RESEARCH ARTICLE

Etv2-miR-130a-Jarid2 cascade regulates vascular patterning during embryogenesis

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Abstract

Remodeling of the primitive vasculature is necessary for the formation of a complex branched vascular architecture. However, the factors that modulate these processes are incompletely defined. Previously, we defined the role of microRNAs (miRNAs) in endothelial specification. In the present study, we further examined the Etv2-Cre mediated ablation of DicerL/L and characterized the perturbed vascular patterning in the embryo proper and yolk-sac. We mechanistically defined an important role for miR-130a, an Etv2 downstream target, in the mediation of vascular patterning and angiogenesis in vitro and in vivo. Inducible overexpression of miR-130a resulted in robust induction of vascular sprouts and angiogenesis with increased uptake of acetylated-LDL. Mechanistically, miR-130a directly regulated Jarid2 expression by binding to its 3’-UTR region. Over-expression of Jarid2 in HUVEC cells led to defective tube formation indicating its inhibitory role in angiogenesis. The knockout of miR-130a showed increased levels of Jarid2 in the ES/EB system. In addition, the levels of Jarid2 transcripts were increased in the Etv2-null embryos at E8.5. In the in vivo settings, injection of miR-130a specific morpholinos in zebrafish embryos resulted in perturbed vascular patterning with reduced levels of endothelial transcripts in the miR-130a morphants. Further, co-injection of miR-130a mimics in the miR-130a morphants rescued the vascular defects during embryogenesis. qPCR and in situ hybridization techniques demonstrated increased expression of jarid2a in the miR-130a morphants in vivo. These findings demonstrate a critical role for Etv2-miR-130a-Jarid2 in vascular patterning both in vitro and in vivo.

Introduction

Endothelial precursors (angioblasts) emerging from the nascent mesoderm proliferate, migrate and coalesce to form the primitive vascular plexus [1]. Remodeling of the primitive vasculature is necessary for the formation of a complex vasculature by the process of angiogenesis [1, 2]. Formation of blood vessels is a complex multistep process that requires precise control and coordination of endothelial cell behavior and other environmental cues [1, 3, 4]. Transcriptional networks and signaling pathways that regulate these processes are not completely known [3, 5, 6].
Given the complexity of the vascular system, a variety of factors and pathways are known to be involved in the modulation of the angiogenic response [1, 3, 7]. Etv2 (Ets-family transcription factor) is expressed in the earliest endothelial progenitors and shown to be essential for both endothelial and hematopoietic lineages during development [8–10]. Studies have shown that Etv2 plays a critical role in mesodermal lineage specification by modulating the transition of Flk1+/Pdgfra− primitive mesodermal lineages [11]. In the absence of Etv2, the formation of Flk1+/Pdgfra− lateral plate mesodermal cell population is perturbed during embryogenesis [11, 12]. The global knockout of Etv2 results in embryonic lethality by E9.5 due to the complete absence of the hematopoietic and endothelial lineages [8, 9, 11]. Previous studies have established that Etv2 gene expression is modulated by Wnt, BMP and Notch signaling factors to regulate hematopoiesis [13]. Multiple studies have demonstrated that regulation of the hematopoietic and endothelial lineage by Etv2 is mediated through its interaction with Gata2, Scl, Lmo2, Tie2, Foxc2 and Vegf signaling [5, 9, 13–16]. We and others have shown that the hierarchical relationship between Etv2, Vegf, p38 MAP kinase and CREB signaling in the modulation of the hematopoietic and endothelial lineages [15–17]. Together these reports establish that Etv2 is a master regulator of hematopoietic and endothelial lineage development [18–20].

Several growth factors and signaling cascades including VEGF, FGF, SHH and MAP kinase have been shown to promote angiogenesis by promoting endothelial cellular (EC) proliferation, cell–cell interaction and migration during development and patho-physiological conditions [21–24]. Other factors including PDGFR-β are indispensable for the synergistic effect of VEGF-A and FGF-2 on neoangiogenesis in adults [21]. Deciphering additional factors that regulate vascular development and angiogenesis would be of intense interest for the field. While the transcriptional regulation of endothelial development is documented, the role of other regulatory mechanisms, including miRNAs, during vascular development is unknown.

MicroRNAs (miRNAs) are a class of small non-coding RNA that suppress gene expression by targeting mRNAs at the 3′-UTR for cleavage and/or degradation [25]. More than 1000 miRNAs have been identified in mammals that are involved in critical steps during development and pathological conditions [26]. Large pre-miRNAs are processed in multiple steps to give rise to a mature miRNA. Maturation of pre-miRNAs into a mature form (~20–22 nucleotides) is mediated by Dicer, a miRNA processing enzyme [27]. Global deletion as well as hypomorphic mutants (deleting exon 1 and 2) of Dicer results in embryonic lethality [27, 28]. Previously, we combinatorially mated Etv2-Cre and DicerL/L mice to generate conditional Dicer knockouts [29]. These studies identified miR-130a as an essential factor in the specification of the endothelial lineage [29]. Furthermore, miR-130 was identified as the first factor to specifically promote hematopoietic and endothelial progenitor divergence to the endothelial lineage [29]. In the present study, we determined that the loss of Dicer in the endothelial precursors resulted in defective vascular remodeling and patterning. We further defined an additional role for miR-130a as an important regulator of angiogenesis and vascular patterning both in vitro and in vivo via its regulation of Jarid2.

