miR-30a Regulates Endothelial Tip Cell Formation and Arteriolar Branching

Qiu Jiang,* Mariana Lagos-Quintana,* Dong Liu, Yu Shi, Christian Helker, Wiebke Herzog, Ferdinand le Noble

Abstract—Microvascular rarefaction increases vascular resistance and pressure in systemic arteries and is a hallmark of fixed essential hypertension. Preventing rarefaction by activation of angiogenic processes could lower blood pressure. Endothelial tip cells in angiogenic sprouts directly bind microvascular networks; the process is regulated by microRNAs, particularly the miR-30 family. We investigated the contribution of miR-30 family members in arteriolar branching morphogenesis via delta-like 4 (Dll4)-Notch signaling in a zebrafish model. The miR-30 family consists of 5 members (miR-30a-e). Loss-of-function experiments showed that only miR-30a reduced growth of intersegmental arterioles involving impaired tip cell function. Overexpression of miR-30a stimulated tip cell behavior resulting in augmented branching of intersegmental arterioles. In vitro and in vivo reporter assays showed that miR-30a directly targets the Notch ligand Dll4, a key inhibitor of tip cell formation. Coadministration of a Dll4 targeting morpholino in miR-30a morphants rescued the branching defects. Conversely, conditional overexpression of Notch intracellular domain restored arteriolar branching in miR-30a gain-of-function embryos. In human endothelial cells, loss of miR-30a increased Dll4 protein levels, activated Notch signaling as indicated in Notch reporter assays, and augmented Notch downstream effector, HEY2 and EFNB2 (ephrin-B2), expression. In spheroid assays, miR-30a loss- and gain-of-function affected tip cell behavior, consistent with miR-30a targeting Dll4. Our data suggest that miR-30a stimulates arteriolar branching by downregulating endothelial Dll4 expression, thereby controlling endothelial tip cell behavior. These findings could have relevance to the rarefaction process and, therefore, to hypertension. (Hypertension. 2013;62:00-00.) ● Online Data Supplement

Key Words: angiogenesis ■ microRNAs ■ Notch receptors

Increased peripheral vascular resistance is the hallmark of fixed essential hypertension. The microcirculation is the site of increased peripheral vascular resistance. Studies in humans and experimental models of hypertension have shown that a reduction in the number of small arterioles and capillaries, rarefaction, commonly occurs. Rarefaction augments peripheral resistance, affects pressure in systemic arteries, and may further contribute to end-organ damage. Rarefaction can be detected in early stages even before the rise in blood pressure, suggesting that hypertension could be caused by defects in the mechanisms that govern blood vessel growth. Exposing young spontaneously hypertensive rats to chronic hypoxia decreases their blood pressure by stimulating small-vessel growth. The underlying molecular mechanism involves vascular endothelial growth factor–mediated proangiogenic responses. Angiogenesis involves the Notch signaling pathway, which is important for cell–cell communication and gene regulation. Both Notch receptors and Notch ligands are membrane-bound proteins. The Notch ligand protein delta-like 4 (Dll4) can bind to the extracellular domain of Notch1 receptors; this interaction triggers Notch activation involving cleavage of the intracellular part of the Notch receptor (Notch intracellular domain) by γ-secretases. The released Notch intracellular domain can interact with corepressors turning gene repression into gene activation. Vascular endothelial growth factor acts upstream of the Dll4-Notch signaling pathway and is the key regulator of angiogenic sprouting.

Angiogenic sprouting and branching involves differentiation of endothelial cells (ECs) into tip and stalk cells. Dll4-Notch signaling acts as an inhibitor of tip cell differentiation in developing vessels. Dll4 dose and location are especially important for proper sprout formation, as either gain- or loss-of-function leads to profound changes in vessel branching. In mice and zebrafish, deletion of Dll4, or inhibiting Notch signaling stimulates angiogenesis and causes hyperbranching of developing vessels. Conversely, overexpressing Dll4 or activating Notch

