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Macrophage β_2 Integrin–Mediated, HuR-Dependent Stabilization of Angiogenic Factor–Encoding mRNAs in Inflammatory Angiogenesis

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HuR is a member of the *Drosophila* Elav protein family that binds mRNA degradation sequences and prevents RNase-mediated degradation. Such HuR-mediated mRNA stabilization, which is stimulated by integrin engagement and is controlled at the level of HuR nuclear export, is critically involved in T-cell cytokine production. However, HuR's role in macrophage soluble factor production, in particular in response to angiogenic stimuli, has not yet been established. We show that the labile transcripts that encode vascular endothelial growth factor and matrix metalloproteinase-9 are stabilized when murine macrophages adhere to the β_2 integrin ligand intercellular adhesion molecule-1. This mRNA stabilization response was absent in bone marrow–derived macrophages obtained from conditional macrophage-specific HuR knockout mice. The microvascular angiogenic response to an inflammatory stimulus (ie, subcutaneous polyvinyl alcohol sponge implantation) was markedly diminished in these macrophage HuR knockout mice despite the equal levels of macrophage localization to those observed in littermate wild-type controls. Furthermore, blood flow recovery and ischemic muscle neovascularization after femoral artery ligation were impaired in the conditional macrophage-specific HuR knockout mice. These results demonstrate that dynamic effects on mRNA, mediated by the RNA-binding and RNA-stabilizing protein HuR, are required for macrophage production of angiogenic factors, which play critical roles in the neovascular responses to a variety of stimuli, including tissue ischemia. ***(Am J Pathol 2012, 180:1751–1760; DOI: 10.1016/j.ajpath.2011.12.025)***

Angiogenesis, including vasculogenesis and arteriogenesis, is a fundamental physiologic process required for normal development, reproduction, wound repair, and response to ischemia.[1] It can also contribute to pathologic states, as in tumor angiogenesis.[2] Inflammation, broadly defined as the recruitment and localized function of leukocytes, has recently been implicated as an angiogenic trigger in response to tissue ischemia. Perivascular inflammatory cells, most notably macrophages, can be identified in tissues early after the onset of ischemia.[3] They can be potent producers of angiogenic factors [eg, vascular endothelial growth factor (VEGF), basic fibroblast growth factor, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor], which stimulate growth of neovessels.[4]

VEGF, in particular the predominant isoform VEGF-A, is a major mediator of physiologic and pathophysiologic angiogenesis.[5] It is essential in many aspects of developmental vasculogenesis and tissue-responsive angiogenesis. VEGF acts in a paracrine manner to stimulate differentiation of VEGFR2$^+$ angioblasts and to promote proliferation and survival of endothelial cells, thereby inducing sprouting of new vessels.[6] Transcription of the

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VEGF gene is strongly hypoxia driven. However, the VEGF transcript is intrinsically labile. Consequently, VEGF mRNA stabilization is an important regulatory point in the modulation of VEGF gene expression.[7]

Steitz and colleagues[8] first established the association between mRNA degradation and HuR, a member of the *Drosophila* Elav protein family. HuR binds to mRNA degradation sequences, adenylate uridylate–rich elements (AREs), in 3′-untranslated regions, thereby preventing ARE-bearing transcripts from RNase-mediated degradation.[9] VEGF is one such transcript. Its strong expression in hypoxic states (including ischemia) is a consequence of transcriptional induction and HuR-dependent mRNA stabilization.[10] Matrix metalloproteinase-9 (MMP-9) is another HuR-regulated transcript. The ubiquitously expressed HuR, but not the Hu family relatives HuB, HuC, and HuD, is the main constituent of complexes binding to ARE motifs of MMP-9's 3′-untranslated region.[11] HuR has been implicated in a diverse array of immune and inflammatory events owing to its apparent binding to and stabilization of numerous inducible transcripts, including those encoding interferon-γ, tumor necrosis factor-α, IL-8, IL-3, and the urokinase-type plasminogen activator.[12]

During leukocyte recruitment and tissue localization, largely chemokine driven, adhesion receptors must be productively engaged to promote firm adhesion and transmigration. Dynamically regulated leukocyte integrins dominate this facet of inflammation. In addition to their adhesive role, β_2 integrins transduce transmembrane signaling events, resulting in a variety of kinase cascades and activation of Rho family small G proteins.[13] We previously demonstrated that engagement of the β_2 integrin lymphocyte function–associated antigen-1 (LFA-1, also referred to as αL, β2, and CD11a/CD18) in T cells results in stabilization of ARE-bearing mRNAs encoding tumor necrosis factor-α and interferon-γ. This occurs through an induced, required, rapid nuclear-to-cytoplasmic translocation of HuR,[14] mediated by a Vav-1/Rac/p38 pathway.[15] We hypothesized that β_2 integrin engagement in cells of the monocyte/macrophage lineage can induce similar mRNA stabilizing signals. Furthermore, we propose that these HuR modulatory events are critical in angiogenic settings dependent on mononuclear cell recruitment and production of angiogenic factors.

In this work, we demonstrate that macrophage β_2 integrin engagement results in HuR-dependent stabilization of angiogenic factor–encoding VEGF and MMP-9 mRNAs. Furthermore, we targeted the murine HuR gene *in vivo* and used a macrophage-specific ($HuR^{fl/fl}$ LysM-Cre) HuR knockout (KO) to document a role for dynamic, HuR-dependent, macrophage-mediated angiogenic factor production in two "inflammatory angiogenesis" models. We discuss the importance of monocyte/macrophage recruitment, adhesion, tissue localization, and activation in angiogenic responses to a foreign body [polyvinyl alcohol (PVA) sponge model] and tissue ischemia (hind limb ischemia model).

