between SPRED1 and ERK phosphorylation in endothelial cells. As a consequence increased miR-126-3p levels stimulate matrix-dependent intercellular interaction of PVC to promote coverage of newly formed vessel and transform immature into mature, less permeable vessels. In contrast, decreased miR-126-3p levels may loosen basement membrane-dependent intercellular connections and support detachment of PVC and EC from preexisting vessels to allow new blood vessel formation. Hence, miR-126-3p modulates basement membrane-dependent PVC migration and cell-cell contacts in the neovasculature. We therefore propose that miR-126-3p is a novel regulator of PVC-mediated vessel stability during neoangiogenesis and in pathological scenarios like tumor angiogenesis.

Acknowledgements

DFG (SFB829-B04, DFG 2304/5-3, 2304/7-1, 2304/9-1), Köln Fortune - University of Cologne (136/2013, 120/2014). We thank Katrin Blumbach for her support in live cell imaging and Manuel Koch and Manuel Keller for providing collagen IV and nidogen-1.
References


Identification of miRNAs regulated in PVC in three-dimensional cocultures with HUVEC

Figures

Figure 1
Identification of miRNAs regulated in PVC in three-dimensional cocultures with HUVEC. Microscopic analysis of HUVEC monocultures and HUVEC/PVC cocultures. (A) Formation of tube-like structures is strongly promoted in three-dimensional cocultures. (B) Detection of PECAM1 by immunofluorescence analysis. (C-F) The miRNA transcriptome between mono- (Mo) and cocultured (Co) PVC or HUVEC was compared using miRNA-array (Agilent) analyses based on the Sanger miRBase release 14. (C) Intensity plot includes all miRNAs detected in PVC. (D) Plot of regulated miRNAs expressed in PVC (fold change ≥ 2, p-value ≥ 0.01) that show a signal intensity above background noise. The nonregulated miR-31 was used for normalization. (E) Intensity plot showing all miRNAs detected in HUVEC. The expression of miR-126 and miR-146a is highlighted (green). (F) Calculated fold changes for miR-126-3p, miR-146a and miR-31 are listed. (G) miRNA and (H) pri-miR-126-3p expression was validated by quantitative PCR (qPCR). Microarray and qPCR analyses were performed in two or three independent biological experiments, respectively. (I) Cell cluster and protrusion formation of miR-126-3p or miR-146a mimic-transfected PVC on matrigel-coated surfaces 16 hours post plating compared to control-transfected PVC.

Figure 2
Characterization of PVC-EC interaction on matrigel
(A) Time lapse images of fluorescently labeled control- or miR-126-3p mimic-transfected PVC (green) added to preformed networks of b.End3 cells. The cumulative tube length of three independent experiments was determined at 14h hours (graph). (B) Coculture of fluorescently labeled control- (green, left) or miR-126-3p mimic-transfected PVC (green, right) and HUVEC (red) on matrigel 16 hours post plating. The HUVEC+ or PVC+ pixel/area of three independent experiments were determined (graph). Representative images are shown.

Figure 3
Analysis of cell morphology and migration of control- or miR-126-3p mimic-transfected PVC
(A) Morphology of transfected PVC on uncoated (glass) or ECM protein-coated surfaces 15 hours after adding the cells to the various substrates. (B) Representative time lapse images of cell attachment and migration on matrigel. Individual cells are numbered. (C) Cell migration parameters like distance, trajectory, speed and time until cell-cell contact were determined for three independent experiments. (D) Scanning electron microscopy illustrates the morphology of control- and miR-126-3p mimic-transfected PVC four hours after plating on matrigel. (E) Aggregate and protrusion formation of control- and miR-126-3p mimic-transfected PVC after siRNA-mediated knock down of ß1-integrin on matrigel-coated surface 4 hours post plating.

