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miR-31 functions as a negative regulator of lymphatic vascular lineage-specific differentiation \textit{in vitro} and vascular development \textit{in vivo}

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Abstract

The lymphatic vascular system maintains tissue fluid homeostasis, helps mediate afferent immune responses and promotes cancer metastasis. To address the role microRNAs (miRNAs) play in the development and function of lymphatic vascular system, we defined the in vitro miRNA expression profiles of primary human lymphatic endothelial cells (LECs) and blood vascular endothelial cells (BVECs) and identified 4 BVEC-signature and 2 LEC-signature miRNAs. Their vascular lineage-specific expression patterns were confirmed in vivo by quantitative real-time PCR (qRT-PCR) and in situ hybridization (ISH). Functional characterization of the BVEC-signature miRNA, miR-31, identified a novel BVEC-specific post-transcriptional regulatory mechanism that inhibits the expression of lymphatic-specific transcripts in vitro. We demonstrate that suppression of lymphatic differentiation is partially mediated via direct repression of PROX1, a transcription factor that functions as a master regulator of lymphatic lineage-specific differentiation. Finally, in vivo studies in Xenopus and zebrafish demonstrated that gain-of-miR-31 function impaired venous sprouting and lymphatic vascular development. Thus, highlighting the importance of miR-31 as a negative regulator of lymphatic development. Collectively, our findings identify miR-31 is a potent regulator of vascular lineage-specific differentiation and development in vertebrates.

Key words: miRNA post-transcriptional regulation, vascular development, lymphangiogenesis, PROX1, miR-31
Introduction

Vertebrates have developed two parallel, but structurally and functionally distinct, vascular systems: the blood and lymphatic vascular systems (1, 7). The lymphatic vascular system controls tissue fluid homeostasis, absorbs lipids and fat-soluble vitamins from the intestine, and mediates afferent immune responses by transporting lymphocytes and antigen-presenting cells to regional lymph nodes (1, 7). In addition, malignant cancers can induce lymphatic vessel activation and growth (lymphangiogenesis) within primary tumors and draining lymph nodes, which enhances cancer metastasis to draining lymph nodes and beyond (1, 22). These findings have fueled a surge of studies aimed at defining the molecular characteristics and functional activities of lymphatic vessels, and identifying molecules that regulate lymphangiogenesis.

Genomic and proteomic studies have identified novel molecular markers and growth factors for lymphatic vessels (2, 23, 48, 52). Genetic mouse models have characterized the transcription factors PROX1 and SOX18 as master regulators of lymphatic vascular development and differentiation in vivo (12, 56, 65). These studies indicate that SOX18 expression in a subset of cardinal vein endothelial cells initiates lymphatic vascular development by inducing PROX1 expression (12). The resulting lymphatic vascular progenitor cells bud off and migrate away from the cardinal vein and form primitive lymph sacs, which subsequently develop into functional lymphatics (12, 56). PROX1 and SOX18 expression in cultured BVECs triggers these cells to adopt lymphatic-specific molecular and phenotypic characteristics (12, 26, 48). Conversely, PROX1 knockdown in LECs inhibits the expression of LEC-signature genes and triggers BVEC-signature gene expression (44 and Shin et al., manuscript in preparation). Despite these advances, a detailed understanding of the mechanisms controlling lymphatic vascular development and cell type-specific differentiation remains elusive.

A potentially crucial aspect of lymphatic vascular biology has remained unexplored to date: the role of miRNA-guided post-transcriptional regulation. miRNAs are genomically encoded, 19-24 nucleotide non-coding RNAs that regulate the flow of genetic information by
limiting protein synthesis (10). This regulation is brought about when mature miRNAs, loaded in the RNA-induced silencing complex (RISC), base-pair with semi-complementary sites within the 3’ untranslated region (3’UTR) of target mRNAs. Once base-paired with its target, the miRNA represses translation and/or induces mRNA degradation (10). Consequently, miRNAs act as novel and potent regulators of the genome. This notion is underscored by recent studies defining critical roles for miRNAs in embryonic development, cell proliferation, cell cycle progression, differentiation, and apoptosis, as well as their contribution to the etiology of several diseases (10, 46, 68).

Interestingly, functional roles for miRNAs in blood vascular development have recently been defined. Down-regulation of the miRNA processing enzymes Dicer and Drosha have been reported to impair angiogenesis (11, 59). Moreover, a few miRNAs have been shown to affect human umbilical vein endothelial cell (HUVEC) migration and proliferation in vitro, regulate nitric oxide synthase expression, promote tumor angiogenesis, control vascular inflammation, and directly contribute to numerous vascular phenotypes (11, 59).

In the study presented here, we identified and addressed the functional relevance of vascular lineage-specific miRNAs. We first defined the miRNA expression profiles of primary human LECs and BVECs and, consequently, identified 4 BVEC- and 2 LEC-signature miRNAs. Their vascular lineage-specific expression was confirmed in normal tissues by qRT-PCR analysis of ex vivo isolated murine LECs and BVECs and by ISH. Interestingly, our findings have further classified the widely-expressed (38), metastasis-associated (63, 64) miRNA, miR-31, as a BVEC-signature miRNA. In vitro functional analysis of miR-31 demonstrated that this miRNA inhibits lymphatic lineage-specific differentiation in BVECs by repressing lymphatic-specific transcript levels. These effects are, in part, due to direct post-transcriptional repression of Prox1, a master regulator of lymphatic development. Finally, in vivo gain-of-function studies in Xenopus and zebrafish embryos established that overexpression of miR-31 impaired lymphatic development and reduced venous sprouting. Taken together, these findings indicate that miR-31 plays a pivotal role in regulating lineage-specific differentiation within the developing vasculature of vertebrates.
Materials and Methods

Cell Culture

Primary human dermal microvascular LECs and BVECs were isolated from neonatal human foreskins and cultured as previously described(23). cLECs and HUVECs were purchased from Cambrex (Verviers, Belgium). Human IMR91 dermal fibroblasts (hdFBs) were obtained from the National Institute on Aging, Bethesda, MD, USA. The immortalized human epidermal keratinocyte line HaCaT was provided by Dr. Norbert Fusenig, German Cancer Research Center, Heidelberg, Germany(4). Cells, except hdFBs, were propagated in supplemented endothelial cell basal medium (EBM; Cambrex) as described(23). hdFBs were propagated in D-MEM (Dulbecco’s modified eagle medium) supplemented as described above and transferred 12 hrs prior to total RNA isolation to EBM supplemented as described above. Primary cells were used at passage 6.

In vitro miRNA expression profiling

The TaqMan microRNA Assays Human Panel Early Access Kit (Applied Biosystems, Foster City, CA), containing 157 individual human TaqMan microRNA assays, was used for qRT-PCR miRNA expression profiling(5). Total RNA was isolated from biological replicates of 80-90% confluent 10-cm tissue culture dishes using the mirVana miRNA isolation kit (Ambion, Austin, TX). Reverse transcription reactions were performed using 2 ng of total RNA and the microRNA Reverse Transcription kit (Applied Biosystems). miRNA expression levels of technical duplicates were determined using a 7900HT Fast Real-Time PCR System (Applied Biosystems) and comparative threshold cycle (Ct) values acquired after 40 cycles using SDS 2.2 software (Applied Biosystems). TaqMan microRNA assays (Applied Biosystems) for hsa-miR-31, hsa-miR-137, hsa-miR-99a, hsa-miR-125b, hsa-miR-95, hsa-miR-326 and human RNU48 were used to confirm lineage-specific expression. For analysis, detection thresholds were set to 0.04 units of fluorescent intensity and when a miRNA Ct value was undetermined in both technical replicates, Ct values of 41 were assigned. Datasets were normalized relative to let-7a and miR-16 using the formula:
AveCt\text{\text{NORM}}=\text{AveCt}_{\text{miRNA}}-(\text{AveCt}_{\text{let-7a/miR-16}}-24), where AveCt_{\text{let-7a/miR-16}} is the combined average Ct value for let-7a and miR-16 from each 96-well plate. RNU48 or sno234 were used to normalize the individual TaqMan microRNA assay datasets using the formula: AveCt\text{\text{NORM}}=\text{AveCt}_{\text{miRNA}}-(\text{AveCt}_{\text{RNU48 or sno234}}-25), where AveCt_{\text{RNU48 or sno234}} is the mean RNU48 or sno234 Ct value (n=3). Relative abundances for LECs and BVECs were calculated from log2 ratios. P-values were calculated using a two-tailed Student’s t-test.