Materials and methods

Generation of Dicer conditional null mice

All studies using animals were approved by the Institutional Animal Care and Use Committee of the University of Minnesota. We characterized the lethality of the conditional knockout embryos by examining the progeny of Etv2Cre+;DicerL/+ and DicerL/L mice. Genotyping was performed using specific PCR primers and standard procedures.
Morphological analysis and whole-mount staining

For morphological analysis, embryos from time-mated females were harvested at distinct stages and imaged using the Zeiss Axios Observer Z1 inverted microscope. Whole-mount staining for the endothelial architecture was performed as described [11, 30]. Briefly, time-mated embryos were fixed in 4% paraformaldehyde (PFA) at 4°C for 6 h, washed in PBT (PBS, 0.1% Tween 20) followed by dehydration through a graded series of methanol (25%, 50%, 80%, and 100%). Dehydrated embryos were rehydrated in decreasing methanol concentration and the endogenous peroxidase activity was quenched with 3% H2O2. Embryos were washed in Pblec [PBS, 1% Tween 20, 1 mM CaCl2, 1 mM MgCl2, and 0.1 mM MnCl2 (pH 6.8)], and incubated with rat anti-endomucin antibody (1:30 dilution; in blocking buffer), followed by incubation with anti-rat Cy3-conjugated secondary antibody (1:100 dilution; in blocking buffer) overnight at 4°C. After washing with PBT (6 times), embryos were post-fixed in 4% PFA and analyzed. For vascular branching analysis, the numbers of angiogenic vessels were counted from the 2D-image. For each genotype, five different embryos were scored and quantified.

Histology and immunohistochemistry of embryos

Stage specific embryos were harvested from time-mated pregnant females. Embryos were fixed in 4% PFA overnight at 4°C and embedded. Histological sectioning was performed according to standard protocols [31]. Immunohistochemistry was performed on paraffin embedded sections (10 μm) using standard procedures [11, 24, 32]. Briefly, sections were rehydrated and heated in antigen retrieval solution (DAKO cytometry) for 20 min at 95°C, and blocked with 10% normal donkey serum at room temperature. Blocked sections were incubated overnight at 4°C with rat anti-endomucin (1:300, Abcam), mouse anti-CD31 (1:200, BD Bioscience) and chicken anti-GFP (1:400, Abcam). Sections were washed and incubated with Cy3- (1:500 dilution, Jackson ImmunoResearch Laboratories), and alexa488- (1:500, Abcam) conjugated secondary antibodies and were imaged using a Zeiss Axios Imager M1 upright microscope and AxioVision software. Vascular density of the transverse sections from 4–5 different embryos was quantified using ImageJ (1.47v) software. Embryo sections at three different levels for each embryo were examined and the vascular density was quantified.

ES cell culture and ES/EB differentiation

Mouse embryonic stem (ES) cell culture and ES/EB differentiation was performed as described elsewhere [15, 16]. The wild-type mouse E14 ES cell line was maintained in Knockout Medium (Invitrogen) supplemented with 15% FBS (Benchmark), 1000 U/ml LIF (Millipore), glutamine (HyClone), 0.1 mM non-essential amino acids (NEAA) and 0.1 mM β-mercaptoethanol (Sigma) in gelatin-coated tissue-culture plates. The ES cells were differentiated into embryoid bodies (EBs) by the hanging drop method using mesodermal differentiation media containing IMDM (Invitrogen), 15% FBS, penicillin/streptomycin, 2 mM GlutaMAX, 50 mg/ml Fe-saturated transferrin (R & D Systems), 0.1 mM monothioglycerol (Sigma), and 50 mg/ml ascorbic acid (Sigma). Immunohistochemical analyses were performed using a standard protocol with anti-CD31 (1:200, BD Pharmingen) sera as previously described [29]. For EdU-incorporation assay, differentiating EBs was treated with EdU (10μM) for a period of 4h prior to harvest time point. The dissociated cells well fixed in 4% PFA for 10 min at room temperature, permeabilized and stained using Click-IT EdU kit (Thermofisher) for analysis.
Flow cytometric analysis and sorting
FACS analysis was performed using BD FACSAria II (BD Biosciences, San Diego, CA, USA) as described previously [9, 17]. Stage specific embryos were separated from yolk sacs, and digested with 0.25% trypsin (Hyclone) to obtain a single cell suspension as previously described [11]. Cells were incubated with antibody cocktails for 30 minutes at 4˚C, washed, and re-suspended in PBS with 2% FBS. Cocktails of antibodies CD31-APC (eBiosciences 25–0311), and VE-cadherin (BD Pharmigen) were used in this study, washed and re-suspended in FACS buffer (PBS/1%FBS). Cells were analyzed or sorted using FACSAria II (BD Biosciences). FACS data were quantified using data obtained from three independent experiments. AnnexinV-FITC labeling was performed according to the manufacturer’s protocol.

RNA isolation and quantitative gene expression analysis
Total RNA was isolated from EBs at various time-points during ES/EB differentiation using miRVANA RNA isolation kit (Ambion), and cDNA was synthesized using superscript cDNA synthesis kit (Invitrogen) as per standard protocol. Quantitative RT-PCR was performed using ABI Taqman probe sets. Probes used include VIC labeled Gapdh: 4352339E, FAM labeled Timp2: Mm00441825_m1; Robo1: Mm00803879_m1; Robo2: Mm00620713_m1; Notch1: Mm00435245_m1; Hoxa5: Mm04213381_s1; Jarid2: Mm00445574_m1; Slit1: Mm01198620_m1; hsa-miR-130a:000454; U6 snRNA:01973.

Matrigel-sandwich angiogenesis assay
For the angiogenesis assays, miR-130a iESCs were differentiated using a previously described protocol [29]. Doxycycline was added to the differentiating EBs from d2-d6 during differentiation. Uninduced and induced EBs were plated on growth factor reduced matrigel (BD Biosciences) containing 5% serum at day 8 of differentiation. Sprouting EBs were imaged at day 3 and day 6 following plating using an inverted microscope (Leica) supported with AxioVison (version 4.8). These assays were performed three times and in triplicate.

Cell transfection and luciferase assays
HEK cells were transfected using Lipofectamine 3000 (Invitrogen) according to the manufacturer’s protocols. Briefly, for the miR-130a target assay, HEK cells were transfected using Lipofectamine 3000 (Invitrogen) with 2 μg of pCMV-miR-130a and 0.1 μg/well of PGK-Luc-Jarid2-3’-UTR and PGK-Luc-Jarid2-3’-UTR mutant constructs in 6-well plate. Transfected cells were harvested after 48 hrs and activities of firefly and Renilla luciferases were measured sequentially using the Dual-Luciferase Reporter Assay system (Promega) and a standard luminometer (Berthold Detection Systems, Sirius). Luciferase activities were expressed in relative light units that were normalized to the transfection efficiency using the Renilla luciferase activity.