Materials and Methods

Generation of Conditional HuR-KO ($HuR^{fl/fl}$ LysM-Cre^{+}) Mice

Exon 2 of the *Elavl1* gene, which encodes the start codon of HuR, was flanked by loxp sites using homologous recombination. A mouse genomic DNA BAC library (Genome Systems Inc., St. Louis, MO) was screened by PCR to isolate a clone containing the *Elavl1* gene. The BamHI/EcoR1 restriction fragments were cloned into pEasyFlox vector (Stratagene, Cedar Creek, TX) to generate the targeting vector (see Supplemental Figure S1A at *http://ajp.amjpathol.org*). This vector was linearized with SfiI and was transfected into SvJ.129 mouse embryonic stem cells. After selection with neomycin and gancyclovir, the embryonic stem clones were transfected with a plasmid-expressing Cre recombinase and were screened for excision of the neomycin cassette. The positive clones were subsequently injected into C57BL/6 blastocysts for chimera production. Mice with germline transmission of the floxed *Elavl1* allele (HuR^{fl}) were crossed with the C57BL/6 background for at least six generations. The resulting mice were intercrossed to generate homozygous $HuR^{fl/fl}$ mice, herein referred to as wild-type (WT) controls. These mice were further crossed with B6.129 P2-$Lyz2^{tm1(cre)Ifo}$/J mice[16] (The Jackson Laboratory, Bar Harbor, ME) to generate $HuR^{fl/fl}$ LysM-Cre^{+} mice, herein referred to as KO mice, which have a myeloid-specific conditional HuR deletion. All the mice were routinely screened for the presence of the HuR^{fl} allele by PCR using primers flanking the loxp site upstream of exon 2: 5′-GGATGACGGCAATGACTAAAA-3′ (F1) and 5′-TTTGGTTTGATTTGGTTGTTCTTG-3′ (R1). Deletion of exon 2 in bone marrow–derived macrophages (BMDMs) was verified by PCR using the forward primer listed in the previous sentence and another reverse primer, 5′-TTCAATTCCCTGCAACCACAT-3′ (R2), downstream of the second loxp site. The presence of the $LysM^{cre}$ allele was verified by PCR following the protocol of The Jackson Laboratory.

Flow Cytometry

Fluorescence staining was performed on ~2 to 5×10^5 cells following standard procedures. Data were acquired using a FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ) and were analyzed using FlowJo software version 9.3.2 (Tree Star Inc., Ashland, OR). The following antibodies were used in the experiments: anti-F4/80-A647 (eBioscience, San Diego, CA), anti-F4/80–fluorescein isothiocyanate (eBioscience), phycoerithtin anti-mouse CD11a (BD Pharmingen, Franklin Lakes, NJ), phosphatidylethanolamine rat anti-mouse CD11b (eBioscience), and phosphatidylethanolamine anti-mouse CD18 (BD Pharmingen). For intracellular HuR staining, cells were fixed with 4% paraformaldehyde, permeabilized with saponin, and stained with mouse anti-HuR antibody (clone 3A2; Santa Cruz Biotechnology, Santa Cruz, CA) or mouse IgG1 isotype control labeled with Zenon Alexa Fluor 488 anti-mouse IgG1 antibody (Invit-

rogen, Carlsbad, CA) and run using the FACSCaliber flow cytometer.

BMDM Isolation and Culture

BMDMs were obtained by *in vitro* differentiation of primary bone marrow cells using an established protocol[17] with the minor variations noted herein. Briefly, femurs and tibiae from WT and KO mice were isolated, cleaned, disinfected in 70% ethanol for ~2 to 5 minutes, washed in PBS, and ground in RPMI 1640 medium, followed by serial washing and centrifugation in cold PBS. Bone marrow cells were then cultured at a density of 4×10^6 cells/mL in 30% L929 cell–conditioned medium and 70% fully supplemented RPMI 1640 medium (Invitrogen). On day 4, fresh medium containing L929 cell–conditioned medium was added, after which cells matured into phenotypic macrophages over 7 days. Cells were recovered with 1 mmol/L EDTA and vigorous pipetting. Flow cytometric analysis confirmed that recovered cells were >97% $F4/80^+$, $CD11b^+$.

mRNA Decay Assay

RNA decay assays and real-time PCR were performed as previously described.[15] A total of 4.5×10^6 cells/mL of the murine macrophage cell line Raw 264.7 or primary BMDMs (WT or KO) was resuspended in the divalent cation–containing "integrin activation buffer" [100 mmol/L Tris-HCl (pH 7.5), 0.9% NaCl, 2 mmol/L $MnCl_2$, 2 mmol/L $MgCl_2$, 5 mmol/L D-glucose, and 1.5% bovine serum albumin] and was plated onto dishes coated with either 0.1 ng/mL recombinant mouse intercellular adhesion molecule 1 (ICAM-1)–Fc (R&D Systems, Minneapolis, MN) or, as a negative control, 20 mg/mL poly-L-lysine (Sigma-Aldrich, St. Louis, MO). At 30 minutes, the integrin activation buffer was replaced with RPMI 1640 medium/10% fetal bovine serum containing 1 nmol/L Phorbol 12-myristate 13-acetate (PMA) to trigger transcription. This concentration of PMA was in all the experimental samples and controls and never, alone, promoted mRNA stabilization. After 2.5 hours (37°C), 0.5 mmol/L 5,6-dichloro-1-β-D-ribobenzimidazole (DRB) (Sigma-Aldrich) was added to arrest transcription. This was considered time 0 in decay assays. Cells were harvested at the indicated time points, and RNA was harvested for analysis by quantitative PCR. mRNA levels at time 0 were designated 100%, to which each subsequent time point mRNA level was compared. In some experiments, the effect of β_2 integrin blockade on induced mRNA stabilization was assessed. Isolated BMDM Fc receptors were first blocked with 0.5 μg/10^7 cells of anti-CD16/CD32 monoclonal antibody (eBioscience) on ice for 15 minutes, followed by saturating concentrations of anti-CD8 monoclonal antibody (clone 53–6.72, IgG2a isotype control), anti-CD11a (clone M17/4.4.11.9, IgG2a), or anti-CD11b (clone M18/2.a.12.7, IgG2a) on ice for 60 minutes. Excess antibody was washed out, and the cells were resuspended in integrin activation buffer, after which the decay assay proceeded as described previously herein.

