MicroRNA-218 Regulates Vascular Patterning by Modulation of Slit-Robo Signaling

Eric M. Small, Lillian B. Sutherland, Kartik N. Rajagopalan, Shusheng Wang, Eric N. Olson

Rationale: Establishment of a functional vasculature requires the interconnection and remodeling of nascent blood vessels. Precise regulation of factors that influence endothelial cell migration and function is essential for these stereotypical vascular patterning events. The secreted Slit ligands and their Robo receptors constitute a critical signaling pathway controlling the directed migration of both neurons and vascular endothelial cells during embryonic development, but the mechanisms of their regulation are incompletely understood.

Objective: To identify microRNAs regulating aspects of the Slit-Robo pathway and vascular patterning.

Methods and Results: Here, we provide evidence that microRNA (miR)-218, which is encoded by an intron of the Slit genes, inhibits the expression of Robo1 and Robo2 and multiple components of the heparan sulfate biosynthetic pathway. Using in vitro and in vivo approaches, we demonstrate that miR-218 directly represses the expression of Robo1, Robo2, and glucuronoyl C5-epimerase (GLCE), and that an intact miR-218–Slit–Robo regulatory network is essential for normal vascularization of the retina. Knockdown of miR-218 results in aberrant regulation of this signaling axis, abnormal endothelial cell migration, and reduced complexity of the retinal vasculature.

Conclusions: Our findings link Slit gene expression to the posttranscriptional regulation of Robo receptors and heparan sulfate biosynthetic enzymes, allowing for precise control over vascular guidance cues influencing the organization of blood vessels during development. (Circ Res. 2010;107:1336-1344.)

Key Words: angiogenesis ■ gene regulation ■ developmental biology

Blood vessels and nerves are organized in nearly identical patterns throughout the body; however, the mechanisms that direct these disparate cell types to follow similar migratory tracts during development have not been fully defined. Recent studies have revealed that vessels and nerves possess a common set of transmembrane receptors that respond to molecular cues governing cell migration and pathfinding. These “axon guidance molecules” influence the behavior of both nerves and endothelial cells (ECs), resulting in similar patterning of each cell type.

Roundabout (Robo) receptors and their Slit ligands function as crucial regulators of axon and vascular guidance. Slit-Robo signaling provides a short-range directional cue for the tangential migration of neurons, ejecting axons from the midline and preventing recrossing. Slit-Robo signaling also controls EC function and the process of vessel sprouting, called angiogenesis, and contributes to the stability of the vascular network. The Robo receptor may impart a positive or negative influence on cell migration and directionality depending on the isoform composition and cellular context. Although it is apparent that the expression and activity of the various Slit and Robo genes exert a major influence on the ultimate form of the vessels and nerves, the rules governing Slit-Robo-dependent cellular behavior are not clear.

The biological activity of many angiogenic growth factors, including Slit, vascular endothelial growth factor (VEGF)-A, fibroblast growth factor-2, and platelet-derived growth factor-BB, is modulated by the sulfation state of heparan sulfate proteoglycans (HSPGs). HSPGs are transmembrane or secreted proteins that are covalently linked to heparan sulfate (HS) chains. HSPGs are essential for Slit-Robo signaling, affecting the diffusion radius of Slit or acting as coreceptors that strengthen the interaction between Slit and Robo. Modification of HSPGs by epimerization and sulfation potentiates Slit binding in vitro, whereas enzymatic digestion of cell surface HS can abolish Slit-Robo activity. Slit-Robo signaling in vivo is blunted by genetic ablation of enzymes involved in the HS biosynthetic pathway.

Recently, microRNAs (miRNAs) have been documented as key contributors to the process of angiogenesis. miRNAs are small noncoding RNAs that inhibit translation or promote mRNA degradation by binding to the 3’ untranslated region (3’UTR) of target mRNAs, resulting in the “fine-tuning” of gene expression. It is becoming evident that a common regulatory mechanism for miRNA action involves the modest repression of many miRNAs with similar function or within a common
molecular pathway by an individual miRNA.28 This creates an additive effect and reduces the dependence on a single molecular target to elicit a functional response. It also appears that miRNAs encoded by introns of protein-coding genes commonly regulate processes related to the host gene function.29 In this way, transcriptional regulation of the host gene results in the posttranscriptional regulation of a set of target genes that enhance or diminish the host gene activity.

