Antagonistic Function of the RNA-binding Protein HuR and miR-200b in Post-transcriptional Regulation of Vascular Endothelial Growth Factor-A Expression and Angiogenesis*

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Background: The role of RNA-binding protein HuR in angiogenesis is not known.
Results: In macrophages, HuR and miR-200b antagonistically regulate VEGF-A and angiogenesis.
Conclusion: Interplay of HuR and miRNAs in the macrophage regulates tumor angiogenesis in the mouse and embryonic vascular development in zebrafish.
Significance: HuR modulation of miRNA function highlights the importance of post-transcriptional gene regulatory mechanisms in biology and disease.

HuR, also known as Elavl1, is an RNA-binding protein that regulates embryonic development, progenitor cell survival, and cell stress responses. The role of HuR in angiogenesis is not known. Using a myeloid-specific HuR knock-out mouse model (Elavl1Mø KO), we show that HuR expression in bone marrow-derived macrophages (BMDMs) is needed to maintain the expression of genes enriched in AU-rich elements and U-rich elements in the 3′-UTR. In addition, BMDMs from Elavl1Mø KO mice also showed alterations in expression of several miRNAs. Interestingly, computational analysis suggested that miR-200b, which is up-regulated in Elavl1Mø KO BMDMs, interacts with myeloid mRNAs very close to the HuR binding sites, suggesting competitive regulation of gene expression. One such mRNA encodes vascular endothelial growth factor (VEGF)-A, a major regulator of angiogenesis. Immunoprecipitation of RNA-protein complexes and luciferase reporter assays indicate that HuR antagonizes the suppressive activity of miR-200b, down-regulates miR-200b expression, and promotes VEGF-A expression. Indeed, Vegfa and other angiogenic regulatory transcripts were down-regulated in Elavl1Mø KO BMDMs. Interestingly, tumor growth, angiogenesis, vascular sprouting, branching, and permeability were significantly attenuated in Elavl1Mø KO mice, suggesting that HuR-regulated myeloid-derived factors modulate tumor angiogenesis in trans. Zebrafish embryos injected with an elavl1 morpholino oligomer or miR-200b mimic showed angiogenesis defects in the subintestinal vein plexus, and elavl1 mRNA rescued the repressive effect of miR-200b. In addition, miR-200b and HuR morpholino oligomer suppressed the activity of a ZVEGF 3′-UTR luciferase reporter construct. Together, these studies reveal an evolutionarily conserved post-transcriptional mechanism involving competitive interactions between HuR and miR-200b that controls angiogenesis.

Post-transcriptional gene regulation is important in a wide array of biological processes including embryonic development, cell stress responses, and oncogenesis. This process is controlled at least in part by RNA-binding proteins (RBPs) and microRNAs (miRNAs) (1, 2). They bind specific regulatory elements on transcripts and control the fate of mRNAs such as processing, stability, and translation. HuR (also known as Elavl1) is an RNA-binding protein that interacts with an AU-rich element (ARE), a regulatory sequence motif located in the 3′-UTR of transcripts (3, 4). Recent findings indicate that HuR association with target mRNA correlates directly with RNA abundance, supporting the role of HuR as an RNA stabilizer (5). mRNA targets that associate with HuR have been identified by several techniques such as RIP-ChIP, PAR-CLIP, and iCLIP (5–9). Consensus HuR binding sequence motifs such as AREs and U-rich elements have been elucidated from such studies. HuR-associated mRNAs regulate numerous cellular processes.

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2 The abbreviations used are: RBP, RNA-binding protein; ARE, AU-rich element; BM, bone marrow; BMDM, bone marrow-derived macrophage; Elavl1Mø KO, myeloid-specific HuR knock-out mouse model; GAIT, IFN-γ-activated inhibitor of translation element; hnRNP, heterogeneous nuclear ribonucleoprotein; hpf, hours postfertilization; LLC, Lewis lung carcinoma; miRNA; microRNA; MO, morpholino antisense oligomer; qPCR, quantitative PCR; RIP, RNA-binding protein immunoprecipitation; SIV, subintestinal vein; SMA, smooth muscle actin; TAM, tumor-associated myeloid.
including inflammation, cell cycle, tumorigenesis, cell survival, and apoptosis.

Recent in vivo studies revealed that Elavl1 knock-out mice are embryonic lethal due to a defect in placentation (10). Our laboratory showed that postnatal deletion of Elavl1 led to rapid lethality due to apoptosis of intestinal and hematopoietic progenitor cells (11). In addition, thymus-specific deletion of Elavl1 resulted in the defective egress of mature thymocytes (12), and overexpression of Elavl1 in murine innate immune cells suppressed inflammatory responses (13). A recent study concluded that HuR suppressed inflammatory processes in myeloid cells and protected mice from colitis-induced intestinal cancer (14). Moreover, cell adhesion-dependent stabilization of mRNAs in macrophages was dependent on HuR (15). These studies suggest that HuR is a key post-transcriptional regulator of numerous biological responses. However, the role of HuR in pathological processes is not well understood.

Angiogenesis, the process by which new blood vessels develop from a pre-existing vascular system, is required for embryonic development, physiological processes such as wound healing and corpus luteum formation, as well as in pathological processes such as tumor progression. Hypoxia, a fundamental angiogenic stimuli, induces key angiogenic cytokines such as vascular endothelial growth factor (VEGF)-A primarily by transcriptional mechanisms. Bone marrow (BM)-derived myeloid cells are also recruited and facilitate tumor growth by producing proangiogenic growth factors, cytokines, and matrix-cellular regulators (16). Generally, tumor vessels are structurally and functionally abnormal, largely as a result of the unbalanced, local overexpression of angiogenic factors such as VEGF-A (17, 18). Tumor vasculature is irregularly organized and contains aberrantly associated mural cells. This abnormality is thought to contribute to the leakiness of vessels and to increase interstitial pressure in the tumor (19, 20). These studies indicate that tight control of VEGF-A expression as well as other angiogenic factors is essential for maintaining normal vascular homeostasis and productive angiogenesis.

Although transcriptional mechanisms are well characterized to regulate the induction of VEGF-A, several post-transcriptional mechanisms are also involved. The Vegf-a 3'-UTR contains several important cis-acting elements, including CA-rich element, IFN-γ-activated inhibitor of translation element (GAIT), and ARE, and is regulated by its RBPs such as AUF, tristetraprolin, heterogeneous nuclear ribonucleoprotein (hnRNP L) and HuR (21–25). ARE-binding proteins such as AUF and tristetraprolin destabilize Vegf-a mRNA in macrophage and tumor cells, respectively (24, 25). Furthermore, hnRNP L and the GAIT complex regulate VEGF-A expression in a mutually exclusive, stimulus-dependent manner through an RNA conformational change known as riboswitch (26). Under hypoxia, hnRNP L overrides GAIT silencing by triggering a VEGF-A mRNA structural change to a translation-permissive RNA conformation, in which the GAIT element is occluded. Finally, HuR stabilizes VEGF-A mRNA under hypoxia (22). Despite these important observations, the molecular details of how HuR regulates VEGF-A expression are not well understood.