**FACS isolation of endothelial cells from mouse colons**

Animal experiments in mice were approved by the Kantonales Veterinäramt Zürich. Colons were excised from sacrificed female FVB mice (Charles River, Sulzbach, Germany; 12-16 weeks old; n=8), opened longitudinally, washed in cold PBS and placed in 1 mM DTT. Mucus was gently removed by scraping. Small tissue pieces were digested with 8 mg/ml collagenase IV (Invitrogen, Carlsbad, CA), 0.5 mg/ml DNaseI (Roche, Rotkreuz, Switzerland) and 5 mM CaCl$_2$ in PBS at 37°C for 15 min. After passing through a 70 µm cell strainer (BD Biosciences, Franklin Lakes, NJ), the resulting cell suspensions were centrifuged at 500 g for 10 min and resuspended in 2% FBS-supplemented PBS, containing 1 mM EDTA.

Antibodies used for FACS sorting were: allophycocyanin (APC)-conjugated rat anti-mouse CD31 (BD Biosciences Pharmingen, San Diego, CA), fluorescein isothiocyanate (FITC)–conjugated rat anti-mouse CD45.2 (BD Biosciences), hamster anti-mouse podoplanin (clone 8.1.1; Developmental Studies Hybridoma Bank, Iowa City, IA), anti-hamster phycoerythrin (PE) (CALTAG/Invitrogen) and isotype control antibodies. FACS sorting was performed using a FACS aria and the FACSDiva software (BD Biosciences). Cells were lysed by sorting directly into RLT Plus lysis buffer (Qiagen, Hilden, Germany) containing β-mercaptoethanol. Total RNA was extracted from LECs (CD45$^-$/CD31$^+$podoplanin$^-$) and BVECs (CD45$^-$/CD31$^+$podoplanin$^+$), using the RNeasy Plus Micro kit (Qiagen, Hilden, Germany). For miRNA expression analyses, 6 ng of total RNA and TaqMan miRNA assays for mmu-miR-31, mmu-miR-326, hsa-miR-137, hsa-miR-99a, hsa-miR-125b and mouse sno234 were used.
**In Situ hybridization and immunofluorescence staining**

miR-31 *in situ* hybridization and Lyve-1/CD31 immunofluorescence were performed on 20 µm serial frozen sections of mouse colons obtained from female FVB mice. *In situ* hybridization for mouse miR-31 was performed using digoxigenin (DIG)-labeled locked nucleic acid (LNA)-modified detection probes (mmu-miR-31, product # 39153-00; hsa/mmu/rno-U6, positive control, # 99002-00); sense miR-159, negative control, # 99003-00; Exiqon, Vedbaek, Denmark) and the formaldehyde/EDC fixation miRNA ISH protocol (47). Briefly, the LNA-modified detection probes were labeled with Digoxigenin (DIG) using the DIG Oligonucleotide Tailing Kit (Roche, Basel, Switzerland) according to the manufacturer’s instructions. Tissue sections were fixed in 4% formaldehyde/Tris buffered saline (TBS) for 10 min and then in EDC solution (47) for 1.5 hours. The sections were acetylated in 1% triethanolamine/0.25% acetic anhydride, washed and prehybridized in hybridization buffer (47) for 1 hour at 53°C. The colon tissue sections were hybridized with 4µM DIG-labeled detection probes overnight at 56°C for miR-31 and 53°C for the controls. Following post-hybridization washing and blocking, the slides were probed with alkaline phosphatase conjugated anti-DIG Fab fragments (Roche). They were then washed in TNT buffer (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Tween 20) and in AP Buffer (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl2). Color developed was performed in Developer solution (AP Buffer with 0.175 mg/mL BCIP, 0.45 mg/ml NBT and 2mM levamisool). All incubations and washing steps were performed at room temperature unless otherwise indicated.

Immunofluorescence stains were performed as described (25, 35) using a rabbit polyclonal antibody against mouse Lyve-1 (AngioBio, Del Mar, CA), a monoclonal rat antibody against mouse CD31 (BD Biosciences) and corresponding secondary antibodies labeled with AlexaFluor488 or AlexaFluor594 (Molecular Probes). Sections were examined on an Axioskop2 microscope (Carl Zeiss, Feldbach, Switzerland), and images were captured at 2.5x (Plan-NEOFLUAR 2.5x/0.075 NA) and 20x (Plan-NEOFLUAR 20x/0.50 Ph2)
Magnification with an AxioCam MRm digital camera (Zeiss). Brightfield and fluorescent channel image acquisition was accomplished using Axio Vision 4.4 software (Zeiss). Adobe Photoshop CS3 (Adobe Systems, San Jose, CA) was used to adjust image brightness.

**Microarray analyses**

All transfections were carried out using the Basic Nucleofector Kit for primary mammalian endothelial cells (Amaxa AG, Cologne, Germany). 500,000 LECs were transfected with 2 µM Pre-miR-31 or Pre-miR-Neg molecules (30) in biological duplicate and total RNA isolated using the mirVana isolation kit 48 hours post-transfection. The transcriptome profiles of these cells were defined using the Applied Biosystems Human Genome Survey Microarray v2.0 as described (54). Briefly, dioxigenin-UTP-labeled cRNA was generated from 1.5 µg of total RNA using the NanoAmp RT-IVT Labeling Kit (Applied Biosystems). 20 µg cRNA were fragmented and hybridized to the microarrays using the Applied Biosystems Chemiluminescence Detection Kit. Signal detection, image acquisition and initial analyses were performed using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer. Microarray data are accessible at http://www.ncbi.nlm.nih.gov/geo (GSE16908).

Raw data were normalized using Quantile normalization available from R/Bioconductor (14). Present calls were defined based on average signal-to-noise ratios (S/N ratio) >3 and quality (error) values <5,000 (54). Feature signal intensities were converted to \( \log_2 \) values. miR-31-repressed genes were identified based on present calls in both Pre-miR-Neg arrays, \( \log_2(\text{Pre31}/\text{PreNeg}) \leq -0.59 \) and p-values <0.05, while miR-31-induced genes were present in both Pre-miR-31 arrays, had \( \log_2(\text{Pre31}/\text{PreNeg}) \geq 0.59 \) and p-values <0.05. P-values were calculated using empirical Bayes statistics for differential expression (55).

**mRNA quantitative RT-PCR analyses**

To confirm the microarray data, the mRNA expression levels of selected candidate miR-31-regulated LEC- and BVEC-signature genes were analyzed in triplicate by
quantitative real-time RT-PCR (qRT-PCR) using dual-labeled TaqMan Gene Expression Assays for TIMP3 (Assay ID: Hs00165951_g1), PPP1R9A (Hs01044146_m1), HOXD10 (Hs00157974_m1), EDNRB (Hs00240752_m1), PROX1 (Hs00160463_m1), NRCAM (Hs00170554_m1), SELE (Hs00950401_m1), ICAM1 (Hs99999152_m1), MMP1 (Hs00899658_m1), RGS4 (Hs00194501_m1), NRG1 (Hs00247620_m1) and LOC554202 (Hs01007340_m1; all from Applied Biosystems). The probe and primers for LYVE-1 were as described(23). 25 ng of cDNA, generated using the High Capacity cDNA Archive Kit (Applied Biosystems) were used. Each reaction was normalized to β-actin expression(54).

**Detailed analysis of PROX1 mRNA and protein levels**

To further characterized miR-31 regulation of PROX1, 500,000 LECs were transfected with 2 µM or 4 µM Pre-miR-31 (n=4) or Pre-miR-Neg (n=4) molecules, or 4 µM Anti-miR-31 (n=2) or Anti-miR-Neg (n=2) molecules(6). Total RNA and whole cell protein lysates were isolated using the mirVana PARIS kit 48 hours post-transfection. qRT-PCR analysis of PROX1 mRNA was preformed as described in the main text.

