Hu Antigen R (HuR) Is a Positive Regulator of the RNA-binding Proteins TDP-43 and FUS/TLS

IMPLICATIONS FOR AMYOTROPHIC LATERAL SCLEROSIS*

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Posttranscriptional gene regulation is governed by a network of RNA-binding proteins (RBPs) that interact with regulatory elements in the mRNA to modulate multiple processes, including splicing, RNA transport, RNA stability, and translation. Mounting evidence indicates that there is a hierarchy within this network whereby certain RBPs cross-regulate other RBPs to coordinate gene expression. HuR, an RNA-binding protein we linked previously to aberrant VEGF mRNA metabolism in models of SOD1-associated amyotrophic lateral sclerosis, has been identified as being high up in this hierarchy, serving as a regulator of RNA regulators. Here we investigated the role of HuR in regulating two RBPs, TDP-43 and FUS/TLS, that have been linked genetically to amyotrophic lateral sclerosis. We found that HuR promotes the expression of both RBPs in primary astrocytes and U251 cells under normal and stressed (hypoxic) conditions. For TDP-43, we found that HuR binds to the 3′ untranslated region (UTR) and regulates its expression through translational efficiency rather than RNA stability. With HuR knockdown, there was a shift of TDP-43 and FUS mRNAs away from polysomes, consistent with translational silencing. The TDP-43 splicing function was attenuated upon HuR knockdown and could be rescued by ectopic TDP-43 lacking the 3′ UTR regulatory elements. Finally, conditioned medium from astrocytes in which HuR or TDP-43 was knocked down produced significant motor neuron and cortical neuron toxicity in vitro. These findings indicate that HuR regulates TDP-43 and FUS/TLS expression and that loss of HuR-mediated RNA processing in astrocytes can alter the molecular and cellular landscape to produce a toxic phenotype.

The genetic linkage of TAR DNA binding protein 43 (TDP-43) and fused in sarcoma/translocated in liposarcoma (FUS/TLS) with amyotrophic lateral sclerosis (ALS)3 has elevated RNA processing to the forefront as a potential mechanism of motor neuron degeneration in ALS (1). These RNA binding proteins contain one or more RNA recognition motifs (RRM) that bind to target mRNA ligands to direct splicing, RNA transport, stabilization, and other processes. They are subject to autoregulation to maintain tight control of expression levels (1–3). Loss of autoregulation with cytoplasmic protein accumulation is a leading hypothesis for cellular toxicity in some models of ALS. In addition to autoregulation, it is well established that many RBPs are subject to cross-regulation by other cellular factors that bind to their mRNAs and modulate splicing and stability (4–6). Although TDP-43 binds to FUS/TLS and positively regulates its mRNA and protein expression (7), little is known about cross-regulatory factors that may influence TDP-43 expression. Several studies have identified Hu antigen R (HuR) as a positive regulator of mRNAs encoding RRMs (5, 8, 9). HuR has three RRMs and regulates its target mRNA ligands through binding U- and AU-rich elements in the 5′ and 3′ UTRs. HuR plays an important role in stress response through a positive regulation of growth and cell survival factors at the level of RNA stability and translational efficiency (10). Similar

3 The abbreviations used are: ALS, amyotrophic lateral sclerosis; ChAT, choline acetyltransferase; CFTR, cystic fibrosis transmembrane conductance regulator; FUS/TLS, fused in sarcoma/translocated in liposarcoma; HuR, Hu antigen R; KSRP, KH-type splicing regulatory protein; RRM, RNA recognition motif; RBP, RNA binding protein; RNA-IP, RNA immunoprecipitation; TDP-43, TAR DNA-binding protein 43; TDPBB, TDP-43 binding region; TIAR, TIAR-1 related protein; TNF-α, tumor necrosis factor α; UTR, untranslated region; VEGF, vascular endothelial growth factor; qRT-PCR, quantitative real-time PCR; ACM, astrocyte-conditioned medium; nt, nucleotide.
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to TDP-43 and FUS/TLS, HuR is predominantly nuclear in location (>90%) but shuttles to the cytoplasm as part of its function in regulating mRNA stability and translational efficiency (11, 12). This translocation can be augmented by cellular stressors such as heat shock or glucose deprivation (12, 13). We demonstrated previously that cellular toxicity related to ALS-associated mutant superoxide dismutase 1 (SOD1) could be reversed with HuR up-regulation (14). Here we show that HuR promotes TDP-43 and FUS/TLS expression by increasing translational efficiency. For TDP-43 mRNA, this regulation is mediated by the 3′ UTR to which HuR binds. Loss of HuR produces a splicing phenotype consistent with attenuated TDP-43 function. Suppression of HuR in primary astrocytes leads to the production of soluble factors that are toxic to cortical and motor neurons, similar to when TDP-43 is directly suppressed. These findings provide evidence that HuR plays a role in regulating ALS-associated RNA binding proteins and raise the possibility that HuR may contribute to motor neuron disease pathology by its unchecked positive regulation of ALS-associated TDP-43 and FUS/TLS.