In the present study, we describe an additional level of control over the Slit-Robo signaling pathway, whereby the expression and activity of the Robo receptors is dependent on a miRNA encoded within an intron of the Slit genes. The Slit2 and Slit3 genes encode miR-218, which directly represses the expression of Robo1, Robo2, and multiple components of the HSPG biosynthetic pathway. Antisense-mediated knockdown of miR-218 relieves repression of Robo1, Robo2, and the HS-modifying enzyme glucuronyl C5-epimerase (GLCE or HSEPI), resulting in alterations in Slit-Robo signaling and, consequently, EC migration. We also show that miR-218 plays a role in retinal angiogenesis, controlling the density of the capillary plexus, at least partially, via promotion of secondary vascular interconnections. Our results reveal an intricate regulatory network between Slit genes, Robo receptors, and HSPG biosynthetic proteins allowing for the precise control over the expression level and activity of Slit-Robo signaling molecules during vascular patterning.

**Methods**

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**RNA Isolation and Analyses**

Total RNA was isolated using TRizol and the protocol of the manufacturer (Invitrogen). microRNA and mRNA levels were quantified using Northern blot or TaqMan real-time probes against miR-218, Robo1, Robo2, and GLCE, Hs3st3B1, Hs6st3, KDR, Flt1, and Hrt1 (Applied Biosystems).

**Cell Culture and Transfection**

Antisense oligonucleotides possessing 2-O'Me modifications on the outermost 6 nucleotides (IDT, final concentration of 10 nmol/L) or LNA-modified antisense oligonucleotides (final concentration of 50 nmol/L) were transfected using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, total RNA or protein was isolated. Sequences were as follows: 2-O'Me modified oligonucleotide sequences: anti-miR-218, 5'-AACCACATGGTTAGATCAAGCACAA-3'; control, 5'-CTCCTTACCTCAGTTACAATTATA-3'; locked nucleic acid (LNA) modified oligonucleotide sequences: anti-miR-218, 5'-GTTAGATCAAGCACAA-3'; control, 5'-CTCCTTACCTCAGTTACAATTATA-3'.

**3'UTR Reporter Construction**

The 3’UTRs of the mouse Robo1, Robo2, and GLCE genes were amplified and cloned into the SacI/HindIII sites of the pMIR-Report luciferase vector. The seed region of the miR-218 target sites in the Robo1 and GLCE 3’UTRs were mutated using the Quickchange II site-directed mutagenesis kit (Stratagene). Primer sequences are in the Online Methods section.

**Western Blot**

Antibodies directed against Robo1 (University of Iowa Hybridoma Bank), Robo2 (Abcam), and GLCE (Abcam) were used to determine protein level by Western blot. GAPDH (Calbiochem) was detected as loading control. Band intensity was quantified using NIH ImageJ software.

**Analysis of Postnatal Retinal Angiogenesis**

In vivo injection and small interfering RNA knockdown in the mouse retina was performed primarily as described previously30 and is detailed in the Online Methods section. Briefly, 1 µL of a 5 mg/mL solution of LNA-modified anti–miR-218 or universal control oligonucleotide was unilaterally injected in the subretinal space of postnatal day (P)2 mice in the ICR background. Mice were allowed to develop for 3 days before RNA and protein isolation at P5, and 5 days before isolation of retinas at P7 for histological analysis. Proliferating cells were visualized by staining for phospho-histone H3 (Cell Signaling). Visualization of the vasculature was performed by isoelectin (Molecular Probes) staining of retinal flat mounts. Quantification of vessel density was performed using NIH ImageJ software. The thickness of the vascular plexus was calculated from confocal z-stacks taken at the vascular migration front. Student’s t tests were used to determine statistical significance between groups.