Northern blot analyses were performed on 1 µg total RNA. PROX1 mRNA was detected using purified PROX1 3’UTR γ32ATP end-labeled probes generated from a NotI-linearized human PROX1 3’UTR plasmid (YH1551; provided by Dr. Young Kwon Hong, University of Southern California, Los Angeles, CA). The membrane was then stripped and β-actin detected using γ32ATP end-labeled human β-actin oligonucleotides (5’-GTGAGGATCTTCATGAGGTAGTCAGTCAGGT-3’).

For western blotting, 25 µg of the protein lysates were resolved by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were probed with rabbit polyclonal anti-human PROX1 (RELIATech, Braunschweig, Germany) and mouse monoclonal anti-human β-actin antibodies (Sigma-Aldrich), detected using horseradish peroxidase (HRP)-conjugated secondary antibodies and standard chemiluminescence(54). QuantityOne software (Bio-Rad) was used to semi-quantitatively analyze the amounts of PROX1 protein relative to β-actin in
each sample. Pre-miR-31 and Pre-miR-Neg PROX1 signal volume averages and standard deviations of the mean were calculated from these normalized values (n=4). P-values were calculated using a two-tailed Student’s t-test.

**DNA Constructs**

pMIR-Luci/miR31BS and psiCHECK-2/miR31BS contain perfect match hsa-miR-31 binding sites (miR31BS). pMIR-Luci/miR31BS was generated by cloning annealed miR-31BS sense (5’– AGCTTGCTGAGCGGCAAGATGCTGGCATAGCTGA–3’) and antisense (5’– CTAGTCAGCTATGCCAGCATCTTGCCGCTCAGCA–3’) oligonucleotides as into the HindIII and SpeI restriction sites of the pMIR-REPORT Luciferase vector (Cheng et al., 2005). psiCHECK-2/miR31BS was constructed by ligating annealed miR31BS/F and miR31BS/R oligonucleotides (Supplementary Table 1) into the XhoI and NotI sites of psiCHECK-2 (Promega, Dübendorf, Switzerland). PROX1 3’UTR and CDS luciferase reporter vectors (Supplementary Table 1) were constructed by PCR amplification from YH1551 using the oligonucleotides listed in Supplementary Table 1. PROX1 3’UTR amplicons were ligated into the XhoI and NotI sites of psiCHECK-2 and the PROX1-CDS amplicon into the PmeI and NotI sites of psiCHECK-2.

**Luciferase Reporter Assays**

For miR-31 overexpression optimization, 500,000 LECs were co-transfected with 0.7 μg pMIR-Luci/miR31BS, 0.7 μg pMIR-REPORT β-Galactosidase (β-gal) control and 0.02 μM, 0.2 μM, 1 μM, 2 μM or 4 μM human miR-31 precursor (Pre-miR-31) or Pre-miR-Neg negative control molecules (Applied Biosystems). Luciferase and β-gal activities were monitored 48 hours after transfection using the Dual-Light Luciferase and β-gal Reporter Gene Assay System (Applied Biosystems). β-gal RLU (Relative Light Units) were used to normalize luciferase RLU.

For the PROXI 3’UTR tethering and PROXI 3’UTR miR-31 binding site mutagenesis assays, 500,000 LEC or HUVECs were co-transfected in triplicate with 4 μM of
miR-31 precursor or inhibitor (6) or the corresponding negative controls and 0.7 µg of the psiCHECK-2 constructs containing the miR-31 binding site (miR31BS), the PROX1 3’UTR fragments (PROX1 FL-F6), the PROX1 coding sequence (PROX1 CDS), or the PROX1 3’UTR miR-31 binding site mutants (PROX1 FLmut or PROX1 F6mut) (Supplementary Table 1). Firefly and Renilla luciferase activities were monitored 48 hours after transfection using the Dual-Luciferase Reporter Assay System (Promega). PROX1 3’UTR/CDS-Renilla luciferase RLUs were normalized to firefly luciferase RLUs. Both the Dual-light and Dual-luciferase assays were performed in triplicate with 20 µL of cell lysate.

**Xenopus microinjection and whole-mount in situ hybridization**

Xenopus studies were conducted under protocols approved by the Veterinary Office of the Canton of Zürich, Switzerland. Xenopus laevis eggs were obtained by hormone-induced laying, fertilized *in vitro* and prepared for microinjection as previously described (21). 2-cell stage embryos were unilaterally microinjected with Pre-miR-31 or Pre-miR-Neg molecules (10-100 ng/blastomere) and 0.2 ng β-galactosidase RNA (lineage tracer). The antisense VEGFC MO (5’-GTAACGCTCCCTCCAGCAAGTACAT-3’) was purchased from Gene Tools (Philomath, OR) and 5 to 10 ng were unilaterally injected into two-cell-stage embryos. When uninjected embryos reached the embryonic stage indicated, the injected embryos were fixed and processed for *in situ* hybridization. Whole-mount *in situ* hybridization, β-galactosidase staining, and bleaching of *Xenopus* embryos were carried out as previously described (29). Digoxigenin-labeled probes were transcribed from linearized plasmids encoding *Xenopus* pecam1 (29), prox1 (GenBank Acc. No. BU903551), and vegfr3 (Kalin et al. 2009). Images were acquired digitally using AxioVision 4.5 (Zeiss) software and an AxioCam color camera (Zeiss) mounted on a Zeiss Stereo Lumar V12 stereoscopic microscope.

**Zebrafish microinjection**

Transgenic *TG(fli1a:gfp)y1* (37) and *plcg1* t26480 zebrafish lines were maintained in the
Hubrecht Institute. Zebrafish experiments were approved by the Animal Experimentation Committee (DEC) of the Royal Netherlands Academy of Arts and Sciences. The \( plcg1^{t26480} \) allele encodes a W1024Stop mutation in the \( plcg1 \) gene (GenBank accession number AY163168).

Morpholino oligomers were ordered from Gene Tools (Philomath, OR). One cell stage \( TG(fli1a:gfp)^{y1}:plcg1^{t26480} \) mutant embryos were injected with 40 ng/embryo of MOs targeting dre-miR-31 (5’-TTAACAGCTATGCCAACATCTTGCC-3’), or 25 ng/embryo of an unrelated control MO (5’-GCATTGACTCTGTAAAACAGACAAT-3’). For miR-31 overexpression, \( TG(fli1a:gfp)^{y1}:plcg1^{t26480} \) mutant embryos were injected with 1 nl of 25 \( \mu \)M human Pre-miR-31 precursor or Pre-miR-Neg control molecules (Applied Biosystems). Venous sprouts were quantified at 48 hpf and statistical significance analyzed using the Student’s t-test. For imaging, embryos were mounted in 0.8% low melting point agarose in a dish with a cover slip replacing the bottom. Imaging was performed with a Leica SP2 confocal microscope (Leica Microsystems) using a 20x objective.
Results

BVEC versus LEC lineage-specific miRNA expression

Using a TaqMan-based qRT-PCR profiling platform(5) we defined the in vitro expression profiles of 157 human miRNAs in primary LECs and BVECs, as well as two non-endothelial cell types (HaCaT keratinocytes and dermal fibroblasts (hDFBs)) (Supplementary Table 2). Following data normalization, expression profiles for each cell-type were defined by setting the Ct value present call cut-off at 34 (Supplementary Table 2). Most of the miRNAs analyzed were expressed at comparable levels in both LECs and BVECs (Supplementary Figure 1). Nevertheless, based on a two-fold or greater differential expression, 16 candidate LEC- and 30 candidate BVEC-signature miRNAs were identified (Table 1). Using a p-value cut-off of ≤0.05, 2 LEC-signature miRNAs (miR-95 and miR-326) and 4 BVEC-signature miRNAs (miR-137, miR-31, miR-125b and miR-99a) were identified (Table 1). Individual TaqMan miRNA assays confirmed the vascular lineage specificity of these miRNAs. miR-95 and miR-326 were, on average, 46-fold and 7-fold higher in LECs than in BVECs, respectively (Figure 1A). Conversely, miR-137 expression was 124-fold higher in BVECs than in LECs, miR-31 was 48-fold higher in BVECs, and miR-125b and miR99a were 3-fold more abundant in BVECs (Figure 1B).

