HuR/Methyl-HuR and AUF1 Regulate the MAT Expressed During Liver Proliferation, Differentiation, and Carcinogenesis

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BACKGROUND & AIMS: Hepatic de-differentiation, liver development, and malignant transformation are processes in which the levels of hepatic S-adenosylmethionine are tightly regulated by 2 genes: *methionine adenosyltransferase 1A* (*MAT1A*) and *methionine adenosyltransferase 2A* (*MAT2A*). *MAT1A* is expressed in the adult liver, whereas *MAT2A* expression primarily is extrahepatic and is associated strongly with liver proliferation. The mechanisms that regulate these expression patterns are not completely understood. **METHODS:** In silico analysis of the 3′ untranslated region of *MAT1A* and *MAT2A* revealed putative binding sites for the RNA-binding proteins AU-rich RNA binding factor 1 (AUF1) and HuR, respectively. We investigated the posttranscriptional regulation of *MAT1A* and *MAT2A* by AUF1, HuR, and methyl-HuR in the aforementioned biological processes. **RESULTS:** During hepatic de-differentiation, the switch between *MAT1A* and *MAT2A* coincided with an increase in HuR and AUF1 expression. S-adenosylmethionine treatment altered this homeostasis by shifting the balance of AUF1 and methyl-HuR/HuR, which was identified as an inhibitor of *MAT2A* messenger RNA (mRNA) stability. We also observed a similar temporal distribution and a functional link between HuR, methyl-HuR, AUF1, and *MAT1A* and *MAT2A* during fetal liver development. Immunofluorescent analysis revealed increased levels of HuR and AUF1, and a decrease in methyl-HuR levels in human livers with hepatocellular carcinoma (HCC). **CONCLUSIONS: Our data strongly support a role for AUF1 and HuR/methyl-HuR in liver de-differentiation, development, and human HCC progression through the posttranslational regulation of *MAT1A* and *MAT2A* mRNAs.**

Keywords: HuR; AUF1; MAT1A; MAT2A.

Methionine adenosyltransferase (MAT) catalyzes the synthesis of S-adenosylmethionine (SAMe), the principal methyl donor and a key regulator of hepatocyte proliferation and differentiation.[1] In mammals, 2 genes encode this enzyme: *MAT1A* and *MAT2A*. *MAT1A* expression is associated with high SAMe levels and represents an excellent adult liver marker.[1] *MAT2A* is related to lower SAMe levels and a more de-differentiated phenotype; it is expressed predominantly in fetal liver, where it is replaced progressively by *MAT1A* as development proceeds. *MAT2A* expression is up-regulated during hepatocyte de-differentiation in liver regeneration,[2,3] human hepatocellular carcinoma (HCC), and cultured liver cancer cells.[3–5] Thus, *MAT1A* expression is related to more restrained cell growth whereas *MAT2A* expression is associated strongly with rapid growth and de-differentiation. MAT1A-deficient mice, with low SAMe levels, have a predisposition to liver injury and overexpress cell proliferation–related genes, including *MAT2A*.[6,7] Overall, these suggest that the *MAT1A* and *MAT2A* expression patterns are tightly regulated in the liver according to tissue type and differentiation stage, and that deregulation occurs during disease and malignant transformation. The mechanisms underlying these processes remain unknown.

Previous reports suggest that *MAT1A* and *MAT2A* expression is associated with a specific pattern of promoter methylation and histone acetylation.[8] Moreover, methionine conversion into SAMe regulates *MAT2A* expression

Abbreviations used in this paper: AICAR, AICA riboside; AUF1, AU-rich RNA binding factor 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GNMT, glycine N-methyltransferase; HGF, hepatocyte growth factor; IP, immunoprecipitation; KO, knockout; MAT, methionine adenosyltransferase; PCR, polymerase chain reaction; RNP, ribonucleoprotein; SAMe, S-adenosylmethionine; $t_{1/2}$, half-life; UTR, untranslated region.

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at the level of messenger RNA (mRNA) turnover.[9] RNA-binding proteins regulate the turnover by recognizing specific RNA sequences.[10,11] The human embryonic lethal abnormal vision proteins are the best-known RNA-binding proteins that selectively recognize and bind to AU-rich elements. HuR, a member of the human embryonic lethal abnormal vision family, is a ubiquitously expressed protein[12] that is localized predominantly (>90%) in the nucleus of unstimulated cells, but becomes cytoplasmic in response to proliferative and stress stimuli, increasing the half-life and/or modulating the translation rate of target mRNAs.[13,14] In contrast, a number of other RNA-binding proteins, including TTP, BRF1, KSRP, and the AU-rich RNA binding factor 1 (AUF1), function as destabilizers of target mRNAs.[15] HuR and AUF1 target mRNAs encode for mitogenic, immune, and stress responses and cell-cycle regulatory proteins (c-fos, interleukins, heat shock protein 70, cyclin D1, and cdc25),[16] and both have been implicated in carcinogenesis.[17,18]

We investigated whether these 2 RNA-binding proteins regulate the expression of the MAT genes during liver de-differentiation, hepatic proliferation, and malignant transformation. We found that HuR associated with the *MAT2A* 3′ untranslated region (UTR) enhanced *MAT2A* mRNA stability and steady-state levels, whereas AUF1 associated with the *MAT1A* 3′ UTR decreased *MAT1A* mRNA stability and steady-state abundance. In addition, we describe a novel function of methyl-HuR as a destabilizer of *MAT2A* mRNA. Finally, we observed a specific expression pattern of HuR and AUF1 mRNAs in correlation with *MAT1A* and *MAT2A* mRNA levels. These findings suggest that HuR/methyl-HuR and AUF1 are important regulators of hepatic SAMe levels during de-differentiation, development, liver proliferation, and carcinogenesis by controlling the switch between *MAT1A* and *MAT2A* expression.

Materials and Methods

Human Samples

Surgically resected liver tumor specimens from 22 cirrhotic patients with HCC (hepatitis C, [n = 10]; alcoholic steatohepatitis, [n = 10]; and nonalcoholic steatohepatitis, [n = 2]) along with 4 normal liver biopsies were examined. Informed consent to all clinical investigations, which were performed in accordance with the principles embodied in the Declaration of Helsinki, was provided. The institutional review board of the Hospital Clínic de Barcelona (Barcelona) approved the protocol.

Mouse and Rat Fetal Liver Samples

Liver samples were harvested and snap-frozen for subsequent analyses from the different animals: 8-month-old male glycine N-methyltransferase (GNMT)-Knockout (KO)[19] and wild-type (C57BI/6J) mice, embryonic days 16 (E16) and 18 (E18), postnatal days 1 (P1) and 5 (P5), and 3-month-old Wistar rats. All animals were treated according to international Institutional Animal Care and Use Committee standards.

Isolation and Culture Hepatocytes

Hepatocytes were isolated from male Wistar rats (200 g) as described previously.[19–21] Adhered cells were maintained in minimum essential medium with 5% fetal bovine serum, and incubated with the test compounds.

RNA Interference

SAMe-deficient cells and H4IIE hepatoma cell lines were transfected with HuR, AUF1, and control short interfering RNA (siRNA) designed and synthesized by either Qiagen (Chatsworth, CA) or Sigma (St. Louis, MO).

Cloning of 3′ UTR of Mouse HuR Complementary DNA and Plasmid Construct

Complementary DNA (cDNA) from SAMe-deficient cells served as a template for polymerase chain reaction (PCR) amplification. A 1400–base pair fragment was obtained, purified by Qiaquik gel extraction (Qiagen), and cloned into the pEGFP-C2 vector (Clontech, Mountain View, CA). The resulting plasmid was verified by sequencing.

Transient Transfection of MLP29 Cells

The mouse liver progenitor cell line (MLP29) was grown in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. For transfection assays, cells were plated into 6 multiwell dishes and 2 mcg of pEGFP-C2–3′ UTR or pEGFP-C2 were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Twenty hours later, cells were treated with SAMe (4 mmol/L) for 4 hours and lysed with RIPA buffer.