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signaling reduces vascular branching during embryogenesis,\textsuperscript{8,10} consistent with Dll4-Notch acting as a branching inhibitor.\textsuperscript{6} The exact regulatory mechanisms controlling Dll4 expression levels are unknown\textsuperscript{11,12} but may involve microRNAs (miRNAs).\textsuperscript{13}

miRNAs are small (22 nucleotides) single-stranded non-coding RNAs that are produced in the nucleus, processed by the enzymes Drosha and Dicer, and incorporated into RNA-induced silencing complexes. miRNAs suppress gene expression at the posttranscriptional level by degrading or inhibiting of target miRNAs.\textsuperscript{14,15} Their role in angiogenesis is established.\textsuperscript{16–19} Because the Dll4-Notch signaling pathway is evolutionarily conserved, we hypothesized the existence of miRNA families capable of targeting Dll4 and influencing vessel branching. The miR-30 family members are highly conserved between human, mouse, and zebrafish with perfect basepair matching. The miR-30 family members are highly conserved between human, mouse, and zebrafish with perfect basepair matching. This raised and maintained under standard conditions. Tg(fli1a:egfp)y1 German animal protection laws. Zebrafish embryos and adult fish were kept and maintained under standard conditions. Tg(fli1a:egfp)y1 German animal protection laws. Zebrafish embryos and adult fish were kept and maintained under standard conditions.

**Methods**

Our local regulatory committee approved the study according to the German animal protection laws. Zebrafish embryos and adult fish were raised and maintained under standard conditions. Tg(fli1a:EGFP)y1, Tg(fli1a-nEGFP)y1, Tg(kdrl:ras-cherry)y506xTg(fli1:nEGFP)y1 double-transgenic embryos (Figure S1A, S1C, and S1D). We found that miR-30a was the most abundant miR-30 family member in the human endothelium, representing ≈2.6% of all known miRNA sequences detected, whereas miR-30c was the most abundant family member in zebrafish endothelium making up ≈1.03% of all miRNAs (Figure S1A). Expression of miR-30a in human and fish ECs was validated by Northern blotting, Taqman analysis, and in situ hybridization (Figure 1H–1J, and Figure S1B and S1C). All 5 miR-30 family members (miR-30a,b,c,d, and e) contain identical seed sequences (Figure S1E), predicted to have the potential to interact with Dll4 3′UTR.

To determine the physiological contribution of the individual miR-30 family members in vessel branching, we knocked down miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e using a morpholino approach in zebrafish embryos and examined their phenotype. Confocal microscopy showed that knockdown of miR-30a was associated with reduced ISV sprouting (Figure 1E, arrowhead). miR-30a overexpression induced ISV hyperbranching at 32 hpf (F and F) and augmented tip cell filopodia (E, arrowhead). miR-30a overexpression induced ISV hyperbranching at 32 hpf (F and F) and augmented tip cell filopodia (E, arrowhead).

### Figure 1.

miR-30a regulates intersegmental vessel (ISV) branching in zebrafish embryos. A, C, and E, Zebrafish embryos at 32 hpf injected with mature miRNA-30a (miR-30a) morpholino (MO) or miR-30a-precursor as indicated. A, C, and E, Confocal stack images of trunk vessels at 24 hpf and B, D, and F at 32 hpf in Tg(fli1a:egfp)y1 embryos. Loss of miR-30a reduced ISV sprouting (D) and reduced filopodia extensions (C, D, arrowhead), miR-30a overexpression induced ISV hyperbranching at 32 hpf (F and F) and augmented tip cell filopodia (E, arrowhead).

Bottom right corner, Fraction of embryos with phenotype similar to image. H, Reduced miR-30a expression after injection of miR-30a MO. I, Mature miR-30a overexpression increased branching. J, Northern blot for miR-30a in control and miR-30a MO-injected embryos. "**P<0.001, Student's t test. A indicates aorta; and DLAV, dorsal longitudinal anastomotic vessel.