EXPERIMENTAL PROCEDURES

Cell Culture, DNA Constructs, and Transfection—U251 MG, U251 Tet-On, U251 SOD1 clones, and doxycycline-inducible U251 HuR clones were maintained as described previously (14, 15). Cortical astrocytes were isolated as described previously (16) from G93A SOD1 mice (C57/B16 background, The Jackson Laboratory), littermate controls, and KSRP(−/−) mice (provided by Dr. Ching-Yi Chen, University of Alabama at Birmingham). The purity of astrocytes was confirmed by immunostaining for glial fibrillary acidic protein. Primary motor neurons were isolated and cultured on the basis of a protocol published previously (17). Motor neurons were plated on 15-mm coverslips (Assistant coverglasses, Carolina) coated with 0.5 mg/ml polyornithine (Sigma) and 0.5 mg/ml laminin (Sigma) and cultured in Neurobasal/B27 medium supplemented with 2% horse serum (Sigma) and 10 ng/ml each BDNF, ciliary neurotrophic factor (PeproTech). The small molecule inhibitor of HuR, MS-444, was provided by Novartis (Dr. Nicole Meisner) and was reconstituted in dimethyl sulfoxide. Cells were treated for 24 h. Rat primary cortical neurons were prepared with the assistance of the University of Alabama Tissue Culture Core Facility on the basis of a protocol published previously (18). The TDP-43 3′ UTR cDNA was provided by Dr. F. E. Baralle (International Centre for Genetic Engineering and Biotechnology). A 900-nt proximal segment, beginning at the stop codon, was cloned downstream from a luciferase open reading frame in pGL2 in the sense (900F) or antisense (900R) orientation using methods described previously (19). The following primers were used: upstream, 5′-TCAAGCTTCCCGGGACAGTGGGGTTGTGG TTGGT-3′; downstream, 5′-TAGACCTTCCCCGGGCGCCAAAGTAAAAAT-3′. A GFP-TDP-43 expression plasmid was provided by Dr. Zuoshang Xu (University of Massachusetts).FLAG-HuR, FLAG-KSRP, and the cystic fibrosis transmembrane conductance regulator (CFTR) hybrid minigene constructs are described elsewhere (19–21). All U251 cells and clones were transfected with Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). Astrocytes were transfected by electroporation using a Neon™ transfection system (Invitrogen, MPK5000) following the protocol of the manufacturer.

Protein Preparation, Western Blotting, Antibodies, and Immunocytochemistry—Whole-cell lysates from cultured cells were prepared in the presence of protease inhibitors and sodium orthovanadate using the M-PER kit (Pierce) and quantitated using a BCA assay (Pierce Endogen). For Western blot analysis, 40 μg of extract were resolved by gel electrophoresis, blotted, and probed with the following antibodies: HuR 3A2 (Santa Cruz Biotechnology); TDP-43 (Cell Signaling Technology), FUS (ProteinTech), HSP-70 (Santa Cruz Biotechnology), cleaved caspase 3 (Cell Signaling Technology, Actin (Sigma), luciferase (Millipore), GAPDH (Cell Signaling Technology), mouse IgG (Santa Cruz Biotechnology), FLAG (Sigma), and KSRP (provided by Dr. Ching-Yi Chen). Band densities were quantified using Quantity One software (Bio-Rad). For motor neuron immunofluorescence, cells were fixed on coverslips with 4% paraformaldehyde for 15 min. Slides were incubated with anti-cleaved caspase 3 (1:500) overnight at 4 °C. The next day, slides were incubated with Alexa Fluor 488 secondary antibody (1:1000, Invitrogen) for 1 h at room temperature. Images were visualized under an Olympus BX41 microscope equipped with a digital camera and processed using Adobe Photoshop (Adobe Systems). Cells were counted in a blinded manner.