Quantitative Real-Time PCR

Total RNA was isolated from cells using the RNeasy mini kit (Qiagen, Germantown, MD) and 1-μg was reverse transcribed using an iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer's protocols. The resulting cDNA template was subjected to real-time PCR analysis by a QuantiTect SYBR Green PCR kit (Qiagen) using an Opticon DNA Engine 2 (MJ Research, Waltham, MA) and the following cycling parameters: 95°C for 15 minutes, then 50 cycles of 95°C for 15 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. VEGF and MMP-9 mRNA levels were normalized to β-actin levels for each sample run in duplicate. Sequences of primers used in quantitative real-time PCR were all synthesized by the Keck Biotechnology Resource Facility at Yale University (New Haven, CT) as follows: VEGF, 5′-GGAGATCCTTCGAGGAGCACTT-3′ (sense) and 5′-GGCGATTTAGCAGCAGATATAAG; AA-3′ (antisense); MMP-9, 5′-CGTCGTGATCCC-CACTTACT-3′ (sense) and 5′-AACACACAGGGTTTGCCTTC-3′ (antisense); and actin, 5′-GTGGGCCGCTCTAGGCACCAA-3′ (sense) and 5′-TGGCTTTAGGGTTCAGG-GGG-3′ (antisense).

ELISA

VEGF levels were measured in supernatants from cultures of BMDMs after 0.5-, 12-, and 24-hour adhesion to ICAM-1– or control poly-L-lysine–coated dishes. VEGF protein was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (PeproTech Inc., Rocky Hill, NJ). VEGF concentrations were normalized to cell number (per 1.5×10^6 cells) and were run in triplicate.

PVA Sponge Implant Model

Two subcutaneous dorsolateral pockets were created through 1.25-cm incisions, each into which a 2-mm-thick, 6-mm-diameter sterile PVA sponge (Ultra Medical Technologies Inc., North Stonington, CT) was inserted. Sponges were harvested at the indicated time points with collagenase digestion for cell [fluorescence-activated cell sorting (FACS)] analysis and VEGF or MMP-9 RT-PCR; frozen section 4% paraformaldehyde fixation (6-μm sections) for VEGF, MMP-9, and F4/80 immunofluorescence; or zinc fixation and paraffin embedding (5-μm sections) for tetrarhodamine isothiocyanate–labeled lectin (*Bandeiraea simplicifolia* 1; Sigma-Aldrich) staining.

Hind Limb Ischemia Model

After anesthesia with ketamine and xylazine, the proximal and distal portions of the femoral artery were ligated, and the distal portion of the saphenous artery was ligated or cauterized, as were all the branches in these ligated segments. Contralateral sham operations of skin incision without femoral artery ligation were performed for control. A deep, penetrating laser Doppler probe (PeriFlux System; Perimed Inc., North Royalton, OH) was placed directly on the gastrocnemius muscle before and after li-

gation and in the contralateral nonischemic limb, with initial flow measurements repeated at 3 days and at 1-week intervals to 4 weeks. Laser Doppler imaging flow results are expressed as the ratio of ischemic to nonischemic (contralateral) hind limb perfusion to avoid the influence of ambient light and temperature. A clinical scoring system was used for limb mobility and tissue injury as previously defined.[18] At 4 weeks, gastrocnemius and soleus muscles from the ischemic and nonischemic control limbs were harvested, zinc fixed, and paraffin embedded. Sections, 5 μmol/L, were stained with tetrarhodamine isothiocyanate–labeled lectin, as described previously herein. In addition, hind limb muscles (gastrocnemius and soleus) were harvested at the indicated time points, and collagenase was digested to obtain cells for FACS analysis.

Immunofluorescence and Morphometry

HuR immunofluorescence and its morphometric assay, have been previously described.[15] WT BMDMs were treated with integrin activation buffer for 60 minutes on coverslips coated with either 0.1 ng/mL recombinant mouse ICAM-1–Fc (R&D Systems) or, as a negative control, 20 mg/mL poly-L-lysine (Sigma-Aldrich). Adhered cells were fixed with 4% paraformaldehyde for 15 minutes at 4°C and were subsequently permeabilized with 0.1% Triton X-100 (Roche Diagnostics GmbH, Mannheim, Germany), followed by blocking with 5% goat serum overnight at 4°C. Cells were immunostained with 5 μg/mL anti-HuR overnight at 4°C and were co-stained with 0.005% DAPI (Invitrogen). For the morphometric assay, 20 cells from each condition in three different fields were included in the analysis. The ratio of HuR translocation was calculated as the area visible with anti-HuR antibodies in the cytoplasm plus the nucleus divided by the nuclear area (by DAPI staining for nuclear definition). ImageJ software version 1.32j (NIH, Bethesda, MD) was used to determine the areas.

Animal Studies

All the animal studies were approved by the Institutional Animal Care and Use Committee of Yale University. For all the procedures on live mice, the animals were anesthetized i.p. with ketamine (80 mg/kg) and xylazine (10 mg/kg).

Statistical Analysis

Statistical significance was determined by two-way analysis of variance (ANOVA) followed by a Bonferroni posttest for multiple comparisons or by an unpaired *t*-test using GraphPad Prism software version 5.01 (GraphPad Software Inc., San Diego, CA). Minimum levels of significance were set at $P < 0.05$.

Results

Targeting of HuR Genes and Characterization of Conditional KO Macrophages

To obtain macrophages with conditional deletion of HuR, we flanked exon 2 of the *Elav1* gene with loxp sites (see Supplemental Figure S1A at *http://ajp.amjpathol.org*) and crossed the resulting HuR^{fl} mice with LysM-Cre mice. Expression of Cre recombinase driven by the LysM promoter resulted in deletion of exon 2 encoding the start codon of HuR in differentiated BMDMs, as evidenced by the product in the PCR with the reverse primer R2 (see Supplemental Figure S1B at *http://ajp.amjpathol.org*). Although some of the genomic DNA remained intact (see Supplemental Figure S1B at *http://ajp.amjpathol.org*), quantitative real-time PCR analyses of the genomic DNA from BMDMs confirmed that the efficiency of deletion approached 95%. BMDMs and splenic lymphocytes were isolated from $HuR^{fl/fl}$ LysM-Cre^{+} (designated KO) and $HuR^{fl/fl}$ (littermate control, designated WT) mice. Immunoblotting revealed effective and specific HuR deletion in gene-targeted BMDMs (Figure 1A). Total splenocyte HuR was only minimally reduced, and none of the AUF-1 (another RNA-binding protein) isoforms were af-