Zebrafish morpholino and in situ hybridization experiments
Zebrafish embryos were injected with 2.5 ng/embryo of mismatch control or miR-130a morpholinos at the one-two cell stage following standard protocols and approved by the UMN IACUC. Mismatch control or miR-130a morpholinos injected transgenic zebrafish Tg(fli1a: EGFP) embryos were analyzed at 48 hpf and 72 hpf. Perturbed ISV were quantified and plotted against total number of ISVs in the trunk region from control and miR-130a injected embryos. Quantification of the number of EGFP+ cells was performed using ImageJ software (NIH) and data obtained as mean intensity of the field reflecting the cell populations in each embryo. For FACS analysis, zebrafish embryos (n = 45) were dissociated using a buffer containing
collagenase (1 mg/ml) for 5 min at 37˚C. Dissociated cells were stained with propidium iodide (PI) and analyzed using FACS (FACSAria II, BD Biosciences). Embryos (n = 20) from each group were quantified and analyzed in triplicate. The following antisense MO oligonucleotides were designed by and obtained from the Gene Tools LLC as previously described [29]. miR-130a-MO: 5’-CAATGCCCTTTATACATTGCACTGC-3’ miR-130a mis-MO-1 (Control MO-1): 5’-CAATCCCaTTTTAAaATaCaATCaC-3’ (lower case represents mismatch bases) and miR-130a mis-MO-2 (Control MO-2): 5’-CAATGaCCaTTTAACaATaGCaTGC-3’ (lower case represents mismatch bases). Whole mount in situ hybridization of 48 hpf zebrafish embryos was performed using standard procedures as described previously [33]. Zebrafish riboprobes included: cdh5, kdrl and jarid2a. For the zebrafish in situ hybridization experiments, 25–30 embryos were examined for each set. For the rescue experiments, miR-130a morpholinos together with negative control oligos or miR-130a mimics were injected at the one-two cell stage following standard protocols and analysed at 72hpf.

**Zebrafish RNA isolation and quantitative gene expression experiments**

Zebrafish embryos were injected with 25 ng/embryo of mismatch control or miR-130a morpholinos at the one-two cell stage [33]. For total RNA isolation, a pool of 25–30 embryos were harvested in lysis buffer using miRVANA RNA isolation kit (Ambion), and cDNA was synthesized using the superscript cDNA synthesis kit (Invitrogen) as per standard protocol. qRT-PCR was performed for jarid2a using specific primers obtained from IDT. Primers specific for gapdh was used as a control.

**Tube formation assay**

Tube formation assays were performed using HUVEC cells as described elsewhere [34]. Briefly, for tube formation assays, HUVECs were transfected with Jarid2 constructs (0.25μg) using a nucleofector kit (Lonza), followed by plating 0.6 x 10⁵ cells on a 24-well plate coated with Low Growth Factor Matrigel (BD Biosciences, San Jose, CA) and supplemented with 50ng/ml of VEGF₁₆₅ (R&D Systems, MN) in serum-free media. The tubes were imaged at 10X magnification and quantified from 4 different fields. Co-transfection of Jarid2 and miR-130a (100nM) was performed as described above.

**miRNA pull down assay**

A biochemical approach was used to confirm the interaction of miR-130a with Jarid2 mRNA as described elsewhere [35]. Briefly, 3’-biotinylated miRCURY LNA miR-130a-3p mimics (cat# 479997–671, design id #713696) and a negative control (cat# 479997–671, design id #713697) were purchased from Exiqon. Mouse ES cells were transfected with 30 pmol of the miRNA mimics using RNAiMAX reagent (cat# 13778–030, Thermo Fisher). After 24 hours of transfection, cells were harvested and cytosolic extracts were obtained in lysis buffer. mRNAs bound to the biotinylated-miRNAs were then pulled down using Streptavidin-Dynabeads (cat# 11205D, Thermo Fisher). The beads were washed and treated with RNase-free DNase to remove any residual DNA. The pulled down RNA was eluted and cDNA was synthesized using the SuperScript™ III First-Strand Synthesis SuperMix (cat# 18080400, Thermo Fisher). The abundance of Jarid2 mRNA in the pulldown samples was assessed using qPCR and normalized to the levels of Gapdh as a negative control.
RNA-electrophoretic mobility shift assay (EMSA)

RNA-EMSAs were performed as described elsewhere [36]. Briefly, RNA oligos were ordered corresponding to the mature form of miR-130a-3p (5’-CAGUGCAAUUGUAAGAGGGCAU-3’), a 21-mer sequence of the Jarid2 3’-UTR (5’-Auagcuacccacauugcacu g-3’) containing the target site for miR-130a and a scrambled control (5’-GGuuAACuCGCCAAu-GAuCCu-3’) from IDT. The miR-130a-3p sequence was labeled with 5’-IRDye 700. Labeled miR-130a-3p probes (200 nM) were incubated with the corresponding target or scrambled RNA molecules in the presence of 10 mM MgCl$_2$, 100 mM NaCl, 50 mM Hepes pH 7.2 and 5% glycerol for 30 minutes at 37˚C. Binding reactions were run in a 12% polyacrylamide gel for 3 h at 120 V (4˚ C) in 1X TBE. Gels were scanned using a LI-COR Odyssey CLx system and assembled.

Statistical analysis

All experiments were repeated at least three times and the data represent the mean ± SEM. Images were analyzed using ImageJ software (NIH). Statistical significance was determined using the Student’s $t$-test and differences are considered significant with a $p$-value of < 0.05 and very significant; $p$ < 0.01.