RNA Isolation, Quantitative Real-time PCR, RNA Decay, and RNA Immunoprecipitation—Total RNA was isolated using a GE Healthcare Illustra RNAspin mini kit and quantitated with a Nanodrop2000 (Thermo Scientific). TDP-43 mRNA was quantitated by qRT-PCR with a ViiATM 7 real-time PCR system using optimized commercial primers and probes (Invitrogen). Primers and probes for luciferase and ß-galactosidase are described elsewhere (16, 22). For RNA degradation analysis, cell cultures were treated with actinomycin D for up to 6 h, as described elsewhere (19). Degradation curves were generated with GraphPad (GraphPad software, San Diego, CA). For quantification of protein-RNA binding, 250 μg of total extracts from cultured cells were prepared, divided equally into three aliquots, and immunoprecipitated with HuR, TDP-43, or mouse IgG (negative control) using methods described previously (23). RNA was eluted from beads using the RNeasy kit (Qiagen) and then analyzed by qRT-PCR for TDP-43 and luciferase mRNA.

Polysome Analysis—U251 Cells were grown to 70% confluence and treated for 3 min at 37 °C and 5% CO2 with 0.1 mg/ml of cycloheximide in complete medium (1× Dulbecco’s modified Eagle’s medium/F12K 50/50, 7% FBS). Cells were washed twice with PBS supplemented with cycloheximide (0.1 mg/ml), scraped in 400 μl of polysome extraction buffer (15 mM Tris-Cl (pH 7.4), 15 mM MgCl2, 0.3 mM NaCl, 1% Triton X-100, 0.1 mg/ml cycloheximide, and 1 mg/ml heparin) and then pelleted. Lysates of U251 cells were prepared and subjected to centrifugation through a linear 10–50% (w/v) sucrose gradient, and fractions were collected while being monitored for UV absorbance (254 nm) with a spectrophotometer. RNA isolation and analysis have been described previously (14).

shRNA and siRNA-mediated HuR Knockdown—For RNA interference, transfections were carried out using Lipofectamine 2000 (Invitrogen) and 30 nm of control scrambled
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small interfering RNA or gene-specific siRNA targeting TDP-43 or FUS (Invitrogen). The siRNA sequences for TDP-43 were as follows: set 1, 5′-GAAUGUGACCAACUAUGAATTT3′ (sense); set 2, 5′-AGACUGAAGCUAAAGCAGCTT3′ (sense). The sequences for control scrambled siRNA were as follows: set 1, 5′-GAAUGUGACCAACUAUGAATTT3′; set 2, 5′-AGACUGAAGCUAAAGCAGCTT3′ (24). Both sets of primers were pooled prior to transfection. SMARTpool siRNAs against HuR have been described elsewhere (27). An ELISA-based RNA binding assay was used to assess RNA binding according to a protocol published previously (26). Briefly, recombinant GST-HuR protein (27) was affixed to the ELISA well and incubated with a biotinylated probe with T7 RNA polymerase, and respective mRNA levels were measured by qRT-PCR. Translational efficiency, luciferase and β-galactosidase activity and respective mRNA levels were measured by qRT-PCR. Translational efficiency was calculated on the basis of the following equation: [Luciferase activity/Luciferase mRNA]/[β-galactosidase activity/β-galactosidase mRNA] as described previously (22).

RNA Binding Assay—The TDP-43 3′ UTR reporter template probe was derived from the 900-nt proximal portion (900F) and used to transcribe a biotin-labeled probe with T7 RNA polymerase, as described previously (26). Templates and synthesis of RNA probes for TNF-α 3′ UTR and pBSK (negative control) have been described elsewhere (27). An ELISA-based RNA binding assay was used to assess RNA binding according to a protocol published previously (26). Briefly, recombinant GST-HuR protein (27) was affixed to the ELISA well and incubated with a biotinylated TDP-43, TNF-α 3′ UTR, or control riboprobe in RNA binding buffer. For cold probe competition, labeled TDP-43 probe (0.02 pmol) was added to the ELISA well in the presence of excess molar amounts of unlabeled TDP-43 3′ UTR or pBSK probes. Binding curves were calculated using GraphPad Prism Software v. 5.0 (GraphPad Software).