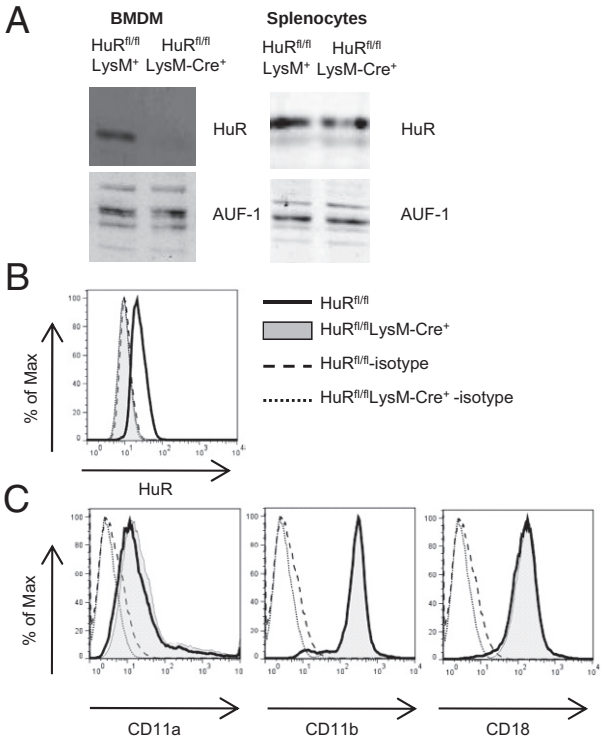

Figure 1. Characterization of conditional HuR KO macrophages. **A:** Lysates obtained from BMDMs and total splenocytes isolated from KO ($HuR^{fl/fl}$ LysM-Cre^{+}) and littermate WT control ($HuR^{fl/fl}$) mice were immunoblotted with anti-HuR or anti-AUF1 antibodies. **B:** BMDMs isolated from KO and WT control mice were fixed and permeabilized, followed by intracellular HuR staining with anti-HuR or mouse IgG1 isotype control and Zenon labeling FITC-conjugated secondary antibody. Five thousand events were FACS-analyzed per sample. **C:** KO and WT control BMDMs were FACS-analyzed for surface CD11a, CD11b, and CD18 expression. Five thousand events were acquired per sample. Data are representative of cells obtained from four different mice.

fected. Flow cytometric analysis of intracellular HuR expression confirmed the loss of HuR protein in KO BMDMs (Figure 1B). Furthermore, maintenance of surface β_2 integrin expression in KO BMDMs was documented (Figure 1C), indicating equivalent levels of LFA-1 and Mac-1 in macrophages derived from WT and KO mice.

To prevent the creation of a lysozyme M–deficient animal, mice with the Cre gene inserted into only one allele of lysozyme M were always used. Use of the lysozyme M promoter to direct macrophage-specific expression of a transgene has been widely discussed. Issues related to timing of promoter activation and specificity are relevant. Neutrophils express lysozyme M. As such, LysM-driven Cre expression can result in neutrophil gene deletion.[16] The $HuR^{fl/fl}$ LysM-Cre mice used in the present studies had only a mild reduction in neutrophil HuR. Circulating monocytes had intermediate levels, whereas tissue macrophages and BMDMs always had much lower levels of HuR. Recent lineage ablation models using LysM-Cre/DTR mice demonstrate much greater depletion of macrophages than do either neutrophils or circulating monocytes.[19] Furthermore, the angiogenesis models we used are much more macrophage than neutrophil dependent.

Effect of β_2 Integrin Engagement and HuR Gene Deletion on Angiogenic Factor Gene Expression

We previously showed that T-cell LFA-1 engagement results in rapid nuclear-to-cytosolic HuR translocation and that this correlates with labile cytokine mRNA stabilization.[14,15] HuR translocation induced by other stimuli, including heat shock, correlates with ARE-bearing transcript stabilization as well.[20] To determine whether β_2 integrin engagement similarly triggers HuR translocation in cells of the monocyte/macrophage lineage, WT BMDMs were plated on ICAM-1 or poly-L-lysine for 60 minutes, after which HuR immunofluorescence was performed. Figure 2A demonstrates a complete overlap of DAPI and HuR in control adhered cells. In contrast, BMDMs adhered to ICAM-I displayed significant extranuclear localization of HuR, shown quantitatively in Figure 2B. Given previous correlations, this result directed the analysis of integrin-stimulated, HuR-dependent effects on labile mRNA decay in macrophages.

VEGF-A is a key angiogenic factor. Its mature transcript is ARE bearing, intrinsically labile, and known to be stabilized by HuR binding.[10] MMP-9 is a zinc-containing endopeptidase family member involved in extracellular matrix degradation and vascular remodeling.[21] MMP-9, provided by bone marrow–derived cells, has been shown to initiate the angiogenic switch, most notably in tumor angiogenesis.[22] The MMP-9 transcript also has AREs in its 3′-untranslated region, although dynamic regulation of its decay and HuR dependence has not been well characterized. To determine whether β_2 integrin engagement promotes angiogenic factor mRNA stabilization, we first performed RNA decay experiments with the immortalized murine macrophage line Raw 264.7. Cells were adhered to either immobilized β_2 integrin ligand

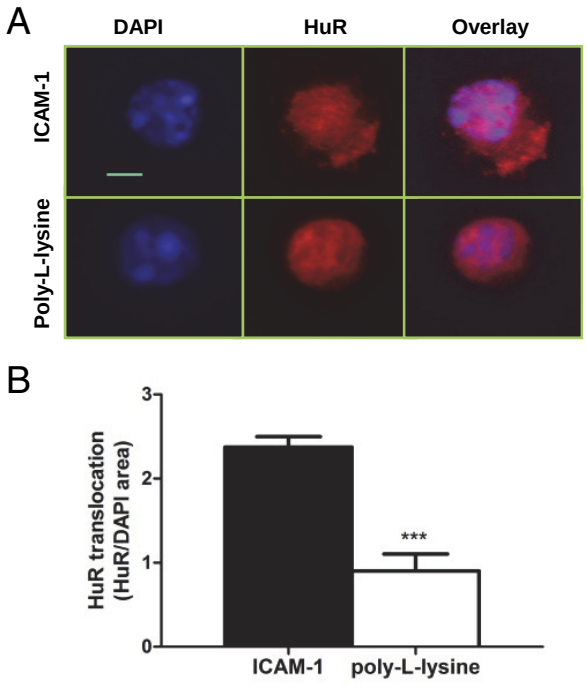

Figure 2. Effect of macrophage β_2 integrin engagement on HuR translocation. **A:** WT BMDMs were adhered to ICAM-1– or control poly-L-lysine–coated coverslips at 37°C for 60 minutes. Cells were fixed and permeabilized, and immunofluorescent co-staining was performed with anti-HuR and with DAPI for nuclear definition. Individual images were overlaid, and merges are displayed as noted. Scale bar = 5 μm. **B:** Quantitative analysis of HuR nuclear-to-cytosolic translocation. HuR translocation was expressed as the ratio of HuR immunofluorescent plus nuclear area divided by the nuclear area only, analyzed by ImageJ software version 1.32j. Twenty cells were analyzed per experimental sample. The data are presented as mean ± SEM. $^{***}P < 0.001$ by ANOVA.