Recent studies suggest that RBPs cooperate with miRNA and control gene expression (1, 27–29). Systems-level bioinformatics analysis of 3'-UTRs have revealed that the ARE motif bound by AUF, tristetraprolin, and HuR is overrepresented near miRNA target sites, suggesting that ARE-binding proteins work in concert with miRNA-dependent mechanisms (30). Moreover, transcriptome-wide mapping of HuR binding sites revealed that HuR frequently binds to transcripts near or overlapping with predicted miRNA sites (5). These observations along with others suggest that ARE-binding proteins such as HuR either antagonize or cooperate with miRNA-dependent gene regulation. For example, HuR allows the expression of the CAT-1 mRNA by counteracting miR-122-dependent repression (31) or, in contrast, represses c-myec mRNA by recruiting let-7 (32). The functional interconnections between HuR and miRNAs may provide additional levels of control in regulating gene expression and thus provide biological robustness in gene regulatory mechanisms. Given the profound phenotypic effects of miRNAs, RBP interactions with miRNAs may be very critical for biological and pathological outcomes.

Little is known about how post-transcriptional gene regulatory mechanisms influence angiogenesis in vivo. Here, we demonstrate that HuR antagonism of miR-200b-mediated gene repression of angiogenic regulators such as VEGF-A controls tumor angiogenesis.

**EXPERIMENTAL PROCEDURES**

**Animals (Mice and Zebrafish)—**Myeloid specific deletion of Elavl1 in mice was generated by crossing Elavl1 floxed mice (11) with mice expressing Cre recombinase driven by the lysozyme M promoter (33). All studies were performed under protocols approved by the Weill Cornell Medical College Animal Care and Use Committee. For subcutaneous tumor isograft model, Lewis lung carcinomas (LLC) cells (10⁶) were injected subcutaneously into Elavl1f/f and Elavl1Mø KO mice which were determined to be >97% C57BL/6 background. 14 days later, tumors were collected and analyzed further. For Matrigel plug experiments, mice were injected subcutaneously with 0.3 ml of Matrigel supplemented with basic FGF (2.5 μg/ml) and 8 units of heparin. At 6 days, Matrigel plugs were harvested and processed for confocal images. For zebrafish husbandry, wild-type zebrafish and fli1:EGFP lines were obtained from the Zebrafish International Research Center, maintained at 28.5 °C and staged as described by Westerfield (53).

**BMDM Culture and ELISA Analysis—**Bone marrow cells obtained by the lavage of femur and tibia from Elavl1f/f and Elavl1Mø KO mice were cultured in complete DMEM with 20% of L929 cell culture medium for 7 days (34). 1 × 10⁶ BMDMs in a 6-well plate were seeded and transfected using Lipofectamine 2000 (Invitrogen) without or with 50 nM antagonir for miR-200b (Ambion). Supernatant was collected the next day and measured for VEGF secretion by ELISA (R&D Systems).

**RNA Isolation, Mouse Exon Chip Array, and qRT-PCR Analysis—**Total RNA was isolated according to the manufacturer's protocol (Clontech), and RNA quality was checked by an Agilent 2100 bioanalyzer (Agilent Technologies). 500 ng was hybridized on the mouse Exon 1.0 ST array (Affymetrix) and
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scanned at the Weill Cornell Medical College core facility. Scanned chip data were analyzed with AltAnalyze (35) using default parameters. To validate mRNA expression by qRT-PCR, 1 μg of RNA was reverse-transcribed with Superscript III reverse transcriptase (Invitrogen), and cDNAs were amplified with specific primers (primer bank) using SYBR Green master mix (Quanta BioSciences).

miRNA Isolation, miRNA High Throughput Sequencing, qRT-PCR Analysis, and miRNA Target Prediction—Small RNAs were isolated according to the manufacturer’s protocol (Clontech). RNAs were ligated to 5’- and 3’- adaptors with RNA ligation and followed by reverse transcription (Illumina Small RNA Sample Preparation kit version 1.5; Illumina). The cDNAs were amplified by PCR for 16 cycles. PCR products were purified on a nondenaturing acrylamide gel with size selected and sequenced for 42 cycles on the Illumina Genome Analyzer II. The resulting sequences were trimmed and mapped to miRBase 627 mouse stem-loop sequences using the Bowtie alignment program (version 0.12.7) allowing up to 1 mismatch. The alignment reads were normalized as proportion of total miRNA reads, and the normalized reads were analyzed for differentially expressed miRNAs using the Fisher’s exact test. miRNA was subject to poly(A) tailing reaction and reverse-transcribed with an oligo(dT) adaptor primer (qScript miRNA cDNA synthesis kit; Quanta) and followed by qPCR amplification with the specific miRNA sequence primer and oligo(dT) adaptor sequence primer (PerfeCTa SYBR Green system, Quanta). Expression of specific miRNAs was normalized to the value of U6 snRNA.

Bioinformatic Motif Analysis and Distance between HuR Binding Site and miRNA Seeding Site—ARE and HuR high affinity sequences were obtained from published studies (9, 36). RNA motif scanning and counting in the 3’-UTRs, 5’-UTRs, exons, introns and the transcribed strand only of ENSEMBL transcript models were performed using previously published programs (37). Motif counts were compared between gene sets using two-tailed Wilcoxon tests; overall motif overrepresentation was evaluated using the hypergeometric distribution. High confidence miRNA target predictions were downloaded from the TargetScan website (release 6.2, June 2012). AREs and U-rich sequences in 3’-UTRs and within 50 nucleotides or less of TargetScan predicted seeds were determined. To examine whether AREs and U-rich sequences/miRNA target sites are closer to each other than expected by chance, we uniformly randomly redistributed the same number of miRNA target sites in the same 3’-UTRs and calculated how often AREs and U-rich sequences are within 50 nucleotides or less of random miRNA targets. We repeated this procedure 100 times and reported how frequently the number of AREs and U-rich sequences within 50 nucleotides or less of random miRNA targets surpassed the true observed value, thus generating a HuR binding site proximity p value for each miRNA.

Luciferase miRNA Target Reporter Assay—PCR products of Vegf-a 3’-UTR fragment (1059–1455) generated using the forward primer 5’-GCGCTAGCATCTTCTCCCTCCAAGGA and the reverse primer 5’-GCCGCGAAGAAGGAATGT-GTGGTGG were cloned into the Nhel and Xhol sites of the multiple cloning site of pmirGLO vector (Promega), a dual luciferase reporter (firefly and Renilla). Zebrafish Vegfaa 3’-UTR (871–1236) was amplified from 24 h postfertilization (hpf) zebrafish embryos cDNA using two primers with adaptors: SacI, 5’-GAGCTCTTTGGTGGAGACATAGGA-GAAATGTG, and XbaI, 5’-TCTAGAGATCTGAGAA-CAATAACG. Fragment was inserted in pmirGLO vector by underlined restriction enzymes. Mutations were introduced in the complementary with the seed region of miR-200b on Vegf-a 3’-UTR (1194–1222). Primers containing mutated target sequences are 5’-GCTCAACACCGCGGTGAAGAAAAC-CCTACTCTTTAAAT and 5’-CCGGGTTTTTTCTACCGC-GTTGGTTAATTTAATTTCAAC. HEK293T cells were co-transfected with 25 ng/ml pLuc vectors containing Vegf-a 3’-UTR or mutant Vegf-a 3’-UTR with 20 nm miR-200b mimics (Ambion) using Lipofectamine-2000 (Invitrogen). After 24 h, the cells were lysed, and luciferase activity was measured using a luminometer. Firefly luciferase values were normalized with Renilla luciferase values.