### Results

**Analysis of miR-30 Expression**

Computational miRNA target prediction programs list Dll4 as a target for miR-30 miRNAs, according to TargetScan version 6.2 (http://www.targetscan.org) and PicTar (pictar.mdc-berlin.de) as shown (Figure S1E). Endothelial miRNA expression profiles were obtained by deep sequencing using human umbilical-artery ECs, human umbilical-vein ECs, and zebrafish ECs. The latter were fluorescence-activated cell-sorted from Tg(fli1a:egfp)y1 embryos at 24 hours post fertilization and from Tg(kdrl:ras-cherry)y506xTg(fli1:nEGFP)y1 double-transgenic embryos (Figure S1A, S1C, and S1D).
the developing vascular network in the trunk (Figure 1, and Figure S2A and S2B). In the trunk of the zebrafish embryo, intersegmental vessels (ISVs) branch from the aorta in a highly stereotyped manner involving tip cell–mediated guidance of the developing ISV sprouts. These vessels express arterial identity markers, will carry arteriolar flow, and are, therefore, considered arterioles during early stages of development. Because in zebrafish, dll4 gain-of-function restricts angiogenic cell behavior, we specifically monitored the development of the intersegmental arterioles that sprout/branch from the aorta in the trunk region of the miR-30 morphants (Figure S2A and S2B). For all miR-30 family members examined, reduced ISV development was most frequently observed after knockdown of miR-30a (Figure 1 and Figure S2B). Given the prominent effects of miR-30a, we examined this family member in more detail in the subsequent loss- and gain-of-function analyses.

miR-30a Stimulates Angiogenesis

We next performed miR-30a loss- and gain-of-function experiments in zebrafish embryos and examined ISV branching morphogenesis (Figure 1; detailed quantification of cell numbers in Figure 2). Morpholino-mediated loss of miR-30a (Figure 1C’, 1D, and 1H) reduced outgrowth, compared with age-matched controls (Figure 1A–1D). After morpholino-mediated knockdown of miR-30a, arteriolar sprouts usually failed to cross the horizontal myoseptum (Figure 1C’ and 1D; additional quantification of ISV length in Figure 2) and as a consequence, formation of the dorsal lateral anastomotic vessel was severely disturbed (Figure 1D). Tip cell filopodia extensions of ISVs appeared smaller and fewer in miR-30a morphants (Figure 1C’ and 1D’). Similar sprouting deficits were observed when we injected a different miR-30a targeting morpholino (Figure S2C).

Injection of miR-30a precursor (miR-30a-pre) increased miR30a expression as evidenced by real-time polymerase chain reaction (Figure 1I); on average mature miR-30a levels were increased by 15-fold at 36 hours post fertilization (Figure 1I). miR-30a overexpression induced hyperbranching, predominantly in the most dorsal aspect of the ISVs (Figure 1E, 1E’, 1F, and 1F’; detailed quantification of cell numbers in Figure 3A–3C). Endothelial tip cell filopodia extensions of vessel sprouts appeared more abundant (Figure 1E”). Both the timing and the anatomic position of the hyperbranched ISVs are reminiscent of dll4 loss-of-function vascular phenotypes reported in zebrafish embryos. We also noted changes in the intestinal vasculature (Figure 1G). miR-30a morphants showed reduced intestinal vascular branching (Figure 1G, middle), whereas miR-30a overexpression embryos showed hyperbranching (Figure 1G, bottom) consistent with previous reports showing that dll4 acts as an inhibitor of intestinal vessel branching. Dll4 has furthermore been implied to restrict lymph-angiogenesis. Consistent with this, miR-30a morphants displayed defective lymph-angioblast migration and a failure to form the thoracic duct (Figure S4). As a consequence 70% of miR-30a knockdown embryos (n=45) developed severe edema; in control morpholino-injected embryos (n=34) edema was never observed (Figure S4). Finally, loss ofDll4 has been shown to affect vessel diameter in mouse and zebrafish embryos. To mimic loss of Dll4, we overexpressed miR-30a and examined the diameter of the aorta and posterior cardinal vein and observed that both were significantly reduced when compared with control embryos (Figure S5).