recombinant ICAM-1 or the polycation control poly-L-lysine. After 30 minutes of adherence, PMA at a low concentration (1 nmol/L) was added to enhance transcription, which was allowed to proceed for 3 hours. The RNA polymerase II inhibitor DRB was then added, and RNA was recovered at multiple time points. Figure 3, A and B, demonstrates that in control poly-L-lysine–adhered cells, VEGF and MMP-9 mRNA decay to ~50% of time 0 levels at 40 minutes. In contrast, adherence onto ICAM-1, ie, β_2 integrin engagement, results in stabilization of both transcripts. This integrin-induced VEGF and MMP-9 mRNA stabilization was also observed in primary murine macrophages obtained from WT but not macrophage HuR KO mice (Figure 3, C and D). That is, the β_2 integrin–stimulated VEGF and MMP-9 transcript stabilization is lost in the absence of HuR. Figure 3E demonstrates the functional significance of this induced extended mRNA half-life. As expected, VEGF protein, as measured by ELISA, accumulated in supernatants of integrin-engaged WT macrophages (>threefold at 24 hours) but minimally in WT cells on poly-L-lysine or KO macrophages on ICAM-1. These data demonstrate that transmembrane integrin signaling drives the stabilization of labile, proangiogenic mRNAs in macrophages and that this posttranscriptional event is HuR dependent.

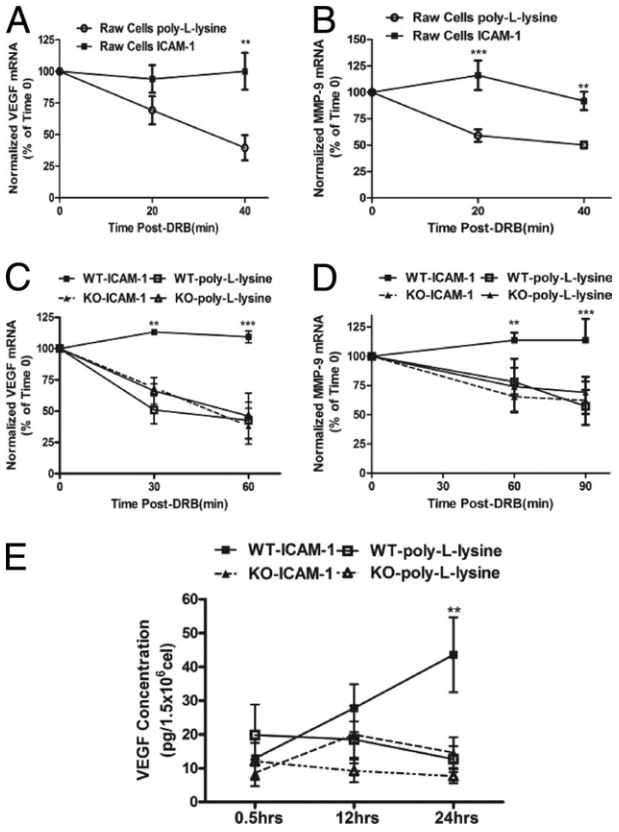

Figure 3. HuR requirement in β_2 integrin–induced angiogenic factor gene expression (mRNA stabilization and protein production). **A** and **B:** Raw 264.7 macrophages were stimulated with 1 ng/mL PMA to induce gene transcription and adhesion and were plated on ICAM-1– or poly-L-lysine–coated plates for 3 hours, after which 0.25 mmol/L DRB was added and mRNA was recovered at 0, 20, and 40 minutes. VEGF (**A**) and MMP-9 (**B**) mRNA levels, normalized to β-actin, were determined by quantitative RT-PCR and are expressed relative to time 0 (the time of DRB addition). Samples were analyzed in triplicate, and the data are presented as mean ± SEM. One representative of three independent experiments is shown. **C** and **D:** BMDMs isolated from WT and KO mice were adhered to ICAM-1– or poly-L-lysine–coated plates for 3 hours, after which they were transcription arrested with 0.25 mmol/L DRB and RNA was harvested at 0, 30, or 60 minutes for VEGF and MMP-9 quantitative RT-PCR analysis. VEGF (**C**) and MMP-9 (**D**) mRNA levels were normalized to β-actin and are expressed relative to time 0 (the time of DRB addition). Data are presented as the combined mean ± SEM of three separate experiments. **E:** BMDMs from WT and KO mice were plated on ICAM-1– or poly-L-lysine–coated plates and were cultured in supplemented RPMI 1640 medium with 2.5 ng/mL macrophage colony-stimulating factor for 0.5, 12, and 24 hours. VEGF levels in the supernatant from the indicated time points were determined by ELISA. Data are displayed as mean ± SEM VEGF level for three separate experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ by ANOVA.