RNA-binding Protein Immunoprecipitation (RIP) Assay—The binding of HuR or Ago-2 with Vegf-a mRNA was determined by immunoprecipitation followed by qRT-PCR as described (9). BMDMs (5 × 10⁵) isolated from two mice were lysed with polysome lysis buffer supplemented with RNase inhibitors and protease inhibitors. The supernatant was incubated overnight at 4 °C with no antibody, control IgG, Ago-2 monoclonal antibody (Wako), or HuR monoclonal antibody (Clonegene) and then incubated with protein G Dynabeads (25 μl) for 4 h at 4 °C. After washing five times, the pellet was treated with 10 units of Dnase I (Promega) in 10 μl of buffer for 10 min at 37 °C and then treated with 5 μg of proteinase K (Roche Applied Science) in 100 μl of buffer for 30 min at 55 °C. The supernatant was collected, and RNA was extracted using acid phenol chloroform (Ambion). Extracted RNA was subjected to qRT-PCR analysis as described above.

Tumor Vessel Leakiness—Mice bearing LLC tumors were anesthetized with ketamine/xylazine and received 0.25 mg of 70-kDa Texas Red-conjugated dextran (Molecular Probes) via tail vein injection. 25 min later, 0.25 mg of 2000-kDa fluorescein isothiocyanate (FITC)-conjugated dextran (Molecular Probes) was injected intravenously. 5 min later, mice were euthanized with CO₂. Tumors were collected and postfixed overnight and embedded in OCT frozen blocks. Blocks were cut into approximately 50-mm thick cryosections using the cryomicrotome (Leica). The intravascular FITC-dextran and extravasated Texas Red-dextran in sections were observed by confocal microscopy (Olympus Fluvioview).

Flow Cytometry and Tumor-associated Macrophage Isolation—Tumors were minced and digested with 2 mg/ml collagenase A (Roche Applied Science) for 45 min at 37 °C, followed by passing through a 40-μm nylon mesh to collect a single cell suspension. The suspensions were centrifuged and washed twice with PBS. For flow cytometry, cells were stained with myeloid-derived suppressor cells, a CD11b antibody, and a Gr1 antibody markers and counted with LSRII flow cytometer (BD Biosciences). The acquired data were analyzed with FlowJo software (Treestar). For tumor-associated myeloid (TAM) cell isolation, suspended single cells were incubated with anti-F4/80 antibody and processed for the positive selection using the Macs column.
(Milenyi Biotec). Collected TAM cells were lysed for RNA purification followed by qRT-PCR analysis or for protein extraction followed by proteome profile angiogenesis array (R&D Systems).

**Immunohistochemistry and Immunofluorescence**—Tumors were fixed in 4% paraformaldehyde and processed for paraffin or frozen sections. Paraffin sections were stained with CD31 antibody (BD Pharmingen) and counterstained with methyl green (Vector Laboratories). Confocal images of tumor vasculature in frozen sections were obtained by co-staining of rat anti-CD31 antibody (BD Pharmingen) and Cy3-conjugated anti-smooth muscle actin (SMA) antibody (Sigma). Zebrafish embryos were fixed in 4% paraformaldehyde overnight and processed for whole mount immunohistochemistry with anti-HuR antibody (Clonogenic), alkaline phosphatase staining (Roche Applied Science), or immunofluorescence staining with anti-GFP antibody (Invitrogen) as described (38). Brightfield images were captured using Zeiss Axioskop2 microscope with an AxioCam digital camera (Zeiss). Immunofluorescence images were taken using FluoView (Olympus) or Zeiss LSM510 upright confocal microscope.

**Morpholino Design, Capped mRNA Synthesis, and Microinjection**—elavl1 morpholino antisense oligomers (MOs) were synthesized by GeneTools: ATG-MO (translational blocking antisense, 5′-TGTGGTCTTCTGTAACCGTTGCACAT-3′) or SPL-MO (splicing antisense, 5′-AGAGCACCTTATGTCAC-3′) and dissolved in distilled water. A full-length elavl1 clone (Open Biosystems) was inserted into the EcoRI and elavl1 ATTACCTT-3′ SPL-MO (splicing antisense, 5′-Prism software. Other statistical tests such as hypergeometric test with two-tailed unpaired and significance was determined by two-tailed unpaired test with Prism software. Other statistical tests such as hypergeometric test were performed using the R statistical software.

**RESULTS**

**Myeloid HuR Regulates the Expression of ARE-bearing mRNAs**—To investigate the specific role of HuR in myeloid cells, we crossed Elavl1f/f KO mice. Similarly, neutrophils (CD11b+Ly6G+/F4/80−) as well as monocytes/macrophages (CD11b+Ly6G−/F4/80+) in blood showed no discernible differences. These data suggest that HuR is not essential for myeloid cell proliferation, survival, or differentiation in vivo.

To gain insights into myeloid genes regulated by HuR, we investigated global gene expression profiles in BMDMs isolated from Elavl1f/f mice and Elavl1MO KO mice using Affymetrix exon-chip microarrays. Among 809 differentially expressed genes in four biological replicates, 71% (575 genes) of differentially expressed genes in Elavl1 KO BMDMs were down-regulated (Fig. 1E). When analyzed in the Ingenuity Pathway Analysis software, cardiovascular system development and function were identified, suggesting a role for HuR in regulating the expression of angiogenic regulatory genes in myeloid cells.

HuR is known as a stabilizer of ARE-bearing mRNAs. Thus, we hypothesized that the loss of HuR in myeloid cells led to destabilization of many transcripts bearing the HuR binding motif in 3′-UTR regions. To identify the relationships between HuR binding sites and mRNA expression, we examined ARE-bearing mRNAs in 809 differentially expressed genes using the ARED search engine (36). 46 genes were identified that contain ARE motifs in their 3′-UTR (Fig. 1F). Interestingly, angiogenic regulatory genes such as, Vegf-a, Jag1 (Jagged 1), Ptgs2 (prostaglandin-endoperoxide synthase 2), Vcam1 (vascular cell adhesion molecule 1), and Nos2 (nitric-oxide synthase 2), which contain ARE motifs, were down-regulated in Elavl1 KO BMDMs, suggesting that HuR stabilizes ARE-containing angiogenic regulatory RNAs in macrophages.

We further examined the enrichment of ARE and HuR high-affinity binding motifs (U-rich sequences (7) in the 3′-UTRs, introns, exons, and 5′-UTRs of all transcripts that changed expression in the microarray studies (Fig. 1G). The average number of AREs in 3′-UTRs of down-regulated genes was significantly higher than that in 3′-UTRs of stable or up-regulated genes (p < 1e-9, Wilcoxon test). Accordingly, 3′-UTRs of down-regulated genes in Elavl1 KO BMDMs are more likely to have higher copies of ARE than 3′-UTRs of stable or up-regulated genes (p < 1e-11, hypergeometric test), indicating significant stabilization of 3′-UTR ARE-containing transcripts by HuR. Similar findings were observed even when the density of ARE sites was considered (data not shown). Further, U-rich sequences that bind HuR with high affinity (7) (Fig. 1H) were also associated with down-regulated transcripts. However, we did not find significant differences of ARE or U-rich sequence enrichment in 5′-UTRs, exons, or introns, indicating that HuR activity is exerted mostly through the regulatory elements present in the 3′-UTRs. Together, these studies suggest that myeloid HuR stabilizes a large number of transcripts including angiogenic regulatory genes bearing ARE or U-rich sequences in their 3′-UTRs.