RNA Isolation and Real-Time PCR (Quantitative PCR)

Total RNA from liver tissue or hepatocytes was isolated with Trizol (Invitrogen) and purified with RNeasy Mini kit (Qiagen). PCR was performed using a BioRad iCycler thermocycler. Cycle threshold values were analyzed as described[22,23] and normalized to the housekeeping transcripts.[24]

Total, Cytosolic, and Nuclear Protein Isolation

Extraction of total protein and cytosolic and membrane lysates from liver tissue and primary hepatocytes has been described previously.[25]

Western Blotting

Proteins were size-fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and were incubated with antibodies recognizing HuR (Santa Cruz Biotechnology, Santa Cruz, CA), AUF1 (BD Pharmingen, San Diego, CA),

MAT II (*MAT2A*; AbCam, Cambridge, UK), MATI/III (*MAT1A*[6]), GFP (Roche Diagnostics, GmbH, Mannheim, Germany), β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma-Aldrich), and methyl-HuR.[29] After incubation with secondary antibodies conjugated to horseradish peroxidase (Santa Cruz Biotechnology), proteins were detected by Western Lightning Chemiluminescence Reagent (PerkinElmer, Foster City, CA). Ponceau S staining was performed to ensure equal loading.

Ribonucleoprotein Immunoprecipitation

For the immunoprecipitation of endogenous ribonucleoprotein (RNP) complexes,[15] whole-cell extracts were processed and analyzed as previously described.[26]

Analysis of Polysomes

Untreated or SAMe-treated rat hepatocytes (5×10^6 cells) were cultured for 24 hours, then incubated with 0.1 mg/mL cycloheximide for 10 minutes. Cytoplasmic extracts were fractionated and collected through sucrose gradients.[26] The RNA of each fraction was isolated with Trizol (Invitrogen), and reverse-transcription quantitative PCR analysis was performed. Protein from each fraction was precipitated with 10% trichloro acetic acid and resuspended in sample buffer for Western blotting.

Biotin Pull-Down Assay

For in vitro synthesis of biotinylated transcripts,[10] total cDNA was used as a template for PCR reactions as described.[26] Biotin pull-down assays were performed as described elsewhere,[15] and bound proteins were analyzed by Western blotting.

Immunofluorescence

Paraffin sections (5-μm thick) of formalin-fixed liver samples were rehydrated, subjected to antigen retrieval in 10 mmol/L sodium citrate buffer (pH 6.0), and avidin-biotin blocked before incubation with primary antibodies (anti-HuR, 1:100; anti-AUF1, 1:100; anti–methyl-HuR, 1:1000) followed by incubation with corresponding secondary antibodies. For quantification of immunofluorescence, images were acquired using 20× or 40× objectives with consistent exposure times for each section. The immunofluorescence intensity of approximately 50 cells from random fields for each sample was quantified using ImageJ software (public domain software, available: rsb.info.nih.gov/ij/) and expressed as relative immunofluorescence intensity. To minimize variations in measurements, all specimens were immunolabeled at the same time and under identical conditions.

Statistical Analysis

All experiments were performed in triplicate with data expressed as means ± standard error of the mean. Representative blots are shown. Statistical significance was estimated with the Student *t* test. A *P* value of less than .05 was considered significant.

Results

MAT2A *and* MAT1A *mRNA Levels Are Stabilized by HuR and AUF1, Respectively*

Hepatocyte growth factor (HGF) up-regulates *MAT2A* mRNA levels.[27] HGF and aminoimidazole carboxamide ribonucleotide (AICAR), activators of adenosyl monophosphate-activated protein kinase, exert a proliferative response in rat hepatocytes by regulating HuR localization and, consequently, the stability of mRNAs encoding proteins involved in cell-cycle proliferation.[25] SAMe inhibits this effect by blocking AMP-activated protein kinase activation and maintaining HuR in the nucleus.

We found that AICAR and HGF up-regulated the expression of *MAT2A* mRNA and this effect was blocked by SAMe (Figure 1*A*). By using actinomycin D, we determined that the half-life ($t_{1/2}$) of the *MAT2A* mRNA was increased by AICAR ($t_{1/2} > 200$ min) compared with actinomycin D alone ($t_{1/2} \cong 77$ min), and that the addition of SAMe limited this stabilization (AICAR + SAMe, $t_{1/2} \cong 173$ min) (Figure 1*B*), suggesting that SAMe decreased MAT2A mRNA stability. These data are consistent with previous reports showing a down-regulation in the half-life of *MAT2A* mRNA in an HCC cell line after SAMe treatment.[28] AICAR treatment did not significantly change *MAT1A* mRNA levels, whereas SAMe treatment ($t_{1/2} \cong 1032$ min) or actinomycin D ($t_{1/2} \cong 693$ min) increased the half-life of *MAT1A* mRNA compared with nontreated hepatocytes ($t_{1/2} \cong 87$ min), suggesting that SAMe increased *MAT1A* mRNA stability.

To directly assess the functional role of the 3′ UTR in the stability of *MAT2A* mRNA, this region was cloned into the expression plasmid pEGFP-C2 and transfected into the MLP29 mouse cell line. After 24 hours in culture, GFP protein expression from pEGFP-C2–3′ UTR mRNA was lower than that expressed by pEGFP-C2 mRNA alone. SAMe treatment decreased the expression of pEGFP-C2–3′ UTR mRNA, while having no effect on pEGFP-C2 mRNA alone (Supplementary Figure 1). These data suggest that the 3′ UTR of *MAT2A* mRNA is responsible for destabilizing GFP mRNA and sensitizing the MLP29 cells to SAMe treatment.

In silico analysis of the *MAT2A* and *MAT1A* 3′ UTRs revealed one computationally predicted hit for an HuR-binding motif in *MAT2A* mRNA at position 2200 and another for AUF1 in *MAT1A* mRNA at position 3012 (Figure 1*C*; the *star* represents the position of the predicted motifs). No AUF1 or HuR binding sites were found in the 3′ UTR of *MAT2A* and *MAT1A*, respectively. To test the possibility that *MAT2A* and *MAT1A* mRNAs were associated with HuR and AUF1, respectively, we used biotinylated transcripts spanning different mRNA regions (Figure 1*C*) and incubated them with hepatocyte lysates. The interaction was assessed by biotin pull-down assays followed by Western blot analysis. As shown in