LEC- and BVEC-signature miRNA expression patterns are maintained in vivo

We next isolated BVECs and LECs from the colons of 8 adult mice by FACS sorting, using the leukocyte marker CD45, the pan-endothelial marker CD31 and the LEC marker podoplanin to differentiate between leukocytes (CD45⁺CD31⁻podoplanin⁻ and CD45⁻CD31⁺podoplanin⁺), BVECs (CD45⁺CD31⁻podoplanin⁻) and LECs (CD45⁻CD31⁺podoplanin⁺)(18). We obtained 1500 to 25000 LECs and 2500 to 55000 BVECs, from which total RNA was extracted and used for ex vivo qRT-PCR miRNA profiling. Based on a 1.5-fold or greater differential expression, miR-31 was indeed more strongly expressed by BVECs than LECs in 6 of 8 mice analyzed (Figure 2A). The degrees of miR-31 differential
expression between mouse BVECs and LECs \textit{in vivo} were less pronounced than those observed between \textit{in vitro} cultured human endothelial cells. Nevertheless, statistical analysis of these \textit{in vivo} data confirmed that the differences in miR-31 expression between mouse BVECs and LECs were statistically significant in 6 out of the 8 mice studied (Figure 2A). The LEC-signature expression pattern of miR-326 and the BVEC-signature classifications of miR-125b and miR-99a were also confirmed, while no major changes were found for miR-137 (Supplementary Figure 2). miR-95 is not present in mice.

Low magnification (2.5x) microscopic analysis of adult mouse colon tissue sections probed for miR-31 expression by ISH revealed strong miR-31 staining throughout the adult mouse colon (Figure 2B and D). Importantly, immunofluorescence staining of serial sections for the panvascular marker CD31 and the lymphatic marker LYVE-1 revealed that miR-31 preferentially co-localized with blood vessels (CD31+LYVE1-) present in the submucosa and mesenteric attachments of the colon, as well as the lamina propria (Figure 2B and D, arrows and arrowheads). In contrast, miR-31 expression was weak or absent in lymphatic vessels (Figure 2B and D, asterisks). Independent CD31 and LYVE1 immunofluorescence staining and negative control (sense-miR-159) \textit{ISH} of serial sections of adult colon tissues (Figure 2H and I), coupled with high magnification (20x) image analysis of the miR-31 \textit{ISH} and the CD31/LYVE1 immunofluorescently labeled serial sections, demonstrated no preferential staining of either the blood and lymphatic vessels in the sense-miR-159 probed sections (Figure 2H and I). Both vessel types developed equivalent, background level signals following ISH processing. By contrast, miR-31 molecules were preferentially associated with colonic blood vessels (CD31+LYVE1--; arrows), while the lymphatic vessels (CD31+LYVE1+) developed no miR-31 signals (asterisks) (Figure 2F and G). Taken together, these \textit{in vitro} and \textit{in vivo} data demonstrate that miR-31 transcripts are rare or absent in LECs, but enriched in BVEC.

\textbf{miR-31 gene synteny is evolutionarily conserved}
miR-31 is encoded within intron 1 of an uncharacterized gene – LOC554202 (GeneID: 554202). This led us to question if miR-31 lineage-specificity results from preferential expression of LOC554202 in BVECs. Indeed, TaqMan-based qRT-PCR analysis demonstrated that LOC554202 transcripts were 54-fold more abundant in BVECs compared to LECs (ΔΔCt=5.78; Supplementary Figure 3A), which is comparable to the degree of differential expression defined for miR-31 in BVECs compared to LECs (ΔΔCt=5.58). Interestingly, further genome and proteome bioinformatic analyses revealed that the gene is not conserved in vertebrates and the putative gene product has no homology to proteins of known function. Thus, LOC554202 might primarily function as a conduit for miR-31 post-transcriptional regulatory activities in BVECs. Sequence alignments of human LOC554202 with 23 eutherian mammals EPO (Enredo Pecan Ortheus) using Ensembl alignment tools (http://www.ensembl.org/index.html) revealed conserved synteny for miR-31 with 6 of the species queried. Moreover, 13 of the eutherian mammals queried are predicted to encode novel miRNA genes at positions aligned with LOC554202 (data not shown). Further alignment analysis of these 20 miRNA genes demonstrated that these 13 novel miRNA genes are, in fact, miR-31 orthologs (Supplementary Figure 3B). The highly conserved synteny suggests that the genomic, transcriptional, and epigenetic factors regulating miR-31 expression have remained conserved during mammalian evolution.

Identification of LEC- and BVEC-signature genes regulated by miR-31

The strong differential expression of miR-31 in vitro and in vivo prompted us to further characterize this BVEC-signature miRNA by defining the transcriptome profile changes in LECs after miR-31 overexpression. Using a luciferase reporter construct containing a miR-31 binding site (pMIR-Luci/miR31BS) and qRT-PCR, we found that high levels of miR-31 gain-of-function could be achieved when LECs were transfected with 2 µM Pre-miR-31 precursor(30) (Supplementary Figure 4). Using ≥1.5-fold differential expression and ≤0.05 p-value thresholds, gene microarray analyses of 2 µM Pre-miR-31 or Pre-miR-Neg transfected LECs identified 548 miR-31-repressed and 335 miR-31-induced genes.
(Supplementary Tables 3 and 4). Comparing the miR-31 targets predicted by TargetScan(17, 39), miRanda(27), miRBase(16) and PicTar(31), 7.2% of the miR-31-repressed genes were predicted targets of miR-31.

*In silico* biological process analysis, using the Panther Classification System(61, 62), was used to assess whether the observed miR-31-mediated reprogramming of LECs specifically affected lymphatic, blood vascular and/or endothelial biological functions. Intriguingly, genes involved in cell communication, signal transduction, cell adhesion, apoptosis and numerous signaling pathways were overrepresented among the miR-31-repressed genes compared to the expected number of genes (Supplementary Table 5). While these biological processes are common to both endothelial cell types, this enrichment suggests a dramatic re-organization of the LECs cell surface characteristics, as well as cell signaling network activity, following miR-31 overexpression. The specific effects miR-31 overexpression had on genes involved in vascular lineage-specific differentiation were then identified by comparing the miR-31-regulated genes to the LEC- (344 genes) and BVEC-signature genes (479 genes) previously identified in vitro (23, 32, 48 and Shin et al., submitted for review). Interestingly, twice as many transcripts encoding LEC-signature molecules (9.6%) than BVEC-signature molecules (4.8%) were reduced following miR-31 overexpression (Figure 3A and B, and Supplementary Table 3). Also, approximately 4 times more BVEC-signature genes (4.6%) were induced/stabilized following miR-31 overexpression compared to LEC-signature genes (1.1%) (Figure 3A and B, and Supplementary Table 4). Corroborating the microarray data, qRT-PCR experiments confirmed that four LEC-signature genes (EDNRB, PROX1, PPP1R9A, HOXD10) and two BVEC-signature genes (ICAM1, SELE) tested were significantly less abundant in the Pre-miR-31 samples compared to Pre-miR-Neg control (Figure 3C and D). Furthermore, we also validated the up-regulation of the miR-31-induced BVEC-signature genes MMP1 and RGS4 (Figure 3E).
miR-31 inhibits PROX1 protein translation

Amongst the miR-31-repressed target genes was PROX1, an essential lymphatic-specific transcription factor (1, 7). qRT-PCR analysis confirmed that transfection of LECs with 4 µM Pre-miR-31 resulted in a ≥60% reduction in PROX1 transcripts (Figure 4A), which was further verified by Northern blotting (Supplementary Figure 5). Importantly, immunoblotting revealed a consistent decrease in PROX1 protein levels by <40% following miR-31 overexpression (Figure 4B and C). Similar, but less consistent, results were observed after transfection with 2 µM Pre-miR-31 (Supplementary Figure 6). Conversely, loss-of miR-31 function in BVECs, via transfection of HUVECs with miR-31 inhibitor molecules (Anti-miR-31), resulted in a 1.7–3.1-fold increase in PROX1 mRNA levels (Figure 4D). PROX1 protein remained undetectable in these samples (data not shown).