**In Situ Hybridization**

Whole mount microRNA in situ hybridization was performed essentially as previously described.31 5’ and 3’ digoxigenin-labeled antisense LNA probes directed against miR-218 and miR-133 (Exiqon) were hybridized overnight at 49°C and 57°C, respectively.

**Institutional Compliance and Animal Care**

All experiments using animals were previously approved by the Institutional Animal Care and Use Committee at University of Texas Southwestern Medical Center.

**Results**

**Expression of miR-218 From Within the Slit2 and Slit3 Genes**

We and others have recently described important roles for intronic miRNAs in the control of cardiovascular development and disease.24,25,29,32 Using a bioinformatics approach, we therefore searched for additional miRNAs within introns of protein-coding genes involved in cardiovascular development and function. We identified miR-218-1 and miR-218-2 within intron 14 of the mouse Slit2 and Slit3 genes, respectively (Figure 1A). The miR-218 stem–loop shares a high level of sequence conservation across species, displaying 100% identity across the mature miRNA from human and mouse to zebrafish and Xenopus (Online Figure I), suggesting this miRNA has an evolutionarily beneficial function.

Northern blot analysis of adult mouse tissues revealed relatively high levels of miR-218 expression in the brain, the

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predominant domain of Slit gene expression. Lower levels of miR-218 were also detected in various endothelial cell (EC)-rich organs and tissues such as the heart and lungs (Figure 1B). miR-218 was detected in the retina and various EC lines (Figure 1C), as previously reported for members of the Slit family. We also found that the expression profile of miR-218 was similar to that of the host genes Slit2 and Slit3, as determined by real-time RT-PCR, although the relative levels of expression sometimes differed (Figure 1D).

Whole mount in situ hybridization was performed on mouse embryos using digoxigenin-labeled antisense oligonucleotides designed to detect miR-218 (Figure 2) or miR-133 as a control for tissue specificity (Online Figure II). This analysis confirmed miR-218 expression in the brain, neural tube, and the eyes (Figure 2A through 2E). At embryonic day (E)9.5, miR-218 displayed robust expression in the dorsal aspects of the midbrain and diencephalon (Figure 2C) and particularly strong expression in the roof plate of the neural tube (Figure 2C). Histological sections of stained embryos further demonstrated specific staining in the roof plate (Figure 2D). Expression of miR-218 was also observed in the embryonic eye (Figure 2B and 2E), persisting in the retinal layer of the eye into the neonatal period (Figure 2F). These expression domains are similar to those of Slit2 and Slit3, which are highly expressed in neural midline structures and the retina. The similar expression and genomic localization of miR-218 and the Slit2 and Slit3 genes strongly suggest that miR-218 is generated by processing of the Slit2/3 pre-mRNAs. The expression levels of miR-218 and the Slit2/3 mRNAs are not identical, however, possibly reflecting variation in processing of the miRNA in different tissues.

**Predicted Targets of miR-218**

To begin to elucidate the potential functions of miR-218, we examined its predicted targets using a bioinformatics approach consisting of algorithms designed to group targets based on functional classification (DIANA-miRPath). Interestingly, the biological pathways that displayed the most significant enrichment in miR-218–targeted mRNAs included proteins involved in axon guidance ($P<10^{-6}$) and HS biosynthesis ($P<4\times10^{-4}$) (Figure 3A). The gene categories predicted to be targets of miR-218 include components of the Slit-Robo pathway, Robo1, Robo2, and SRGAP2 and the HS biosynthetic molecules Hs3st3b1, Hs6st3, and GLCE. We examined the efficiency of interaction with and evolutionary conservation of predicted targets using Targetscan5.0. Most of the predicted targets from within the top 2 most enriched groups contained at least 1 highly conserved 8-mer binding site for miR-218 within the 3’UTR (Figure 3B). Like miR-218, the top predicted targets are conserved from humans to Xenopus (Online Figure III). These results suggested the possibility that miR-218 might link the expression of the Slit genes to posttranscriptional regulation of components of the Slit-Robo signaling axis.
Repression of Robo and HS Biosynthetic Genes
by miR-218

We next conducted reporter assays to determine whether miR-218 can directly inhibit predicted target genes. Plasmids consisting of the 3′UTR of predicted targets linked to a luciferase reporter driven by a constitutive promoter were transfected into COS cells along with increasing amounts of a miR-218 expression plasmid. The activity of the luciferase reporter linked to the Robo1, Robo2, and GLCE 3′UTRs, normalized to β-galactosidase activity, displayed dose-dependent repression by miR-218 (Figure 3C). This effect was dependent on the miR-218 target site, because mutation of this site attenuated repression by miR-218 (Figure 3C).