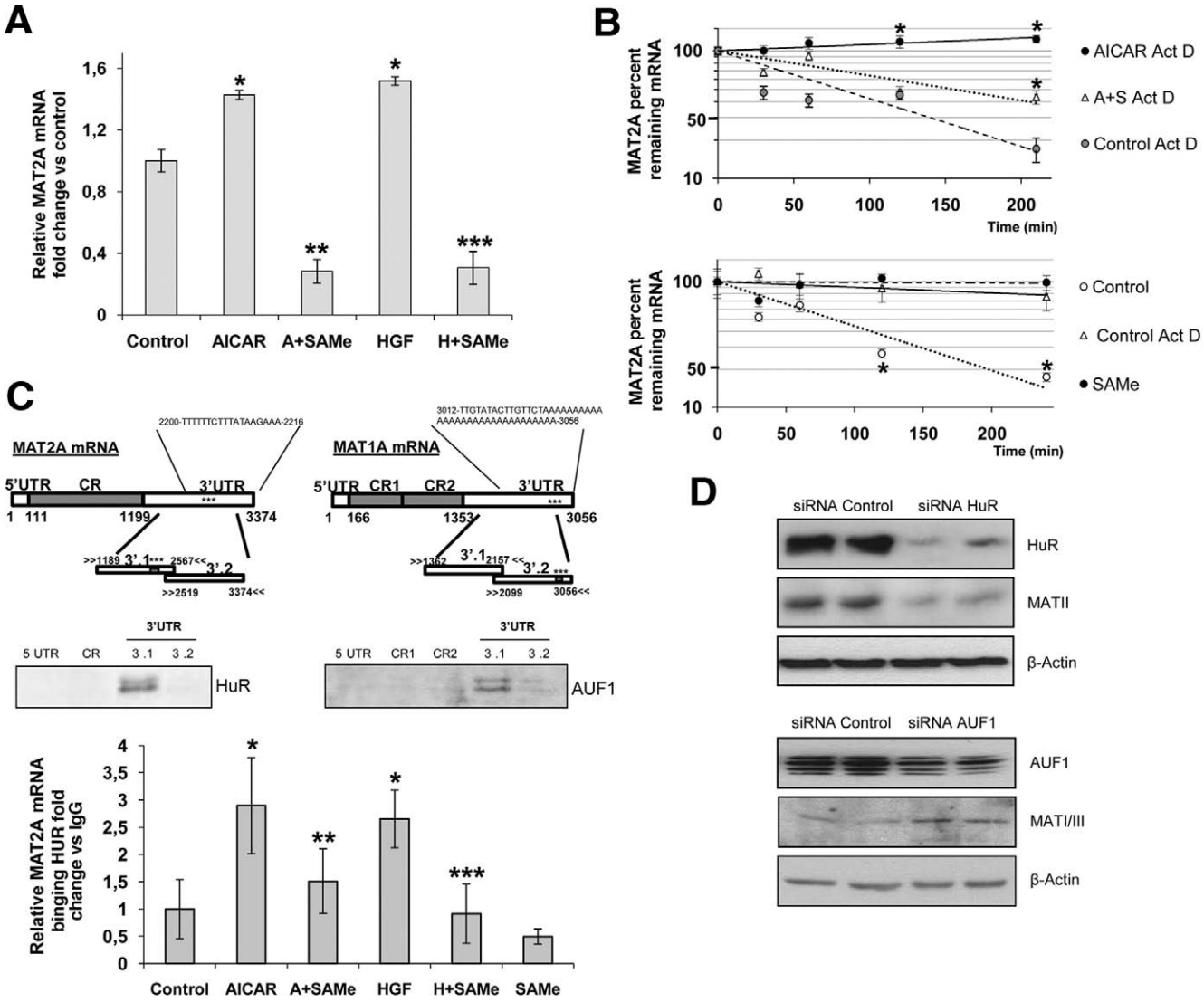

Figure 1. HuR stabilizes *MAT2A* mRNA and AUF1 destabilizes *MAT1A* mRNA. (*A*) Analysis of *MAT2A* mRNA from rat hepatocytes treated with AICAR (2 mmol/L), AICAR and SAMe (4 mmol/L), HGF (40 ng/mL), or HGF and SAMe for 4 hours. Treatments were performed in triplicate ($P < .05$, *AICAR or HGF vs control, **SAMe and AICAR vs AICAR, ***SAMe and HGF vs HGF). (*B*) After AICAR, SAMe, or AICAR and SAMe treatments, rat hepatocytes were incubated with actinomycin D (Act D; 2 mcg/mL) for 4 hours. The levels of *MAT2A* and *MAT1A* mRNAs were normalized to *GAPDH* mRNA and represented on a semilogarithmic scale. *Top graph*, *$P < .05$, AICAR Act D or A and S Act D vs Act D; *bottom graph*, *$P < .05$, SAMe vs control. (*C*) *Upper panel*, *MAT2A* and *MAT1A* mRNA showing the biotinylated transcripts (5′ UTR, coding region [CR], 3′ UTR) and the predicted HuR and AUF1 motifs. Western blots show the association between HuR or AUF1 with biotinylated *MAT2A* and *MAT1A* fragments. Biotin pull-down assays were performed in triplicate using rat hepatocyte lysates. *Bottom panel*, RNP-IP analysis of *MAT2A* mRNA bound to HuR after SAMe, AICAR, AICAR and SAMe, HGF, and HGF and SAMe treatments. The enrichment was calculated from triplicate ($P < .05$, *AICAR or HGF vs control, **SAMe and AICAR vs AICAR, ***SAMe and HGF vs HGF). (*D*) Three days after siRNA transfection, SAM-D and H4IIE cells were harvested to monitor the protein expression of HuR and MAT2A, or AUF1 and MAT1A, respectively. Western blots are representative of 3 independent experiments.

Figure 1*C* (*bottom panels*), HuR only formed complexes with the 3′ UTR-*MAT2A*, having a stronger association with 3′ (1) UTR-*MAT2A* than with 3′ (2) UTR-*MAT2A*. No interaction was observed with the 3′ UTR-*MAT1A* (not shown). Biotinylated GAPDH 3′ UTR was used as a negative control and no signal was detected (data not shown). In the case of AUF1, complexes were observed only with the 3′ UTR-*MAT1A*, specifically with the 3′ (1) UTR-*MAT1A* although the computationally predicted site was in the 3′ (2) UTR. In silico predictions are not always biological hits, most likely because other RNA-binding proteins have greater affinity for the predicted site. Finally, we analyzed whether HGF, AICAR, and SAMe regulated the affinity of HuR for *MAT2A* mRNA by RNP immunoprecipitation (IP) assays (Figure 1*C*, *lower panel*). The results showed that AICAR and HGF increased the binding of HuR to *MAT2A* mRNA, whereas SAMe treatment prevented the formation of this com-

plex. Silencing of HuR markedly decreased the expression of MAT II (66%), the protein encoded by *MAT2A* (Figure 1*D*). In contrast, silencing of AUF1 markedly decreased the expression of MAT I/III (37%), the protein encoded by MAT1A mRNA (Figure 1*D*).

Coordinated Expression of MAT2A *and* MAT1A*, and Their Respective Regulators, HuR and AUF1, During De-Differentiation of Cultured Hepatocytes*

De-differentiation of cultured primary hepatocytes results in a switch in expression from *MAT1A* to *MAT2A*, an effect that is blocked by SAMe supplementation, which maintains the adult phenotype.[29] We found that, similar to *MAT2A*, *HuR* mRNA levels also increased during culture, an effect also blocked by SAMe treatment (Figure 2*A*, *upper* and *lower panel*). Previous reports have implicated the methylation of arginine 217 in modulating HuR levels.[26] We analyzed HuR and methyl-HuR protein levels in cultured hepatocytes, with or without SAMe treatment, over time and found that HuR levels remained stable at 6 and 12 hours in the presence of SAMe and decreased at 24 hours. Methyl-HuR levels, however, changed only slightly during this de-differentiation process (Figure 2*B*).

To assess whether the 2 HuR forms bind *MAT2A* mRNA differentially, RNP-IP assays were performed over different time points in culture hepatocytes, with or without SAMe. As shown in Figure 2*C*, *MAT2A* mRNA was enriched in HuR-IP samples compared with control immunoglobulin (Ig)G-IP samples, with peak enrichment at 12 hours (Supplementary Table 1). Interestingly, treatment with SAMe decreased the levels of *MAT2A* mRNA and largely suppressed the (HuR-*MAT2A* mRNA) RNP complexes (Figure 2*A* and *C*). In contrast, methyl-HuR was bound to *MAT2A* mRNA only in the presence of SAMe (Figure 2*D* and Supplementary Table 2). This sug-