Results

Etv2-Cre-mediated Dicer deletion results in vascular patterning defects

Etv2 (Ets-family transcription factor) marks the earliest hematopoietic and endothelial progenitors [9, 20]. Homozygous global deletion of Etv2 results in complete loss of hematopoietic and endothelial lineage and embryonic lethality [8, 9]. We previously generated Etv2$^{Cre+;Dicer^{L/L}}$ progeny and observed that these conditional mutant embryos were lethal by E12.5 [29]. Therefore, our initial analysis focused on the lethality of the Etv2$^{Cre+;Dicer^{L/L}}$ embryos. Analysis of the stage specific Dicer$^{L/L}$ and Etv2$^{Cre+;Dicer^{L/L}}$ embryos were indistinguishable at E8.0, however, we found significantly reduced size at E9.5 as compared to the control littermates (S1A and S1B Fig). We next performed immunohistochemical analysis using endomucin antibodies at two developmental stages, E9.5 and E10.5, to visualize the endothelial development in Dicer$^{L/L}$ and Etv2$^{Cre+;Dicer^{L/L}}$ embryos. Immunohistochemical analysis of E9.5 embryos (whole mount imaging as well as tissue sections) revealed the presence of major vessels including the dorsal aorta (da) and cardinal vein (cv) in both Dicer$^{L/L}$ and Etv2$^{Cre+;Dicer^{L/L}}$ embryos (Fig 1A–1D). Although the initial vascular structures were present in the mutant embryos, higher magnification revealed poorly developed vascular plexuses with reduced vascular branching in Etv2$^{Cre+;Dicer^{L/L}}$ as compared to Dicer$^{L/L}$ embryos (Fig 1A’ and 1B’). Reduced plexus formation was also evident in the transverse sections of E9.5 embryos with fewer endomucin-positive vessels in the mutant embryos (Fig 1C and 1D). Quantitative analysis within the paraxial mesoderm revealed significantly reduced vascular density in the Etv2$^{Cre+;Dicer^{L/L}}$ embryos relative to the Dicer$^{L/L}$ control littermates (Fig 1E). To verify these results, we undertook immunohistochemical analysis using anti-CD31 antibodies using Dicer$^{L/L}$ and Etv2$^{Cre+;Dicer^{L/L}}$ embryo sections at E9.5. Although the initial vascular structures were present in the mutant embryos, higher magnification revealed poorly developed vascular plexuses with reduced vascular branching in Etv2$^{Cre+;Dicer^{L/L}}$ as compared to Dicer$^{L/L}$ embryos (Fig 1A’ and 1B’). Reduced plexus formation was also evident in the transverse sections of E9.5 embryos with fewer endomucin-positive vessels in the mutant embryos (Fig 1C and 1D). Quantitative analysis within the paraxial mesoderm revealed significantly reduced vascular density in the Etv2$^{Cre+;Dicer^{L/L}}$ embryos relative to the Dicer$^{L/L}$ control littermates (Fig 1E). To verify these results, we undertook immunohistochemical analysis using anti-CD31 antibodies using Dicer$^{L/L}$ and Etv2$^{Cre+;Dicer^{L/L}}$ embryo sections at E9.5. Consistent with the endomucin staining, we observed reduced levels of CD31 staining in the Etv2$^{Cre+;Dicer^{L/L}}$ sections as compared to Dicer$^{L/L}$ sections (S1C, S1C’, S1D and S1D’ Fig). To further validate these results, we crossed Etv2$^{Cre+;Dicer^{L/L}}$ Etv2-EYFP:Dicer$^{L/L}$ lines and performed immunohistochemical analysis using anti-CD31 and anti-GFP antibodies at the somite level to visualize inter-somitic vessels. Our analysis revealed a marked reduction in the expression of both EYFP and CD31 in these regions of Etv2$^{Cre+;Dicer^{L/L}}$ embryos as compared to Dicer$^{L/L}$ embryos (S1E–S1H Fig). By E10.5, we observed a reduced number of vessels...
Fig 1. Etv2-Cre-mediated Dicer deletion results in vascular abnormalities. A-D. Representative images of whole-mount embryos (A, B) and transverse sections (C, D) labeled with endomucin antibody (red) and counterstained with DAPI (blue) in the developing embryos at E9.5. Higher magnification (A', B') of the boxed region in panel A and B. E. Quantification of the boxed areas in panel C and D. F-K. Whole-mount endomucin labeled (red) images of wild-type (F, I) and mutant (G, J) embryos at E10.5 in the trunk (F, G [yellow brackets]) and middle cranial region (I, J). H, K. Quantitative analyses of vascular branching and cranial vessels at E10.5. Error bars indicate SEM (n = 4; *p < 0.05). Scale bar: 500 μm (A, B, F, G, I and J); 200 μm (A', B', C and D).

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in the trunk-region and perturbed branching of cranial vessels in the \textit{Etv2}^{Cre/+};\textit{Dicer}^{L/L} embryos (Fig 1F–1K).

To examine whether the vascular defects were due to altered endothelial lineages, we undertook FACS analysis to assess the endothelial cell populations in the \textit{Etv2}^{Cre/+};\textit{Dicer}^{L/L} and \textit{Dicer}^{L/L} embryos. FACS analyses at E10.5 using CD31 and VE-cadherin antibodies showed significantly reduced number of endothelial cell populations in the \textit{Etv2}^{Cre/+};\textit{Dicer}^{L/L} embryos compared to the wild-type littermates (Fig 2A–2C). We next evaluated vascular patterning in the developing yolk-sac to further characterize these \textit{Etv2}^{Cre/+};\textit{Dicer}^{L/L} embryos. Similar to the embryo proper, the whole-mount imaging of the yolk-sac showed perturbed vascular development and were pale colored consistent with decreased blood content (Fig 2D). Further, whole-mount endomucin immunohistochemistry of the yolk sac revealed poorly developed vascular plexuses in the mutants (Fig 2E).

These observations suggested that the loss of \textit{Dicer} in the endothelial precursors resulted in defective vascular remodeling. Overall, these results supported the hypothesis that Dicer and \textit{miRNAs} were required for angiogenesis during development.

