

A long non-coding RNA, *APOA4-AS*, regulates *APOA4* expression depending on HuR in mice

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Received January 28, 2016; Revised April 15, 2016; Accepted April 18, 2016

ABSTRACT

Long non-coding RNAs (lncRNAs) have been shown to be critical biomarkers or therapeutic targets for human diseases. However, only a small number of lncRNAs were screened and characterized. Here, we identified 15 lncRNAs, which are associated with fatty liver disease. Among them, *APOA4-AS* is shown to be a concordant regulator of Apolipoprotein A-IV (*APOA4*) expression. *APOA4-AS* has a similar expression pattern with *APOA4* gene. The expressions of *APOA4-AS* and *APOA4* are both abnormally elevated in the liver of *ob/ob* mice and patients with fatty liver disease. Knockdown of *APOA4-AS* reduces *APOA4* expression both *in vitro* and *in vivo* and leads to decreased levels of plasma triglyceride and total cholesterol in *ob/ob* mice. Mechanistically, *APOA4-AS* directly interacts with mRNA stabilizing protein HuR and stabilizes *APOA4* mRNA. Deletion of HuR dramatically reduces both *APOA4-AS* and *APOA4* transcripts. This study uncovers an antisense lncRNA (*APOA4-AS*), which is co-expressed with *APOA4*, and concordantly and specifically regulates *APOA4* expression both *in vitro* and *in vivo* with the involvement of HuR.

INTRODUCTION

Long non-coding RNAs (lncRNAs) are transcripts longer than 200 nucleotides, which do not have protein-coding potential. According to the genomic location, lncRNAs can be further categorized into intergenic, intronic, antisense and enhancer lncRNA (1). The antisense lncRNAs are transcribed from the antisense strand of protein-coding genes

and often partially overlap with opposite strand mRNA. They may regulate sense-strand mRNAs in a positive (concordant) or negative (discordant) manner at transcriptional or post-transcriptional levels to carry out a wide range of biological and cellular functions (2,3). Emerging evidence has shown that lncRNAs are involved in multiple aspects of human health and diseases such as cancer (4) and diabetes (5,6).

APOA4 is a plasma lipoprotein, which is involved in the regulation of many metabolic pathways such as lipid and glucose metabolism. In rodents, *APOA4* is primarily synthesized by the liver and small intestine, followed by secretion into blood (7). The plasma *APOA4* level correlates to the high-density lipoproteins levels and has anti-oxidative and anti-inflammatory properties (8–10). *APOA4* mutations, which are associated with plasma lipid levels, have been identified in many populations (11–15). *In vivo* study has shown that *APOA4* enhances triglyceride (TG) secretion from the liver (16). *APOA4* could also enhance glucose stimulated insulin secretion (17) and inhibit glucose production in the liver (18). *APOA4* is closely linked to the obesity and type 2 diabetes in both mice and humans (16,19). Multiple transcription factors such as HNF-4 α , CREB, CREBH, ERR- α , PPAR α and SERBP1 regulate *APOA4* expression (7,16,20,21). However, whether lncRNA regulates *APOA4* expression is largely unknown.

In the present study, we report an antisense lncRNA, *APOA4-AS*, which is transcribed from the reverse strand of *APOA4* gene, partially overlaps with 3' end of *APOA4*, and plays an essential role in regulating *APOA4* gene expression. Short hairpin RNA (shRNA)-mediated knockdown of *APOA4-AS* leads to a reduction of *APOA4* mRNA and protein abundance both *in vitro* and *in vivo*. In the genetic (*ob/ob*) obesity mice model, the elevated *APOA4-AS* expression contributes to the induction of *APOA4* level in

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the liver and the hypertriglyceridemia in the plasma. Moreover, the protein–RNA complex (*APOA4*-AS–HuR) is involved in *APOA4* expression; and furthermore, the deletion of HuR leads to a significant reduction of both *APOA4*-AS and *APOA4* transcripts, indicating that *APOA4*-AS transcript may stabilize *APOA4* mRNA through HuR. Results from current investigation present a novel antisense lncRNA, *APOA4*-AS that preserves the specific and potential biological function in regulating *APOA4* expression and plasma TG levels, providing novel molecular mechanisms that can be applied to biomedical and therapeutic application for metabolic diseases such as type 2 diabetes.