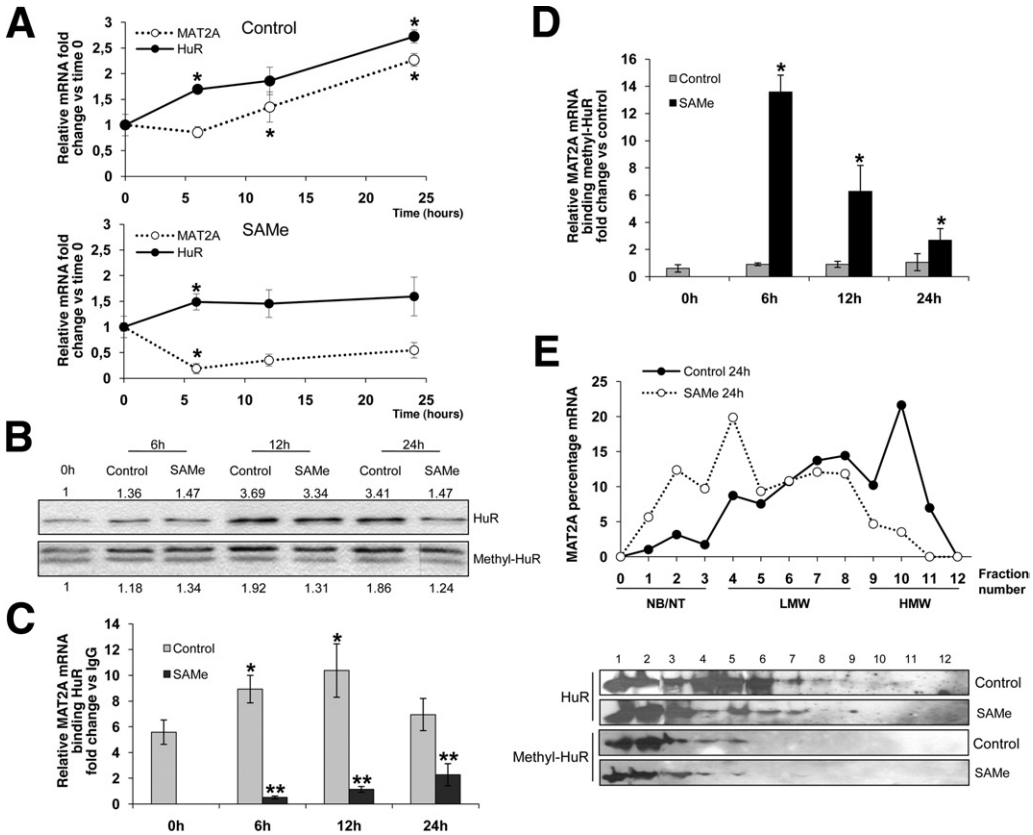

Figure 2. *MAT2A* expression during de-differentiation of cultured hepatocytes. (*A*) Expression of *MAT2A* mRNA over a time course in rat hepatocytes in the presence/absence of SAMe (4 mmol/L). *$P < .05$, time of treatment vs previous time. (*B*) Representative Western blots of HuR and methyl-HuR in rat hepatocytes during de-differentiation. HuR vs time 0 hours and methyl-HuR vs time 0 ratios from densitometric analysis are presented; each assay was performed in triplicate. (*C*) The association of HuR with *MAT2A* mRNA was assayed by RNP-IP analysis using cytoplasmic fractions of rat hepatocytes incubated as described earlier. *MAT2A* mRNA was normalized to *GAPDH* mRNA in HuR-IPs and represented relative to the levels of *MAT2A* mRNA in IgG-IP samples. $P < .05$, *time of treatment vs time 0 hours, **SAMe vs control. (*D*) RNP-IP analysis of *MAT2A* mRNA bound to methyl-HuR; the enrichment was calculated from triplicate samples. *$P < .05$, SAMe vs control. (*E*) Polysome gradient analysis in rat hepatocytes cultured for 24 hours in the presence/absence of SAMe. *MAT2A* mRNA levels were plotted as a percentage of the total *MAT2A* mRNA levels. The translational activity of each fraction is as follows: NB, not bound polysomes; NT, not translated; moderately translated (LMW, low-molecular-weight polysomes); and actively translated (HMW, high-molecular-weight polysomes) (*upper panel*). HuR and methyl-HuR protein in each fraction were analyzed by Western blot analysis. Each assay was performed in triplicate.

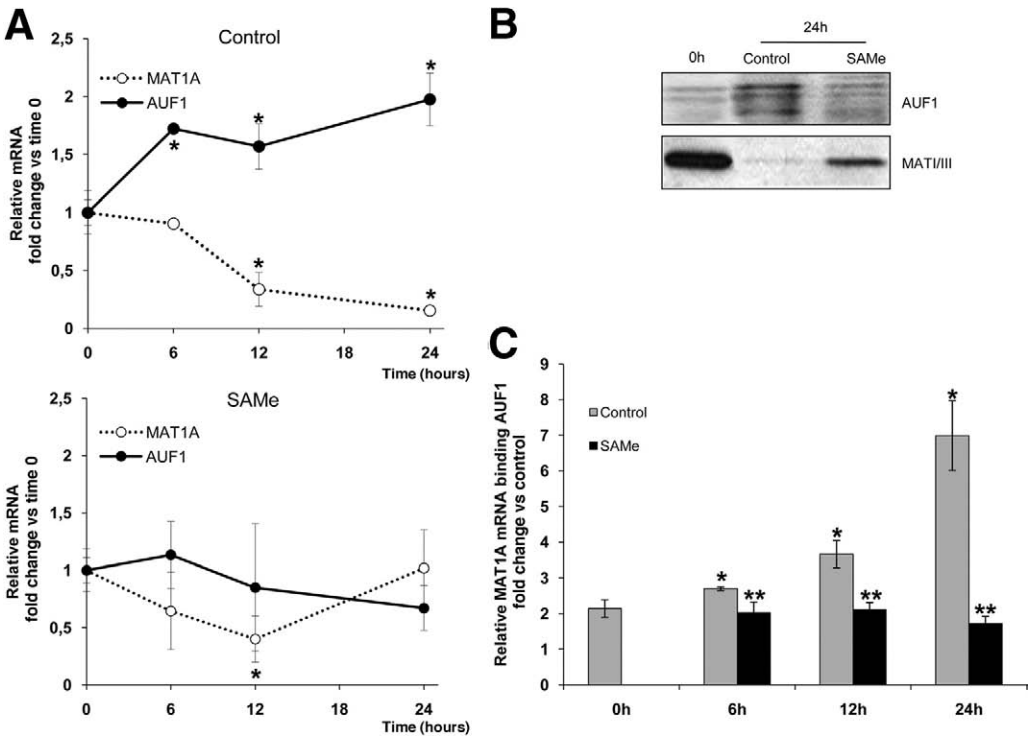

Figure 3. *MAT1A* expression during de-differentiation of cultured hepatocytes. (*A*) *MAT1A* mRNA expression in rat hepatocytes at the indicated times. *$P < .05$, time of treatment vs time 0 hours. (*B*) Representative Western blot analyses of AUF1 and MATI/III levels in rat hepatocytes undergoing de-differentiation; data are representative of 3 independent experiments. (*C*) RNP-IP analysis of the association of AUF1 with *MAT1A* mRNA in cytoplasmic fractions of rat hepatocytes incubated as indicated. The enrichment of *MAT1A* mRNA in AUF1-IPs was calculated as described in Figure 2*C*. $P < .05$, *time of treatment vs time 0 hours, **SAMe vs control.

gests that HuR and methyl-HuR have opposing effects on *MAT2A* regulation; HuR stabilizes *MAT2A* mRNA and methyl-HuR likely destabilizes *MAT2A* mRNA. Our results indicate that the ratio between HuR/methyl-HuR might function as a sensor mechanism to control specific targets such as *MAT2A* mRNA during the de-differentiation process of hepatocytes.

To address the functional role of methyl-HuR in *MAT2A* mRNA destabilization, a HuR mutant was generated with lysine substitutions at the argine 217 methylation site (HuR[R217K]), and its interaction with *MAT2A* mRNA was analyzed in MLP29 cells. We found that the association with HuR wild type (WT) was constitutively lower in the presence of SAMe, whereas the association with HuR(R217K) remained unaltered (Supplementary Figure 2). Furthermore, we failed to detect *MAT2A* mRNA in AUF1-IP samples from hepatocytes treated with SAMe (data not shown), suggesting that AUF1 is not involved in the methyl-HuR–mediated mRNA destabilization.