miR-30a Targets Dll4

The miR-30a loss- and gain-of-function experiments suggested that miR-30a acts as a positive regulator of vessel branching. Dll4-Notch signaling is an inhibitor of branching in this setting and in silico analysis predicted binding of miR-30a with zebrafish dll4 3’ UTR (PicTar, TargetScan;
Figure 3. Hyperbranching in miR-30a gain-of-function embryos involves loss of Notch. A and B, Imaging of endothelial nuclei in control and embryos overexpressing miR-30a using Tg(fli1a:egfp)y1 x Tg(kdrl:hras-mCherry)hsp double-transgenic embryos at 32 hpf. Numbers denote endothelial cell (EC) nuclei, vessels in red. C, Quantification of EC numbers per intersegmental vessel (ISV). D, Quantification of hyperproliferation. E, Time-lapse images of miR-30a overexpression embryos; time (hpf) indicated in top right corner; nuclei are numbered in black and decimals indicate daughter cells arising from cell division. F, Confocal images of ISVs in control, G, miR-30a overexpression, and H combination of miR-30a overexpression and conditional overexpression of notch intracellular domain (notch1a-ICD) using Tg(hsp70:Gal4) x Tg(clus: notch1a-ICD) embryos. I, Quantification of EC numbers per ISV. Data are presented as mean±SE; measurements from 4 adjacent ISVs/embryo, n=20 embryos. **P<0.01; and ***P<0.001, Student t test.

Figure S1E). Given the similarities between the vascular phenotypes of miR-30a loss- and gain-of-function embryos, and the dll4-notch gain- and loss-of-function, respectively, and the predicted interaction of miR-30a with dll4 3′ UTR (Figure 4A), we hypothesized that miR-30a influences vessel branching by modulating DLL4-Notch signaling.

To demonstrate functional interaction of miR-30a with dll4 3′ UTR, we injected zebraphish embryos with a GFP-dll4 3′ UTR- or a GFP-dll4 truncated 3′ UTR RNA (lacking a miR-30a binding site), together with a mCherry injection control RNA and miR-30a precursor or control (Figure 4A–4C). In the GFP-dll4 3′ UTR RNA-injected embryos, overexpression of miR-30a reduced GFP expression as evidenced by in vivo imaging analysis (Figure 4C), and by GFP and mCherry Western blot analysis (Figure 4D). Deleting the miR-30a binding site in the dll4 3′ UTR annihilated this response in vivo (Figure 4C, right column; Western blot in Figure 4D), furthermore confirmed by in vitro reporter assays (Figure 4E) suggesting that miR-30a can directly target the dll4 3′ UTR.

Taken together, these data indicate that knockdown of miR-30a may augment dll4 and subsequently lead to a restriction of angiogenic cell behavior. If the mechanism indeed involves elevated dll4, reducing dll4 levels should restore angiogenic potential. To test this idea directly, we knocked down dll4 using a morpholino approach in miR-30a morphants and examined ISV length and EC numbers (Figure 2). miR-30a morphants showed reduced sprout length and reduced EC numbers (Figure 2A–2D, 2G, and 2H), consistent with dll4-notch gain of function. Knockdown of dll4 in this setting greatly normalized both sprout length, dorsal lateral anastomotic vessel formation, and ISV cell numbers (Figure 2E–2H).

Conversely, if miR-30a targets dll4, overexpression of miR-30a should result in loss of Notch signaling. To substantiate that the vascular phenotype in miR-30a overexpression embryos indeed involves loss of Notch signaling, we performed a rescue experiment and conditionally overexpressed Notch intracellular domain (notch1a-ICD) in miR-30a overexpression embryos. Consistent with loss of Notch signaling, we observed hyperbranching and augmented EC numbers in developing vessels of miR-30a overexpression embryos (Figure 3A–3D). Furthermore, time-lapse imaging showed proliferation of ECs at the tip of developing ISVs (Figure 3E), a feature consistent with loss of notch signaling in this setting. Conditional overexpression of notch1a-ICD in miR-30a overexpression embryos rescued hypoproliferation and partially normalized ISV EC numbers (Figure 3F–3I).