Figure 4
Identification of miR-126-3p-dependent target genes and signaling pathways in PVC
(A) Interaction of miR-126-3p with the intact or mutated (mut) putative binding site in the 3'-UTR of the indicated target genes. Fold changes of Renilla to Firefly luciferase activity in miR-126-3p mimic-transfected cells compared to control-transfected cells are presented. (B) Heatmap to illustrate the expression of miR-126-3p target genes in mono- (Mo) or cocultured (Co) PVC analyzed by mRNA microarray. (C) Extracts of control- and miR-126-3p mimic-transfected PVC analyzed for the presence of SPRED1, PLK2 and IRS1 by immunoblotting. GAPDH detection was used as loading control. Fold change of SPRED1, PLK2 or IRS1 to GAPDH ratio in miR-126-3p mimic-transfected cells compared to control-(graph). (D) Cell cluster and protrusion formation of control-, miR-126-3p mimic- and siRNA-transfected PVC on matrigel-coated surface 16 hours post plating. The individual target...
genes of each siRNA are indicated. The cumulative tube length of three independent experiments was quantified (graph).

Figure 5
Silencing of target genes in PVC on matrigel-coated surfaces and consequences for ERK1/2 and AKT signaling
(A) Immunoblot analysis of ERK1/2 signaling pathway activation 15 minutes after serum stimulation in control-, miR-126-3p mimic- or siRNA-transfected PVC on plastic. The molecular weight of ERK1 and ERK2 is given. The fold change in phosphorylation normalized to total ERK1/2 in miR-126-3p mimic-transfected cells compared to control for five independent experiments is shown (graph). (B) Representative immunoblot showing phosphorylated (p) ERK1/2, total ERK1/2 (upper panel), pAKT and total AKT (lower panel) in control- and miR-126-3p mimic-transfected PVC after serum stimulation. The fold change in phosphorylation in miR-126-3p mimic-transfected cells compared to control-transfected cells normalized to total ERK1/2 or AKT was determined. The results of four or more independent experiments 15 minutes after stimulation are summarized (graph). (C) Aggregate and protrusion formation of control- and miR-126-3p mimic-transfected PVC in the presence of 10µM U0126 or 2µM Wortmannin on matrigel-coated surface 16 hours post plating. (D) Immunoblot analysis of ERK1/2 and AKT phosphorylation in treated PVC 15 minutes after serum stimulation.

Figure 6
Determination of transcriptome changes in miR-126-3p mimic-transfected PVC on matrigel-coated surfaces
The mRNA transcriptome of control- and miR-126-3p mimic-transfected PVC was compared four hours post plating on matrigel. Four independent experiments were analyzed using whole genome mRNA-arrays (Agilent). (A) Intensity expression plot of differentially expressed genes. The fold change (FC) is shown. (B) Aggregate and network formation of control-, miR-126-3p mimic- and siRNA-transfected PVC on a matrigel-coated surface 16 hours post plating. The individual target genes of each siRNA are indicated. The cumulative tube length of three independent experiments was determined (graph).

Figure 7
Characterization of proangiogenic effects by miR-126-3p mimic-transfected PVC in vivo
(A) Microscopic assessment of neoangiogenesis in subcutaneously implanted silicone tubes (angioreactors) filled with control- or miR-126-3p mimic-transfected cells. Angioreactors were isolated from immunodeficient mice 14-17 days after implantation. Three representative angioreactors for control- and mimic-transfected PVC are shown. (B) FITC-Dextran-70 solution was injected intravenously 30 minutes prior isolation of the angioreactors to stain newly formed vessels. Fluorescence microscopy analysis was used to determine the distribution and permeability of newly formed vessels. All images were simultaneously adjusted in brightness, contrast and color. (C) Quantification of FITC-Dextran-70 in the supernatant of dispase-digested angioreactors by fluorescence spectrometry.
Figure 1

A  HUVEC  HUVEC + PVC

B  PECAM1

C  intensity values

D  $p < 0.01$, fold change > 2

E  intensity values

F  microarray

G  qPCR

H  qPCR

I  control  miR-126-3p  miR-146a

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Figure 2

A

15min  7h  14h

b.End5 + PVC control

miR-126-3p

B

HUVEC + PVC control  HUVEC + PVC miR-126-3p

200µm

HUVEC pixel area

PVC pixel area

control  miR-126-3p

control  miR-126-3p

*  **
Figure 3

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C

- Distance (μm/cell)
- Trajectory (μm/cell)
- Speed (μm/s/cell)
- Time cell contact (h)

D

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Figure 4

(A) Fold change to control for Spred1, Spred1 mut, Pkl2, Pkl2 mut, Irs1, Irs1 mut, Pkl3R2, Pkl3R2 mut, Rgs3, and Rgs3 mut.