Finally, hepatocytes, cultured for 24 hours in the presence or absence of SAMe, were used to prepare polysomes by sucrose gradient fractionation. *MAT2A* mRNA levels were increased in the molecular-weight fractions 9–11, containing the actively translating polysomes of untreated cells. After SAMe treatment, *MAT2A* mRNA was localized to the lighter fractions (1–8), which had either very limited or no translational activity (Figure 2*E*, *upper panel*). HuR protein was detected in fractions 1–8, whereas in SAMe-treated hepatocytes, HuR was more abundant in fractions characterized as having limited translational activity (1–3). Methyl-HuR signals were found almost exclusively in the low-molecular weight fractions irrespective of SAMe treatment (Figure 2*E*, *lower panel*). Taken together, these data suggest that HuR-bound *MAT2A* mRNA is stabilized and actively translated during the de-differentiation of the hepatocytes, whereas in the presence of SAMe, only methyl-HuR remains co-localized with untranslated *MAT2A* mRNA.

We also found that *MAT1A* mRNA and MATI/III protein decreased during the culture period, concomitant with an increase in AUF1 mRNA and protein levels (Figure 3*A*, *upper panel*, Figure 3*B*). This response was inhibited by SAMe treatment (Figure 3*A*, *lower panel*). AUF1-*MAT1A* mRNA RNP complexes also increased in a time-dependent manner and, predictably, this effect was blocked by SAMe treatment (Figure 3*C*). These results suggest a functional link between AUF1 expression and *MAT1A* mRNA levels during the de-differentiation process, which is ablated by SAMe treatment.

Role of HuR, Methyl-HuR, and AUF1 During Liver Development

The aforementioned data raise the question of whether these 2 RNA-binding proteins also are involved

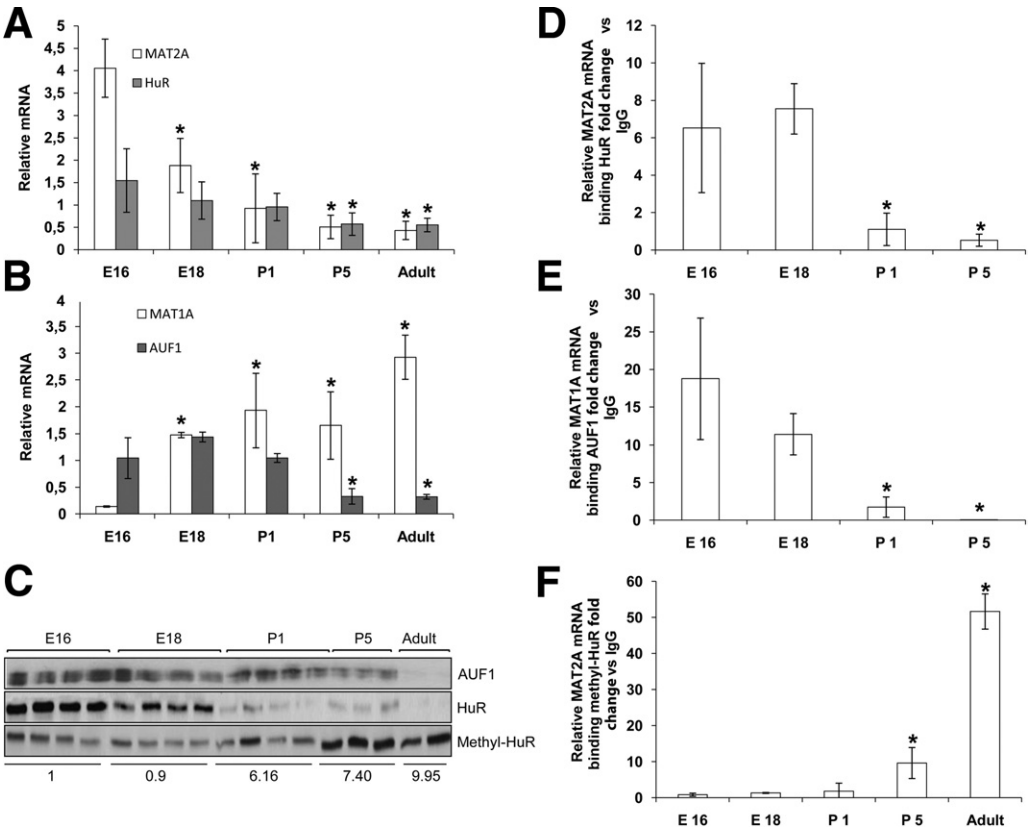

Figure 4. Role of HuR, methyl-HuR, and AUF1 during liver development. (*A* and *B*) mRNA expression of *MAT2A*, *HuR*, *MAT1A*, and *AUF1* in fetal livers (E16, E18), and livers from pups (P1 and P5) and adult rats (3 months), normalized to *GAPDH* mRNA. $P < .05$, *ages of development vs E16. (*C*) Levels of HuR, methyl-HuR, and AUF1 as evaluated by Western blot analysis. Ponceau S staining was used as loading control (Supplementary Figure 3). The ratio of methyl-HuR/HuR was calculated. *$P < .05$, ages of development vs E16. (*D* and *E*) Binding of HuR or AUF1 to target mRNAs during liver development, as assessed by RNP-IP and real-time PCR analysis. *$P < .05$, ages of development vs E16. (*F*) RNP-IP analysis of *MAT2A* mRNA bound to methyl-HuR; enrichment represents the average from triplicate experiments. *$P < .05$, ages of development vs E16.

in liver differentiation. In rats and human beings, *MAT1A* is expressed only in the adult liver, whereas *MAT2A* is found predominantly in fetal liver with only minimal expression in the adult organ.[2,31] We examined the expression levels of *MAT2A*, *MAT1A*, *HuR*, and *AUF1* mRNAs in livers from fetuses (E16, E18), neonates (P1 and P5), and adult rats (3 mo) (Figure 4*A* and *B*). *MAT2A* and *HuR* mRNA levels decreased during liver development to minimal levels in adult rats (Figure 4*A*). In contrast, *MAT1A* mRNA reached its maximum expression in adult liver, whereas *AUF1* mRNA decreased as the rats aged (Figure 4*B*). There was a sharp decrease in AUF1 and total HuR protein levels with development, whereas methyl-HuR levels increased (Figure 4*C*). The methyl-HuR/HuR ratio revealed an increase of the methyl-HuR that correlated with an observed decrease in *MAT2A* mRNA levels.

A sharp decrease in the HuR-*MAT2A* mRNA RNP complexes was observed during the different stages of liver development (Figure 4*D*). There was a reduction in the levels of (AUF1-*MAT1A* mRNA) RNP complexes, which correlated with increased *MAT1A* mRNA levels (Figure 4*E*). Finally, an increase in the binding of methyl-HuR to *MAT2A* mRNA was observed with development (Figure 4*F*), suggesting that methyl-HuR might destabilize *MAT2A* mRNA and/or inhibit its translation. Taken together, these results strongly suggest that a balance between methyl-HuR, HuR, and AUF1 is required to regulate the levels of *MAT2A* and *MAT1A* mRNAs during liver differentiation.