**miR-130a regulates angiogenesis in vitro**

Previously, we identified \textit{miR-130a} as an endothelial enriched \textit{miRNA} and established its role in endothelial specification [29]. To further examine the role of \textit{miR-130a} during the endothelial development, we first utilized the Dox-inducible mouse ES cell line to over-express \textit{miR-130a} and determined whether \textit{miR-130a} could modulate angiogenic functions using a matrigel sandwich sprouting assay. Uninduced and induced \textit{miR-130a} iES/EBs at d6 were plated on a growth factor-reduced Matrigel sandwich supplemented with VEGF (50ng/ml) and 5% serum medium (Fig 3A). Dox-mediated induction of \textit{miR-130a} resulted in a robust increase of EBs with angiogenic sprouts (35–40%) relative to the uninduced EBs (12–15%) [Fig 3B and 3C]. We next performed immunohistochemical analysis to determine whether the sprouts were endothelial populations. Our immunohistochemical analysis using CD31 antibodies confirmed the presence of endothelial cells in the sprouting EBs (Fig 3B). We then quantified the spraying EBs at two different time points, d3 and d6, of plating to evaluate the role of \textit{miR-130a} in the angiogenic response. Quantitative analysis revealed extensive sprout formation both at d3 (3-fold) and d6 (2.5-fold) in the presence of Dox (+Dox) as compared to the controls (-Dox) (Fig 3C). To determine whether these angiogenic sprouts consisted of functionally active endothelial cells, we performed an acetylated low-density lipoprotein (Ac-LDL) incorporation assay. Our fluorescence microscopic analysis revealed Ac-LDL uptake by the migrating endothelial cells, further confirming the endothelial nature of these angiogenic sprouts (Fig 3D). These results confirmed the role of \textit{miR-130a} in angiogenesis. To evaluate whether the increased spraying was due to the prevention of cell death, we performed a cell death assay using Annexin V staining using the differentiating EBs. FACS analysis of d6 uninduced and induced EBs revealed no significant differences in the percentage of Annexin V staining (Fig 3E and 3F). Furthermore, fluorescence microscopy did not show any difference in the number of Annexin V stained cells (S2 Fig). We then monitored the proliferative response following induction of \textit{miR-130a} in the differentiating EBs. FACS analysis using a ClickIT-EdU assay kit revealed that Dox-mediated induction of \textit{miR-130a} resulted in a modest but significant increase in the percent of EdU+ cells as compared to uninduced EBs (Fig 3G and 3H).

Overall, these results indicated that the increased angiogenesis and spraying following the induction of \textit{miR-130a} was not solely due to the prevention of cell death or enhanced cellular proliferation.
Fig 2. Etv2-Cre-mediated Dicer deletion results in vascular abnormalities. A, FACS profile of endothelial populations (VE-cadherin and CD31) from Dicer^L/L and Etv2-Cre/Dicer^L/L embryos at E10.5. B, C, Quantitative analysis of endothelial markers from Dicer^L/L and Etv2-Cre/Dicer^L/L embryos. Note the reduced percentage and absolute number of endothelial populations in Cre^+ embryos. D, Whole-mount imaging of yolk-sac from Dicer^L/L and Etv2-Cre/Dicer^L/L yolk-sacs. The absence of defined blood vessels were apparent in the Etv2-Cre/Dicer^L/L yolk-sac. E, Whole-mount endomucin labeled images of wild-type and mutant yolk-sac at E10.5. Note the defective vasculature in the yolk-sac of Etv2-Cre/Dicer^L/L embryos. Error bars indicate SEM (n = 3; *p < 0.05).

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**Fig 3. miR-130a regulates angiogenic response in vitro.**

**A,** Schematic showing the matrigel sandwich angiogenesis assay. **B,** Representative images of sprouting EBs from -Dox and +Dox conditions 6-days after plating are shown. Extensive endothelial [CD31 (red)] sprouting was observed in +Dox condition. **C,** Quantification of sprouting EBs at day 3 and day 6 of plating revealed significantly increased percentage of sprout formation upon induction of miR-130a. **D,** Ac-LDL uptake assay in EBs differentiated in the absence and presence of Dox. The cells migrating out of EBs incorporated Ac-LDL (red), indicating enhanced angiogenic response upon induction of miR-130a. Merged panel shows overlay with phase contrast images. **E, F,** FACS analysis and quantification of Annexin V-FITC labeled cells in the absence (-Dox) and presence (+Dox) of doxycycline. **G, H** FACS analysis and quantification of EdU labeled cells in the absence (-Dox) and presence (+Dox) of doxycycline. Error bars indicate SEM (n = 4; *p < 0.05, **p < 0.01).
**miR-130a regulates endothelial patterning in vivo**

Having established the role of miR-130a in promoting endothelial differentiation and angiogenic response in vitro, we assessed the in vivo function of miR-130a using morpholino (MO) oligonucleotide-mediated knockdown experiments in zebrafish embryos. We designed specific MOs and injected control mismatch and miR-130a MOs (25 ng/embryo) at the 1–2 cell stage and first performed qPCR analysis using RNA isolated from 60-pooled embryos from each condition for the endothelial transcripts at 48hpf. As compared to control morphants, qPCR experiments revealed significantly reduced expression of kdrl and tek in miR-130a morphants (Fig 4A and 4B). To validate these results, we performed in situ hybridization experiments using kdrl and cdh5 probes in the control and miR-130a morphants at 48 hpf. In situ hybridization experiments for kdrl and cdh5 showed perturbed vasculature with reduced expression of both kdrl and cdh5 in the inter-somitic vessels (ISVs) in the miR-130a morphants (Fig 4C). These results indicated an important role of miR-130a in vascular patterning in vivo. To further evaluate its function during the endothelial patterning, we utilized the fli1a:EGFP transgenic reporter line \[Tg(fli1a:EGFP)\], which expresses GFP in the developing vasculature [37]. As compared to control morphants, the injection of miR-130a MO did not cause detectable change in gross morphology of the developing embryos (Fig 4D and 4E). We did not observe any defects in the developing vasculature in the control morphants (Fig 4F and 4H). Although gross morphology of the developing embryos was not significantly changed (Fig 4D and 4E), we found multiple defective embryos with perturbed vasculature (Fig 4F–4I). Additionally, we found incompletely formed inter-somitic vessels (ISVs) in the miR-130a MO injected embryos at both 48 hpf and 72 hpf (Fig 4F–4K). These defects were observed in 70% ± 5% and 60% ± 7% (n = 30 embryos per experiment repeated three times) of the miR-130a MO injected embryos at 48 hpf and 72 hpf, respectively (Fig 4I and 4K). To evaluate the specificity of the injected MO, we designed a second control MO (control-MO-2) and performed similar experiments and observed the same results as the control-MO-1 injections (S3A–S3G Fig). These findings indicated the critical role of miR-130a in angiogenesis, vascular remodeling and sprouting process during development. Quantitative analysis of EGFP* using ImageJ software (NIH) revealed significantly reduced mean EGFP intensity in the miR-130a morphants as compared to control morphants (Fig 4L). Next, we performed FACS analysis to quantify the EGFP* endothelial populations following injection with control and miR-130a morpholinos at 48hpf. As observed with the qPCR and ImageJ analyses, FACS profiling of the Tg(fli1a:EGFP) zebrafish embryos revealed significantly reduced number of EGFP counts in the miR-130a morphants as compared to the control morphants (Fig 4M). To further demonstrate the role of miR-130a during vascular development, we performed rescue experiments using Tg(fli1a:EGFP) transgenic lines. We injected LNA-modified scrambled oligos and miR-130a mimics together with miR-130a morpholino at the 1–2 cell stage. Confocal microscopic analysis at 72hpf revealed that co-injection of miR-130a morpholino with scrambled oligos resulted in perturbed vasculature. These defects were rescued by co-injecting miR-130a morpholinos and miR-130a mimics (Fig 4N and 4O). Quantitative analysis at 72hpf showed 30% ± 2% (n = 70 embryos) embryos with severely defective vasculature in the miR-130a morpholino with scrambled mimic groups whereas, none were found to have defects in the miR-130a morpholino with miR-130a mimic groups (Fig 4O).