Because miR-30a has the same seed as the other miR-30 family members, we next tested whether overexpression of other miR-30 family members can phenocopy the vascular phenotypes observed in miR-30a gain-of-function embryos. We selected miR-30e and in line with the computational prediction, overexpression of miR-30e consistently induced hyperbranching of ISVs both in Tg(fli1a:egfp)y1 embryos and in Tg(fli1a:egfp)y1 x Tg(kdrl:hras-mCherry)hsp double transgenic zebrafish embryos (Figure S3A–S3D, quantification in Figure S3E). Similar to miR-30a gain-of-function, miR-30e
gain-of-function embryos showed more ECs per ISV (Figure S3). Furthermore, overexpression of miR-30a in GFP-dll4-3′ UTR RNA-injected embryos reduced GFP expression (Figure S3F; compare column 2 middle with column 1), and elimination of the miR-30 binding site in the dll4-3′ UTR reporter annihilated this response (compare column 4 middle with column 3), thus confirming that the response was specific for the miR-30 binding site. Taken together, miR-30a and miR-30e share the same seed, and both can target dll4 and stimulate angiogenic cell behavior in zebrafish embryos. Similar effects were reported for miR-30b and miR-30c overexpression.25 These data suggest that because all miR-30 family members share an identical seed, they all have the potential of targeting dll4 in gain-of-function experiments.

miR-30a Targets Dll4-Notch Signaling in Human ECs

The miR-30 family is conserved between human and zebrafish (Figure S1E), and so are the binding sites for miR-30a in the dll4-3′ UTR (Figure 4A and Figure S1E). On the basis of this high degree of sequence conservation, we postulated that similar to zebrafish, in human ECs miR-30a targets DLL4 and modulate angiogenesis. To test this, we performed loss-and gain-of-function experiments in human ECs (Figure 5). Consistent with our zebrafish data, we found that overexpression of miR-30a enhanced angiogenic cell behavior, whereas miR-30a knockdown results in reduced sprouting (Figure 5A–5J). Knockdown of miR-30a augmented DLL4 protein expression =2-fold, compared with controls (Figure 5K) and activated downstream Notch signaling as indicated by Notch reporter assays using 2 independent (TP1 and CBF1) reporter constructs (Figure 5L and 5M). Specific mutations in the CBF1 response elements in the Notch promoter annihilated the activating response indicating that the effect of miR-30a is NOTCH specific (Figure 5M). Activation of NOTCH signaling on loss of miR-30a was, furthermore, demonstrated by upregulation of the NOTCH downstream targets HEY2 and ephrin-B2 (EFNB2; Figure 5N and 5O). These data are consistent with a model in which loss of miR-30a in human ECs induces DLL4, resulting in activation of NOTCH receptor signaling and restriction of angiogenic cell behavior.

Discussion

Rafectration of small arterioles and capillaries increases peripheral resistance. An increase in peripheral resistance is the main cause of increased arterial blood pressure in hypertension. Activation of angiogenesis reduces peripheral resistance and stimulation of angiogenesis in young spontaneously hypertensive rats normalizes pressure in adult spontaneously hypertensive rats, suggesting that angiogenesis could be a therapeutic target for hypertension.1 We investigated whether or not we can modulate angiogenesis by interfering with miRNAs that target DLL4, a conserved key inhibitor of angiogenic cell behavior and arteriolar branching, in a notch-dependent manner. Conversely, loss of miR-30a increases dll4 and reduces branching morphogenesis. The influence of miR-30a on branching could be traced to an effect on tip cell differentiation. Loss of miR-30a impairs tip cell formation, whereas miR-30a overexpression induces hyperactive endothelial tip cells, displaying numerous filopodia extensions culminating into enhanced vessel branching. In time-lapse imaging,
Optimal levels of miR-30 in tip/stalk cells and their members may enable customized outputs in microvascular network formation. Given its importance, it may not be surprising that in ECs miR-30a can be regulated or fine-tuned by small changes in Dll4 dosage. In human umbilical vein EC, loss of miR-30a augments DLL4 protein levels and activates NOTCH signaling via increased Dll4 levels, thereby contributing to rarefaction in essential hypertension. Given that overexpression of DLL4 and induces hyperactivity upstream of the Dll4-Notch signaling pathway and is an important therapeutic target in many cancers. Hypertension has been commonly reported in all clinical trials testing inhibitors of angiogenesis with an incidence ranging from 11% to 43% in all studies. The mechanism of elevated blood pressure in patients treated with antiangiogenic agents is not fully understood but probably involves endothelial dysfunction and capillary rarefaction. Taken together, our findings have therapeutic implications for treatment strategies in hypertension and modulation of miRNA activity using antagonirs (miRNA inhibitor), or lipid-based nanoparticle delivery of miRNAs seems efficient.