Repression of endogenous target proteins by administration of 2-O-Me modified oligonucleotides that mimic miR-218 activity was next examined in cultured cells. For these experiments, we used the DLD1 colon cancer cell line, which expresses GLCE but not miR-218. Transfection of miR-218 mimic oligonucleotides resulted in significantly reduced levels of endogenous GLCE protein, as demonstrated by Western blot (Figure 3D and 3E). These results indicate that miR-218 may impinge on Slit-Robo regulated processes such as EC migration and angiogenesis.

miR-218 Affects Endothelial Cell Migration

miR-218 expression was detected to varying degrees in human umbilical vein endothelial cells (HUVECs), MS1 cells, and retinal ECs (Figure 1C and Figure 4A). In addition to Robo1, which has previously been documented in ECs,11 we also detected endogenous expression of GLCE in ECs (data not shown).

Figure 2. Embryonic expression of miR-218. In situ hybridization of E9.5 mouse embryos reveals the expression of miR-218. A, Lateral view reveals miR-218 expression in the eye, midbrain, and hindbrain. B, Rostral view reveals miR-218 expression in the eyes, roof plate of the neural tube, tectum, and commissural plate. C, Ventral view reveals miR-218 expression in the rhombic lip and roof plate of the neural tube. Histological sections of miR-218-stained embryos demonstrating expression in the roof plate (D) and eye (E). Arrows point to staining. Probe trapping is observed in lumen. F, Histological section of the neonatal eye demonstrates miR-218 expression in the retina. cp indicates commissural plate; ey, eye; on, optic nerve; rl, rhombic lip; rp, roof plate; rt, retina; tc, tectum; vt, vitreous.

Figure 3. Identification of predicted miR-218 targets. A, Top 2 most enriched biological pathways containing an over-representation of genes predicted to be targeted by miR-218 using DIANA-lab analysis. B, Components of the axon guidance and HS biosynthesis pathways were analyzed by TargetScan 5.0. Number and quality of sites within the various predicted targets and target function are highlighted. C, COS cells were transfected with a luciferase reporter linked to designated 3′UTRs, increasing amounts of miR-218 expression vector, and 20 ng of lacZ expression vector. Total plasmid amount was kept constant with empty pcDNA3.1 vector, and results were normalized to β-galactosidase activity. Mut designates 3′UTR with a mutation in the seed region of the miR-218 target site. D, Real-time RT-PCR reveals overexpression of miR-218 in DLD1 cells on transfection with 2-O-Me modified miR-218 mimic oligonucleotides. miR-218 expression is normalized to U6. E, Western blot to detect GLCE in DLD1 cells transfected with 2-O-Me modified miR-218 mimic or control oligonucleotide. GAPDH is detected as loading control. Numbers refer to GLCE band intensity, normalized to GAPDH and relative to control.

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These results confirm reports of a role for Slit-Robo signaling in EC function\textsuperscript{10,11} and suggest miR-218 might impinge on this process. To begin to decipher the potential influence of miR-218 on EC function, we used LNA-modified oligonucleotides to knockdown the expression of miR-218 and 2-O\textsuperscript{Me} modified mimic oligonucleotides to overexpress miR-218 in HUVECs and MS1 cells (Figure 4A and data not shown). Because the Slit-Robo pathway modulates EC migration across a scratch “wound,” we examined the effect of altering miR-218 levels on this process. After miR-218 knockdown, we observed an increase in the distance of EC migration in a monolayer scratch wounding assay (Figure 4B). Quantification of the distance of migration revealed a significant increase on miR-218 knockdown in both HUVECs and MS1 cells (Figure 4C and 4D). Conversely, overexpression of miR-218 resulted in a significant reduction of HUVEC migration (Figure 4D). Initial filopodia extension and actin stress fiber formation was not noticeably altered by miR-218 knockdown (Online Figure IV). These observations support our hypothesis that modulation of Slit-Robo signaling by miR-218 affects EC migration. Furthermore, our results suggest that Robo1/2 and GLCE positively influence the migration rate of EC cells in culture and that miR-218 counteracts this process.