Regulation of MAT2A *in an In Vivo Model of Chronic Excess of Hepatic SAMe*

The maintenance of adequate hepatic SAMe levels for proper liver function is critical.[32] GNMT is the enzyme responsible for the catabolism of SAMe. The GNMT KO mouse has a chronic excess of SAMe and spontaneously develops steatosis, fibrosis, and HCC.[20] *MAT2A* mRNA levels were significantly lower in GNMT mutant livers compared with WT mice (Figure 5*A*) whereas methyl-HuR protein levels were significantly higher in GNMT mutant livers compared with WT animals (Figure 5*B*). In contrast, although SAMe levels were high, GNMT KO mice did not show reduced HuR levels, possibly owing to the highly proliferative status of the livers in these mice.[20] In addition, the amount of (HuR-*MAT2A* mRNA) RNP

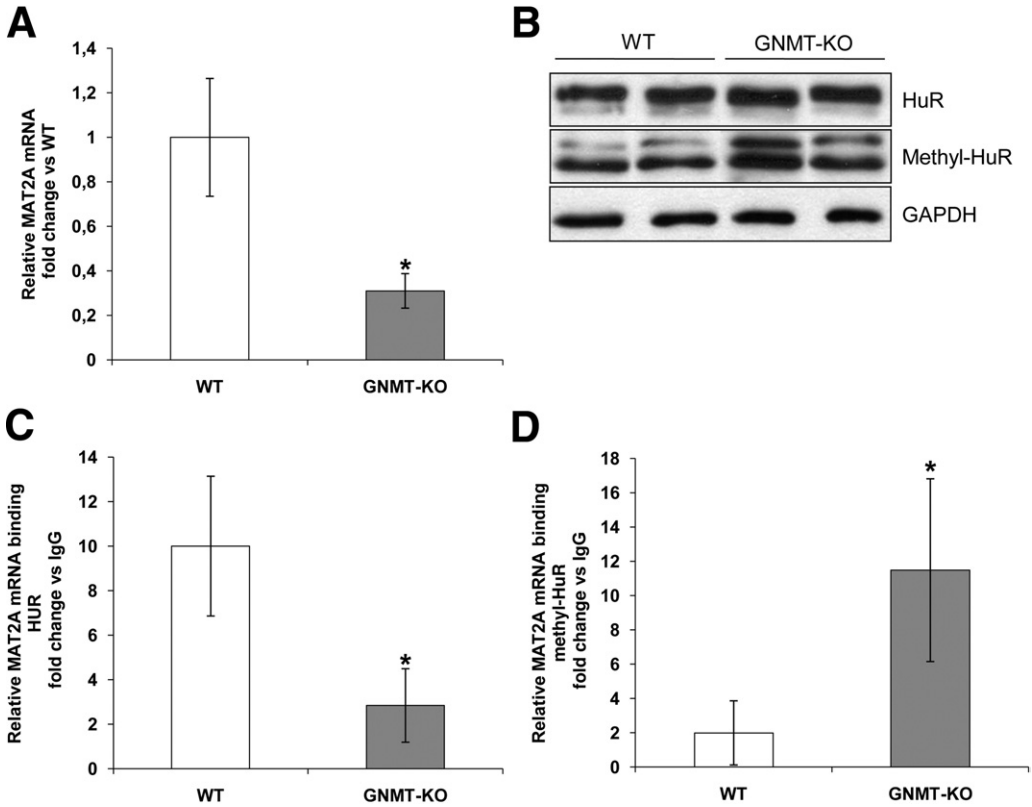

Figure 5. The levels of *MAT2A* mRNA are regulated by HuR in GNMT KO mice. (*A*) *MAT2A* mRNA expression in GNMT KO mice expressed as fold-change vs WT (*$P < .05$). (*B*) Levels of HuR, methyl-HuR, and loading control GAPDH in total extracts. (*C*) RNP-IP analysis of HuR binding to *MAT2A* mRNA. (*D*) RNP-IP analysis of *MAT2A* mRNA bound to methyl-HuR. *$P < .05$, GNMT KO vs WT.

complexes was lower in KO mice compared with WTs, whereas (methyl-HuR-*MAT2A* mRNA) complexes were increased in KO animals (Figure 5*C* and *D*). The low levels of hepatic *MAT2A* mRNA observed in the GNMT KO mice could be owing to an increase in the ratio of methyl-HuR/HuR, the reduction of (HuR-*MAT2A* mRNA) RNP complexes, and the enhanced levels of (methyl-HuR-*MAT2A* mRNA) complexes.

HuR and AUF1 Levels in Human HCC

The switch between MAT1A and MAT2A genes was investigated in human hepatoma cell lines and HCC tissues resected from patients.[33] Immunofluorescent analyses of healthy vs cancerous livers revealed that HuR and AUF1 were expressed at low levels in normal human liver samples whereas liver tumors were strongly positive for HuR and AUF1 staining (Figure 6). In contrast, the signal for methyl-HuR was stronger in healthy livers than in livers containing tumors (Figure 6).

Discussion

MAT is the sole enzyme responsible for SAMe synthesis, and the proper regulation of SAMe levels is critical for the maintenance of liver function.[1,32] In adult and quiescent hepatocytes, *MAT1A* is the gene expressed, encoding the isoform MATI/III. However, in fetal liver or during periods of proliferation and malignant transformation, it is the MATII isoform, encoded by the *MAT2A* gene, that predominates.[2] In hepatocytes, SAMe controls development, de-differentiation, and proliferation.[2,22,25,29] Our data indicate that HuR and AUF1 could be pivotal regulators of this process through their influence on the posttranscriptional expression of *MAT2A* and *MAT1A*. Specifically, we found correlative shifts between HuR and AUF1 abundance and *MAT2A* and *MAT1A* expression during these processes. In addition, the observation that SAMe regulates the balance between methyl-HuR and HuR, thereby modulating *MAT2A* mRNA levels, led us to propose that methyl-HuR could function as a destabilizer of *MAT2A* mRNA, potentially influencing hepatocyte proliferation.

There is great interest in understanding the genetic changes that occur during malignant transformation of the liver. Hepatocytes isolated from healthy rat livers lose the expression of their liver-specific genes when maintained in culture[34] and thus recapitulate certain characteristics of the transformation process. A switch between *MAT1A*/*MAT2A* mRNA occurs during the de-differentiation of cultured hepatocytes. SAMe prevented changes in the expression of these and other genes while maintaining homeostasis in one-carbon metabolism in functional hepatocytes.[29] Our findings revealed that *HuR* and *AUF1*

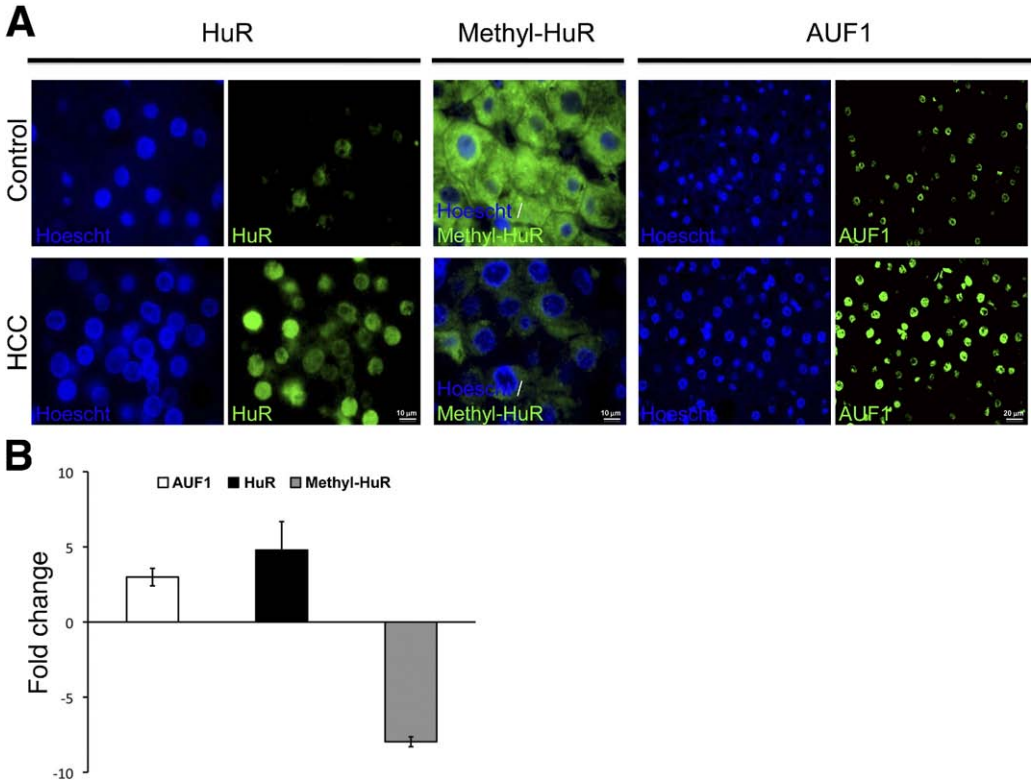

Figure 6. HuR, methyl-HuR, and AUF1 detection in human HCC. (*A*) Representative immunofluorescence analysis of HuR, methyl-HuR, and AUF1 protein in normal and human HCC samples. (*B*) The relative immunofluorescence intensity in cancer tissues was calculated using Image J software and expressed as fold-change of the relative immunofluorescence intensity in normal tissues. Data are representative of experiments realized in 22 HCC patients and 4 normal biopsies, and fold-changes are significantly different (*$P < .05$).