Figure 5. Loss of miR-30a upregulates delta-like 4 (DLL4) and activates NOTCH signaling in human endothelial cells (ECs). A–D, Images of human umbilical vein endothelial cell spheroids after transfection with an inhibitor of miR-30a (miR-30a-LNA) or miR-30a-precursor (ctrl-precursor). E–H, Confocal micrographs of spheroids transfected as (A–D) stained with calcein (green) and 4′,6-diamidino-2-phenylindole (DAPI; blue) for quantification of nuclei number. I. Number of ECs in angiogenic front. J, Taqman analysis of miR-30a expression after transfection with miR-30a inhibitor (miR-30a-LNA) and miR-30a-precursor compared with controls. K, Western blot analysis of DLL4 after treatment with miR-30a inhibitor or control; note upregulated DLL4 in miR-30a knocked down group. L, TP1 promoter construct driving luciferase and (M) CBF1 promoter construct driving luciferase; to verify NOTCH specificity CBF1 elements were mutated in CBF1-mut. NOTCH signaling is upregulated by miR-30a inhibition. N and O, Relative mRNA expression of NOTCH downstream targets Hey2 and EFNB2. Both are upregulated in cells treated with miR-30a inhibitor (miR-30a-LNA). *P<0.05; **P<0.01; and ***P<0.001, Student t test. NT indicates not treated.

Perspectives
Our work draws attention to Dll4-Notch as a possible signaling mechanism involved in angiogenesis and possibly in rarefaction. We identify a miRNA, miR-30a, that when stimulated

we observe proliferation of tip cells in developing arteriolar branches all resembling the vascular phenotypes previously reported in dll4-notch loss- and gain-of-function embryos. In human umbilical vein EC, loss of miR-30a augments DLL4 protein levels and activates NOTCH receptor signaling pathways. In developing sprouts, angiogenic cell behavior largely depends on the Notch signaling status. These data suggest that in ECs miR-30a may act cell-autonomously to fine-tune DLL4 levels, thereby influencing Notch signaling status, and EC fate.

All miR-30 family members have an identical seed sequence allowing adequate physiological control of angiogenesis and vessel branching. At present, information on the contribution of these miRNA in microvascular remodeling events associated with hypertension is lacking. Conceivably, miRNA expression could be investigated in early developmental stages, before the onset of hypertension, in experimental animal models and man. In secondary forms of hypertension, in which case microvascular rarefaction could be an adaptive response to increased systemic pressure and serves to autoregulate tissue flow, changes in local hemodynamics factors may alter miRNA expression, allowing vascular regression involving Notch signaling.

We believe these findings are highly clinically relevant because the converse experiment has been performed, even in man. Vascular endothelial growth factor signals angiogenesis upstream of the Dll4-Notch signaling pathway and is an important therapeutic target in many cancers. Hypertension has been commonly reported in all clinical trials testing inhibitors of angiogenesis with an incidence ranging from 11% to 43% in all studies. The mechanism of elevated blood pressure in patients treated with antiangiogenic agents is not fully understood but probably involves endothelial dysfunction and capillary rarefaction. Taken together, our findings have therapeutic implications for treatment strategies in hypertension and modulation of miRNA activity using antagonirs (miRNA inhibitor), or lipid-based nanoparticle delivery of miRNAs seems efficient.
inhibits DLL4, permitting more branching. These findings draw attention to novel mechanisms and potential hypertension-relevant targets, accrued from models in which blood pressure cannot even be measured.