miR-218 Contributes to Retinal Angiogenesis

The previous results suggested that miR-218 might influence EC migration during vascular patterning. To further address this potential role, we perturbed miR-218 expression in the retina of neonatal mice. Retinal vascular development is a well-characterized model of angiogenesis that is highly amenable to small interfering RNA–mediated knockdown of gene expression.\textsuperscript{30} Importantly, miR-218 is highly expressed in the retina, as demonstrated by real-time RT-PCR and in situ hybridization (Figure 1C and Figure 2F). We therefore used LNA-modified antisense oligonucleotides to silence the expression of miR-218 during early postnatal development by injection into the subretinal space. Injection was performed 2 days after birth (P2), and retinas were isolated for RNA and protein analyses 3 days later (P5). An additional subset of animals was allowed to develop until P7, and retinas were isolated for immunohistochemistry to examine the developing vascular network (Figure 5A).
Retinal injection of LNA anti–miR-218 resulted in ≈90% reduction in miR-218 levels compared with either noninjected or control LNA oligonucleotide-injected retinas (Figure 5B). LNA miR-218 injection did not inhibit the unrelated miRNA, miR-126, nor did injection of an LNA-modified oligonucleotide directed against miR-126 inhibit miR-218 expression in the retina (Figure 5B). These results demonstrate a high level of efficacy and specificity of LNA oligonucleotides and imply limited potential for off-target effects.

Importantly, the mRNA and protein levels of Robo1, Robo2, and GLCE were enhanced by miR-218 knockdown in the retina (Figure 5C and 5D), although Robo2 mRNA levels were not significantly affected by miR-218 knockdown (data not shown). Quantification of Western band intensity confirmed the significant enrichment of Robo1, Robo2, and GLCE protein levels on silencing of miR-218 (Figure 5F). The predicted miR-218 targets, Hs3st3b1 and Hs6st3, did not change at the mRNA level (Online Figure V), although we did not have an adequate antibody to examine the protein levels. The alterations in Slit-Robo pathway genes was not accompanied by changes in the Notch target Hr1t or the VEGF receptors KDR and Flt1 (Online Figure VI).

To dissect the cell type responsible for Slit-Robo modulation by miR-218, we used a retinal EC line. Transfection of retinal ECs with antisense LNA-modified miR-218 oligonucleotides resulted in elevated levels of Robo1 and GLCE protein levels (Figure 5E and 5F), implying miR-218 expression in ECs contributes to the effect on Slit-Robo pathway gene expression.

The modulation of the Slit-Robo axis by miR-218 in the mammalian retina suggests this regulatory network may constitute a conserved mechanism controlling blood vessel patterning. Indeed, gross examination revealed vascular leakage in LNA anti–miR-218–injected retinas, whereas control injected retinas rarely exhibited hemorrhage (Figure 6A). We therefore harvested LNA anti–miR-218 and control–injected retinas at 5 days postinjection (P7) and examined the vascular network by staining of retinal flat mounts with isolectin (Figure 6B). This experiment revealed that antisense-mediated knockdown of miR-218 levels in the retina during the neonatal period resulted in aberrant patterning and reduced complexity of the retinal vascular plexus (Figure 6B [compare a through c with d through f]). Quantification of vascular density and the thickness of the vascular bed revealed significant attenuation of both in anti–miR-218–injected retinas (Figure 6C and 6D).