**HuR Regulates the Expression of miRNAs in BMDMs**—We next characterized the miRNA expression profile in WT and Elavl1MO KO BMDM by high throughput RNA sequencing. Two biological replicates of WT and Elavl1MO KO BMDM miRNA preparations were sequenced, resulting in >32 million total reads that perfectly aligned to 627 mouse miRNA loci.
described in miRBase. We further analyzed miRNA species that were most abundant (top 45% in both WT and KO). Of 282 miRNA species, 10 miRNAs (miR-126-3p, 143-3p, 196a-5p, 100, 199a-3p, 126-5p, 20b, 200b, 199b, 199a-5p) were up-regulated, and two miRNAs (miR-1249, 3108) were down-regulated (Fig. 2A). Up-regulation of miR-126-3p, 143-3p, 196a-5p, 199a-3p, 200b, 199a-5p and down-regulation of miR-1249, 3108 were further tested and confirmed by qRT-PCR analysis (Fig. 2B). These data suggest that lack of HuR in BMDMs results in alteration in abundance of certain miRNAs, which may have profound effects on gene expression in the myeloid cells and may influence some of their phenotypes.

We next explored the hypothesis that miRNA suppression of gene expression via the RNA-induced silencing complex may be influenced by HuR interaction with mRNAs targeted by these miRNAs. We therefore used bioinformatic tools to search for targets of HuR-regulated miRNAs. Using TargetScan, miRNA targets were identified for five of the up-regulated miRNAs we validated by qRT-PCR (the other three up-regulated miRNAs did not have any predicted high confidence targets in the June 2012 TargetScan release (6.2)) (Fig. 2C). Of the five miRNAs, only miR-200b showed significant presence of ARE or U-rich HuR binding sites in close proximity (≤50 nucleotides) from the miRNA seed sequence in the 3′-UTR of transcripts. For example, 257 mRNAs showed the presence of adjacent ARE and miR-200b binding sites whereas 276 mRNAs contained adjacent U-rich and miR-200b binding sites (Fig. 2C). We further observed that 12 of these mRNAs (Vegfa, Cited2, Mkl2, Ephb2, Gja1, Pten, Pkd1, Reck, Srf, Qk, Vezf1, and Zfp42) are known to be involved in vascular development. These bioinformatic analyses suggest that interaction between miR-200b and HuR on mRNAs regulates the expression of angiogenic regulatory genes.

Myeloid HuR and miR-200b Regulate VEGF-A Expression in a Competitive Manner—Vegf-a, one of the strongly down-regulated genes in Elavl1 Mø KO BMDMs and a key angiogenic growth factor, was chosen for further analysis. Significantly attenuated expression of Vegf-a mRNA and secretion of VEGF-A protein were observed in Elavl1 Mø KO BMDMs

![Graph A](image1.png)

**Figure 1.** Deletion of Elavl1 in myeloid cells results in down-regulation of ARE-bearing mRNAs. A and B, efficiency of Elavl1 deletion in isolated BMDMs as determined by quantitative RT-PCR (A) and Western blotting (B). Data are representative of at least five independent experiments. C and D, flow cytometry analysis. Bone marrow cells isolated from Elavl1f/f and Elavl1 Mø KO mice were stained for immature myeloid (CD11bGr1low) and mature myeloid cells (CD11bGr1high). Blood cells were stained for neutrophils (CD11bLy6G+) and monocytes/macrophages (CD11bLy6G-F4/80). n = 3 mice/group. E, microarray analysis indicating that 575 genes were down-regulated and 234 genes were up-regulated in Elavl1 Mø KO BMDMs. -fold change >1.4, n = 4 mice/group. F, heat map of differentially expressed ARE-containing mRNAs. Differentially expressed genes in Elavl1 Mø KO BMDMs are color-coded based on the -fold change relative to Elavl1f/f BMDMs (blue, down-regulated genes; red, up-regulated genes). The heat map was produced by open source software, Matrix2png. G, the average number of ARE (AUUUA) in each region (3′-UTRs, 5′-UTRs, exons, and introns) of genes detected in the microarray as above.
region in the Vegf-α 3′-UTR sequence (1194–1222) contains a putative HuR binding site (U-rich 7-mer) in proximity to a highly conserved mir-200b seed (predicted as an miR-200n target site) by all four programs (Fig. 3C). This region is conserved between mouse and human VEGF-A (hg18: chr6: 43,861,550–43,861,596) and was identified as a HuR interaction site in HEK293 cells by ELAVLI PAR-CLIP analysis (Fig. 3D) (7). We therefore tested whether HuR and mir-200b compete for the same regulatory element in the Vegf-α 3′-UTR.

To test whether the Vegf-α regulatory element (1194–1222) containing the U-rich 7-mer and the mir-200b seed is functionally important for expression, we generated a reporter construct bearing Vegf-α 3′-UTR (1059–1455) or a mutant reporter in which the region of complementarity with mir-200b seed sequence was mutated (Fig. 3E). Transfection of mir-200b mimic reduced the luciferase activity in HEK293T cells expressing Vegf-α 3′-UTR reporter but not the mutant or the empty vector (Fig. 3E). Interestingly, in HuR knockdown 293T cells, mir-200b further reduced the luciferase activity of a reporter bearing Vegf-α 3′-UTR, suggesting that the absence of HuR may potentiate the suppression of Vegf-α mRNA by mir-200b (Fig. 3F). These data suggest that the (1194–1222) regulatory element of the Vegf-α 3′-UTR is subject to negative regulation by mir-200b and positive regulation by HuR.

To further examine whether the mir-200b-RISC complex interacts with the Vegf-α 3′-UTR and whether this interaction is competed by HuR binding, we immunoprecipitated Elavl1fl/fl and Elavl1Mø KO BMDMs lysates with the anti-Ago-2 antibody (an essential component of RISC) and examined the level of Vegf-α mRNA by qRT-PCR analysis (Fig. 3G). Expression of Ago-2 polypeptide was similar between Elavl1f/f BMDMs and Elavl1f/f KO BMDMs (data not shown). Higher levels of Vegf-α mRNA were associated with the Ago-2 complex in Elavl1Mø KO BMDMs compared with that in Elavl1f/f BMDMs, indicating that HuR blocks the ability of RISC complex to engage Vegf-α transcript and suppress VEGF-A expression.

Furthermore, inhibition of mir-200b activity by a specific antagonist in Elavl1Mø KO BMDMs partially rescued the repression of VEGF-A, whereas treatment of Elavl1f/f BMDMs (which have high HuR expression) with the miR-200b antagonist did not affect VEGF-A expression (Fig. 3H). Together, these data strongly suggest that Vegf-α mRNA in macrophages is subject to post-transcriptional regulation by miR-200b and HuR in a competitive manner.