(B) Heat map showing expression levels of Spred1, Pkl2, and Irs1 for different conditions.

(C) Western blots showing expression of SPRED1, PLK2, and IRS1 with GAPDH as a loading control.

(D) Images showing the effect of miR-126-3p, siSpred1, siPkl2, and silrs1 on cumulative tube length.
Figure 5

A. Western blots showing the levels of pERK1 (~44kDa), pERK2 (~42kDa), ERK1 (44kDa), and ERK2 (42kDa) in control and miR-126-3p, asSpred-1, avpLQ, and siIrs1 conditions. The bar graph on the right shows the fold change in pERK1/ERK12 levels.

B. Western blots showing the levels of pAKT and AKT at different time points (0min, 5min, 15min, 30min) in control and miR-126-3p, asSpred-1, avpLQ, and siIrs1 conditions. The bar graph on the right shows the fold change in pAKT/ AKT levels at 15min.

C. Light micrographs showing cells treated with no inhibitor, U0126, and Wortmannin in control and miR-126-3p conditions.

D. Western blots showing the levels of pERK1 (~44kDa), pERK2 (~42kDa), ERK1 (44kDa), and ERK2 (42kDa) in control and U0126, Wortmannin conditions. The bar graph on the right shows the fold change in pAKT/AKT levels.
Figure 6

A

![Heatmap showing gene expression levels for control and miR-126-3p conditions.](image)

B

![Images showing cell morphology under different conditions.](image)

![Graph showing cumulative tube length.](image)
Figure 7

A

control

miR-126-3p

B

control

miR-126-3p

C

Emission (a.u.)

0

1000

2000

3000

4000

control

miR-126-3p

*
Supplemental Figure 1
Characterization of the purity of separated PVC and HUVEC from three-dimensional cocultures

(A) Microscopic analysis of PVC and HUVEC monocultures and HUVEC/PVC cocultures. Formation of tube-like structures is strongly promoted in three-dimensional cocultures. (B-E) Detection of EC-specific (PECAM1 (CD31)) and PVC-specific (NG2) marker gene expression by immunofluorescence in PVC and HUVEC monocultures (B, C, Mo) and in immunomagnetically separated PVC and HUVEC from cocultures (D, E, Co) 24 hours after separation and cultivation on plastic. (F) Microarray analysis of selected marker genes in
purified PVC. Normalized intensity values of selected genes in whole genome transcriptome analysis are displayed for cocultured cells. Counts correspond to the number of detected probes for a specific gene. Transcripts specific for hypertrophic chondrocytes (Col10a1) or leukocytes (Ptpc (CD45)) define the background signal of expression. Genes coding for endothelial cell markers Pecam1 (CD31) and perivascular cell markers (Cspg4 (Ng2), Acta2 (a-Sma), Pdgfrb and Anxa5) are shown. The analysis confirms that PVC and HUVEC can successfully be separated from cocultures and used in downstream experiments.
Supplemental Figure 2
Phenotypic analysis of control- or mimic-transfected cells
(A) Cell cluster and protrusion formation of control, miR-126-3p mimic or inhibitor-transfected PVC (anti-miR-126-3p) on matrigel-coated surfaces 16 hours post plating compared to control-transfected PVC. (B) Time lapse images of control- or miR-126-3p mimic-transfected PVC and MC3T3 cells. The cumulative tube length was determined in three independent experiments (graph). Representative images are shown.
Supplemental Figure 3
Expression of recombinant SPRED1 inhibits aggregate and protrusion formation in miR-126-3p mimic-transfected PVC on matrigel-coated surfaces

