ONLINE DATA SUPPLEMENT

MIR-30A REGULATES ENDOTHELIAL TIP CELL FORMATION AND ARTERIOLAR BRANCHING

Qiu Jiang,*1, Mariana Lagos-Quintana, Ph.D.*,1, Dong Liu1, Ph.D., Yu Shi1, Ph.D., Christian Helker2, Wiebke Herzog2,3, Ph.D., Ferdinand le Noble1,4, Ph.D.
*equal contribution

1. Dept. of Angiogenesis and Cardiovascular Pathology
Max Delbrück Center for Molecular Medicine (MDC), D13125 Berlin, Germany.

2. University of Muenster, D48149 Muenster, Germany.

3. Max Planck Institute for Molecular Biomedicine, D48149 Muenster, Germany

4. Center for Stroke Research (CSB), Charite, D10117 Berlin, Germany

Address for correspondence:
Ferdinand le Noble, Ph.D.
Max Delbrück Center for Molecular Medicine (MDC)
Dept. of Angiogenesis and Cardiovascular Pathology
Robert Roessle Strasse 10
D13125, Berlin, Germany
Tel: +49-30-9406-3892
Fax: +49-30-9406-3850
Email: lenoble@mdc-berlin.de
Supplemental Materials and Methods:

Zebrafish experiments:
The study was conducted conforming to the local institutional laws, and the German law for the Protection of Animals. Zebrafish embryos and adult fish were raised and maintained under standard conditions. Tg(fli1a:egfpEGFP)y1, Tg(fli1:negfpEGFP)y7, Tg(kdrl:hras-mCherry)s896, Tg(hsp70:Gal4)xTg(uas:notch1a-ICD), and Tg(kdrl:ras-cherry)s916xTg(flt1BAC:yfp) double transgenic zebrafish lines were used as described. Activation of Notch signaling was performed using Tg(hsp70:Gal4)xTg(uas:notch1a-ICD) double transgenic embryos which were heat shocked at 40°C for 20 minutes at the 16-18-somite stage and then kept at 28°C for further experiments.

Injection of morpholinos and microRNA precursor
Morpholino antisense oligomers (MO, Gene Tools) were prepared according to the manufacturer’s protocol. The MO sequences used are the following:
dre-miR-30a-MO (15 ng), 5’-CTTCCAGTCGGAATGTTTACAACACT-3’;
dre-miR-30a-MO-2 (15 ng), 5’-CAACCTCCAGTCGGAATGTTTACACT-3’;
dre-miR-30b-MO (15 ng), 5’-AGTGTAGGATGTTTACAGCGACTAC-3’;
dre-miR-30c-MO (3.5 ng), 5’-CAACTTCCAGTCGGAATGTTTACATCA-3’;
dre-miR-30d-MO (15 ng), 5’-GGGATGTACGGCATGAAACC-3’;
dre-miR-30e-MO (15 ng), 5’-CTTCCAGTCGGAATGTTTACAGAC-3’;
dll4-MO (7 ng), 5’-GTTCCAGTACGCGCCACCCAAG-3’;
control-MO (15 ng), 5’-CTTCCAGTACGCGCCACCAAATTA-3’.
miR-30a precursor (ID:PM11062, Ambion), 0.025pmol was injected in all transgenic lines except for the Notch-ICD rescue experiments using the Tg(hsp70:Gal4)xTg(uas:notch1a-ICD) double transgenic line. Here we used 0.02pmol (at higher dosage, embryos died). Injections were performed at 1-2-cell stage.

microRNA sensor assay
Whole-embryo microRNA sensor assay in zebrafish was carried out as described. Briefly, egfp or mcherry were cloned into the pCS2+ vector. The pCS2-egfp-dll4-3’UTR construct was generated by cloning nucleotides 2362 to 2975 of the zebrafish dll4 mRNA (accession NM_001079835) into the pCS2-egfp vector, while pCS2-egfp-dll4-3’UTR (truncated) was generated by inserting only nucleotides 2455-2975 of the dll4 mRNA. The truncated construct lacks the fraction of the dll4-3’UTR containing the miR-30 binding site. As an injection control pCS2-mcherry vector was used. The three plasmids were linearized with NotI and used as templates to synthesize the capped mRNAs using mMessage Machine (Ambion). The RNAs mentioned above were injected into the cytoplasm of 1-2-cell-stage embryos (75 pg/embryo).