Effect of Macrophage HuR Gene Deletion on Inflammatory Angiogenesis in Vivo

Little is known about HuR-dependent mechanisms regulating angiogenic factor mRNA *in vivo*. We focused on angiogenesis models that we believe to depend, in part, on the recruitment of mononuclear leukocytes. As such, we used a PVA sponge implantation mouse model, which exhibits many features of wound healing, including monocyte/macrophage localization of microvessel formation. On days 7, 10, and 14 after subcutaneous implantation, PVA sponges were excised and evaluated for leukocyte localization, VEGF and MMP-9 mRNA expression, and angiogenesis. Flow cytometric analysis demonstrated equal percentages of macrophages ($F4/80^+$) extracted from excised sponges in WT and KO mice (Figure 4B). Total cell numbers extracted were similar, indicating nearly identical numbers of macrophages localized to PVA sponges in WT and KO mice. Macrophages recovered from KO PVA sponges were, indeed, HuR deleted, as determined by flow cytometric intracellular HuR analysis (Figure 4D). Immunofluorescent micrographs confirmed the lack of a defect in macrophage localization to sponges in macrophage HuR KO mice (Figure 4, A and E). However, there was substantially less detectable VEGF and MMP-9 observed in these micrographs (Figure 4, A and E, respectively). Of note is that merged images displayed mostly detectable VEGF and MMP-9 in and around $F4/80^+$ cells. This finding supports the concept that recruited mononuclear leukocytes are the primary sources of the angiogenic factors VEGF and MMP-9 in this model. Levels of VEGF and MMP-9 mRNA, extracted from excised sponges and analyzed by quantitative PCR, confirmed a significant reduction in KO sponges at 2 weeks and 3 days, respectively (Figure 4, C and F). Most important, there was a dramatic reduction in endothelial-specific lectin reactivity and, therefore, microvessel formation in sponges excised from macrophage HuR KO mice (Figure 4G, with quantification in Figure 4H). This correlated with a reduction in $CD45^-$, $CD31^+$ (endothelial) cells extracted from excised KO sponges at all time points tested (see Supplemental Figure S2 at *http://ajp.amjpathol.org*). These results demonstrate that monocyte/macrophages are the primary source of the angiogenic factors VEGF and MMP-9 in this inflammatory angiogenesis model and that macrophage HuR-dependent posttranscriptional mechanisms are required for neovessel formation.

Clearly, other cells produce VEGF in response to wounding. However, the present data are consistent with those generated in wound repair models in which macrophages have been selectively depleted.[23] In the absence of macrophages, neovascularization is disturbed and overall wound healing is impaired.

Effect of Macrophage-Specific HuR Gene Deletion on Flow Recovery to Hind Limb Ischemia

Numerous studies support at least a role for the recruitment and localization of bone marrow–derived cells in adaptive responses to tissue ischemia.[24] To determine whether the defects in inflammatory angiogenesis, described previously herein, are relevant in more widely applicable and relevant pathologic models, femoral artery ligation with consequent hind limb ischemia was performed in WT and macrophage-specific HuR KO mice, following which the indices of angiogenesis, blood flow, and clinical scoring were examined. A deep, penetrating laser Doppler probe was used to measure gastrocnemius blood flow immediately, 3 days, and 1, 2, 3, and 4 weeks after ligation in the ischemic and control contralateral nonischemic hind limbs. Significant attenu-

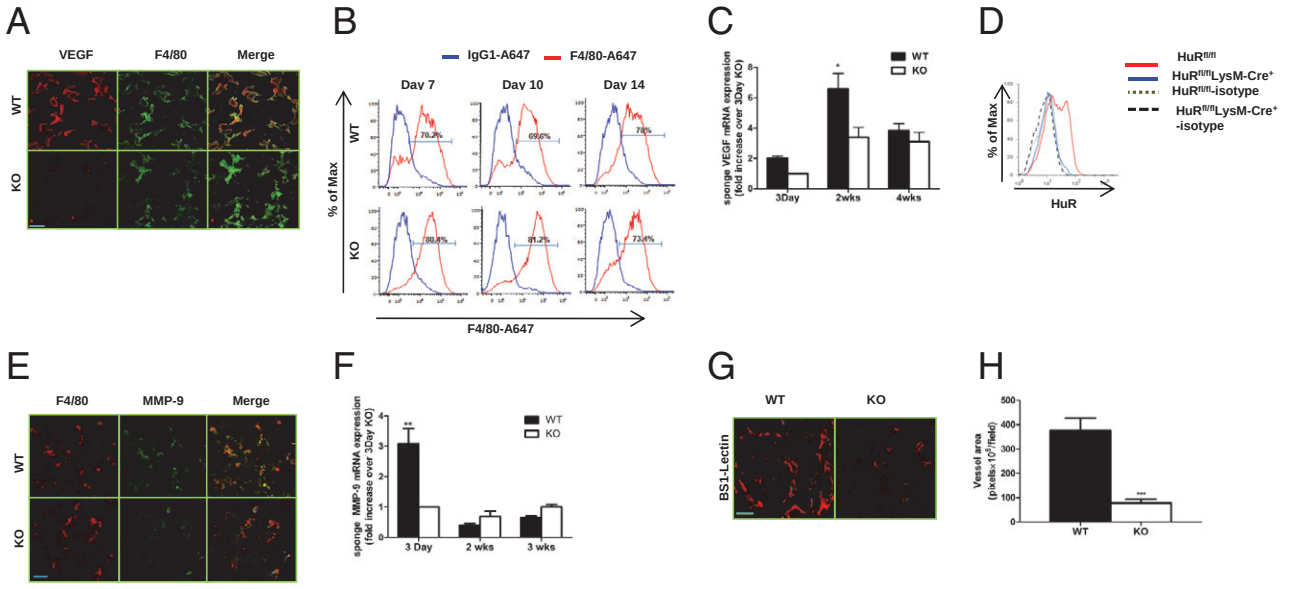

Figure 4. Effect of macrophage HuR gene deletion on inflammatory angiogenesis *in vivo*. **A:** Two weeks after subcutaneous PVA sponge implantation into macrophage HuR KO ($HuR^{fl/fl}$ LysM-Cre^{+}) or WT ($HuR^{fl/fl}$) mice, sponges were excised, sectioned, and immunostained for the localization of macrophages ($F4/80^{+}$) and the production of VEGF, with notable digitally merged images. Scale bar = 50 μm. **B:** Implanted PVA sponges were excised at the indicated time points, and cells extracted by collagense digestion were phenotyped by FACS for macrophages ($F4/80^{+}$). Five thousand events were acquired per sample. **C:** RNA was isolated from cells extracted from implanted PVA sponges excised at the indicated time points. VEGF mRNA levels were determined by quantitative RT-PCR, displayed as fold increase over 3-day KO level, defined as 1.0. Data are mean ± SEM; $n = 3$ mice per group at each time point. $^{*}P < 0.05$ by ANOVA. **D:** PVA sponges were excised 4 weeks after implantation. Cells were recovered by collagenase digestion, and the macrophages ($F4/80^{+}$ cells) were analyzed by FACS for intracellular HuR expression. FACS profiles represent cells stained with anti-HuR or isotype control IgG1 as indicated. Five thousand events were acquired per sample. **E:** Using the same process applied in **A,** we assessed MMP-9. Scale bar = 50 μm. **F:** Using the same process applied in **C**, we assessed MMP-9. Data are mean ± SEM; $n = 3$ mice per group at each time point. $^{**}P < 0.01$ by ANOVA. **G:** Implanted PVA sponges were excised at 6 weeks, sectioned, and stained with tetrarhodamine isothiocyanate–conjugated endothelial-specific lectin (*Bandeiraea simplicifolia* 1). Scale bar = 40 μm. **H:** Quantification as determined by ImageJ 1.32j analysis. Data are mean ± SEM; $n = 4$ animals per group. $^{***}P < 0.001$ by ANOVA.