MATERIALS AND METHODS

RNA-seq and gene enrichment (GO) analysis

RNA-seq data (GSE43314, NCBI GEO database) from wide type (WT) and *db/db* mice were analyzed to identify differentially expressed lncRNAs in the liver with Tophat, Cufflinks and CummeRbund (22). To link the lncRNA to its associated protein-coding gene, lncRNA neighboring mRNAs were extracted and gene enrichment (GO) analysis was performed (<http://genontology.org/>).

Animals

C57BL/6 mice were purchased from Vital River (A Charles River Company, Beijing, China), and ob+/- mice were from Dr. Liangyou Rui (University of Michigan, Ann Arbor, MI, USA) (23). All animals were maintained on 12-h light/12-h dark cycles in a clean facility at Northeast Normal University. This study was carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and was approved by the Institutional Animal Care and Use Committee or Animal Experimental Ethics Committee of Northeast Normal University (NENU/IACUC).

Human liver tissues

Human fatty and normal liver tissues were obtained from Shanghai Tongji Hospital based on liver biopsy. The study was approved by Shanghai Tongji Hospital ethical committees and individual permission was obtained using standard informed consent procedures. The investigation conforms to the principles that are outlined in the Declaration of Helsinki regarding the use of human tissues.

Rapid Amplification of cDNA Ends

RACE experiment was performed using Smart RACE cDNA Amplification Kit (Clontech) according to manufacturer's instruction. The 5' RACE specific primer for *APOA4*-AS is 5' GCCACTTGAGCTTCCTGGAGAAGA G 3'. The 5' ends of *APOA4*-AS cDNA were polymerase chain reaction (PCR) amplified and sequenced.

Northern blot

Total RNAs from the small intestine, liver, brain, heart and kidney of C57BL/6 mice were extracted using TriPure

Isolation Reagent (Roche, Mannheim, Germany). RNAs were treated with DNase I to remove potential chromosomal DNA contamination. A 683-nt *APOA4*-AS probe was synthesized utilizing the PCR DIG Probes Synthesis Kit (Roche, Mannheim, Germany). Northern blot hybridization was performed using the DIG-labeled *APOA4*-AS probes, and the signals were detected by a DIG Nucleic Acid Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instruction. The primers used for *APOA4*-AS probe synthesis were listed: northern-F: 5'-GCAACCCGATGGGGCTAA-3'; northern-R: 5'-GGGC GTGCAGGAGAAACT-3'.

Primary hepatocyte cultures and adenoviral infection

Primary hepatocytes were isolated from male mice as described previously (24,25). Primary hepatocytes were cultured in Dulbecco's modified Eagle's medium supplemented with 2% fetal bovine serum, 100 units/ml penicillin and 100 µg/ml streptomycin. For knockdown of *APOA4*-AS, two target sequences (shAPOA4-AS1: CACCAGAGCCTTGT TGAACAT and shAPOA4-AS2: AACTTGTCTTTAA GTTGGTG) were selected. Primary hepatocytes were infected with control adenovirus (ad-scramble) or two different adenoviral vectors encoding *APOA4*-AS shRNAs (2 × 10⁹ viral particles/well) for 2 h, and cultured for 36 h. Cells were then collected for quantitative real time (qRT)-PCR or immunoblotting.

APOA4-AS knockdown in the liver of WT or ob/ob mice

C57BL/6 (8 weeks old) or *ob/ob* male mice (8 weeks old) were injected with ad-scramble or ad-shRNAs (1 × 10¹¹ viral particles/mouse) via tail vein for 10–14 days. Liver sections were stained with hematoxylin-eosin. Plasma TG levels were measured using a TG assay kit (Pointe Scientific Inc., Canton, MI, USA). Plasma total cholesterol (TC) levels were measured using a TC assay kit (Dongou Diagnosis, Zhejiang, China).