CFTR Minigene Assay—A CFTR minigene (21) was cotransfected with TDP-43, HuR, or control GFP siRNA into U251 cells using Lipofectamine 2000 (Invitrogen). The mRNA was assessed by RT-PCR for CFTR exon 9 splicing using the following primers: 5′-CAACCTTCAGCTCCTAGCCACTGCG-3′ and 5′-TGGGGCCTCACCAGGAAGTTGGTGAATTC-3′.

Cell Viability and Neuronal Toxicity Assays—Cell viability/proliferation of astrocytes was assessed by Vialight assay (Lonza). For motor neuron and cortical neuron toxicity, cells were treated with 10% conditioned medium from HuR, TDP-43, or control GFP knockdown astrocytes. For motor neuron and cortical neuron cultures. In treatment groups and controls, the yield of purified motor neurons was equivalent. Cleaved caspase 3-positive cells were counted in ten high-power fields in a blinded manner and expressed as a percentage of total cells. For cortical neurons, caspase 3/7 activity was measured using a SensoLyte AMC kit (AnaSpec).

Statistics—All statistical analyses were performed with GraphPad Prism V. 5 software using two-tailed Student’s t test.

RESULTS

HuR Regulates TDP-43 and FUS/TLS—We used shRNA to knock down HuR in U251 clones expressing mutant or wild-type SOD1 and in primary astrocytes (14, 28). These clones have been used previously by our laboratory to assess dysregulation of VEGF mRNA processing in the presence of mutant SOD1 (14, 28). We achieved excellent knockdown of HuR compared with control shRNA, as determined by Western blot analysis (Fig. 1, A and B). In the U251 clones, there was a concomitant 3- to 4-fold attenuation of TDP-43 and FUS/TLS. Hsp70, another RNA binding protein, was not affected, indicat-
ing a specificity of regulation (29). In astrocytes, the attenuation of FUS/TLS was ~3-fold in WT and G93A SOD1 astrocytes, whereas TDP-43 was suppressed more than 5-fold compared with control shRNA. We then assessed the effect of HuR overexpression using doxycycline-inducible HuR U251 clones described previously (14). We observed up-regulation of TDP-43 (2-fold) and FUS/TLS (3- to 6-fold) with induction of HuR (Fig. 1C). Again, there was no change in Hsp70 expression, indicating a specificity of the effect. As another method of HuR suppression, we used the small molecule inhibitor MS-444, which blocks HuR function through inhibition of its dimerization and cytoplasmic translocation (30). Astrocytes or U251 clones were treated for 24 h and then harvested. We observed more than 50% attenuation of TDP-43 and FUS/TLS in the

FIGURE 2. The HuR inhibitor MS-444 attenuates TDP-43 and FUS/TLS expression. A and B, Western blot analysis of wild-type and mutant SOD1 U251 clones (A) or cortical astrocytes (B) after treatment with MS-444 for 24 h (50 μM for astrocytes and 100 μM for U251 clones). Antibodies are shown to the left. Bottom panels, densitometric quantification of bands shown in the blots. Values for each protein were adjusted to the loading controls (GAPDH or actin) and are shown as a fold change over dimethyl sulfoxide (DMSO) vehicle control. The results are representative of three independent experiments.

FIGURE 3. Hypoxia induction of TDP-43 and FUS/TLS is blocked with HuR silencing. A, primary astrocytes from WT or G93A mice were subjected to 1% O2 or normoxic conditions for 48 h and assessed by Western blot analysis. Antibodies are shown to the left of the blots. B, primary astrocytes were treated with siHuR or siGFP (control) RNAs and subjected to hypoxic conditions as in A. Bottom panels, quantitative densitometry was calculated as described in Fig. 1. Results are representative of three independent experiments.
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FIGURE 4. Knockdown of TDP-43, FUS/TLS, or KSRP does not affect HuR or other RBP expression in astrocytes. A, Western blot analysis of primary astrocytes after transfection with TDP-43 or control (siGFP) siRNAs. B, astrocytes were knocked with the siRNA shown, and extracts were assessed for HuR or FUS/TLS expression. C, extracts were prepared from KSRP−/− and KSRP+/+ (littermate) mouse astrocytes and assessed by Western blot analysis. Antibodies are shown to the left of the blots.