ation of blood flow recovery was observed in the macrophage HuR KO mice compared with in WT controls (Figure 5A). This correlated well with clinical recovery. Ischemic hind limbs became paretic in WT and KO mice and largely recovered function over a 4-week period in WT, but not in KO, mice. Figure 5B displays a much greater degree of necrosis at 2 weeks in the paws of KO mice compared with those of WT mice. This finding is quantified using the clinical scoring system[18] in Figure 5C. Furthermore, capillary number per muscle fiber, as determined by endothelial-specific lectin reactivity, was significantly lower in hind limb muscle (gastrocnemius and soleus) obtained from macrophage HuR KO mice compared with from WT controls 4 weeks after ligation (Figure 5D, with quantification in Figure 5E). There were no differences in baseline or nonischemic muscle capillary (Figure 5, D and E) density. This difference was not due to impaired recruitment and localization to ischemic muscles as there were equal numbers of $F4/80^{+}$ cells in WT and KO ischemic muscle (see Supplemental Figure S3A at *http://ajp.amjpathol.org*, with quantification in Supplemental Figure S3B) and there were equal percentages of macrophages ($F4/80^{+}$) extracted from excised hind limb muscle in WT and KO mice by flow cytometric analysis (see Supplemental Figure S3C at *http://ajp.amjpathol.org*, with quantification in Figure 5F). These data demonstrate that dynamic posttranscriptional mechanisms, specifically in macrophages, play a major role in neovessel formation and flow recovery in acutely ischemic tissues.

Discussion

The mRNA-binding Hu family proteins are involved in a diverse array of biological processes, including neuronal differentiation and plasticity. HuR is ubiquitously expressed and has been convincingly demonstrated to play a pivotal role in cellular stress responses.[25] It provides a dynamic level of gene expression control from effects on RNA splicing to translation.[26] This includes labile mRNA half-life extension, allowing a burst of transcript accumulation and consequent protein production. This is highly relevant not only in development but also in adaptive responses to metabolic, immune/inflammatory, and ischemic perturbations.

Ischemic neovascularization, including microvessel angiogenesis and arteriogenesis, is a complex process requiring contributions of numerous growth factors, cytokines, and proteases, including VEGF, placental growth factor, fibroblast growth factor, insulinlike growth factor 1, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, angiopoietins, and MMPs.[27–29] The relative importance of a given soluble factor depends on the exact context, including the stimulus and target tissue. VEGF is the prototype angiogenic factor that is involved, to some degree, in most neovascular responses. It is hypoxia/ischemia stimulated, is encoded by a very labile transcript(s) that is known to be HuR bound, and can be produced by inflammatory cells. MMP-9 is a zinc-dependent metalloproteinase involved in degrading the extracellular matrix, a function important in