Immunoblotting

Mice were anesthetized and sacrificed after fasting for 20–24 h. Livers were isolated and homogenized in a lysis buffer (50 mM Tris HCl, pH 7.5, 1% TRITON-X 100, 150 mM NaCl, 2 mM EGTA, 100 mM NaF, 1 mM PMSF, 10 µg/ml aprotinin and 10 µg/ml leupeptin). The extracts from the liver, primary hepatocytes or HEK293T cells were immunoblotted with anti-APOA4 (Cell Signaling), anti-tubulin (Santa Cruz), anti-HuR and anti-albumin (Proteintech Group, Inc.) or anti-actin (BD Transduction Laboratories™).

qRT-PCR analysis

Total RNAs were extracted using TriPure Isolation Reagent (Roche, Mannheim, Germany). RNAs were treated with DNase I to remove potential chromosomal DNA contamination before cDNA synthesis. The first-strand cDNAs were synthesized using random primers and M-MLV reverse transcriptase (Promega, Madison, WI, USA). RNA

abundance was measured using ABsolute qRT-PCR SYBR Mix (Life technologies) and Roche LightCycler 480 real-time PCR system (Roche, Mannheim, Germany). The expression of individual genes was normalized to the expression of *36B4*, a house-keeping gene. Primers for real time qRT-PCR were listed in Supplementary Table S1.

In vitro translation assay

In vitro translation assay was performed using Transcend™ Non-Radioactive Translation Detection Systems (Promega, Madison, WI, USA). *APOA4-AS* transcript was subjected to *in vitro* translation assay, and luciferase serves as a positive control. Translational products were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis followed by western blot. Strep-HRP detected the translational incorporation of biotinylated lysine. The same membrane was stained by Ponceau S.

CRISPR/Cas9 mediated gene editing

Two guide RNA sequences are selected (sgRNA1: 5'-CUUAUUCGGGAUAAAGU AGCAGG-3'; sgRNA2: 5'-CCGUUUGUCAAACCGGAUAAAGG-3'). The production of viral vectors Ad-Cas9, Ad-sgRNA1 and Ad-sgRNA2 was carried out as described elsewhere (26,27). Primary hepatocytes were isolated and infected with Ad-Cas9, or Ad-Cas9 and two Ad-sgRNAs (Ad-sgRNA1 and sgRNA2) adenovirus for 36 h. Immunoblotting and qRT-PCR analysis were performed.

RNA-protein interaction assays

HEK293T cells were transiently transfected with Flag-HuR and *APOA4-AS* (WT, T1 or T2) alone or in combination. Immunoprecipitation (IP) was performed by using anti-Flag agarose beads (Sigma). After washing, RNA was extracted with TriPure Isolation Reagent (Roche, Mannheim, Germany), treated with RNase-free DNase and analyzed by semi-quantitative RT-PCR or qRT-PCR. For reverse precipitation, StA-*APOA4-AS* (WT, T1 or T2) was precipitated from total lysates prepared from transfected HEK293T cells using streptavidin agarose beads. *APOA4-AS* binding proteins were eluted and analyzed by immunoblotting.

Statistical analysis

Figures were prepared by using GraphPad Prism software (La Jolla, CA, USA). Data were presented as means \pm SEM. Differences between groups were analyzed by two-tailed Student's *t*-tests. $P < 0.05$ was considered statistically significant. For statistical analysis of RNA levels, data were calculated by the $2^{-\Delta\Delta Ct}$ method and represented as the means \pm SEM. Differences between groups were analyzed by two-tailed Student's *t*-tests. For Figure 6C and D, one-way ANOVA analysis was performed. $P < 0.05$ was considered statistically significant.

RESULTS

Identification and characterization of an antisense lncRNA (*APOA4-AS*) at *APOA4* gene locus

In an attempt to investigate the lncRNA expression profile in the liver, specifically those associated with lipid metabolisms, we analyzed a previously published RNA-seq dataset from the WT and *db/db* mice liver, which were originally designed to identify genes associated with glucose metabolism and involved in the development of type 2 diabetes (28). Here, both RNA-seq reads were mapped to mouse genome (GRCm38/mm10) by TopHat and assembled against annotated lncRNA library (gencode vM2 long non-coding RNAs library) by Cufflinks (22,29). Cuffdiff and CummeRbund were further utilized to analyze the differentially regulated lncRNAs in the liver between WT and *db/db* mice. Fifteen uncharacterized lncRNAs were then identified and shown to be dysregulated in the liver of *db/db* mice (Figure 1A). In an effort to analyze the systemic network and pathways in which lncRNA-associated mRNAs were involved, lncRNA-neighboring protein coding genes were selected to perform gene enrichment analysis (Gene Ontology: <http://geneontology.org/>). GO analysis focuses on the biological process, molecular function and cellular component that lncRNA-neighboring mRNAs are involved in. GO analysis showed that the lncRNA-neighboring mRNAs are associated with the lipid metabolism (Supplementary Figure S1).