Imaging
Zebrafish embryos were anesthetized with egg water/tricaine/PTU (0.016% tricaine (MS-222); 0.003% PTU, Sigma) solution, embedded in 0.4-0.6% low-melt agarose (Invitrogen). In vivo confocal imaging was performed with Zeiss-510-NLO or Leica-SP5 confocal microscopes. The analysis was done using Zeiss-ZEN, Leica-LAS-AF, and ImageJ software. For the microRNA sensor assay images we used a LEICA-MZ-16FA fluo-microscope and MetaVue software.
**In situ hybridization for miR-30**

To visualize the expression of miR-30a on sections we used digoxigenin-labeled RNA probes labeling primary miR-30a as described previously. The primers used for cloning the probe are listed in Table S1.

**Gene and microRNA expression analysis by TaqMan PCR**

Total RNA of zebrafish embryos was isolated with TRIZOL (Invitrogen). Quantity and quality of extracted RNA were analyzed by using Nanodrop and Agilent 2100 Bioanalyzer (Agilent Technologies) according to the manufacturer’s instructions, followed by cDNA synthesis using Thermoscript First-Strand Synthesis System (Invitrogen). microRNA TaqMan® Gene Expression Assays were purchased from Applied Biosystems. Amplification was carried out in the ABI Prism 7000 thermocycler (Applied Biosystems). Gene expression data were normalized against Elongation factor 1-alpha (Efl-α). microRNA TaqMan® MicroRNA Assays and Taqman® microRNA Reverse Transcription (RT) kit (Applied Biosystems) were used for microRNA analysis according to the manufacturer’s instructions. For examining the miR-30a expression in zebrafish embryos, dre-miR-21 was used for normalization. For examining miR-30a expression of human U6 snRNA assay was used.

**Fluorescence-activated cell sorting (FACS) and flow cytometry analysis**

Tg(fli1:egfp)\textsuperscript{1} or Tg(kdrl:ras-cherry)\textsuperscript{\textregistered}×Tg(fli1\textsuperscript{YAC}:yfp) embryos were kept in egg water to the indicated developmental stage and dechorionated with 0.5 mg/mL pronase (Roche). Embryos were transferred into a 15 mL falcon tube with 5 mL phosphahate buffered saline (PBS) containing 0.25% trypsin and incubated for 60 min at 28°C during which they were triturated with a pipette tip every 15 min. After centrifuging for 5 min at 800 g at 4°C, cells were resuspended in PBS containing 0.25% trypsin and 15% fetal calf serum (FCS) to stop the digestion and centrifuged for 5 min at 800 g at 4°C. Cells were rinsed with PBS containing 2% FCS for 2 times and resuspended in PBS at 10\textsuperscript{7} cells/mL. FACS was performed on a FACS Aria2 (BD Biosciences), and approximately 1x10\textsuperscript{6} positive cells were collected for deep sequencing or quantitative PCR.

**Endothelial cell culture**

Human umbilical vein endothelial cells (HUVECs) and human umbilical artery endothelial cells (HUAECs) were purchased from PromoCell and cultured in EBM-2 media with SingleQuots supplements and Growth factors according to the protocol provide by manufacturer (Lonza).

**Plasmids and transfections**

The NOTCH-regulated reporter gene constructs TP1 and 4×CBF1 were a generous gift from M. Potente.\textsuperscript{10} Transient transfections of HUVECs were carried out by electroporation with Amaxa HUVEC nucleofector Kit (Lonza). 1.6x10\textsuperscript{6} HUVECs were electroporated according to the manufacturer’s protocol.

**Luciferase assays**

psiCheck2-zebrafish dll4-3’UTR reporter assay was transfected into HeLa cells with or without miR-30a-pre or miR-30a antagonir, Ambion (Ambion-Anti-miR-microRNA Inhibitor). Note: HeLa cells expressed miR-30a under baseline conditions (miR-30a/miR-21 expression ratio is 0.2). Reporter assays in HUVECs were
performed with the Dual-Luciferase Reporter Assay System (Promega) in a Tecan Infinite 200 Pro plate reader. Shortly, 24hrs after co-transfection with the NOTCH luciferase reporters, the constitutive Renilla luciferase reporter pGL4.74hRluc/TK (Promega) and miR-30a-LNA, cells were lysed and Luciferase activity measured as indicated by the manufacturer. Reporter activity was adjusted for the internal Renilla luciferase controls and is expressed as relative to control.