Myeloid HuR Regulates Tumor Growth and Angiogenesis—We next investigated the role of myeloid HuR in tumor growth and angiogenesis. We implanted LLC cells subcutaneously and studied tumor growth and the formation of intratumoral vessels. Tumor growth in Elavl1Mø KO mice was significantly attenuated compared with Elavl1f/f mice (Fig. 4A). Tumor cell apoptosis by TUNEL staining was significantly increased in tumors of Elavl1Mø KO mice whereas tumor cell proliferation was not altered (Fig. 4, B and C), indicating that the loss of myeloid HuR resulted in tumor cell death and reduced tumor mass.

Tumor growth is known to be supported by the BMDM cells recruited to the tumor microenvironment (40). To examine whether the loss of myeloid HuR perturbs recruitment of mye-
loid cells to the tumor, we immunostained tumors for F4/80⁺ (macrophages) or CD11b⁺Gr1⁻ (myeloid-derived suppressor cells) (Fig. 4, D and E). The population of myeloid-derived suppressor cells (CD11b⁺Gr1⁻) by flow cytometry and F4/80⁺ macrophages by immunostaining in tumors of Elavl1f/f mice and Elavl1Mø KO mice did not show significant differences. This suggests that myeloid HuR function is not required for recruitment/migration or egress of myeloid cells into the tumor microenvironment.

CD31 immunohistochemistry staining of tumor sections indicates that Elavl1Mø KO mice had reduced vascular density, sprouting and branching, and contained vessels with dilated lumens compared with the WT counterparts (Fig. 5A). To examine the pericyte coverage of tumor vessels, tumor tissue sections were immunostained for CD31 and α-SMA. As shown in Fig. 5, B and C, intratumoral vessels in the Elavl1Mø KO mice contained more SMA⁺ mural cells. We also examined vascular perfusion and leakiness by injecting fluorescent dextrans (70-kDa Texas Red-conjugated and 2000-kDa FITC-conjugated) intravenously and imaging the tumor sections by confocal fluorescence microscopy. Tumor vessels in Elavl1f/f mice were highly permeable because a significant amount of 70-kDa dextran was found in extravascular regions (Fig. 5D). In sharp contrast, most 70-kDa dextran remained in the intravascular regions in tumors from Elavl1Mø KO mice. The 2000-kDa FITC-dextran decorated the outline of the intratumoral vessels. These data indicate that tumor vascular permeability in Elavl1Mø KO mice was markedly decreased, suggesting the
phenotypic alteration of tumor vasculature in *Elavl1* KO mice. Mature and stable vessels with better pericyte coverage and reduced branching were also observed in growth factor-induced angiogenesis in Matrigel plugs implanted subcutaneously in *Elavl1* KO mice (Fig. 5E). In addition, the increased synthesis of vascular maturation markers such as angipoi- etin 1, fibronectin, and type IV collagen a2 were observed in *Elavl1* KO BMDMs (Fig. 5, F and G). Together, the findings strongly suggest that myeloid HuR is critically important for tumor angiogenesis, vascular phenotype alterations, and tumor growth.

**HuR and miR-200b Regulate Angiogenesis in the Subintestinal Vein Plexus in Zebrafish**—To investigate whether interplay between HuR and miR-200b regulates developmental angiogenesis, we utilized the zebrafish model. The zebrafish genome encodes a HuR homologue (*elavl1*) that is highly conserved (85% identity between fish and mouse) (41). In addition, miR-200b is completely invariant between fish and mammals. (85% identity between fish and mouse) (41). In addition, miR-200b is completely invariant between fish and mammals.

Because mammalian HuR antibody detects the zebrafish homolog specifically, we used whole mount immunostaining to determine the embryonic expression pattern of HuR. *elavl1* was highly and ubiquitously expressed in zebrafish embryos at 24 and 72 hpf (Fig. 6A). We determined that two different MOs targeting *elavl1* (ATG-MO, translational blocking MO; SPL-MO, splice blocker MO) both suppressed HuR protein synthesis in a dose-dependent manner at 24 hpf, thus validating specificity for knockdown (Fig. 6A).

In zebrafish, the *vegfa* 3′-UTR contains HuR- and miR-200b binding sites that are only 9 bases apart (Fig. 6). To test whether *elavl1* MO and miR-200b regulate *vegfa* expression, we cloned the *vegfa* 3′-UTR (from nucleotides 871–1236) downstream of the luciferase ORF. As shown in Fig. 6C, miR-200b suppressed expression from the *vegfa* 3′-UTR reporter in a dose-dependent manner in HEK293 cells; moreover, siRNA against HuR further suppressed reporter expression.

To examine the role of HuR in angiogenesis, we knocked down *elavl1* expression in *tg(fli1:EGFP)* vascular reporter embryos (Fig. 6E, left). Injection of embryos with *elavl1*MO (2 ng/embryo) resulted in a severe impairment in subintestinal vein (SIV) vascular development at 72 hpf, although other vessels including intersegmental vessels were not discernibly affected. The miR-200b mimic (57 pg/embryo) yielded similar defects in SIV vascular development, suggesting an antiangiogenic role of miR-200b in SIV plexus development. Alkaline phosphatase-stained images (Fig. 6E, right) further confirmed impaired vascular development in the SIV plexus in 94% of embryos injected with *elavl1*MO (68/72) and 88% of embryos injected with miR-200b mimics (53/60). Importantly, when embryos injected with the anti-angiogenic dose of miR-200b (57pg) were co-injected with *elavl1* mRNA (200 pg/embryo) 71% of the embryos (40/56) displayed normal SIV plexus development, suggesting that HuR relieved miR-200b mediated anti-angiogenesis in the SIV plexus. Furthermore, at a suboptimal dose of *elavl1*MO (1 ng) or miR-200b mimic (14 pg), the embryos develop with little or no vascular defects detected at 72 hpf (57/63, and 54/58 embryos, respectively; Fig. 6F). However, when these same doses of *elavl1*MO and miR-200b mimic were simultaneously administered, 61% of the embryos (38/62) exhibited a severe defect in SIV plexus angiogenesis, again consistent with the two factors competitively regulating common angiogenic targets during embryogenesis.

**DISCUSSION**

A major finding of this work is that HuR plays a critical role regulating myeloid cell gene expression at the post-transcriptional level. HuR is not essential for myeloid cell survival, proliferation, differentiation, or trafficking. This is in sharp contrast to the essential role of HuR in hematopoietic progenitor cell survival (11). Thus, once hematopoietic progenitors are committed to the myeloid lineage, the HuR function switches from an essential prosurvival role to that of myeloid phenotype switch.