Among the dysregulated lncRNAs, *Gm10680* is highly upregulated in *db/db* mice liver. Retrieving the sequence and genome wide location from the Ensembl Genome Browser (www.ensembl.org/) database, the putative *GM10680* (– strand) is transcribed from the opposite strand of *APOA4* locus (+ strand), partially overlaps with *APOA4* exon 3 and 3'-UTR (chr9:46 242 621–46 243 529) and contains two exons and one intron (Supplementary Figure S2). Therefore, we named *GM10680* as *APOA4* antisense transcript (*APOA4-AS*).

APOA4-AS was identified to be highly induced in diabetic mouse liver. To measure the expression level of *APOA4-AS*, we designed *APOA4-AS*-specific qPCR primers, spanning the intron region. Our results showed that our qPCR primers specifically detected the non-adenylated *APOA4-AS* (Supplementary Figure S3A and B). Furthermore, we measured the *APOA4-AS* expression level in both WT and obese mice liver showing that *APOA4-AS* transcript was increased by ~ 7.5 -fold (Figure 1B) and concordantly, *APOA4* mRNA level was increased by ~ 12 -fold in *ob/ob* mice compared with that of WT mice (Figure 1C). *APOA4* protein level was also dramatically increased (Figure 1D).

Human *APOA4* expression and synthesis are only confined to the intestine (30). Compared with rodents, such as mouse and rat, *APOA4* expression is barely detected in the human liver (30). However, we demonstrated that *APOA4* expression was highly induced in the liver of human with fatty liver disease (Figure 1E). Thus, we speculated that whether the human *APOA4-AS* transcript may exist and its expression also correlates with that of *APOA4*. By analyzing the human liver RNA-seq data from database