PROX1 is a direct target of miR-31 post-transcriptional regulation

PROX1 was not predicted to be a target of miR-31 by TargetScan (17, 39), miRanda (27), miRBase (16) or PicTar (31), but human cells express two isoforms of PROX1 mRNA: A 7.9Kb isoform, which contains a 5.4Kb 3’UTR; and a 3.1Kb isoform that has a much shorter 602bp 3’UTR (57). Only the short 3’UTR has thus far been used for miRNA binding site predictions. In agreement with previous studies (25, 26, 49), our LECs expressed the longer, 7.9Kb isoform of PROX1 (Supplementary Figure 5). Therefore, standard nucleic acid alignment techniques (SIM alignment tools) and independent Targetscan 5.0 analyses were used to identify potential miR-31 binding sites in the 5.4Kb 3’UTR. SIM alignment identified 5 potential miR-31 recognition sites and the Targetscan search identified one of these sites, nucleotides (n.t.) 949-971, as a 7mer-m8 binding site (Figure 5A and Supplementary Table 6).

To test the functional relevance of these candidate miR-31 binding sites luciferase reporter genes containing a full-length PROX1 3’UTR (PROX1 FL), 6 PROX1 3’UTR fragment (PROX1 F1-6) (Figure 5A), or a PROX1 coding sequence (CDS) were constructed.
The activities of these chimeras were monitored following miR-31 gain-of-function in LECs (Figure 5B) and loss-of-function in HUVECs (Figure 5C). Confirming Pre-miR-31 overexpression and Anti-miR-31 knockdown activity, miR-31 binding site (miR-31 BS) luciferase reporter gene activity decreased or increased by <40% when co-transfected with Pre-miR-31 or Anti-miR-31 molecules, respectively (Figure 5B and C). The luciferase activities of PROX1 FL and PROX1 F2 reporter genes, which contain the 7mer-m8 site, decreased significantly (>35% and >45%, respectively) following miR-31 overexpression (Figure 5B). Conversely, their activities increased >14-fold and 3-fold, respectively, after miR-31 inhibition (Figure 5C). While PROXI 3’UTR F1 and F4 reporter gene activities increased after miR-31 knockdown, reciprocal responses following overexpression were not observed. Together, these findings confirm the direct post-transcriptional regulation of PROXI by miR-31, and suggested that this regulation is mediated via n.t. 949-971 of the 5.4Kb 3’UTR.

To validate this, the seed sequence (n.t. 964-971) and 3’ compensatory site interacting nucleotides (n.t. 954-960) the of the PROXI 3’UTR were mutated to match the miR-31 sequence in both the PROXI FL and PROXI F2 luciferase reporter plasmids, thus eliminating the predicted PROXI:miR-31 interaction. While the luciferase activities of the wild-type the PROXI FL and PROXI F2 constructs increased or decreased after miR-31 overexpression or knockdown, respectively (Figure 5D), the activities of the mutant full-length and F2 fragment luciferase reporter genes did not change significantly under either condition. Thus, mapping a bona fide, biologically active miR-31 binding site to n.t 949-971 of the PROXI 3’UTR.

**miR-31 overexpression inhibits PROXI target genes**

Numerous studies have demonstrated the PROXI transcriptional activities help dictate the molecular characteristics and functional activities of vascular endothelial cells(12, 26, 44, 48 and Shin et al., manuscript in preparation). Therefore, our characterization of
**miR-31 regulates vascular development in vivo**

During embryogenesis PROX1 is expressed in a subpopulation of cardinal vein endothelial cells that give rise to the mammalian lymphatic vascular system (1, 7). The BVEC-specific expression of miR-31, together with its ability post-transcriptionally repress numerous BVEC- and LEC-signature genes (Supplementary Tables 3 and 4), including Prox1, suggested that this miRNA might play a role in vascular development. As many of the BVEC- and LEC-signature genes targeted by miR-31 also play a major roles in *Xenopus* vascular development (9, 20, 45), we reasoned that ectopic expression of miR-31 in early *Xenopus* embryos might interfere with lymphatic vascular development. To investigate this, 2-cell stage *Xenopus* embryos were unilaterally microinjected with human Pre-miR-31 or Pre-miR-Neg molecules. Lymphatic and blood vascular system development were then monitored in stage 39 embryos using whole-mount in situ hybridization (ISH) for specific lymphatic and blood vascular marker genes (28, 29, 45).

No gross developmental defects or externally visible phenotypes were observed following Pre-miR-Neg or Pre-miR-31 microinjection into *Xenopus* embryos (Figure 6). Moreover, prox1 and vegfr3 marker gene analysis demonstrated that lymphatic vascular...
development progressed normally in 95% and 79% of the Pre-miR-Neg control embryos, respectively (Table 2). The embryos had well-defined and clearly visible lymph hearts, lymph vessels, and punctate patches of LECs in their tails (Figure 6A and B). In contrast, a dose-dependent increase in lymphatic vascular defects was observed in Pre-miR-31 injected embryos. Specifically, 

vefgr3 ISH demonstrated that the percentage of embryos with lymphatic vascular defects as monitored by the loss of vefgr3-positive lymphatics sprouting from the lymph hearts progressively increased from 6.1 to 76.2% as the amounts of Pre-miR-31 molecules increased from 1 ng to 50 ng (Table 2). Generally, lymph hearts were present in these embryos but appeared smaller and less well-defined compared to control embryos (Figure 6A and B) or the uninjected side of the Pre-miR-31 embryos (data not shown). Moreover, lymphangiogenesis, scored by the presence of vefgr3-expressing lymphatic vessels sprouting from the lymph heart, was either strongly reduced or absent in the presence of excess miR-31 (Figure 6A and B). These phenotypes were similar to those observed following morpholino inhibition of vefgc, where lymphangiogenesis was disrupted in the lymph heart region of 67-100% (N=3, total embryos analyzed: 71) of the injected Xenopus embryos (Supplementary Figure 7).

Pecam1 expression was used to monitor blood vascular system development in Pre-miR-Neg and Pre-miR-31 microinjected Xenopus embryos. In control embryos, all of the major blood vascular structures such as the posterior cardinal veins and the dorsal aorta were clearly visible, and angiogenic sprouting of intersomitic veins occurred normally in 73% of the Pre-miR-Neg injected embryos (Figure 6C and Table 2). By comparison, the percentage of embryos displaying unilateral intersomitic vein growth and/or guidance defects progressively increased from 0% to 76% with increasing amounts of Pre-miR-31 injected (Figure 6C and Table II).

Gain-of-function phenotypes can occasionally be attributed to the off-target effects associated with non-physiological expression levels of an siRNA or miRNA(41, 51). We, therefore, sought to confirm the Xenopus miR-31 overexpression phenotypes in zebrafish, another highly relevant vertebrate model organism for studying blood and lymphatic vascular
development(40). To facilitate the quantification of venous sprouting from the posterior cardinal vein (PCV)(24, 33, 67), we used phospholipase C gamma-1 (plcg1) mutant embryos in the TG(fli1a:gfp)y1 background(36) (TG(fli1a:gfp)y1;plcg1t26480), in which only the venous sprouts contributing to both the blood and lymphatic vasculature are visible. Venous sprouting was quantified 48 hpf following injection of 170 pg or 340 pg of either human Pre-miR-31 precursor or Pre-miR-Neg control molecules (Table 3 and Figure 7A). In agreement with our Xenopus studies, a significant dose-dependent reduction in venous sprouting was observed in Pre-miR-31 injected embryos compared to embryos injected with 340 pg Pre-miR-Neg molecules or to uninjected controls (Table 3 and Figure 7). The increase in venous sprouting observed in Pre-miR-Neg injected embryos (Figure 7B and C) appears to be a stress response, which we have also observed in a number of unrelated control injections (data not shown). Conversely and importantly, miR-31 overexpression led to a highly significant reduction in venous sprouting and no other developmental defects were observed in these embryos (Table 3 and Figure 7A and D).