High-resolution confocal imaging of the endothelial tip cells at the migratory front revealed relatively normal filopodia formation; however, the number and diameter of capillaries within the vascular plexus appeared to be dramatically diminished following miR-218 knockdown (Figure 6B [compare c with f]). EC protrusions emanated from the stalk cells within the migrating front of the vascular plexus (arrowheads in Figure 6B, f), but there was a reduction in interconnections between
adjacent capillaries, which may explain the lower density and reduced vascular thickness observed in miR-218–deficient retinas. Taken together, our study suggests miR-218 represents a regulatory link between the Slit genes and other components of the Slit-Robo signaling pathway during EC pathfinding.

Discussion

Here, we describe an evolutionarily conserved miRNA located within an intron of the Slit genes, miR-218, which controls the expression of various Slit-Robo pathway components during development. Dysregulation of Slit-Robo signaling by altered miR-218 levels affects EC function in vitro. We also provide evidence that deficiency in miR-218–dependent Slit-Robo regulation results in aberrant vascular patterning in the mouse retina. Taken together, this study identifies miR-218 as an important regulatory hub, linking transcriptional regulation of Slit genes to posttranscriptional regulation of multiple components of the Slit-Robo pathway, which ultimately influence angiogenesis (Figure 7).

These data are consistent with previous reports of intronic miRNA function, in which the miRNA regulates the same biological process as the protein encoded by the host gene. miR-218 may contribute to “fine-tuning” of Slit-Robo pathway genes or generate negative feedback in response to Slit gene activation. It is interesting to speculate that miR-218 may serve to repress the expression of the Robo1/2 receptors in the Slit ligand–expressing cells, thereby spatially separating ligand from receptor. Because Robo4 is not a target of miR-218 regulation, it also is possible that miR-218 affects the ratio of Robo1/2 and Robo4 proteins, thereby influencing vascular patterning.

It is currently debated whether the Robo1 and -2 receptors provide a positive or negative influence on EC migration, although Robo4 is generally thought of as a repulsive or stabilizing cue during vascular pathfinding. In our hands, it appears that repression of Robo1/2 and HSPG biosynthetic molecules by miR-218 negatively affects EC migration. In line with these findings, miR-218 was recently reported to also
inhibit tumor cell migration and metastasis via repression of Robo1.38 The disorganization and reduction of retinal vascular density observed on miR-218 knockdown appears to be a consequence of altered migration or cellular adhesion because we did not detect aberrant proliferation (Online Figure VII).

The second most enriched biological process targeted by miR-218 includes components of the HS biosynthetic pathway. Here, we show that miR-218 directly targets the CS epimerase, GLCE, for degradation during vascular development. miR-218 is also predicted to repress 2-O and 6-O sulfotransferases, both of which are critical enzymes, along with GLCE, in regulating the biological activity of HSPGs.13 HSPGs interact with and regulate the function of many angiogenic growth factors. For example, HSPGs contribute to transport of signaling molecules through the extracellular matrix, formation of growth factor gradients, and stability of ligand–receptor interactions, including Wnt, VEGF, fibroblast growth factor, and Slit-Robo pathways.19,21,39–41 It is conceivable that miR-218–dependent modulation of HSPGs may influence Slit-Robo signaling via multiple mechanisms, including alteration of Slit-Robo affinity or modulating the diffusion radius of Slit from the secreting cell.

Another level of HSPG-dependent regulation involves the localization of HS in the cellular microenvironment. Recent evidence has revealed an important distinction between HSPG presentation to a transmembrane receptor in cis or in trans. For example, HS in trans potentiates VEGF receptor–mediated angiogenesis; it is hypothesized that HS presented by an adjacent cell delays VEGF receptor internalization and increases the efficiency and duration of activity of the bound receptor.14 It is interesting to speculate that miR-218 might influence the HSPG microenvironment, thereby affecting the activity of various growth factor receptors on neighboring cells.