Overall, these in vivo experiments demonstrated the critical role of miR-130a in vascular remodeling and sprouting process during embryonic development.
Fig 4. miR-130a regulates endothelial patterning in vivo. A, B, qPCR analysis of endothelial transcripts, kdr1 and tek at 48hpf using RNA from control and miR-130a morphants. C, Whole-mount in situ hybridization images.
miR-130a targets Jarid2 expression during development

miRNAs can regulate the expression of multiple genes by binding to the 3′-UTR of the target genes [25]. To decipher the mechanism by which miR-130a regulates embryonic vascular development, we utilized three miRNA target prediction tools including TargetScan 6.2, PicTar and miRANDA to mine common predicted targets between mouse and zebrafish genomes. Among the several targets, we identified Jarid2 as a top-ranked candidate with a high percentile score using TargetScan 6.2 in both mouse and zebrafish genomes (Fig 5A and S1 Table). To validate these predicted targets, we utilized wild-type miR-130a inducible and CRISPR/Cas9-mediated miR-130a knockout ES/EB system and performed qPCR analysis at d6 of differentiation. Besides Jarid2, we also evaluated the expression of multiple miR-130a predicted targets including HoxA5, Robo-Slit signaling, Timp2 and Notch, that are shown to have a role in endothelial patterning using RNA isolated from wild-type EBs, miR-130a iEBs and miR-130a Δd6 EBs (S4A–S4F Fig). As expected the levels of HoxA5 (a known target of miR-130a [38]) was decreased upon Dox-mediated induction of miR-130a (S4A Fig). Accordingly, the levels of HoxA5 were increased in the miR-130a-null EBs relative to the wild-type EBs (S4A Fig). These results supported the previously defined role of miR-130a in the regulation of anti-angiogenic molecules (HoxA5) [38]. We found a similar expression pattern for Jarid2, Robo1 and Robo2 transcripts, whereas the expression of Slit1, Temp2 and Notch1 did not show any such trend in the wild-type EBs, miR-130a iEBs and miR-130a Δd6 EBs (Fig 5B and S4B–S4F Fig). Based on the bioinformatics analysis and qPCR results, we hypothesized that miR-130a target Jarid2, Robo1 and Robo2 transcripts by binding to the 3′ UTR of these genes in the regulation of endothelial patterning. An earlier study has demonstrated the role of miR-218 in vascular patterning by modulating Robo-Slit signaling [39]. Therefore, we focused on Jarid2 expression, as little is known about its role in vascular patterning and its miRNA-mediated regulation during development. Sequence alignment using Multiz align (UCSC genome browser/mm9) revealed two highly conserved miR-130a seed-sequences in the Jarid2-3′-UTR region (Fig 5A). To decipher whether miR-130a could bind to the Jarid2-3′-UTR region and regulate its expression, we performed luciferase assays using a reporter construct harboring the miR-130a binding motif of the Jarid2-3′-UTR region (PGK-Luc-Jarid2-3′-UTR). Co-transfection of HEK cells with PGK-Luc-Jarid2-3′-UTR and pCMV-miR-130a construct led to a robust and significant reduction (~35%) in luciferase activity. In contrast, co-transfection with mutated Jarid2 3′-UTR (Jarid2-3′ UTR mut), the pCMV-miR-130a construct resulted in unaltered luciferase activity (Fig 5C). Next, we performed miRNA pull down assay using biotinylated-miR-130a and demonstrated an interaction between miR-130a and Jarid2 mRNA. Our data showed ~1.75-fold enrichment of Jarid2 3′-UTR in the miR-130a transfected cells as compared to the scrambled control (Fig 5D). To further validate these results, we performed in vitro
miRNA regulates angiogenesis

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Figure A shows the regulation of angiogenesis by miRNA. Figure B illustrates the expression levels of miR-130a and its effects on angiogenesis. Figure C demonstrates the relative luciferase units for different constructs. Figure D shows the fold enrichment of Jarid2 in Wild-type EBs and BIO-miR-130a EBs. Figure E depicts the complex and unbound states of Jarid2. Figure F and G compare the expression of Jarid2 and miR-130a in Ev2 WT and Ev2 KO. Figure H and I show the expression levels of eGFP and jard2a. Figure K and L illustrate the expression of hoxa5 and jarid2a in Control MO, miR-130a MO, Control-MO, and Anti-MO conditions. Figure M and N depict the effects of miR-130a on angiogenesis in zebrafish embryos. Figure O shows the quantification of jarid2a expression levels. Figure P outlines the regulatory network involving miR-130a and Jarid2 in angiogenesis.
RNA-EMSA to demonstrate direct binding of miR-130a and Jarid2 3'-UTR region. We found that co-incubation of Jarid2 3'-UTR oligo with miR-130a resulted in a complex formation, whereas, the scrambled oligo did not show any mobility shift (Fig 5E). These results indicated that miR-130a directly binds to Jarid2 mRNA and regulates its expression.