**RNA interference and microRNA inhibition and overexpression**

miR-30a was inhibited through transfection of hsa-miR-30a-LNA in vivo inhibitor and a scrambled control (Exiqon), or with miR-30a antagonist (miR-30a antago, Ambion® Anti-miR™ microRNA Inhibitor) Life Technologies, as indicated. For miR-30a overexpression miR-30a-precurser was purchased from Life Technologies (Ambion, Pre-miR™ microRNA precursor), and miR-30e was overexpressed using miR-30e duplex from IDT, see Table S1 for sequences. HUVECs were transfected with the indicated microRNA inhibitors or precursors (50 nanomol/L) with Lipofectamine RNAiMAX (Invitrogen) according to manufacturer’s protocol.

**Western blot analysis and antibodies**

SDS–PAGE and western blot analyses were performed according to standard procedures and detected with the ECL detection kit (GE Health Care Bio-Sciences). DLL4 antibody (2589S) was purchased from Cell Signaling. The GFP and mCherry antibodies were purchased from Abcam and Clontech, respectively. Quantification of Western blot signal was performed using ImageJ software.

**Spheroid assay**

Cell spheroids of defined cell number were generated as described. In short, 24hrs after transfection, HUVECs were suspended in culture medium containing 20% (wt/vol) carboxymethylcellulose (Sigma-Aldrich) and seeded in non-adherent round-bottom 96-well plates (Greiner Bio-One). Under these conditions, all suspended cells contribute to the formation of a single spheroid per well of defined cell number (400 cells/spheroid). Spheroids were generated overnight, after which they were embedded into collagen gels. 500µL of spheroid-containing gel was transferred into prewarmed 24-well plates and allowed to polymerize (30 min), after which 50µL endothelial growth medium containing 10x SingleQuot supplements and growth factors (Lonza) was added on top of the gel. To stimulate sprouting, 20ng recombinant human VEGF was added. After 24hrs, spheroids were stained with 50 ng/mL calcein AM (Invitrogen) for 3hrs, then fixed with 4% PFA for 3hrs, and finally DAPI stained, for 16hrs. Spheroids were imaged by confocal microscopy with a Zeiss-LSM-510 microscope (Carl Zeiss MicroImaging). About 7 spheroids were analyzed per experimental group and experiments were repeated 3-5 times.

**Northern blot for miR-30a in human endothelial cells and zebrafish embryos**

Total RNA was extracted from zebrafish embryos or HUVEC cells using Trizol (Invitrogen). Northern blotting for miR-30a was performed as described previously. Briefly, 7.5 µg of human, or 10 µg of zebrafish RNA were loaded onto an 12% acrylamide gel with 8 mol/L urea, transferred to positively charged Nylon membrane (GE Healthcare), and hybridized with 32P-end-labeled species-specific LNA probes (Exiqon) at 50°C for 16hrs. For loading control the gel was stained with ethidium bromide and visualized under UV light.
Supplement References


### Supplement Table S1. Primer sequences.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence 5' – 3'</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>in situ</em> pri-miR-30a F</td>
<td>TGTGTGGGTGGTCTAGTGG</td>
</tr>
<tr>
<td><em>in situ</em> pri-miR-30a R</td>
<td>GGGAAACCTGGACTAACAG</td>
</tr>
<tr>
<td>dll4-3'UTR F</td>
<td>CCGGAATTCTAGAGGAGGAGACGCAA (EcoR1)</td>
</tr>
<tr>
<td>dll4-3'UTR R</td>
<td>CCGCTCGAGTGGGCAAAACATAGCCTC (Xho1)</td>
</tr>
<tr>
<td>dll4-3'UTR (truncated) F</td>
<td>CCGGAATTCCTGCTGAGGAGAAAACACAAGAACAAAGCAGCAAAGGACAGACGCAAAA (EcoR1)</td>
</tr>
<tr>
<td>miR-30e duplex-guide</td>
<td>UGUAAACAUUCCUUGACUG</td>
</tr>
<tr>
<td>miR-30e duplex-passenger</td>
<td>CUUCCAGUCAGGAGGAUU</td>
</tr>
<tr>
<td>miR-30 mismatch control-guide</td>
<td>CUUCAAGUCAAGCAUAG</td>
</tr>
<tr>
<td>miR-30 mismatch-passerenger</td>
<td>UAUUCAUAGUCUUGACUU</td>
</tr>
</tbody>
</table>
Supplement Figure S1. Endothelial expression of miR-30