mRNA levels were up-regulated in a time-dependent manner during the de-differentiation process. HuR became methylated in the presence of SAMe, and, unexpectedly, the association of methyl-HuR with *MAT2A* mRNA appeared to promote the decay of *MAT2A* mRNA. Previous reports have considered HuR as a negative translational regulator in mammalian cells,[30] and this effect of HuR is observed in cooperation with other RNA-binding proteins or micro-RNAs.[35] In our studies, we focused on the consequences of the possible involvement of AUF1 in the decay of *MAT2A* mRNA associated with methyl-HuR in the presence of SAMe. However, RNP-IP analysis in the presence of SAMe showed no interaction, suggesting that AUF1 was not implicated in the loss of *MAT2A* mRNA.

Although many aspects of the regulation of HuR and methyl-HuR binding activities remain to be elucidated, a model emerges from our results whereby HuR promotes the accumulation of *MAT2A* mRNA, whereas methyl-HuR inhibits it. In the presence of high levels of SAMe, the balance between the 2 HuR forms ultimately determines the steady-state abundance of *MAT2A* mRNA. This mechanism is highly relevant in vivo because HuR would be expected to influence the process of hepatocyte de-differentiation, leading to a loss of MAT homeostasis and impaired liver function, which then might enhance malignant transformation in the liver.

During embryonic development there is a switch in expression from *MAT2A* to *MAT1A*.[2] During active liver proliferation, there is a reduction in HuR and an increase in methyl-HuR levels, thereby reducing *MAT2A* mRNA expression. In contrast, *MAT1A* mRNA levels showed an inverse correlation with AUF1 abundance, consistent with its decay-promoting function, its increased association with *MAT1A* mRNA during de-differentiation, and its reduced interaction in the presence of SAMe. Taken together, our results suggest a mechanism whereby the relative abundance of HuR, methyl-HuR, and AUF1 can drive the differentiation of hepatocytes in a process dependent on SAMe levels. The preservation of *MAT1A* mRNA expression and MAT activity is a fundamental feature of healthy and differentiated hepatocytes. In this regard, AUF1 and HuR could be considered pivotal regulators of this essential biological process.

Finally, in human HCC, a switch from *MAT1A* mRNA to *MAT2A* mRNA expression facilitates cancer cell growth. Although a transcriptional regulation has been described for this switch in gene expression pattern,[36] our results also suggest a posttranscriptional regulation of *MAT2A* and *MAT1A* mRNA levels by HuR, methyl-HuR, and AUF1 in HCC. HuR is up-regulated commonly in most tumor types and the deregulation of AUF1 promotes tumorigenesis.[17,37] Normally, AUF1 is found only in very

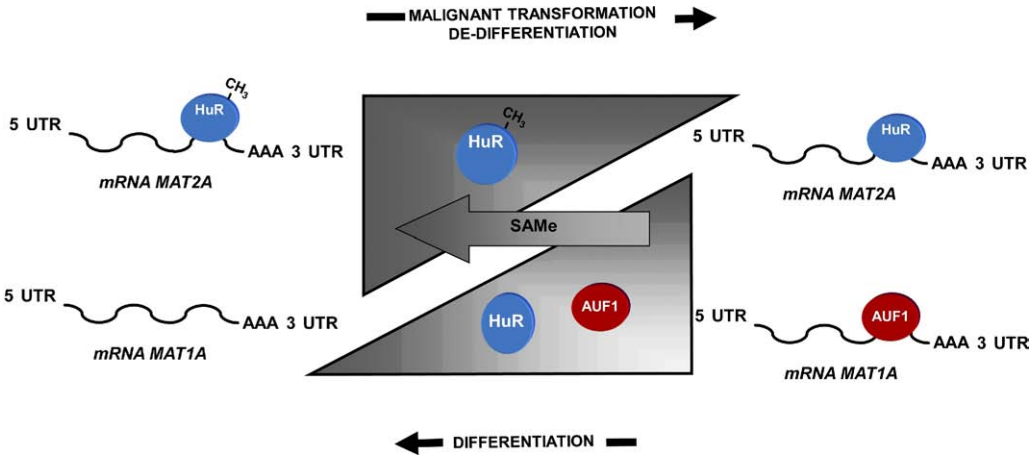

Figure 7. Diagram of *MAT2A*, *MAT1A*, and their posttranslational regulators HuR, methyl-HuR, and AUF1 in hepatocyte de-differentiation, development, and malignant transformation. Mature hepatocytes express high levels of *MAT1A* mRNA and low levels of AUF1, whereas *MAT2A* mRNA is in low abundance owing to its negative regulator, methyl-HuR. During de-differentiation, the levels of *AUF1* mRNA in hepatocytes increase and the ratio of methyl-HuR/HuR decreases. This leads to a switch from *MAT1A* to *MAT2A* mRNA expression. SAMe treatment of hepatocytes prevents these changes. Malignant transformation of hepatocytes is accompanied by similar expression patterns for AUF1, HuR, and methyl-HuR, as well as for *MAT1A* and *MAT2A* mRNAs. During liver development, the opposite is observed, with decreased AUF1 levels and increased methyl-HuR/HuR ratios.

low levels in adult liver.[38] We were able to detect the presence of both HuR and AUF1 in resected samples from HCC patients with different etiologies, whereas methyl-HuR consistently was found in low abundance. By contrast, in normal human control samples HuR and AUF1 were expressed at low levels, whereas methyl-HuR was expressed at high levels. Our results allow us to postulate that increased levels of HuR and AUF1 and reduced expression of methyl-HuR observed in surgically resected HCC might be the hallmarks of the transformation of hepatocytes into cancer cells. Such an imbalance between HuR and AUF1 can underlie the deregulation of *MAT2A*/*MAT1A* homeostasis, the decrease in SAMe levels, and the proliferation of liver cancer cells.

In conclusion, our results provide a model for HuR, methyl-HuR, and AUF1 function on the posttranslational regulation of *MAT2A* and *MAT1A* mRNAs in a SAMe-dependent manner during essential biological processes such as hepatocyte differentiation, de-differentiation, and malignant transformation (Figure 7). There is a similar temporal distribution of both RNA-binding proteins, suggesting that they regulate cell growth and differentiation through their opposing functions. Finally, the observation that methyl-HuR binds to *MAT2A* mRNA in correlation with its enhanced decay is a novel finding that points to a mechanism through which SAMe may regulate liver functionality. These results are significant because they reveal critical new insight into the molecular mechanisms underlying the switch between *MAT1A* and *MAT2A* expression, which is observed consistently during malignant hepatic transformation, and facilitates the development and progression of HCC.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.01.032.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Cloning of 3′ UTR of Mouse HuR cDNA and Plasmid Construct

SAMe-D cells were lysed in Trizol (Invitrogen). RNA was prepared according to the manufacturer's protocol. cDNA was synthesized using SuperScript II reverse transcriptase and random primers (Invitrogen, Carlsbad, CA). This material served as a template for PCR amplification. As expected, a fragment of 1400 base pairs was obtained, purified by Qiaquik gel extraction (Qiagen), and cloned into the pEGFP-C2 vector (Clontech), which contains the GFP gene, within the Xho I and Hind III restriction sites. The resulting plasmid was verified by restriction enzyme analysis and by sequencing.