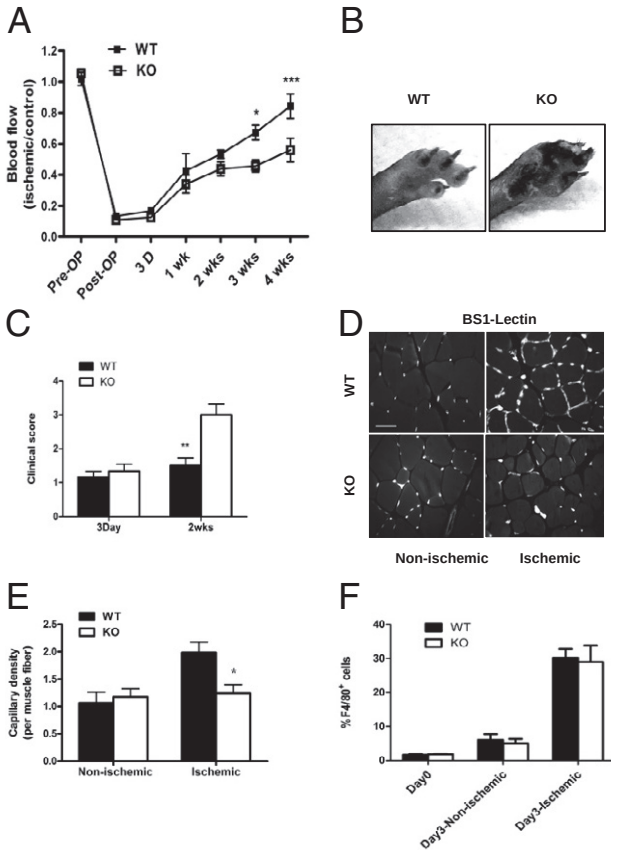

Figure 5. Effect of macrophage HuR gene deletion on blood flow recovery to hind limb ischemia. **A:** Left femoral artery ligation or sham contralateral surgery was performed on WT ($HuR^{fl/fl}$) and HuR KO ($HuR^{fl/fl}$ LysM-Cre^{+}) mice ($n = 6$ in each experimental group). Laser Doppler imaging assessment of gastrocnemius blood flow was performed before (Pre-OP), immediately after (Post-OP), and at the indicated time points after ligation. Data are expressed as a ratio of the left (ischemic) to right (control) limb perfusion ± SEM. $n = 6$ animals per experimental group. $^{*}P < 0.05$, $^{***}P < 0.001$ by ANOVA. **B:** Photographs of paws distal to femoral artery ligation at 2 weeks in WT and HuR KO mice, with severity of necrosis (black) displayed. **C:** Clinical scoring of ischemic hind limbs in WT and HuR KO mice at the indicated time points. As an index of limb ischemia severity, scores are designated as follows: 0, normal; 1, pale foot and/or gait abnormalities; 2, less than half of paw necrotic; 3, more than half of paw necrotic without lower limb necrosis; and 4, more than half of paw necrotic with some lower limb necrosis. Data are expressed as mean ± SEM, $n = 6$ per experimental group. $^{***}P < 0.01$ by ANOVA. **D:** Photomicrographs of endothelial-specific lectin staining from representative sections of gastrocnemius and soleus muscles 4 weeks after femoral artery ligation in WT and HuR KO mice. Scale bar = 80 μm. **E:** Per lectin staining, quantitative data are presented as capillary density or number per muscle fiber, which was counted in eight randomly selected fields from three to four sections per mouse in each experimental group ($n = 3$ animals per group). Data are expressed as mean ± SEM. $^{*}P < 0.05$ by ANOVA. **F:** Quantification of macrophage localization to ischemic muscle. Gastrocnemius and soleus muscles were excised at the indicated time points, and cells extracted by collagense digestion were phenotyped by FACS for macrophages ($F4/80^{+}$). Five thousand events were acquired per sample, with histograms displaying the percentage of F4/80-positive cells (mean ± SEM) in each experimental group ($n = 3$ animals per group).

angiogenesis and vascular remodeling. MMP-9 has been shown to be important in regulating smooth muscle cells function such that silencing MMP-9 gene expression inhibits smooth muscle cells migration.[30] The present *in vitro* data demonstrate macrophage β_2 integrin–stimulated, HuR-dependent stabilization of the VEGF and MMP-9 transcripts. Furthermore, the reduction in VEGF and MMP-9 (RNA and protein) in both angiogenesis models, when using the macrophage-specific HuR gene–deleted mouse, with a correlative impairment in neovascularization, not only documents the importance of the aforementioned posttranscriptional mechanisms in these responses but also defines recruited inflammatory cells as critical producers of soluble factors contributing to angiogenesis. This adds mechanistic data to the long-standing notion that macrophages play an important role in wound repair.[31] Recent work has carefully quantified the contributions of macrophages to distinct phases of wound repair, some of which include an angiogenic response.[23,32,33]

ICAM-1 is the most widely expressed ligand for β_2 integrins, productively engaging LFA-1 and Mac-1. In the context of wound healing and angiogenesis, ICAM-1 is basally expressed on all endothelial cells, as well as on fibroblasts and melanocytes. Its expression is induced or up-regulated in ischemic muscle cells,[34] in viable myocardium on the border zone of infarcted heart muscle,[35] and in many cells in the setting of inflammatory cytokine release. In the present models, it is likely that basal and induced ICAM-1 expressed in capillary and postcapillary venular endothelium engages monocyte/macrophage β_2 integrin, promoting firm adhesion and transmigration and triggering the critical signal transduction resulting in the noted posttranscriptional regulatory responses. Few T lymphocytes express Mac-1 (CD11b/CD18). As such, our previous ICAM-1–binding, integrin-engagement experiments clearly reflected LFA-1 transmembrane signaling. Macrophages express high levels of LFA-1 and Mac-1. Interaction between LFA-1 and ICAM-1 occurs through LFA-1's insertion domain and ICAM-1's first Ig-like domain.[36] In contrast, Mac-1's insertion domain interacts with ICAM-1's first Ig-like domain.[37] Given this difference, it is possible that one of these two interactions more productively stimulates the kinase cascade known to result in HuR translocation. Supplemental Figure S4 (available at *http://ajp.amjpathol.org*) demonstrates that anti-CD11a, but not anti-CD11b, monoclonal antibody efficiently blocks ICAM-1–stimulated BMDM VEGF mRNA stabilization, suggesting that, as in T cells, engagement of LFA-1 drives the HuR-dependent mRNA stabilization response.

The role of β_2 integrins in chemokine-driven leukocyte recruitment, adhesion, and transmigration has been extensively studied. Using a similar hind limb ischemia model, Chavakis et al[38] previously demonstrated significantly impaired limb perfusion recovery in β_2 integrin gene–deleted mice compared with in WT controls. In the present work, HuR conditional KO macrophages expressed typical membrane levels of LFA-1 (Figure 1C) and adhered normally to the β_2 integrin ligand ICAM-1 (data not shown). Consistent with this, equal numbers of HuR KO and WT macrophages were recruited and localized to responsive tissues in both of the *in vivo* models. Given the dramatic reduction in macrophage β_2 integrin–stimulated VEGF and MMP-9 mRNA stabilization and protein production in HuR KO macrophages, we believe that, on recruitment, adhesion, and transmigration, integrin-triggered signals are initiated, but a major downstream consequence, that of HuR-dependent posttranscriptional

(mRNA stability) regulation, is defective. Limiting quantities of proangiogenic factors are produced, with reduction in induced neovessel formation and limb perfusion recovery.

In addition to beneficial angiogenesis required in adaptive responses to tissue ischemia and in wound healing, pathologic angiogenesis driving tumor growth and metastasis can also be macrophage dependent. Monocyte-depleted mice have less-vascularized tumors,[39] and the degree of angiogenesis correlates with the number of CD68-positive macrophages in human tumors.[40] Tumor-associated macrophages produce VEGF and MMP-9, which are both required for full-amplitude angiogenesis and the growth of some tumors.[41] Furthermore, greater levels of cytoplasmic HuR correlate with the aggressiveness and regional metastasis of some breast cancers.[42] It is likely that the link between macrophage recruitment/localization, signals driving HuR translocation, stabilization of otherwise labile transcripts encoding angiogenic molecules, macrophage production of these soluble factors, and tumor angiogenesis follows the same pattern as in the adaptive models.

Many of the potential angiogenic factors described herein are encoded by ARE-bearing transcripts and, as such, would also require similar posttranscriptional controls. It is likely that a significant deficiency of multiple macrophage-secreted factors plays a role in the impaired responses we observed. We are currently characterizing the spectrum of relevant macrophage transcripts that are deficient in these models. The findings presented herein are the first to document a role of posttranscriptional mechanisms, mediated by a ubiquitous RNA-binding protein and required selectively in macrophages, in adaptive angiogenic responses, including that observed in ischemia.

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