FIGURE 5. HuR Regulates TDP-43 via the 3′ UTR. A, schematic of the luciferase reporter constructs used to assess the TDP-43 3′ UTR. The portion of the 3′ UTR (900F) used in these experiments was cloned downstream of the luciferase coding region at the PflM1 site. Potential HuR binding sites are shown below (33, 34). A fragment representing the reverse complement (900R) was used as a control. TDPBR, TDP-43 binding region as defined by Ayala et al. (35). B, top panel, luciferase constructs were transfected into U251 cells along with TDP-43 or control siRNA. Luciferase activity was normalized to an internal transfection control (-galactosidase). Bottom panel, Western blot analysis of protein extract from the same transfection experiment. C, the same as B, except HuR was knocked down with siRNA. Some cells (control) were not treated with any siRNA. D, U251 cells were transfected with the 900F construct and treated with vehicle (dimethyl sulfoxide (DMSO)) or MS-444 at the doses shown for 24 h. E, luciferase constructs were transfected into a U251 clone that can be induced to express HuR with doxycycline treatment (shaded boxes). Luciferase activity was normalized to the internal transfection control. All results are mean ± S.E. of at least three independent tests. **, p < 0.005; ***, p < 0.001.
U251 clones and primary astrocytes, without an obvious difference between mutant SOD1-expressing cells and wild-type controls (Fig. 2). For primary astrocytes, we checked hsp70 and did not see any attenuation with HuR knockdown. Overall, the effects seen in U251 clones and primary astrocytes were similar, which is consistent with our previous observations that these cell models have substantial overlap (15). To examine the effects of HuR knockdown on the stress response, we subjected wild-type and G93A SOD1 mouse astrocytes to hypoxia (1% O2) for 48 h (Fig. 3). We observed a 3- to 5-fold induction of TDP-43 and FUS/TLS by Western blot analysis, but not of Hsp70, in both cell types (Fig. 3A). HuR showed up-regulation as well, greater in wild-type than mutant astrocytes (1.5-fold versus 3-fold). With HuR silencing, we observed no induction of TDP-43 and FUS/TLS. Rather, there was an ∼60% attenuation of TDP-43 and ∼90% with FUS/TLS compared with control siRNA (Fig. 3B). With the control siRNA, we observed the induction of each RBP. Hsp70 did not show any appreciable differences. Therefore, depletion of HuR blunted the physiological up-regulation of TDP-43 and FUS/TLS in response to hypoxia in wild-type and mutant SOD1 astrocytes. To determine whether there was any reciprocal effect on HuR expression, we silenced TDP-43 or FUS/TLS in primary astrocytes (Fig. 4, A and B) but did not observe any change in HuR expression. Interestingly, we did not see a change in FUS/TLS with TDP-43 knockdown as reported elsewhere (7). As an additional control, we assessed primary astrocytes from a mouse in which the RNA binding protein KSRP (which contains KH-type RNA binding domains) was knocked out (16, 31). We observed no change in TDP-43 or FUS/TLS expression compared with astrocytes from littermate controls (Fig. 4C). These findings suggest that the cross-regulation is specific but not bidirectional and that HuR is an upstream regulator of TDP-43 and FUS/TLS.

**HuR Modulates Gene Expression via the 3′ UTR of TDP-43**—The 3′ UTR of TDP-43 is lengthy (∼3.0 kb) and displays alternative splicing with different polyadenylation sites that produce short and long transcripts (7, 32). The proximal portion (initial 900 nt) of the 3′ UTR contains a number of cis elements that represent potential binding sites for HuR (Fig. 5A) (33, 34). We subcloned this part of the 3′ UTR (900F) downstream from the luciferase open reading frame to determine its impact on reporter expression. The reverse complement of this sequence was used as a control (900R). Plasmids were transfected into U251 cells along with a β-galactosidase control plasmid. Luciferase and β-galactosidase activity and mRNA levels were measured, and translational efficiency was calculated by the following equation: [Luciferase activity / Luciferase mRNA] / [β-galactosidase activity / β-galactosidase mRNA] (see “Experimental Procedures”). All data points represent the mean ± S.E. of at least three independent tests. "p < 0.0005.