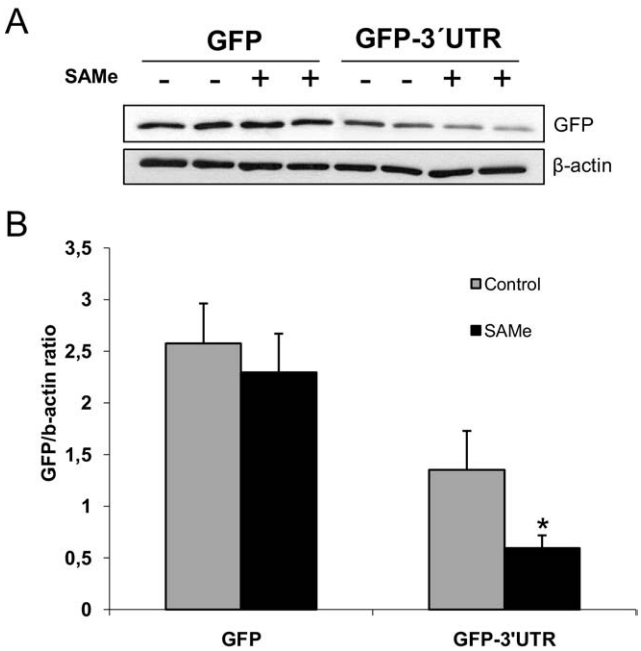
Cloning of Mouse HuR WT and HuR(R217K) and Plasmid Construction

The full-length mouse HuR cDNA was obtained from RZPD Deutsches Ressourcenzentrum für Genomforschung GmbH. The HuR(WT)-V5 was constructed by PCR amplification using the 5′ oligonucleotide containing the V5 tag sequence and subcloned into pCDNA 3.3 TOPO vector (Invitrogen). The HuR(R217K)-V5 mutants were constructed by using the QuickChange kit for site-directed mutagenesis (Stratagene, La Jolla, CA) with 2 complementary oligonucleotides and the pCDNA-HuR(WT)-V5 plasmid as template.

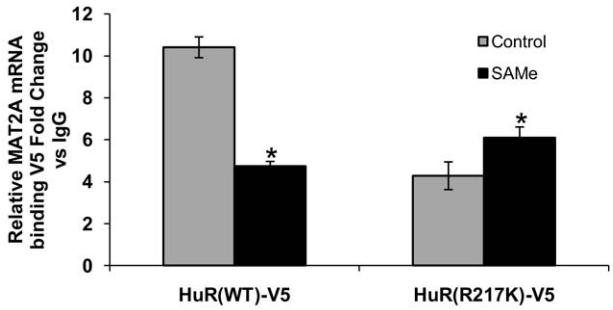
Transient Transfection of MLP29 Cells

The mouse cell line MLP29 was cultured in Dulbecco's modified Eagle medium containing 10% fetal bovine serum. For transfection assays, cells were plated into 6 multiwell dishes and 2 mcg of pEGFP-C2–3′ UTR or pEGFP-C2 were transfected in OPTI-MEM I media using Lipofectamine 2000 (Invitrogen). Twenty hours later, cells were treated with SAMe (4 mmol/L) for 4 hours. Cell extracts were prepared using RIPA buffer and used for Western blot analysis.

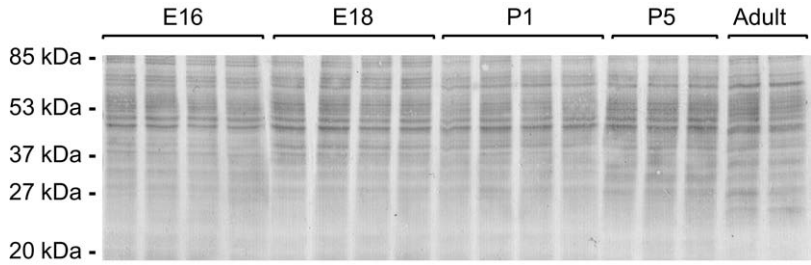
For HuR(WT)-V5 and HuR(R217K)-V5 transfection, MLP29 cells were seeded into 100-mm dishes and transfected with 2 mcg of plasmid DNA using Lipofectamine 2000 in OPTI-MEM media. Cells then were treated with SAMe (4 mmol/L) for 12 hours before RNP IP analysis.



Supplementary Figure 1. The 3′ UTR of *MAT2A* confers instability to *GFP* mRNA. The 3′ UTR of *MAT2A* was cloned into the expression vector pEGFP-C2–3′ UTR and the resulting construct was transfected in the MLP29 cell line. Cells were treated with SAMe (4 mmol/L) for 4 hours. (*A*) Western blot analysis of GFP expression. The results are representative of 3 independent experiments. (*B*) Quantification of the expression levels for GFP and GFP–3′ UTR. There was no significant difference in GFP expression with or without SAMe; the decrease in protein expression was significant ($P < .05$) when the cells were transfected with GFP–3′ UTR.



Supplementary Figure 2. HuR(R217K)-V5 binding to *MAT2A* mRNA is not altered in the presence of SAMe. The binding of HuR(WT)-V5 and HuR(R217K)-V5 to MAT2A was assayed by RNP IP in MLP29 cells treated with or without SAMe (4 mmol/L). The levels of *MAT2A* mRNA were first normalized to the levels of *GAPDH* mRNA, and expressed relative to the levels of *MAT2A* mRNA in IgG IPs. *$P < .05$, SAMe vs control.



Supplementary Figure 3. Ponceau S staining of fetal liver extracts. Ponceau S staining of the nitrocellulose membranes was performed to ensure equal loading of protein samples in fetal liver Western blot experiments.

Supplementary Table 1. Raw Data of RNP IP Analysis of *MAT2A* mRNA Bound to HuR Using Cytoplasmic Fractions of Rat Hepatocytes Treated With SAMe for 24 Hours

	MAT2A-IP IgG	MAT2A-IP HuR	Average Fold Change (IP HuR/IP IgG)	Std Dev
Control 0h	0.10	0.59	5.93	0.85
Control 6h	0.25	2.16	8.60	1.13
Control 12h	0.46	4.73	10.20	2.05
Control 24h	0.50	3.39	6.78	1.20
SAM 6h	1.22	0.58	0.47	0.11
SAM 12 h	0.30	0.39	1.31	0.21
SAM 24h	0.16	0.36	2.23	0.86

MAT2A mRNA levels were normalized to *GAPDH* mRNA levels, and calculated relative to the levels of *MAT2A* mRNA in IgG IP samples. Data are the average of 3 independent experiments performed in triplicate.

Supplementary Table 2. Raw Data of RNP IP Analysis of *MAT2A* mRNA Bound to Methylated HuR Using Cytoplasmic Fractions of Rat Hepatocytes Treated With SAMe for 24 Hours

	MAT2A-IP IgG	MAT2A-IP methyl-HuR	Average Fold Change (IP met-HuR/IP IgG)	Std Dev
Control 0h	0.04	0.03	0.65	0.3
Control 6h	0.06	0.07	1.02	0.2
Control 12h	0.09	0.08	0.87	0.2
Control 24h	0.06	0.06	1.09	0.6
SAM 6h	0.01	0.14	13.71	1.2
SAM 12 h	0.12	0.74	6.19	1.9
SAM 24h	0.12	0.34	2.77	0.8

MAT2A mRNA levels in methylated HuR IP samples were normalized to *GAPDH* mRNA levels in the same IP samples, and expressed relative to the levels of *MAT2A* mRNA in IgG IP samples. Data are the average of 3 independent experiments performed in triplicate.