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**Disclosures**
None.

**References**

**Novelty and Significance**

**What Is Known?**

- Activation of blood vessel growth, angiogenesis, can restore blood pressure in hypertension models.
- MicroRNAs (miRs) contribute to angiogenesis and vessel remodeling.
- Angiogenesis involves formation of endothelial tip cells.
- The delta-like 4/Notch signaling pathway is a key regulator of tip cell formation and angiogenesis.

**What Is Relevant?**

- The miR-30 family member miR-30a is expressed in vascular endothelial cells.
- Knockdown of miR-30a reduces angiogenesis.
- Overexpression of miR-30a stimulates angiogenesis and vessel branching.
- miR-30a controls tip cell differentiation allowing vessel branching morphogenesis by targeting delta-like 4 and Notch signaling.

**Summary**

Microvascular rarefaction is a hallmark of essential hypertension. Preventing rarefaction by activation of angiogenic processes associates with lower arterial pressure. Angiogenesis involves differentiation of endothelial cells into tip cells and is modulated by miRs. Delta-like 4-Notch signaling acts as an inhibitor of tip cell differentiation. We show that miR-30a targets delta-like 4, determining Notch signaling status and angiogenesis. The data indicate that miR-30 mimetic may be used to stimulate angiogenesis and arteriolar branching, which has relevance to the rarefaction process and, therefore, to hypertension.
miR-30a Regulates Endothelial Tip Cell Formation and Arteriolar Branching
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MIR-30A REGULATES ENDOTHELIAL TIP CELL FORMATION AND ARTERIOLAR BRANCHING

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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)\(^{\gamma1}\), Tg(fli1:negfpEGFP)\(^{\gamma7}\), Tg(kdrl:hras\(-\)mCherry)\(^{s896}\), Tg(hsp70:Gal4)\(x\)Tg(uas:notch1a-ICD), and Tg(kdrl:ras\(-\)cherry)\(^{s916}\)xTg(flt1\(\text{BAC}\):yfp) double transgenic zebrafish lines were used as described.\(^1\) 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’-CTTCCAGTCGGGAATGTTTACAACT-3’;
dre\(-\)miR\(-\)30a-MO-2 (15 ng), 5’-CAACTTCCAGTCGGGAATGTTTACA-3’;
dre\(-\)miR\(-\)30b-MO (15 ng), 5’-AGTGTAGGATGTTTACAGCGACTAC-3’;
dre\(-\)miR\(-\)30c-MO (15 ng), 5’-CAGCTGAGAGTGTAGGATGTTTACA-3’;
dre\(-\)miR\(-\)30d-MO (3.5 ng), 5’-GGGATGTTTACAGGCATGAACAACC-3’;
dre\(-\)miR\(-\)30e-MO (15 ng), 5’-CTTCCAGTCGGGAATGTTTACA-3’;
dll4-MO (7 ng), 5’-GTTCGAGCTTACCGGCCACCCAAAG-3’;
control-MO (15 ng), 5’-CTCTTACCTCAGTTACAATTATA-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.\(^7\), \(^8\) 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 Not1 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). \textit{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)y1 or Tg(kdr:ras-cherry)s916xTg(fli1BAC: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 phospahate 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 10^7 cells/mL. FACS was performed on a FACS Aria2 (BD Biosciences), and approximately 1x10^6 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. Transient transfections of HUVECs were carried out by electroporation with Amaxa HUVEC nucleofector Kit (Lonza). 1.6x10^6 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 antagonim (miR-30a antago, Ambion® Anti-miR™ microRNA Inhibitor) Life Technologies, as indicated. For miR-30a overexpression miR-30a-precursor 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.

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<th>Primer</th>
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<td><em>in situ</em> pri-miR-30a F</td>
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<td><em>in situ</em> pri-miR-30a R</td>
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<td>dll4-3’UTR R</td>
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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-30a 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:egfp)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:egfp)y7xTg(kdrt: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).