**FIGURE 6.** HuR does not affect TDP-43 mRNA expression or stability but suppresses translational efficiency. A and B, HuR was knocked down (A) or overexpressed (B) in U251 clones as indicated, and TDP-43 mRNA was measured by qRT-PCR and expressed as a percentage of the housekeeping gene S9. C, analysis of TDP-43 mRNA decay in U251 cells after transfection with siRNA to TDP-43, HuR, or GFP. Cells were treated with actinomycin D for the time interval indicated, followed by measurement of TDP-43 mRNA levels. RNA values are expressed as a percentage of the baseline value prior to actinomycin D treatment (time 0). D, the 900F luciferase construct (Fig. 5A) and a β-galactosidase control were transfected into U251 cells along with siRNA to HuR, GFP, or no siRNA (−). Luciferase and β-galactosidase activity and mRNA levels were measured, and translational efficiency was calculated by the following equation: [Luciferase activity / Luciferase mRNA] / [β-galactosidase activity / β-galactosidase mRNA] (see “Experimental Procedures”). All data points represent the mean ± S.E. of at least three independent tests. ***, p < 0.0005.
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Knockdown of HuR Affects the Translational Efficiency of TDP-43 mRNA—We next sought to determine how HuR regulates the expression of TDP-43. We first assessed the impact of HuR silencing or transgenic up-regulation on TDP-43 mRNA in the U251 clones. With HuR knockdown, we saw no significant changes in mRNA levels compared with shControl (Fig. 6A and B). Fractions were collected and analyzed by qRT-PCR (Fig. 7, C and D). We observed a prominent shift of TDP-43 and FUS/TLS mRNAs away from the polysome fractions in shHuR-treated cells versus the control. GAPDH mRNA was unaffected and localized predominantly to the polysome fractions. These data are consistent with the luciferase reporter results and support the conclusion that HuR promotes translational efficiency of TDP-43 and FUS/TLS.

HuR Binds to the TDP-43 3' UTR—The data so far indicate that HuR can modulate TDP-43 expression via the portion of the 3' UTR that contains AU-rich elements. We next deter-
We performed RNA immunoprecipitation (RNA-IP) of U251 mRNA, but 5-fold less. To confirm that the pull-down was with an anti-TDP-43 antibody also pulled down TDP-43 with a HuR antibody compared with the IgG control. RNA-IP control with a known HuR target (TNF-α 3′ UTR) was negative for HuR, whereas the biotinylated probe (pBSK) was positive. The HuR antibody yielded a 3-fold increase (Fig. 9B). Finally, unlabeled TDP-43 3′ UTR could compete for HuR binding, whereas unlabeled control 3′ UTR could not (Fig. 9C). In summary, HuR can specifically bind to the TDP-43 3′ UTR with a pattern similar to RNA ligands associated previously with HuR (38, 39).

**Knockdown of HuR Alters a Downstream Function of TDP-43**—Because we established that TDP-43 expression is dependent on HuR, we next determined whether a downstream function of TDP-43 would be attenuated with HuR knockdown. A major role of TDP-43 is regulating alternative splicing, and exon 9 exclusion in the CFTR gene was the initial splicing function linked to TDP-43 (40, 41). We used a CFTR minigene (Fig. 10A) to assess splicing after HuR knockdown. U251 cells were treated with siRNA for HuR, TDP-43, or control and then transfected with the minigene. We assessed exon 9 inclusion/exclusion by RT-PCR as described previously (42). We observed a significant increase in exon 9 inclusion when either TDP-43 or HuR was knocked down compared with control siRNA (Fig. 10B). This molecular phenotype could be reversed by cotransfecting a GFP-TDP-43 expression plasmid that lacks the 3′ UTR regulatory elements (Fig. 10C). Therefore, knockdown of HuR reduced a downstream splicing function of TDP-43.