**FIGURE 3.** *HuR* and miR-200b regulate Vegf-a expression in a competitive manner. A, reduced expression of VEGF-A in *Elavl1*KO BMDMs. Expression of Vegf-a transcript was determined by qRT-PCR (left) and VEGF-A secretion in the supernatant of cultured BMDMs analyzed by ELISA (right). Data are shown as -fold change relative to *Elavl1*mut BMDMs. Data reflect mean ± S.E. (error bars) Left; n = 2 mice/group; p = 0.008. Right; n = 7 mice/group; p = 0.001. VEGF secretion varied between 20 and 100 pg/ml between experiments. B, HuR binding to Vegf-a mRNA in Raw264.7 cells in a dose-dependent manner. Cell lysates were immunoprecipitated with anti-HuR antibody (0.1, 0.5, and 2.5 µg) or control IgG antibody (0.1, 0.5, and 2.5 µg) followed by qRT-PCR to measure the level of Vegf-a mRNA. Data are presented as relative level of Vegf-a mRNA normalized to Hprt mRNA. *, p = 0.026. C, schematic of murine Vegf-a 3′-UTR elements. HSR, hypoxia stability region; inset, region used to generate a reporter construct. The black box in the inset is expanded to show the sequence containing HuR binding site and seed region of miR-200b. Note the fragment (hg18/chr6:43,861,550 – 43,861,596) which was identified as a HuR target by PAR-CLIP analysis (7) is conserved. Lower panel shows the complete conservation of miR-200b sequences between human, mouse, rat, and zebrafish. E, miR-200b inhibits the expression of a reporter bearing Vegf-a 3′-UTR. Schematic of reporter plasmids; pLuc, pLuc-Vegf-a (3′) reporter bearing the fragment of Vegf-a 3′-UTR (1059–1455), and pLuc-Vegf-a (3′ mut) reporter bearing six mutant nucleotides in the seed region of miR-200b is shown. Each plasmid (25 ng/ml) was transfected into HEK293T cells with miR-200b (5 or 20 nM) or control miRNA, and 24 h later, luciferase activity was measured. Firefly luciferase values were divided by Renilla luciferase values to normalize variation in transfection efficiency. Data are presented as -fold change compared with control miRNA treatment. Data are mean ± S.E., each point is average of four independent experiments. ***, p < 0.001 (Ago-2, 1 µg of Elavl1 versus Ago-2, 1 µg Elavl1 Me KO). H, supernatants of BMDMs treated with miR-200b antagonist (50 nM) were analyzed with VEGF-A ELISA. Data are shown as -fold change relative to Elavl1mut BMDMs with control treatment averaged from four independent experiments. **, p = 0.019 (control Elavl1mut versus Elavl1 KO).
Using the myeloid-specific Elavl1 (HuR) knock-out model, we analyzed the transcriptome of primary bone marrow-derived macrophages. Significant down-regulation of ARE- and U-rich-motif containing transcripts was observed. These data strongly suggest that an important function of HuR is to stabilize transcripts in macrophages. mRNAs bearing ARE sequences, which function as instability elements in 3’UTR, constitute approximately 8% of the transcribed genome (36). These encode mostly immediate early genes, inflammatory cytokines, and growth factors, suggesting a role for post-transcriptional regulation in processes such as inflammation and oncogenesis.

Our analysis of primary macrophages clearly showed the positive correlation of the number of putative HuR binding sites (AREs and U-rich sequences) in 3’UTR/transcript and degree of HuR-dependent RNA stabilization, a finding that was echoed in other systems (5).

Second, our data show that miRNA expression profiles in BMDM were altered by lack of HuR expression. In particular, we found that several miRNAs (miR-126-3p, 143-3p, 196a-5p, 199a-5p, 200b, and 199a-3p) were up-regulated, and two (miR-3108, 1249) were down-regulated in BMDMs that lack HuR. Multiple mechanisms could account for such changes in miRNA expression in the absence of HuR. For example, RBPs are shown to regulate the biogenesis of miRNAs. A recent study found that HuR interaction with H19 primary transcripts in the nucleus suppresses miR-675 generation from the first exon of H19 lincRNA (54). The KH-type splicing regulatory protein (KSRP), a key mediator of mRNA decay by interacting with ARE-containing mRNAs, promotes the biogenesis of a subset of miRNAs (42). Some miRNA precursors reside in introns, and their expression often correlates with the transcription of the host gene or can be also regulated by RBPs during the precursor processing. miR-7 resides in an intron of HNRNPK, and the biogenesis of mature miR-7 is derepressed in siRNA-mediated knockdown of HuR in HeLa cells (6). Like miR-7, the miR-200b precursors reside in the first intron of Tll10-001 gene, and HuR binding to its intron and the flanking exons may directly influence the efficiency of miRNA generation.
Deletion of *Elavl1* in myeloid cells results in normalized vasculature. 

A, representative images of CD31 immunostaining of tumors. *n* = 3 mice/group.  

B and C, confocal fluorescence microscopy images of endothelial cells (CD31, green) and pericytes (smooth muscle actin, red) in LLC tumors. *n* = 5 mice/group.  

D, tumor vascular permeability. Fluorescent microscopy images of FITC-dextran (2000 kDa) and Texas Red-dextran (70 kDa) angiography on LLC isografts are shown. Arrows indicate extravasated Texas Red-dextran, indicating vascular leakage. *Elavl1* mice, *n* = 8; *Elavl1*Mø KO mice, *n* = 6.  

E, representative images of CD31 and SMA co-immunostaining of neovessels in growth factor impregnated-Matrigel plugs. *n* = 3 mice/group.  

F, up-regulated expression of angiopoietin 1 in *Elavl1*Mø KO BMDMs as determined by qRT-PCR and Western blotting.  

G, increased mRNA expression of fibronectin and type IV collagen α2.

Data represent mean ± S.E. (error bars). *n* = 4 mice/group.
HuR-regulated genes could be involved in up-regulation of miRNA gene transcription or stability. Nevertheless, because a single miRNA can have profound phenotypic effects, we explored the possibility that HuR interaction with miRNA function is involved in post-transcriptional gene regulatory circuits.

Many genes involved in the angiogenic response were altered in the Elavl1 MO KO mice, suggesting a functional role in blood vessel development. For example, Vegf-a, Jag1, Vcam1, and Nos2 were down-regulated. However, HuR in the macrophages is unlikely to play a role in developmental angiogenesis because Elavl1 MO KO mice did not show developmental or blood vessel defects. Thus, we hypothesized that macrophage HuR plays a role in the postnatal process regulated by macrophages such as tumor angiogenesis. In particular, we focused on Vegf-a, which is a major angiogenic factor important in tumor angiogenesis. Although the Vegf-a gene is strongly induced by hypoxia and cytokines at the level of transcription, it is also regulated at the post-transcriptional level by RNA-binding proteins and miRNAs such as AUF, hnRNP L, miR-15b, 16, and 20a/b (24, 26, 43). HuR is known to bind directly to Vegf-a mRNA (Fig. 3B and Ref. 7). Our data show that in the absence of HuR, basal expression of Vegf-a mRNA and polypeptide was signifi-
cantly suppressed. We also describe a novel site on the Vegf-a 3’-UTR where HuR and the miR-200b-RISC complex competitively regulate VEGF-A. HuR and miR-200b binding sites overlap, and the ability of miR-200b to suppress Vegf-a expression was antagonized by HuR. Importantly, more Ago-2-containing RISC complex was associated with Vegf-a mRNA in the absence of HuR. Functionally, miR-200b suppressed Vegf-a 3’-UTR luciferase reporter expression, and an antagonist of miR-200b restored VEGF expression in macrophages that lack HuR. These data strongly suggest that competitive interaction between HuR and miR-200b at the 3’-UTR of Vegf-a constitutes an RNA regulon that is important for the expression of VEGF-A.