(A) Microscopy and flow cytometry analysis of GFP expression in pmaxGFP-transfected PVC. (B) Representative immunoblot showing the overexpression of SPRED1 in pcDNA 3.1 6xHis-Spred1-transfected PVC. (C) Time lapse images of mock or pcDNA 3.1 6xHis-Spred1 transfected PVC (SPRED1) containing control or miR-126-3p mimics. The cumulative tube length was determined for three independent experiments (graph). Representative images four and seven hours post plating are shown.
Supplemental Figure 4
Transcriptome analysis of control and miR-126-3p mimic-transfected PVC
The mRNA transcriptome of control- and miR-126-3p mimic-transfected PVC was compared four hours post plating on matrigel using whole genome mRNA arrays (Agilent). (A) Intensity expression plot of vascular basement membrane entities. The values for four individual experiments are shown. (B) String database analysis plot of expressed and regulated mRNA (fold change ≥ 2, p-value ≤ 0.01).
Supplemental figure 5
Characterization of aggregate and protrusion formation on collagen I gel and matrigel-coated surfaces
Cell cluster and protrusion formation of miR-126-3p control- or mimic- transfected PVC on 16μl of collagen I gel or matrigel. Representative images are shown.
Supplemental Figure 6
Analysis of transfection efficiency, proliferation and viability in control- or miR-126-3p mimic-transfected PVC as well as characterization of network formation on growth factor-depleted matrigel
(A) Transfection efficiency and interaction of miR-126-3p with its target sequences was confirmed by luciferase reporter assays. The vector map depicts the coding sequence of Renilla luciferase (hRluc), the multiple cloning site and the Firefly luciferase (hluc) normalizer (left). qPCR analysis of miR-126-3p expression in control- and mimic-transfected cells (center). The ratio of Renilla to Firefly luciferase activity in control- and miR-126-3p mimic-transfected cells was determined (right). (B) Cell proliferation was determined as the...
production of a colored formazan product in metabolically active cells two, 24, 48 and 72 hours after transfection. (C) Cell viability was characterized by flow cytometry. The proportion of AnxA5-Alexa488/PI+ viable, AnxA5-Alexa488+/PI+ apoptotic and AnxA5-Alexa488+/PI+ necrotic cells 24, 48 and 72 hours post transfection was determined. (D) Cell cluster and protrusion formation of miR-126-3p control- or mimic- transfected PVC on growth factor reduced matrigel. Representative images are shown. The results of three independent experiments were analyzed.
A

**SPRED1**

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Supplemental Figure 7
Analysis of transfection efficiency in control-, miR-126-3p mimic- or siRNA-transfected PVC as well as characterization of network formation on matrigel
Extracts of control-, miRNA-126-3p mimic-, siSpred1-, siPlk2- and siirs1-transfected PVC analyzed by immunoblotting. Two siRNA were transfected for each target gene. Fold change compared to control is given (graph). GAPDH was used for normalization. (B) Cell cluster and protrusion formation of control-, miR-126-3p mimic- or siRNA-transfected PVC on matrigel-coated surface 14 hours post plating. Representative images are shown. (C) Flow cytometry or qPCR analysis of β1 integrin or Ccl5 knock down in transfected PVC. (D, left) Cell cluster and protrusion formation of control-, miR-126-3p-mimic or sITlr3-transfected PVC on matrigel coated surfaces 14 hours post plating. Representative images are shown. (D, right) qPCR analysis of siRNA-mediated knock down in miR-126-3p mimic-transfected cells. Three independent experiments were analyzed.
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**Supplemental Table 1**

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<td>gcgcgcAATAAGTACCCGACCATCAAACCTGCTCCCATC</td>
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<tr>
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<tr>
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<td>gcgcgcATAGGTTCTGACCTACTACTACTACTACTACTACTACTACTAC</td>
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