**Knockdown of HuR in Astrocytes Produces Motor Neuron and Cortical Neuron Toxicity**—We have shown previously that posttranscriptional gene regulation can inhibit secretion of soluble factors by astrocytes, including inflammatory cytokines (16). The regulation of TDP-43 and FUS/TLS by HuR, as shown here, would have an additional impact because they can modulate (positively or negatively) a number of mRNA targets encoding secreted proteins (7, 43). Therefore, we investigated whether HuR knockdown in astrocytes could impact motor neuron viability. We collected astrocyte-conditioned medium (ACM) following knockdown with HuR, TDP-43, or control siRNA and added it to primary motor neurons in culture. The purity of motor neurons was determined by staining with anti-choline acetyltransferase antibodies (Fig. 11A). After 24 h, apoptosis was assessed by staining motor neurons with cleaved caspase 3 antibody (Fig. 11B and C). We observed a significant 4-fold increase in apoptosis with siHuR ACM versus the control (p < 0.001). A similar toxic pattern was seen with TDP-43 knockdown. We also tested primary cortical neurons and found a 3- to 5-fold increase in apoptotic cells with siTDP-43 and siHuR ACM versus the control (Fig. 12, A and B). This was accompanied by a significant increase in caspase 3 and 7 activ-
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To determine whether HuR or TDP-43 knockdown was inducing astrocyte cell death (and releasing toxic factors), we assessed cell viability by an ATP-based viability assay (Fig. 12C). We found no significant effect on astrocyte viability with any of the siRNAs compared with untreated cells, indicating that the toxic effect of the conditioned media was neural-specific. In summary, attenuation of HuR or TDP-43 shifted the astrocytes to a "toxic" phenotype.

DISCUSSION

HuR plays a major role in regulating gene programs that govern a range of cellular functions, including stress response, inflammation, proliferation, and senescence (11, 44). The far-reaching effects of HuR on RNA metabolism stem, in part, from its regulation of other RNA regulators (5, 8, 9). Here we show that HuR regulates two RNA binding proteins, TDP-43 and FUS/TLS, which together regulate thousands of splicing events in coding and non-coding RNA targets (7, 43, 45). Indeed, following HuR knockdown, there was a significant attenuation of exon 9 exclusion in the CFTR gene, which is the first splicing function linked to TDP-43 (Fig. 10) (40). The cross-regulation observed here was unidirectional because neither TDP-43 nor FUS/TLS knockdown affected HuR expression. The hierarchy of RNA regulators is further underscored by a recent observation that TDP-43 binds to and regulates FUS/TLS mRNA in the brain (7). Interestingly, hnRNP A1, another RBP recently linked to ALS, was also identified as a possible mRNA target of HuR.
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Although the effect on its posttranscriptional regulation has not been investigated (8, 43). These findings play into the concept of the ribonome in which RNA regulators are interconnected and coordinate the dynamic expression of a broad set of functionally related genes (46, 47). The auto- and cross-regulation of RBPs is necessary to maintain the proper balance for these coordinated responses.

Many of the downstream targets of TDP-43 and FUS/TLS, typically characterized by long introns, play vital roles in synaptic transmission, neurodevelopment, and neurological disease (7, 43, 45). The biological consequences of HuR knockdown in astrocytes, which would also affect non-TDP-43 and FUS/TLS mRNA targets, are likely complex. We did not observe any alterations in astrocyte viability, but HuR-silenced astrocytes produced soluble factors that were toxic to motor neurons (Fig. 11). Astrocytes play an important role in CNS homeostasis, including synaptic maintenance, blood-brain barrier and blood flow, neuroprotection, and energy production (48). The broad range of functions is attributable to a versatile molecular phenotype, including the array of factors capable of being secreted, which vary depending on the microenvironment and signals therein (e.g. hypoxia, inflammation, and tissue injury) (49, 50). The astrocyte may also contribute to disease pathology (49). In ALS, the astrocyte has been implicated in the propagation of motor neuron death (and, therefore, clinical disease), possibly through the secretion of toxic factors (51–56).

Our data suggest that perturbation of posttranscriptional regulation by silencing HuR shifts the secretome of the astrocyte to a toxic one for motor neurons but not astrocytes themselves (Figs. 11 and 12). Because TDP-43 knockdown produced a similar toxic effect, it is possible that the phenotype of HuR knockdown resulted from concomitant TDP-43 attenuation. Indeed, a recent report by Yang et al. (55) found that partial in vivo knockdown of TDP-43 in glial cells in the spinal cord, without loss of expression in motor neurons, led to significant motor neuron cell loss. Another report, however, observed no motor neuron toxicity following TDP43 knockout in astrocytes, although a different model was used (on the basis of in vitro differentiated astrocytes from glial restricted precursor cells) (57). We also observed cortical neuron toxicity from these knockdowns, which potentially extends the ramifications of our findings to other neurodegenerative diseases (Fig. 12). A similar impact of altered posttranscriptional regulation in astrocytes has been described recently in our laboratory, where deletion of the RNA destabilizer KSRP led to toxicity of cortical neurons (16). Taken together, disruption of RNA homeostasis through alterations of RBPs can have a significant impact on astrocyte phenotype and neuronal maintenance. The nature of the toxic phenotype observed in this study has not yet been characterized.