Venous sprouting and lymphangiogenesis were also monitored in zebrafish embryos following injection of increasing concentrations of morpholino oligonucleotides (MO) targeting both mature and precursor dre-miR-31. Significant vascular phenotypes could not be specifically attributed to loss-of miR-31 activity in these embryos (data not shown). Taken together, our miR-31 gain-of-function studies in Xenopus and zebrafish embryos indicate that appropriate expression levels of miR-31 during vertebrate embryogenesis are required for normal lymphatic and blood vascular development.
Discussion

In the study presented here we first defined the *in vitro* expression profiles of 157 human miRNAs in primary human LECs and BVECs using a TaqMan-based qRT-PCR profiling platform, whose increased sensitivity facilitated the detection of at least twice as many miRNAs in HUVECs as previously reported (19, 34, 50, 58, 66). We also found that one of the most highly expressed HUVEC miRNAs, miR-126 (19, 34, 50, 58, 66), was >600 times more abundant in both endothelial cell types than in either keratinocytes or fibroblasts. Comparative analysis identified 4 BVEC- and 2 LEC-signature miRNAs. Of the 4 BVEC-signature miRNAs, 3 were previously reported as highly expressed in HUVECs (19, 34, 50, 58, 66), and a very recent study has demonstrated that TNF treatment augments miR-31 expression in HUVECs (60: 2010). Moreover, our miRNA profiling study has further classified the widely-expressed (38), metastasis-associated (63, 64) miRNA miR-31 as a BVEC-signature miRNA. Finally, in agreement with their LEC-specific expression, neither miR-95 nor miR-326 were detected in the previous studies.

Importantly, further analysis of miR-31, miR-326, miR-125b and miR-99a in adult mouse tissues confirmed that their vascular lineage-specific expression patterns were maintained *in vivo*. The degrees of lineage-specific expression differences *in vivo* were, however, usually less pronounced and more variable than those observed *in vitro*. This is likely due to the mixed populations of BVECs and LECs isolated from the multiple vessel types present in the colon tissue (capillaries, postcapillary venules, lymphatic capillaries, lymphatic collecting vessels etc.), which likely exhibit different gene expression patterns. Moreover, their relative contributions to the isolated total RNA might vary, thus contributing to larger variability in miRNA expression patterns. In addition, the *ex vivo* miRNA expression profiling studies were technically challenging as the whole process took more than 2 hours and only a few thousand endothelial cells could be isolated by high-speed cell sorting. Consequently, the smaller amounts of isolated total RNA, reduced RNA quality and possible gene expression changes incurred during the 2-hour isolation procedures likely contributed to
the observed differences of in vivo and in vitro miR-31 expression, as well as to the observed inter-individual variability of miR-31 expression. Surprisingly, we were unable to confirm the differential expression patterns of miR-137 in vivo. This is likely because miR-137 expression levels were very low in the adult tissues analyzed here as indicated by the late qRT-PCR detection (Ct > 35) and high standard deviations between technical replicates. ISH analysis of chicken embryos revealed that miR-137 is expressed in blood vessels and cardinal veins at stage 25 of embryonic development(8), demonstrating that miR-137 expression is associated with the developing blood vasculature.

The identification of vascular lineage-specific miRNAs suggested that they might regulate fundamental and lineage-specific endothelial cell functions and/or differentiation processes. Indeed, overexpression of the BVEC-specific miRNA, miR-31, in LECs induced the preferential degradation of LEC-signature genes, including the well-characterized lymphatic transcription factors PROX1 and FOXC2. As these lymphatic-specific molecules act as molecular switches, their preferential suppression suggests that BVEC-specific post-transcriptional regulatory mechanisms help maintain BVEC phenotypes by suppressing lymphatic-specific transcription programs. This concept was supported by our findings that ectopic overexpression of miR-31 in LECs preferentially repressed LEC-signature gene and induced BVEC-signature gene expression. In this respect, our identification and validation of PROX1 as a direct miR-31 target is an intriguing finding as BVEC-specific post-transcriptional regulation of PROX1 could, at least in part, explain these in vitro miR-31-mediated reprogramming events on the molecular level. Indeed, previous studies have demonstrated that PROX1 overexpression in BVECs induces the expression of lymphatic vascular markers and suppresses blood vascular markers(26, 48), whereas PROX1 knockdown in LECs inhibits LEC-signature gene expression and triggers BVEC-signature gene expression (44 and Shin et al., manuscript in preparation). Moreover, the overlaps between the miR-31-regulated genes identified here and a PROX1 loss-of-function dataset, further indicate that transcriptional reprogramming events observed following miR-31 overexpression in LECs were, in part, mediated by miR-31 repression of Prox1. Additional
experiments are required to determine which of the miR-31-regulated candidate Prox1 target genes may also be direct targets of miR-31.

While PROX1 was not a predicted target gene of miR-31, our manual miR-31 site prediction analyses of the 5.4 Kb PROX1 3’UTR and subsequent luciferase 3’UTR tethering assays identified a bona fide miR-31 binding site between nucleotides 949-971 of the PROX1 3’UTR. Interestingly, similar manual miR-31 prediction analyses of the chimpanzee, mouse, rat, chicken, Xenopus and zebrafish PROX1 3’UTRs revealed that this site is evolutionarily conserved in vertebrates, and identified additional, potentially functional miR-31 binding sites (Supplementary Table 6). Taken together, our transcriptome profiling and biochemical studies have revealed a novel, highly conserved BVEC-specific post-transcriptional regulatory mechanism that suppresses PROX1 expression in the blood vasculature.

Our findings also suggested that miR-31 expression in the developing blood vascular endothelium could regulate the acquisition of lymphatic-specific characteristics and, thus, vascular development in vivo. Multiple miR-31 loss-of-function studies using morpholino oligonucleotides were performed in both wild-type and plcg1 mutant zebrafish embryos. Statistically significant vascular phenotypes were not observed in zebrafish embryos injected with low to moderate (≤10 ng) amounts of MOs (data not shown). This suggests that the miR31-mediated regulation of vascular development identified here is redundant. This is not surprising, since miRNAs frequently function cooperatively, which in turn complicates the attribution of specific functions to individual miRNAs. In contrast, miRNA gain-of-function experiments have proven very informative and have defined important biological functions for several miRNAs. For example, overexpression studies in Xenopus embryos have demonstrated that miR-15 and miR-16 restrict in vivo the size of the Spemann’s organizer by targeting the Nodal-type II receptor acrvr2a. We, therefore, carried out miR-31 overexpression studies in Xenopus and zebrafish embryos to determine the effect of miR-31 on cells and tissues that normally do not express miR-31, such as the lymphatic vasculature.
Our gain-of-function experiments clearly demonstrated that miR-31 expression is incompatible with normal lymphatic vascular development in *Xenopus* and, to a lesser extent, zebrafish embryos. The analysis in *Xenopus* embryos suggests that some aspects of lymphatic vascular development, such as specification of lymph hearts and LECs in the tail, are unaffected by miR-31 overexpression. Lymphangiogenesis and the development of an extensive lymphatic vasculature in the embryonic trunk is, however, clearly reduced and/or disrupted. Furthermore, we demonstrated that these observed lymphatic defects were reminiscent of those observed following MO-mediated inhibition of vegfc. These phenotypic similarities indicate that miR-31 overexpression interferes with an early step of lymphatic development. The identification of evolutionarily conserved miR-31 binding sites in *PROX1* 3’UTRs (Supplementary Table 6) suggests that miR-31 overexpression may directly target and interfere with *PROX1* transcripts *in vivo*. Moreover, the abnormal or disrupted intersomitic vein sprouting in *Xenopus* and zebrafish embryos (data not shown) following miR-31 overexpression, implies that miR-31 also regulates BVEC responsiveness to the environmental stimuli directing blood vascular growth and maturation. Interestingly, several genes involved in the Slit/Robo, netrin, and ephrin signaling pathways (Supplementary Table 3), which provide crucial guidance cues during blood vascular development(1), were repressed following miR-31 gain-of-function *in vitro*. *In vivo* post-transcriptional regulation of any one of these molecules by miR-31 could contribute to the observed blood vascular maturation defects. Taken together, our results indicate that appropriate expression of miR-31 during vertebrate embryogenesis is required for both lymphatic vascular development and blood vascular growth and maturation. Interestingly, our *in vivo* studies also correlate well with a recent study demonstrating that miR-31 controls the invasive capacity of breast cancer cells(63, 64). Collectively, these studies suggest roles for miR-31 in the regulation of cell migratory behavior during embryonic normal development and under pathological conditions in the adult body.