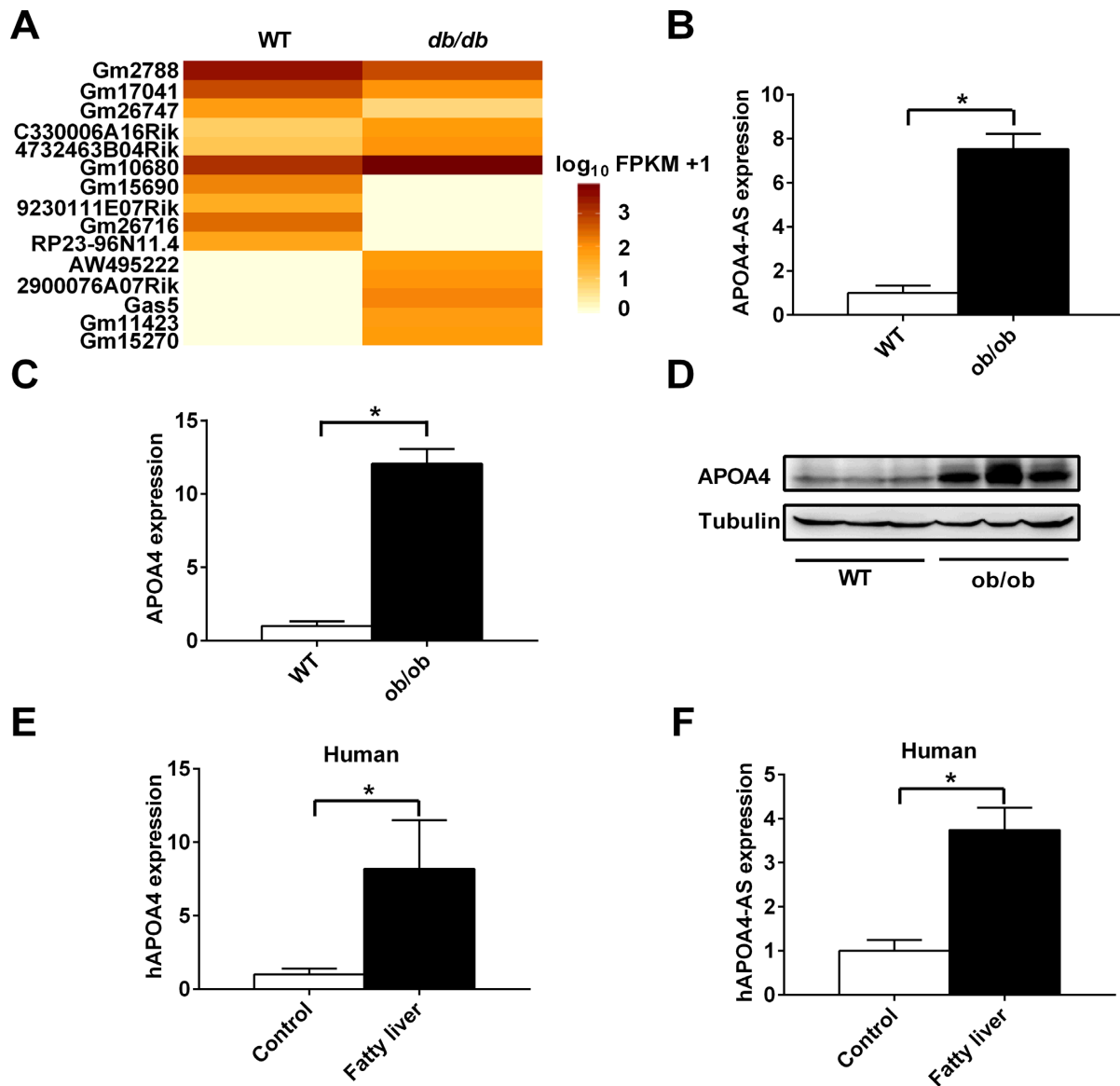


Figure 1. Identification of lncRNAs differentially expressed in the liver of obese mice. (A) The cluster heat map shows lncRNAs with expression change fold >5 from RNA-seq data ($P < 0.05$). (B–D) WT and *ob/ob* mice were fed with normal chow diet for 14 weeks. *APOA4-AS* (B) and *APOA4* transcripts (C) in the liver were measured by qRT-PCR. *APOA4* protein level in the liver was measured by Western blot (D). Expression analysis of *hAPOA4* (E) and *hAPOA4-AS* (F) in human fatty and normal liver tissues. $n = 5–8$. * $P < 0.05$.

(<https://www.encodeproject.org/>), we observed the sequencing peaks in Integrative Genomics Viewer (Data access number: ENCSR285LXD) from the opposite strand of *APOA4* in a similar region of mouse *APOA4-AS* (data not shown). To further characterize the expression of *APOA4-AS* in human liver samples, we showed that *hAPOA4-AS* transcript level was also significantly increased in the liver of human with fatty liver disease (Figure 1F). These data demonstrate that both *hAPOA4-AS* and *hAPOA4* can be induced by lipid accumulation, and could potentially be used as biomarkers for fatty liver disease.

Putative *APOA4-AS* sequence in both Ensembl and UCSC Genome Database contains two exons and one intron. However, by performing coding potential analysis, the predicted *APOA4-AS* (previously annotated as *GM10680*)

cDNA sequence in both database has coding potential assessed by Coding Potential Assessment Tool (CPAT) (Supplementary Table S2). Next, we performed 5' RACE to determine whether *APOA4-AS* cDNA contains the intron. It was shown that in C57B/L6 mouse liver, *APOA4-AS* transcript contains intron and the intron-containing full length transcript (blue color highlighted intron sequencing from the database does present in our RACE determined new *APOA4-AS* transcript sequencing in Supplementary Figure S2) is an ~900-nt non-coding RNA assessed by CPAT (Supplementary Table S2) while the protein coding mRNA *APOA4*, *APOC3* and *APOA5* were predicted to have protein-coding potential utilizing the same prediction setting for CPAT (Supplementary Table S2). Furthermore,

the full-length *APOA4*-AS failed to express any protein using *in vitro* translation assay (Figure 2A).