(A) Expression of miR-30 family members, indicated as percentage of known microRNAs identified by deep sequencing microRNA profiling in HUVECs, HUAECs and zebrafish ECs FAC-sorted from Tg(fli1:egfp)y1 embryos. White bar (miR-30 family) shows added percentages of the individual miR-30 family microRNAs. (B) Expression of the primary pri-miR-30a transcript (pri-miR-30a) in a 24hpf zebrafish embryo as detected by in situ hybridization, in whole-mount (top panel, higher magnification of blue boxed area is presented in bottom left panel) and cross-section (anatomical position indicated by red line in top panel, in bottom right panel). The high magnification lateral view (bottom left) shows miR-30a expression in the dorsal aorta (da). (C) Expression of arterial markers dll4, flt1 and venous marker ephb4a in FAC-sorted cells. (D) Expression of arterial markers dll4, flt1 and venous marker ephb4a in FAC-sorted cells. (E) Alignment of the mature miR-30 family from zebrafish and human miR-30a. Note the conserved sequences across species. Abbreviations: nt, neural tube; nc, notochord; da, dorsal aorta; cv, cardinal vein; pd, pronephric duct. **P<0.01; ***P<0.001, Student’s t-test.
Supplement Figure S2. Vascular phenotypes after knockdown of miR-30 in zebrafish embryos.

(A) Representative light microscopic images of Tg(fli1:egfp)y1 zebrafish embryos injected with control-MO, or injected with miR-30a, b, c, d, or e morpholino as indicated. (B) Frequency of ISV phenotypes in Tg(fli1:egfp)y1 zebrafish embryos injected with control-MO, or injected with miR-30a, b, c, d, or e morpholino as indicated. The vascular phenotypes are color indicated and schematically represented in the top panel part. Note that reduced ISV sprouting is most prominent in the miR-30a morphants. Measurements from n=40-60 embryos/group. (C) Tg(fli1:egfp)y1 embryos injected with miR-30a-MO2 show reduced ISV sprouting. Left panels, light microscopic images, confocal images of the boxed areas are shown at high magnification in the right panels. Numbers at bottom right indicate the fraction of embryos showing the phenotype presented in the image.
Supplement Figure S3. miR-30e overexpression promotes branching similar to miR-30a. (A,B) Confocal images of Tg(fli1:negfp)y7xTg(kdrl:hras-mcherry)s896 double transgenic embryos at 32hpf, after injection of control or miR-30e-pre. EC nuclei in green; vessels in red, arrowheads indicate hyperbranching. Ratio in lower right corner: fraction of embryos showing phenotype similar to the image. Note hyperbranching in miR-30e-pre-injected embryos. Numbers denote cell nuclei of representative ISVs. On average, control ISVs showed 3 nuclei, whereas embryos overexpressing miR-30e showed 5.8 nuclei/ISV. (C,D) Confocal images of Tg(fli1:egfp)y1 embryos injected with control or miR-30e-pre. Quantification in (E). Arrowheads indicate hyperbranching. (E) Quantification of hypersprouting events in control, and after overexpression of miR-30a or miR-30e. Note that both microRNAs caused hypersprouting.; n=80 embryos/group. (F) dll4-3'UTR sensor assays. GFP sensors were co-injected with mCherry control as indicated. miR-30e-pre injection reduced GFP levels in GFP-dll4-3'UTR sensor (second column) while mCherry levels were unchanged. In the absence of a miR-30 binding site, GFP expression was not greatly affected by miR-30e overexpression (fourth column).
Supplement Figure S4. Disturbed lymphangiogenesis in miR-30a morphants.

(A,B) lateral view of control and miR-30a morphant. (A’,B’) ventral view of control and miR-30a morphant; note general edema in miR-30a MO. (C-F) Confocal stack images of trunk vessels in Tg(fli1:egfp)y1 embryos, in control (C,E) and miR-30a morphants (D,G). At 3dpf, in control MO injected embryos (C) parachordal lymphangioblasts (PLs) align along the horizontal myoseptum (arrowhead) whereas in miR-30a MO (D) PLs fail to migrate (asterisk). (E) At 5.5dpf, parachordal vessel (yellow arrowhead) and thoracic duct (blue arrow) are clearly visible in control but appear interrupted in miR-30a morphants.
Supplement Figure S5. Overexpression of *miR-30a* reduces diameter of aorta and cardinal vein.

Confocal images of *Tg(fli1:negfp)y7xTg(kdrl:hras-mcherry)s896* double transgenic embryos; lateral (A) and cross-sectional (A’) view of axial vessels. (B) lateral and cross-sectional (B’) view of axial vessels. Drawings are schematic representations of axial vessels in control (top panel) and after overexpression of *miR-30a* (bottom panel). Note the reduced vessel diameters in *miR-30a* overexpression embryos. (C) Statistical analysis of DA and PCV diameters in control and *miR-30a* overexpression embryos. ***, p<0.001 in t-test. Dorsal aorta (DA), Posterior Cardinal Vein (PCV).