On the basis of the *in vitro* studies, we postulate that PROX1 transcripts represent one of the key targets of miR-31. This repression would prevent in appropriate and/or premature
transcriptional activation of lymphatic differentiation in the developing blood vasculature. While this notion is an attractive model, it is however important to stress that miR-31 targets several other LEC-signature genes. It is, therefore, unlikely that post-transcriptional repression of PROX1 by miR-31 is solely responsible for the vascular developmental defects observed in Xenopus and zebrafish embryos overexpressing miR-31. For example, miR-31-mediated repression of FOXC2, a transcription factor that is required for specification of the lymphatic capillaries versus collecting lymphatic vessels at later stages of embryogenesis(1,7), may also contribute to the vascular defects. Another miR-31 candidate target is RAMP2, a calcitonin receptor-like receptor associated receptor activity-modifying protein that triggers lymphangiogenesis in response to adrenomedullin signaling(13). Finally, other LEC-signature molecules subject to miR-31 regulation, whose lymphatic-specific functions have not yet been characterized, could also enhance the effects miR-31 has on lymphatic and blood vascular development.

The miRNAs profiled in the present study represent approximately only 25% of all known human miRNAs. Thus, more comprehensive and global miRNA profiling studies along the lines outlined in the present studies may result in the identification of additional endothelial lineage-specific miRNAs. In summary, we have defined the first vascular lineage-specific miRNAs and identified with miR-31 a novel miRNA-mediated regulatory mechanism that inhibits LEC phenotype acquisition in vitro and vascular development in vivo. From a therapeutic perspective, it remains to be investigated whether the ectopic expression of miR-31 might also inhibit malignant tumor-associated (lymph)angiogenesis, thus preventing tumor growth and cancer metastasis.
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D.M.L.P. and T.K. designed and performed research experiments, analyzed the data and wrote the manuscript. V.D., G.J., G.V., D.H., and R.E.K., designed and performed research experiments, and analyzed the data. D.M., J.W.S., S.L., and P.C. performed research experiments and analyzed the data. M.D., A.W.B., and S.S-M. designed research experiments, analyzed the data, and wrote the manuscript.
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<td>2.8737</td>
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<td>miR-151b</td>
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<td>23.9781</td>
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<td>miR-154*</td>
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<td>28.1490</td>
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<td>let-7i</td>
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<td>24.3335</td>
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16 candidate LEC-signature miRNAs and 30 BVEC-signature miRNAs were identified among the 157 human miRNAs profile in primary human LECs and BVECs based on a > 2-fold difference in relative expression between endothelial cell types. Let-7a/miR-16 normalized average LEC (LEC Ct Ave) and BVEC (BVEC Ct Ave) Ct values, the corresponding relative differences (ΔCt LEC/BVEC) and absolute fold changes are shown. P-values were calculated using a two-tailed Student’s t-test.
### Table 2: Analysis of *Xenopus* embryos injected with Pre-miR-31 and Pre-miR-Neg.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival At Stage 39-40 (%)</th>
<th>ISH Marker</th>
<th>n</th>
<th># Embryos Analyzed</th>
<th>Embryos with Normal Vasculature (%)</th>
<th>Embryos with Vascular Defects (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1ng Pre-miR-Neg</td>
<td>84 prox1 61 60 91.7 8.3</td>
<td>vegfr3 55 52 94.1 5.9</td>
<td>pecam1 41 23 95.7 4.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10ng Pre-miR-Neg</td>
<td>84 prox1 48 46 91.3 8.7</td>
<td>vegfr3 59 57 94.7 5.3</td>
<td>pecam1 49 44 100 0.0</td>
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</tr>
<tr>
<td>100ng Pre-miR-Neg</td>
<td>79 prox1 21 21 95.2 4.8</td>
<td>vegfr3 19 19 78.9 21.1</td>
<td>pecam1 16 15 73.3 26.7</td>
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<td></td>
</tr>
<tr>
<td>1ng Pre-miR-31</td>
<td>74 prox1 70 68 94.1 5.9</td>
<td>vegfr3 89 82 93.9 6.1</td>
<td>pecam1 64 63 100.0 0.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10ng Pre-miR-31</td>
<td>57 prox1 50 48 93.8 6.3</td>
<td>vegfr3 60 56 87.5 12.5</td>
<td>pecam1 35 29 86.2 13.8</td>
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<tr>
<td>25ng Pre-miR-31</td>
<td>66 prox1 36 31 64.5 35.5</td>
<td>vegfr3 34 31 64.5 35.5</td>
<td>pecam1 37 29 55.2 44.8</td>
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<tr>
<td>50ng Pre-miR-31</td>
<td>52 prox1 34 29 48.3 51.7</td>
<td>vegfr3 31 21 23.8 76.2</td>
<td>pecam1 32 25 24.0 76.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Lymphatic and blood vascular development was monitored by whole mount *in situ* hybridization of embryos microinjected with Pre-miR-31 and Pre-miR-Neg. The number and frequency of lymphatic and blood vascular developmental defects, as scored based on the presence of several lymphatic and blood vascular structures, are presented.
Table 3: Analysis of zebrafish embryos injected with Pre-miR-31 and Pre-miR-Neg.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative number of venous sprouts</th>
<th>SD</th>
<th>n</th>
<th>p-value</th>
<th>% of UIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>UIC</td>
<td>1.000</td>
<td>0.348</td>
<td>120</td>
<td></td>
<td>100</td>
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<tr>
<td>Pre-miR-Neg 340 pg</td>
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<td>0.477</td>
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<tr>
<td>Pre-miR31 170 pg</td>
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<tr>
<td>Pre-miR31 340 pg</td>
<td>0.140</td>
<td>0.175</td>
<td>46</td>
<td>2.86825E-35</td>
<td>14</td>
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</table>

Venous sprouting was monitored in TG(fli1a:gfp)^y1:plcg1^26480 mutant zebrafish embryos injected with Pre-miR-31 and Pre-miR-Neg by fluorescence confocal microscopy. The number of venous sprouts were normalized to the UICs and represented as relative numbers of venous sprouts.
Figure 1

A

LEC-signature miRNAs

Relative Abundance

**

miR-95

miR-326

B

BVEC-signature miRNAs

Relative Abundance

**

miR-137

miR-31

**

miR-125b

miR-99a
Figure 5

A  PROX1 3'UTR

![Diagram of PROX1 3'UTR and miR-31 binding sites]

PROX1 3'UTR fragments

B  miR-31 Overexpression PROX1 3'UTR Tethering Assay

![Bar graph showing relative abundance of miR-31 and PROX1 constructs]

C  miR-31 Knockdown PROX1 3'UTR Tethering Assay

![Bar graph showing relative abundance of miR-31 and PROX1 constructs]

D  Mutagenic PROX1 3'UTR Tethering Assay

![Bar graph showing relative abundance of miR-31 and PROX1 constructs]
Figure 6

A  
Pre-miR-Neg  Pre-miR-31

prox1

B  
Pre-miR-Neg  Pre-miR-31

vegr3

C  
Pre-miR-Neg  Pre-miR-31

pecan1
Figure 7

A

Relative number of venous sprouts

<table>
<thead>
<tr>
<th></th>
<th>UIC</th>
<th>Pre-miR-Neg</th>
<th>Pre-miR-31 170 pg</th>
<th>Pre-miR-31 340 pg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.0</td>
<td>2.0</td>
<td>***</td>
<td>***</td>
</tr>
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</table>

B

PCV

C

PCV

D

PCV

Pre-miR-Neg

Pre-miR-31
**Figure Legends**

**Figure 1:** Identification of vascular lineage-specific miRNAs. qRT-PCR using individual TaqMan miRNA Assays confirmed the lymphatic-specific expression of miR-95 and miR-326 (A), and the BVEC-specific expression of miR-137, miR-31, miR-125b and miR-99a (B). Data were normalized using RNU48 and are shown as mean relative abundances +/- SD (n=4/group). * p ≤ 0.05.