The Slit-Robo signaling pathway was initially identified as a guidance cue controlling the crossing of the midline by axons.42 Multiple studies report the interdependence of vascular and neural patterning during development.1 Although our study reveals a direct role of miR-218 in EC migration in vitro, it remains possible that the effect of miR-218 on retinal angiogenesis is at least partially attributable to an influence on axonal pathfinding. It is also possible that miR-218 contributes to the dynamic regulation of the Robo receptors at the neural midline, directly influencing axonal pathfinding. Although miR-218 is robustly expressed in the brain and eye, it is interesting to note that miR-218 is expressed at much lower levels than the Slit1 and -2 genes in bladder, kidney, and lungs. This suggests the possibility that miR-218 is inefficiently processed in these tissues, which may result in altered miR-218-dependent Slit-Robo activity.

Slit1 and Robo1 were recently identified as part of the transcriptional network activated during the switch to tumor angiogenesis.39 This process appears to be especially sensitive to miRNA control. In addition to the present study, miRNA-126, -378, -296, and the 17-92 cluster have been shown to regulate various aspects of vessel formation, remodeling, and tumor angiogenesis.44 Other physiological processes that might respond to alterations in miR-218 levels and Slit-Robo signaling include oxygen-induced retinopathy, light-induced choroidal angiogenesis, VEGF-induced vascular permeability, and tumor angiogenesis. Further analysis of miR-218 during vascular pathologies and tumor angiogenesis may define miR-218 as an intriguing candidate for therapeutic manipulation in the treatment of vascular diseases.

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Disclosures
E.N.O. holds equity in miRagen Therapeutics, which is developing miRNA-based therapies for muscle disease.

References
Novelty and Significance

What Is Known?
- microRNAs are small noncoding RNAs that inhibit the expression of target mRNAs.
- Individual microRNAs are often predicted to repress large numbers of target mRNAs.
- Control of gene expression by microRNAs influences many physiological processes.

What New Information Does This Article Contribute?
- miR-218 is expressed within an intron of the Slt genes.
- miR-218 regulates the expression of multiple genes that contribute to the Slt-Robo signaling pathway.
- miR-218-dependent regulation of Slt-Robo signaling controls vascular patterning in the retina.

microRNAs are short noncoding RNAs that often inhibit the expression of a large number of mRNA targets via interaction with complimentary sequences in the 3’ untranslated region. It is hypothesized that a common mode of microRNA action involves targeting multiple microRNAs that encode proteins contributing to the same biological process. Here, we describe the repression of multiple components of the Slt-Robo signaling pathway by miR-218, which is encoded by an intron of the Slt genes. Furthermore, we demonstrate that the miR-218–Slt-Robo signaling pathway is necessary for normal patterning of the retinal vasculature in mice. Taken together, our study describes the coordinated function of a microRNA and its host gene and advances the understanding of how microRNAs control biological processes by modest inhibition of an organized array of target mRNAs.
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SUPPLEMENTAL MATERIAL

Detailed Methods

Northern Blot
Ten µg of total RNA was loaded onto a denaturing 20% polyacrylamide gel and transferred to a Zetaprobe GT membrane (Bio-Rad), crosslinked by UV irradiation, and baked at 85°C. Blots were hybridized overnight at 39°C with 32P-labeled antisense STARFIRE probes directed against the mature sequence of miR-218 (Integrated DNA Technologies). U6 RNA levels were detected as loading control.

Cell culture and transfection
Retinal endothelial cells (RECs) and human umbilical vein endothelial cells (HUVECs) were cultured in EGM2 medium (Lonza) supplemented with penicillin/streptomycin at 31°C and 37°C, respectively. DLD1 cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin.

A scratch wound was produced 48 hrs after transfection with a P200 pipet tip. Images of the wound were taken 12 hrs after scratching. Scratch width was quantified using NIH ImageJ software.

COS cells were transfected using Fugene 6.0 (Roche) according to manufacturer’s protocol. For 3’UTR target assays, 20ng of pMIR-Report luciferase vector was transfected with increasing amounts of CMV6-miR-218 expression vector. Empty CMV6 expression vector was used as control and to keep total plasmid DNA amounts constant.