Transcriptome-wide mapping analysis of the spatial relationship between HuR binding sites and Ago binding sites suggested that the functional antagonism of HuR is most likely due to competition for physical access at proximal sites (5). Dnd1, another RBP, also relieves miRNA activity by binding its target 3’-UTR at a location that overlaps with miRNA binding sites and blocking miRNA association with its target sites (44). hnRNP L, another RBP, also competes with miR-297/299 for the CA-rich element in the Vegf-a 3’-UTR (26). Thus, the antagonism of RBP in proximal miRNA-mediated repression may be a general mechanism in post-transcriptional gene regulation. A recent study demonstrates that HuR leads to the dissociation of miRNA-RISC complex from target miRNAs irrespective of the distance between miRNA-RISC sites and HuR binding site, suggesting that the HuR effect is unlikely due to steric hindrance of miRNA-RISC (45).

Interestingly, we found that post-transcriptional gene regulation by myeloid HuR is critical in tumor angiogenesis in mice. Subcutaneous LLC implants in myeloid Elavl1 KO mice showed reduced vascular density, vessel branching, vascular leakage, and attenuated tumor growth, suggesting that HuR promotes the proangiogenic phenotype in macrophages that lack HuR. These data strongly suggest that competitive interaction between HuR and miR-200b at the 3’-UTR of Vegf-a constitutes an RNA regulon that is important for the expression of VEGF-A.

In conclusion, HuR promotes angiogenesis by limiting the anti-angiogenic effect of miR-200b. It suppresses miR-200b expression and antagonizes its suppressive action on VEGF-A mRNA. Complex post-transcriptional control of VEGF-A expression may be necessary to achieve precise spatial and temporal control of this critical angiogenic factor. We speculate that the complex interplay between RBPs and miRNAs allow specificity, precision, and robustness of post-transcriptional gene regulation.