**Figure 2:** miR-31 is preferentially expressed by BVECs in vivo. (A) Mouse BVECs (CD45–CD31+podoplanin–) and LECs (CD45–CD31+podoplanin+) were isolated from adult mouse colons (n=8) by FACS sorting. TaqMan qRT-PCR analysis of mmu-miR-31 demonstrated that miR-31 was at least 1.5-fold more abundant in BVECs from 6 of the 8 mice. Data were normalized using sno234 and are shown as relative abundances +/- SD. * p ≤ 0.05; **  p ≤ 0.01; ***  p ≤ 0.001; ns, not statistically significant. (B-I) miR-31 (B, D and F), negative control (G; sense-miR-159) ISH and double immunofluorescence (C, E, H and I) of serial sections of adult mouse colon tissues imaged at low magnification (2.5x; B-E) and high magnification (20x; F-I). Low magnification of ISH of adult mouse colon revealed strong miR-31 staining in the majority of the cells (B and D), while double immunofluorescence analysis of serial sections (C and E), and comparison of miR-31 and sense-miR-159 probed sections at high magnification (F-I), demonstrated that miR-31 preferentially co-localized with blood vessels (CD31+LYVE1-) present in the submucosa and mesenteric attachments of the colon (arrows), as well as the lamina propria (arrowheads). In contrast, lymphatic vessels (CD31+LYVE1+) displayed weak or no miR-31 signals (asterisks). Panels F and G are high magnification images of the boxed regions in panels D and E, respectively. Corresponding blood and lymphatic vessels in panels F-I are numbered. Scale bars = 200 µm (B-E) and 50 µm (F-I).

**Figure 3:** miR-31 overexpression in LECs modulates the expression of BVEC- and LEC-signature genes. (A-B) miR-31 was overexpressed in LECs via transfection with 2µM Pre-miR-31 precursor (n=2) or Pre-miR-Neg (n=2). Microarray analysis after 48 hours demonstrated that miR-31 overexpression repressed the expression of 33 LEC-signature genes (A) and 23 BVEC-signature genes (B). In addition, 4 LEC-signature genes and 22 BVEC-signature genes were induced. (C-E) TaqMan-based qRT-PCR analyses confirmed statistically significant miR-31-mediated repression of 4 of the LEC-signature genes studied (C) and of 2 of the BVEC-signature genes studied (D), as well as statistically significant induction of 2 of the BVEC-signature genes studied (E). Data were normalized using β-actin and are shown as relative abundances +/- SD. * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001).

**Figure 4:** miR-31 gain-of and loss-of function modulates PROX1 mRNA and protein levels. (A-C) Transfection of LECs with 4 µM Pre-miR-31 precursor (n=4) or Pre-miR-Neg (n=4). (A) qRT-PCR analysis revealed a ≥60% decrease in PROX1 transcripts after 48 hours. (B) Immunoblotting of cell lysates with α-PROX1 and α-β-actin antibodies (loading control) demonstrated decreased PROX1 protein levels following miR-31 gain-of-function. (C) Semi-quantitative analysis of PROX1 immunoblot signal intensities, relative to β-actin, defined an <40% reduction of PROX1 protein following miR-31 overexpression. Data are shown as relative abundances +/- SD (n=4/group). (D) qRT-PCR analysis showed that transfection of HUVECs with 4 µM Anti-miR-31 inhibitor (n=2) or Anti-miR-Neg (n=2) induced a ≥1.69-fold increase in PROX1 mRNA levels after 48 hours. Data were normalized using β-actin and are shown as mean relative abundances +/- SD. *** p ≤ 0.001.

**Figure 5:** Direct post-transcriptional regulation of PROX1 by miR-31. (A) Schematic representation of the full-length PROX1 3’UTR and the consecutive fragments present in the PROX1 3’UTR-luciferase reporter constructs (PROX1 FL-F6). Candidate miR-31 binding
sites identified using standard nucleic acid alignment techniques (gray) and TargetScan 5.0 (black) are indicated. The PROX1:miR-31 base-pairing is shown for the 7mer-m8 TargetScan predicted binding site. (B-D) LECs or HUVECs were co-transfected with the plasmids containing a miR-31 binding site (miR-31BS), the PROX1 3′UTR (FL-F6), the PROX1 coding sequence (CDS), or PROX1 3′UTR miR-31 binding site mutants (FLmut or F2mut) and Pre-miR-31 precursor, Anti-miR-31 inhibitor or negative control molecules. (B) The activities of luciferase constructs containing miR-31BS, PROX1 FL and PROX1 F2 decreased significantly following miR-31 overexpression (Pre-miR-31; n=3) in LECs compared to negative controls (Pre-miR-Neg; n=3). (C) miR-31BS and PROX1 FL reporter gene activities increased significantly following miR-31 inhibition (Anti-miR-31; n=3) in HUVECs compared to negative controls (Anti-miR-Neg; n=3). miR-31 loss-of-function also enhanced the activities of PROX1 F1 and PROX1 F2 constructs, but these differences were not statistically significant. (D) miR-31 mutant binding site full-length and F2 PROX1 3′UTR (PROX1 FLmut and PROX1 F2mut) luciferase activities showed no major change following miR-31 overexpression (Pre-miR-31, n=3) or knockdown (Anti-miR-31, n=3). Data were normalized to firefly luciferase activities and are shown as mean relative abundances +/- SE. ns, not significant; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001).

Figure 6: miR-31 overexpression in Xenopus impairs lymphatic vessel sprouting from the lymph heart. 2-cell stage Xenopus embryos were co-injected with Pre-miR-31 precursor (50 ng) or Pre-miR-Neg (100 ng) control molecules and 0.2 ng of β-gal mRNA (lineage tracer) and were raised to stage 39. (A-B) Lymphatic vascular system development was monitored by whole-mount ISH for prox1 (A) and vegfr3 (B). Pre-miR-31 injected embryos exhibited marked defects in lymphatic vascular development, the most striking of which was impaired lymphatic vessel sprouting (arrowheads) from the lymph heart (arrow). (C) Pecam1 ISH showed that intersomitic vein growth (arrowheads) was unilaterally misguided or delayed in embryos with elevated levels of miR-31.

Figure 7: miR-31 overexpression decreases venous sprouting in zebrafish embryos. (A) plcg1+1t26480 mutant embryos in the TG(fli1a:gfp)y1 background were injected with 340 pg of negative control Pre-miR-Neg (n=71) or 340 pg (n=46) or 170 pg (n=24) of human Pre-miR-31 precursor. Venous sprouts were quantified at 48 hpf. UIC, uninjected control (n=120). Data are shown as mean +/- SD relative to the UIC of the same clutch. *** p ≤ 0.001. (B-F) Lateral views of plcg1+1t26480 mutant embryos in TG(fli1a:gfp)y1 background. Dorsal aorta formation and sprouting of intersegmental arteries is suppressed in mutant embryos and therefore only venous sprouts (examples indicated with arrows) and parachordal lymphangioblasts (asterisks) are visible in the dorsal trunk. Uninjected control plcg1+1t26480 mutant embryo (B) and plcg1+1t26480 mutant embryos injected with 340 pg negative control Pre-miR-Neg (C) or 340 pg human Pre-miR-31 precursor (D) at day 2 postfertilization. PCV, posterior cardinal vein.