Primer sequences
Robo1 3’UTR: For 5’-GAGCTCAAGACAAACCAGAGGAGGCTTAC-3’;
Rev 5’-AAGCTTGGTCTCCCTAGAAGTGCACAT-3’;
Robo2 3’UTR: For 5’-GAGCTCCTGATATGTCTACGAGACTCC-3’;
Rev 5’-AAGCTTCTAGAAGTGCACATGAGCCT-3’;
GLCE 3’UTR: For 5’-GAGCTCAAGAGCCCCAAAATGTCACATT-3’;
Rev 5’-AAGCTTCAAGAGGACAGCAAGGACAGA-3’.

mutRobo1 : 5’-CTGTTTCTAAATCTTTTTTTTTTGGTTTTAAATCAGAATCAGTAAACTTTATTTG-3’;
mutGLCE: 5’-CGTGCAGGTTACGCGACATACATAATCAGAACTGAAATG-3’;

Analysis of retinal angiogenesis
1 µl of a 5 mg/ml solution of LNA modified anti-miR-218 or universal control oligonucleotides was unilaterally injected in the subretinal space of postnatal day (P) 2 mice in the ICR background. We found that LNA modified oligonucleotides did not require electroporation for efficient uptake into the retina and knockdown of gene expression. Mice were allowed to develop for 3 days prior to RNA and protein isolation at P5, and 5 days prior to isolation of retinas at P7 for histological analysis. Visualization
of retinal vasculature was performed as previously described. Briefly, retinas dissected from injected mice were fixed in 4% paraformaldehyde (PFA) for 1hr, permeabilized, and stained with 40 µg/ml FITC conjugated isolectin from Bandeiraea simplicifolia (Sigma-Aldrich) at 4°C overnight. Retinas were then washed five times with PBS and post fixed with 4% PFA prior to flatmounting. Quantification of vessel density was performed on 20-25 random fields at the vascular migration front from 7 control and 5 anti miR-218 injected retinas using NIH ImageJ software. The thickness of the vascular plexus was calculated from z-stacks of images taken at the vascular migration front of 6 control and 6 anti miR-218 injected retinas. The radial length of the vascular network was calculated by measuring the distance from the optic disc to the periphery of the vascular plexus in four quadrants of 12 control and 13 anti miR-218 injected retinas. Student’s t-tests were used to determine statistical significance between groups.

**Supplemental Figure and Figure Legends**
Online Figure I

Evolutionary conservation of miR-218-1 within Intron 14 of the Slit2 (A) and miR-218-2 within intron 14 of the Slit3 gene (B). Sequence comparison of the pre-miR-218 is shown at bottom of image with mature miR-218 sequence highlighted in blue revealing 100% identity from human to zebrafish.
Online Figure II

In situ hybridization of E9.5 mouse embryos reveals specific expression of miR-133 in the heart and somites (A). Histological sections of the embryo shown in (A) demonstrating heart (B) and somite (C) expression. Atrium (at) heart (ht), outflow tract (ot), somite (sm), ventricle (vt).
Online Figure III

Evolutionary conservation of miR-218 target sites within the 3'UTRs of the Robo1 (A), Robo2 (B), and GLCE (C) genes. Human (Hs), Mouse (Mm), Rat (Rn), Opossum (Md), Chicken (Gg), Xenopus (Xt).
Online Figure IV.

Phalloidin staining of the actin cytoskeleton following transfection of control or anti miR-218 oligonucleotides in HUVECs demonstrates stress fiber formation and lamellapodial extension.
Online Figure V

Retinal expression of heparan sulfotransferases after miR-218 knockdown. Real time RT-PCR on pools of 3 control and 3 anti-miR-218 injected retinas.
Online Figure VI

Real time RT-PCR demonstrates expression of VEGF and Notch pathway genes. The VEGF receptors, KDR and Flt1, and Hrt1 do not display significant alterations upon miR-218 knockdown in the retina.
Online Figure VII.

Normal proliferation in anti miR-218 injected retinas. Phospho-histone H3 / isolectin staining demonstrate proliferating cells and the retinal vasculature 5 days after anti miR-218 injection (P7).