Previously, we have demonstrated Etv2 as an upstream regulator of miR-130a [29]. Etv2 marks the earliest endothelial precursors [9]. To monitor the expression of Jarid2 in the angioblasts and endothelial cells, we sorted EGFP+ and EGFP* cells using Etv2-EYFP transgenic wild-type mouse embryos at E8.5 and performed qPCR analysis. Our data showed that Jarid2 transcripts were expressed in both EGFP+ as well as EGFP* cells, however, the levels of Jarid2 was lower in the EGFP* cells as compared to EGFP+ cells (S5A–S5D Fig). Next, we investigated whether the expression of Jarid2 was altered in the Etv2-null embryos during development. Our qPCR analysis using RNA isolated from the wild-type and Etv2+/- embryos revealed significantly increased expression of Jarid2 mRNAs in the Etv2+/- embryos as compared to the age-matched wild-type embryos (Fig 5F). In order to determine which cell populations have increased levels of Jarid2 mRNA in the Etv2+/- embryos, we analysed the microarray datasets obtained from EGFP+ and EGFP* sorted cells using wild-type and Etv2+/- embryos at E8.5 [11]. We did not find any differences in the levels of Jarid2 mRNA within the EYFP+ population; however, the levels of Jarid2 transcripts were increased in the EYFP+ populations in the Etv2+/- embryos as compared to the wild-type embryos (S5E Fig). To decipher whether miR-130a expression was altered in the Etv2+/- embryos, we performed qPCR experiments for miR-130a using RNA isolated from the wild-type and Etv2+/- embryos. Our analysis indicated that the level of miR-130a was decreased in the Etv2+/- embryos as compared to wild-type littermates (Fig 5G). Based on the reciprocal expression of miR-130a and Jarid2 in the wild-type vs. Etv2+/- embryos, we propose that the levels of Jarid2 are modulated by miR-130a in the angiogenic response. To test this hypothesis, we utilized HUVEC cells and performed tube formation assays following the over-expression of Jarid2. We observed extensive tube formation in the vector transfected cells, whereas, over-expression of Jarid2 resulted in defective tube formation. These results suggested an inhibitory role of Jarid2 during vascular development (S6A and S6B Fig). We then co-expressed Jarid2 and miR-130a mimics to examine whether miR-130a could rescue the defective tube formation phenotype. Our data revealed that expression of Jarid2 together with miR-130a mimics resulted in significant restoration of the tube formation (S6C Fig). Collectively, these results supported the notion that Jarid2 and miR-130a interacted and impacted vascular development.
Next, we examined whether miR-130a could regulate the expression of jarid2a transcripts in vivo. Initially, we designed jarid2a qPCR probes and examined the expression of jarid2a in the endothelial populations of the developing zebrafish embryos. qPCR analysis using RNA isolated from the sorted EGFP- and EGFP+ cells at 72hpf revealed that jarid2a was expressed in both EGFP- as well as EGFP+ cells, however, at lower levels in the EGFP+ cells relative to EGFP- cells (Fig 5H and 5I). We next evaluated the expression of miR-130a in these sorted cell populations. We found that comparable to the mouse endothelium, miR-130a was highly enriched in the EGFP+ cells as compared to EGFP- cells (Fig 5J). These results supported the notion that miR-130a plays an important role in the regulation of jarid2a in the developing vasculature. To analyse this hypothesis, we performed qPCR analysis using RNA isolated from control and miR-130a morphants at 72hpf. qPCR analysis for hoxa5a transcripts (a known target of miR-130a [38]) showed enrichment of hoxa5a mRNAs in the miR-130a morphants relative to control morphants, indicating a functional repression of miR-130a in the injected morphants (Fig 5K). Next, our qPCR analysis revealed significant enrichment of jarid2a transcripts in the miR-130a morphants relative to the control morphants (Fig 5L). To validate these results, we injected control and miR-130a morpholinos at the one-cell stage and performed in situ hybridization using jarid2a probes at 72hpf of the developing zebrafish embryos (Fig 5M). We found no detectable expression of jarid2a transcripts within the developing vasculature in the control embryos, whereas, injection of miR-130a morpholinos showed an increased expression of jarid2a in the miR-130a morphants within the developing vasculature (Fig 5N). Quantitative analysis further revealed an increased number of the embryos with enriched jarid2a expression in the miR-130a morphants relative to the controls (Fig 5O).

These results support the hypothesis that miR-130a regulates jarid2 transcripts both in vitro and in vivo, thereby regulating vascular patterning.

Discussion

During vascular development, coordinated endothelial cell behavior is critical for functional blood vessel formation [1, 2, 22]. Deciphering the transcriptional and post-translational mechanisms that fine-tune key regulatory network to control vasculogenesis and angiogenesis is critical for embryogenesis. Several miRNAs have been shown to play an essential role by regulating the expression of multiple components of complex biological pathways [25, 26]. Here, we have uncovered an essential role of miRNAs in endothelial patterning. Specifically, we showed the importance of miR-130a in the vascular and angiogenic response during embryonic development. Mechanistically, miR-130a binds to the 3' UTR of Jarid2 and modulates transcript expression and vascular patterning during embryogenesis.

miRNAs (small non-coding RNAs) biogenesis and their maturation is mediated by Dicer (a RNA binding protein) [25, 27]. Global as well as hypomorphic mutants of Dicer are embryonic lethal, however, only a few studies have described the functional role of Dicer in a lineage specific fashion [27, 28]. Fox example, Nkx2.5-Cre mediated deletion of Dicer resulted in embryonic lethality [40], whereas, the deletion by Tie2- or VE-Cadherin-Cre produced viable progeny [41]. In our previous study, we demonstrated that Dicer and miRNA function in the endothelial progenitors (angioblasts) were essential for vascular development [29]. The present study further extends the role of miRNAs in the later stages of vascular development in the regulation of endothelial patterning. Since Tie2- or VE-Cadherin-Cre mediated Dicer-floxed deletion resulted in a viable phenotype whereas Etv2-Cre-mediated Dicer-floxed deletion led to embryonic lethality [29], we hypothesize that miRNAs expressed at an earlier stage are essential for endothelial precursors and survival. It is also possible that during the late stages of embryonic development, Dicer functions are not essential for survival; however it is required for the vascular integrity to
facilitate the process of angiogenesis and vascular patterning. These findings suggest developmental stage and lineage specific role of miRNAs during embryogenesis.