Our data show that HuR positively regulates gene expression through the TDP-43 3’ UTR and that negative autoregulation could be overrun with increasing HuR expression (Fig. 5). Because of the proximity of AU-rich elements with the TDP-43 binding region, it is possible that the two proteins may compete in some way, although our findings indicate that HuR binding does not overlap a major binding locus for TDP-43 (Fig. 8) (35). Our data imply that there is a balance between the positive effects of HuR and the negative autoregulatory effects of TDP-43 to control expression. This balance could be perturbed with HuR overexpression, knockdown, or inhibition, resulting in the up- or down-regulation of TDP-43 (Figs. 1–3 and 5). Likewise, FUS/TLS expression levels could be altered with changes in HuR expression. This finding is relevant to neurodegenerative disease, where cytoplasmic mislocalization of mutant TDP-43 or FUS/TLS can circumvent the normal autoregulatory pathways that curtail expression (3, 58). Therefore, a possibly unfettered positive regulation by HuR in these pathological states could contribute to the accumulation of cytosolic TDP-43 of FUS/TLS.

Interestingly, knockdown of HuR did not alter the TDP-43 mRNA level or half-life. Rather, there was a loss of translational efficiency (Figs. 6 and 7). RNA stability and transla-
tional efficiency, although closely connected, are distinct levels of regulation, and HuR can modulate one process without affecting the other for a given RNA target (10, 59). A similar dissociation of RNA stability from translational efficiency was observed with cytochrome c, where HuR augmented protein expression but did not affect mRNA levels (60). The role of HuR in regulating translation is complex, with evidence of both positive and negative effects either through the 5′ or 3′ UTR (11, 60–63). Enhanced translation is often linked to HuR localization to the cytosol, its association with polysomes, and/or competition with silencing miRNAs (10, 12, 64). Although we did not test the role of the 3′ UTR in HuR regulation of FUS/TLS, the pattern of polysome dissociation with HuR knockdown was similar to TDP-43 mRNA, suggesting that HuR regulates its translational efficiency. The 3′ UTR of FUS/TLS contains many putative AU-rich elements and was identified in a recent analysis of 3′ UTRs as a possible binding target of HuR (8).

There was no obvious impact of mutant SOD1 on HuR regulation of TDP-43 or FUS/TLS in U251 cells or astrocytes, unlike with endothelial growth factor (VEGF), where mutant SOD1 suppressed RNA and protein expression and shortened the RNA half-life (14, 15). Therefore, the biochemical effect of mutant SOD1 is target-specific despite its capacity to bind different AU-rich 3′ UTRs (37).

HuR plays an important role in the cellular responses to stress, including heat shock, UV irradiation, oxidative stress, glucose deprivation, and hypoxia (10, 12, 13, 65). With hypoxia, we saw an up-regulation of HuR in astrocytes and a concomitant increase in TDP-43 and FUS/TLS (Fig. 3), indicating a physiological stressor in which this cross-regulation comes into play. With hypoxic exposure, HuR promotes the expression of key response genes, such as VEGF and Hif-1α, through post-transcriptional pathways (65). Both TDP-43 and FUS/TLS are also stress response proteins that can translocate to the cytoplasm and assemble into stress granules with different triggers, including heat shock, oxidants, and osmotic shifts (66–69). These granules are considered part of a survival mechanism whereby the translational apparatus is broken down for certain mRNAs in favor of those that promote damage repair and stress response (70). The downstream impact of hypoxia-induced TDP-43 and FUS/TLS up-regulation via HuR in astrocytes remains to be determined.

In summary, we show that HuR is a regulator of ALS-associated TDP-43 and FUS/TLS and, as a consequence, their downstream functions. Our findings reinforce the concept that there is a balance within the RNA world that is maintained by tightly regulated networks of RNA binding proteins. Disruption of this balance can have devastating consequences, possibly aggravat-
ing the accumulation of toxic proteins or, as in this report, creating a toxic astrocyte phenotype.

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