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REFERENCES
1. Keene, J. D. (2007) RNA regulons: coordination of post-transcriptional events. Nat. Rev. Genet. 8, 533–543
2. Moore, M. J. (2005) From birth to death: the complex lives of eukaryotic miRNAs. Science 309, 1514–1518
3. Shaw, G., and Kamen, R. (1986) A conserved AU sequence from the 3’ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. Cell 46, 659–667
4. Brennan, C. M., and Steitz, J. A. (2001) HuR and mRNA stability. Cell. Mol. Life Sci. 58, 266–277
5. Mukherjee, N., Corcoran, D. L., Nusbaum, J. D., Reid, D. W., Georgiev, S., Hafern, M., Ascano, M., Jr., Tuschl, T., Ohler, U., and Keene, J. D. (2011) Integrative regulatory mapping indicates that the RNA-binding protein HuR couples pre-mRNA processing and mRNA stability. Mol. Cell 43, 327–339
6. Lebedeva, S., Jens, M., Theil, K., Schwanhäusser, B., Selbach, M., Landthaler, M., and Rajewsky, N. (2011) Transcriptome-wide analysis of regulatory interactions of the RNA-binding protein HuR. Mol. Cell 43, 340–352
7. Kishore, S., Jaskiewicz, L., Burger, L., Haussler, J., Khorshid, M., and Zavolan, M. (2011) A quantitative analysis of CLIP methods for identifying binding sites of RNA-binding proteins. Nat. Methods 8, 559–564
8. Uren, P. I., Burns, S. C., Ruan, J., Singh, K. K., Smith, A. D., and Penalva, L. O. (2011) Genomic analyses of the RNA-binding protein Hu antigen R (HuR) identify a complex network of target genes and novel characteristics of its binding sites. J. Biol. Chem. 286, 37063–37066
9. López de Silanes, I., Zhan, M., Lal, A., Yang, X., and Gorospe, M. (2004) Identification of a target RNA motif for RNA-binding protein HuR. Proc. Natl. Acad. Sci. U.S.A. 101, 2987–2992
10. Katsanou, V., Milatos, S., Yiakouvaki, A., Sgantzi, N., Kotsoni, A., Alexiou, M., Harokopos, V., Aidinis, V., Hemberger, M., and Kontoyiannis, D. L. (2009) The RNA-binding protein Elavl1/HuR is essential for placental branching morphogenesis and embryonic development. Mol. Cell. Biol. 29, 2762–2776
11. Ghosh, M., Aguila, H. L., Michaud, J., Ai, Y., Wu, M. T., Hemmes, A., Ristic, L., Guo, C., Furneaux, H., and Hla, T. (2009) Essential role of the RNA-binding protein HuR in progenitor cell survival in mice. J. Clin. Invest. 119, 3530–3543
12. Papadaki, O., Milatos, S., Grammenoudi, S., Mukherjee, N., Keene, J. D., and Kontoyiannis, D. L. (2009) Control of thymic T cell maturation, deletion and egress by the RNA-binding protein HuR. J. Immunol. 182, 6779–6788
13. Katsanou, V., Papadaki, O., Milatos, S., Blackshear, P. J., Anderson, P., Kollias, G., and Kontoyiannis, D. L. (2005) HuR as a negative post-transcriptional modulator in inflammation. Mol. Cell 19, 777–789
14. Yiakouvaki, A., Dimitriou, M., Karakasiliotis, I., Efthychi, C., Theocharis, S., and Kontoyiannis, D. L. (2012) Myeloid cell expression of the RNA-bind-
ing protein HuR protects mice from pathologic inflammation and colorectal carcinogenesis. J Clin Invest. 122, 88–81
15. Zhang, J., Modì, Y., Yarovinsky, T., Yu, J., Collinge, M., Kyriakides, T., Zhu, Y., Sessa, W. C., Pardi, R., and Bender, I. R. (2012) Macrophage $\gamma$ integrin-mediated stabilization of angiogenic factor-encoding mRNAs in inflammatory angiogenesis. Am. J. Pathol. 180, 1751–1760
16. Du, R., Lu, K. V., Petritsch, C., Liu, P., Ganss, R., Passegué, É., Song, H., Vandenberg, S., Johnson, R. S., Werb, Z., and Bergers, G. (2008) HIF1α induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell 13, 206–220
17. Dvorak, H. F. (2002) Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. J. Clin. Oncol. 20, 4368–4380
18. Jain, R. K. (2003) Molecular regulation of vessel maturation. Nat. Med. 9, 685–693
19. Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O’Shea, K. S., Powell-Braxton, L., Hillan, K. J., and Moore, M. W. (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380, 439–442
20. Oosthuysse, B., Moons, L., Starkebaum, G., Beck, H., Nuyens, D., Brussel, M., Oosthuyse, B., Moons, L., Storkebaum, E., Beck, H., Nuyens, D., Brussel, M., K. R., Lee, S. W., and Detmar, M. (1998) Identification of a human VPF/H11032 target for diagnosis and therapy. J. Biol. Chem. 273, 6417–6423
21. Claffey, K. P., Shih, S. C., Mullen, A., Dziennis, S., Cusick, J. L., Abrams, K. R., Lee, S. W., and Detmar, M. (1998) Identification of a human VPF/H11032 target for diagnosis and therapy. J. Biol. Chem. 273, 6417–6423
22. Hua, Z., Lv, Q., Ye, W., Dong, Y., Li, J., Deng, Y., Zhu, L., Grzesik, D. A., Qian, F., Plate, K. H., Robberecht, W., Herbert, J. M., Collen, D., and Carmeliet, P. (2001) Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat. Genet. 28, 131–138
23. Fellows, A., Griffin, M. E., Petrella, B. L., Zhong, L., Parvin-Nejad, F., F.P., Fava, R., Morganelli, P., Robey, R. B., and Nichols, R. C. (2012) AUFI/hnRN D2 represses expression of VEGF in macrophages. Mol. Biol. Cell 23, 1414–1422
24. Essafi-Benkhdhir, K., Onesto, C., Stebe, E., Moroni, C., and Paqes, G. (2007) Tristetraprolin inhibits Ras-dependent tumor vascularization by inducing vascular endothelial growth factor mRNA degradation. Mol. Biol. Cell 18, 4648–4658
25. Jafarifar, F., Yao, P., Eswarappa, S. M., and Fox, P. L. (2011) Repression of VEGFA by CA-rich element-binding microRNAs is modulated by hnRN P L. EMBO J. 30, 1324–1334
26. Chang, S. H., and Hla, T. (2011) Gene regulation by RNA-binding proteins and microRNAs in angiogenesis. Trends Mol. Med. 17, 650–658
27. Filipowicz, W., Bhattacharrya, S. N., and Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. 9, 102–114
28. Chan, C. S., Elemento, O., and Tavazoie, S. (2005) Revealing posttranscriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. 9, 102–114
29. Zygmont, T., Gay, C. M., Blondelle, S., Singh, M. K., Flaherty, K. M., Means, P. C., Herwig, L., Krudewig, A., Beltung, H. G., Afholter, M., Epstein, I. A., and Torres-Vázquez, J. (2011) Semaphorin-PlexinD1 signaling limits angiogenic potential via the VEGF decoy receptor sFlt. Dev. Cell 21, 301–314
30. Avraham-David, I., Ely, Y., Pham, V. N., Castranova, D., Grunspan, M., Malkinson, G., Gibbs-Bar, L., Mayseless, O., Allmog, G., Lo, B., Warren, C. M., Chen, T. T., Unos, J., Kidd, K., Shaw, K., Rogachev, I., Wan, W., Murphy, P. M., Farber, S. A., Carmel, L., Shelsness, G. S., Iruela-Arispe, M. L., Weinstein, B. M., and Yaniv, K. (2012) ApoB-containing lipoproteins regulate angiogenesis by modulating expression of VEGF receptor 1. Nat. Med. 18, 967–973
31. Qin, B. Z., and Pollard, J. W. (2010) Macrophage diversity enhances tumor progression and metastasis. Cell 141, 39–51
32. Good, P. J. (1995) A conserved family of elav-like genes in vertebrates. Proc. Natl. Acad. Sci. U.S.A. 92, 4557–4561
33. Trabucchi, M., Briata, P., Garcia-Mayoral, M., Haase, A. D., Filipowicz, W., Ramos, A., Gherzi, R., and Rosenfeld, M. G. (2009) The RNA-binding protein KSRP promotes the biogenesis of a subset of microRNAs. Nature 459, 1010–1014
34. Hua, Z., Lv, Q., Ye, W., Dong, Y., Cai, G., Gu, D., Ji, Y., Zhao, C., Wang, J., Yang, B. B., and Zhang, Y. (2006) miRNA-directed regulation of VEGF and other angiogenic factors under hypoxia. PLoS One 1, e116
35. Kedde, M., Strasser, M. J., Boldajipour, B., Oude Vrielink, J. A., Slanchev, K., Strasser, M. J., Boldajipour, B., Oude Vrielink, J. A., Slanchev, K., Seigler, M., Williams, B. R., and Khabar, K. S. (2006) ARED 3.0: the large non-coding RNA database. Nucleic Acids Res. 34, D101–D104
36. Bakheet, T., Williams, B. R., and Khabar, K. S. (2006) ARED 3.0: the large and diverse AU-rich transcriptome. Nucleic Acids Res. 34, D111–D114
37. Essafi-Benkhadir, K., Onesto, C., Stebe, E., Moroni, C., and Pagés, G. (2007) Tristetraprolin inhibits Ras-dependent tumor vascularization by inducing vascular endothelial growth factor mRNA degradation. Mol. Biol. Cell 18, 4648–4658
38. Clausen, A., Wen, J., Marks, D. S., and Krogh, A. (2010) Signatures of bone marrow-derived macrophages (BMM): isolation and application. Cold Spring Harb. Protoc. 2008, pdb.prot5080
39. Almeida, O., Slanchev, K., le Sage, C., Nagel, R., Voorhoeve, P. M., van Duijse, J., Ørom, U. A., puerto, Y., Perrakis, A., Raz, E., and Agami, R. (2007) RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. Cell 131, 1273–1286
40. Kundu, P., Fabian, M. R., Sonenberg, N., Bhattacharya, S. N., and Filipowicz, W. (2012) HuR protein attenuates miRNA-mediated repression by promoting miRISC dissociation from the target RNA. Nucleic Acids Res. 40, 5088–5100
41. Lin, E. Y., Li, F., Gnatovskiy, L., Deng, Y., Zhu, L., Grzesik, D. A., Qian, H., Xue, X. N., and Pollard, J. W. (2006) Macrophages regulate the angiogenic switch in a mouse model of breast cancer. Cancer Res. 66, 11238–11246
42. Galbán, S., Kuwano, Y., Pullmann, R., Jr., Martindale, J. L., Kim, H. H., Lal, A., Abdelmohsen, K., Yang, X., Dang, Y., Liu, J. O., Lewis, S. M., Holcik, M., and Gorospe, M. (2008) RNA-binding proteins HuR and PTEB promote the translation of hypoxia-inducible factor 1α. Mol. Cell. Biol. 28, 93–107
43. McArthur, K., Feng, B., Wu, Y., Chen, S., and Chakraborti, S. (2011) MicroRNA-200b regulates vascular endothelial growth factor-mediated alterations in diabetic retinopathy. Diabetes 60, 1314–1323
44. Chan, Y. C., Roy, S., Khanna, S., and Sen, C. K. (2012) Down-regulation of endothelial microRNA-200b supports cutaneous Wound angiogenesis by desilencing GATA binding protein 2 and vascular endothelial growth factor receptor 2. Arterioscler. Thromb. Vasc. Biol. 32, 1372–1382
45. Chan, Y. C., Khanna, S., Roy, S., and Sen, C. K. (2011) miR-200b targets Ets-1 and is down-regulated by hypoxia to induce angiogenic response of endothelial cells. J. Biol. Chem. 286, 2047–2056

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51. Takeda, Y., Mishima, Y., Fujiwara, T., Sakamoto, H., and Inoue, K. (2009) DAZL relieves miRNA-mediated repression of germ-line mRNAs by controlling poly(A) tail length in zebrafish. *PLoS One* **4**, e7513

52. Maragkakis, M., Vergoulis, T., Alexiou, P., Reczko, M., Plomaritou, K., Gousis, M., Kourtis, K., Koziris, N., Dalamagas, T., and Hatziiogeiou, A. G. (2011) DIANA-microT Web server upgrade supports Fly and Worm miRNA target prediction and bibliographic miRNA to disease association. *Nucleic Acids Res.* **39**, W145–148

53. Westerfield, M. (1994) in The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Brachydanio rerio), 2.1 Ed., Institute of Neuroscience, University of Oregon, Eugene, OR

54. Keniry, A., Oxley, D., Monnier, P., Kyba, M., Dandolo, L., Smits, G., and Reik, W. (2012) The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and IGF1R. *Nat. Cell Biol.* **14**, 659–665