A previous study from our laboratory identified an Etv2-miR-130a-Pdgfra cascade in the regulation of mesodermal specification [29]. In this study, we report yet another function of miR-130a in the regulation of vascular patterning and angiogenesis both in vitro and in vivo. Several other miRNA clusters including miR-126, miR-218, miR-23/27 clusters are documented in the regulation of angiogenesis [39, 42–44], whether, all these families of miRNAs function independently or in concert are unclear. Many other growth factors including VEGF, FGF, PDGFR-B are known to promote angiogenesis and regeneration [21, 22]. It is possible that miRNAs including miR-130a modulate the angiogenic response mediated via interacting with these signaling cascades. Therapeutically, miR-130a-mediated induction of neoangiogenesis might be useful in pathophysiological conditions such as ischemic heart disease, whereas, specific antagonizers may be required for antiangiogenic therapy. We found that the action of miR-130a is mediated through the modulation of Jarid2 expression both in mouse and zebrafish, however, the possibility of other regulatory mechanisms can not be ruled out and warrant further study in the future. Jarid2 is a member of the Jumonji C (JmjC) and Arid-domain protein family and serves as a transcriptional repressor by facilitating the binding of Polycomb Repressive Complex (PRC) 2 to the target genes [45, 46]. Based on our results, we proposed that miR-130a promotes the angiogenic response by inhibiting the suppressor such as Jarid2 in the endothelium. Suppression of Jarid2 could switch on the activation of genes responsible for the angiogenic response; however, we realize that involvement of additional pathways cannot be ruled out (Fig 5P). It has been shown that the levels of Jarid2 manifest the proper differentiation of ES cells and embryonic development as the Jarid2-mutant mice are embryonic lethal due to cardiovascular defects [46, 47]. Tie2-Cre-mediated deletion of Jarid2 (Jarid2−/−) phenocopies the Jarid2 knockout, indicating its essential role in the endothelial lineage during embryogenesis [46]. This study supports that fine-tuning the levels of Jarid2 through miR-130a in the endothelial lineages is one of the mechanisms to control the angiogenic response during development.

Supporting information

S1 Fig. Etv2-Cre-mediated Dicer deletion results in vascular abnormalities. A, B, Representative images of whole-mount embryos (A) and quantification (B) of Dicer−/− and Etv2Cre+/−;Dicer−/− embryos. Note the reduced embryo size in the Etv2Cre+/−;Dicer−/− embryos. C, D, Immunostaining using anti-CD31 antibodies of the transverse sections of Dicer−/− and Etv2Cre+/−;Dicer−/− embryos at E9.5. Panel C’ and D’ shows higher magnification of the boxed area in panel C and D. E-H, Immunostaining using EYFP (green; E, G) and CD31 (red; F, H) antibodies of the parasagittal sections of Dicer−/− and Etv2Cre+/−;Dicer−/− embryos at E9.5. Note the reduced vascular plexus in Etv2Cre+/−;Dicer−/−. Nuclei were stained with DAPI (blue). Error bars indicate SEM (*p<0.05). (TIF)

S2 Fig. Induction of miR-130a has no impact on cellular death. Representative images of Annexin V-FITC labeled differentiating cells in the absence (-Dox) and presence (+Dox) of doxycycline. (TIF)

S3 Fig. miR-130a regulates endothelial patterning in vivo. A, B, Brightfield microscopic images revealed no major changes in gross morphology of mismatch control-2 and miR-130a morphants. C-F, Lateral fluorescence images of Tg(fli1a:EGFP) zebrafish lines revealed defective vasculature in miR-130a morphants (white arrowheads) at 48 hpf (C, D) and 72 hpf (E, F)
time periods. **G,** Quantitative analysis of the number of defective zebrafish embryos with perturbed inter-somitic vessels (ISVs) at 48 hpf and 72 hpf. Error bars indicate SEM ("p<0.01). (TIF)

**S4 Fig. miR-130a regulates Jarid2 expression.** **A-F,** qPCR analysis of HoxA5, Robo1, Robo2, Slit1, Timp2 and Notch1 expression using RNA isolated from wild-type EBs, miR-130a iEBs and miR-130a-null EBs at d6 of differentiation. Error bars indicate SEM ("p<0.05). (TIF)

**S5 Fig. Jarid2 is expressed in the endothelial population.** **A-D,** qPCR analysis of Etv2, Tie2, CD31 and Jarid2 expression using RNA isolated from the EYFP⁻ and EYFP⁺ cell populations using Etv2-EYFP transgenic mouse embryos at E8.5. Note that Jarid2 is expressed in both EYFP⁻ and EYFP⁺ cell populations. **E,** Heatmap showing expression of Pdgfra and Jarid2 obtained from microarray analysis of EYFP⁻ and EYFP⁺ cell populations from wild-type and Etv2⁻/⁻ embryos at E8.5. Note that the enrichment of Jarid2 in the Etv2⁻/⁻ embryos is restricted to EYFP⁺ populations. Error bars indicate SEM ("p<0.01; "p<0.05). (TIF)

**S6 Fig. Jarid2 over-expression inhibits vascular development.** **A,** Tube formation assay using HUVEC cells following transfection with the vector and Jarid2 constructs, respectively. White arrow indicates the defective tube formation **B,** Quantitative analysis of the number of vascular tubes per field at 10x magnification. Note the decreased number of tubes following over-expression of Jarid2. **C,** Quantitative analysis of tube formation following co-injection of Jarid2 and miR-130a mimics. Note the restoration of tube formation upon co-expression of Jarid2 and miR-130a mimics. Error bars indicate SEM ("p<0.05). (TIF)

**S1 Table. miR-130a target prediction.** Top 100 common miR-130a predicted gene targets between mouse and zebrafish. List of the targets were ranked on the basis of the average context score. (DOCX)

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