Next, we examined the expression profile of both *APOA4* and *APOA4*-AS across 10 tissues by qRT-PCR, showing that *APOA4* is majorly detected in both the small intestine and liver (Figure 2B) as reported before (7), and *APOA4*-AS is also significantly enriched in the small intestine and liver, while it is barely detected in other tissues, such as the brain, heart and kidney (Figure 2C). Meanwhile, our northern blot also confirmed that *APOA4*-AS is an ~900-nt transcript that is only expressed in the small intestine and liver (Supplementary Figure S4). Both *APOA4*-AS and *APOA4* show a similar expression pattern, suggesting that functionally related mechanisms may present between these two transcripts.

To further determine the cellular localization of *APOA4*-AS transcript, the nuclear and cytosolic RNAs were isolated from primary hepatocytes, and the expressions of *APOA4*-AS and *APOA4* transcripts in both subcellular locations were measured. qRT-PCR data show that both *APOA4*-AS and *APOA4* transcripts are highly expressed in the cytosol compared with that in the nucleus (Figure 2D and E). As control, *TfIIb* mRNA is specifically located in the cytosol (Figure 2F), whereas 45S ribosomal RNA precursor is primarily located in the nucleus (Figure 2G).

APOA4*-AS is an essential component to maintain *APOA4* expression both *in vitro* and *in vivo

We have shown that both *APOA4*-AS and *APOA4* transcripts are localized in cytosol. To investigate whether the *APOA4*-AS transcript is associated with the expression of *APOA4*, we designed two distinct adenovirus-based shRNAs targeting to *APOA4*-AS transcript. Adenovirus-mediated expression of the sh*APOA4*-AS1 or sh*APOA4*-AS2 in primary hepatocytes resulted in a significant reduction of not only the targeted *APOA4*-AS transcript (Figure 3A) but also *APOA4* mRNA and protein levels (Figure 3B and C). Two distinct *APOA4*-AS shRNA molecules that specifically target to the *APOA4*-AS transcript resulted in concomitant reduction of *APOA4* expression, which suggests that *APOA4*-AS may regulate the expression of *APOA4* at both the mRNA and the protein levels in primary hepatocytes.

To further determine whether *APOA4*-AS regulates *APOA4* mRNA and protein levels *in vivo*, we generated liver-specific *APOA4*-AS knockdown mice by tail-vein injection of two individual purified adenovirus particles carrying *APOA4*-AS shRNA (sh*APOA4*-AS1 or sh*APOA4*-AS2). Consistently, either sh*APOA4*-AS1 or sh*APOA4*-AS2 significantly reduced *APOA4*-AS transcript level as well as *APOA4* mRNA and protein levels in mouse liver (Figure 3D–F). Furthermore, the plasma *APOA4* protein level in *APOA4*-AS liver-specific knockdown mice was significantly reduced (Figure 3F). Therefore, these data demonstrate that *APOA4*-AS is an essential component to stabilize *APOA4* expression both *in vitro* and *in vivo*.

APOA4 gene is a part of the gene cluster that also includes *APOA1*, *APOC3* and *APOA5* involving in lipid metabolism (13,15). Recent study has shown that *APOA1*-AS could negatively regulate *APOA1*, *APOA4* and *APOC3* expression

at transcription level (31). Here, we showed that manipulation of *APOA4*-AS expression does not affect the expression level of *APOA1*, *APOC3* or *APOA5* mRNA both *in vitro* and *in vivo* (Supplementary Figure S5), providing strong evidence to support the specificity of *APOA4*-AS to regulate *APOA4* expression.

Liver-specific knockdown of *APOA4*-AS decreases *APOA4* expression and reduces plasma TG and TC level in *ob/ob* mice

Consistent with the observation we made in WT mice, we also noted that knockdown of *APOA4*-AS in the liver of *ob/ob* mice by shRNA dramatically decreased *APOA4* mRNA and protein levels (Figure 4A and B). However, the expression of other lipid metabolism associated genes (e.g. *FASN*, *SCD1*, *APOB*, *MTP*, *CPT1 α* and *MCAD*) was not altered (Figure 4B). These data demonstrate that similar regulatory mechanisms may exist between *APOA4*-AS and *APOA4* in both healthy and obese conditions.

It has been shown before that *APOA4* regulates hepatic TG secretion (16). To assess whether *APOA4*-AS may regulate hepatic TG homeostasis, we measured liver TG, plasma TG and TC levels in liver-specific *APOA4*-AS knockdown *ob/ob* mice and control *ob/ob* mice. We did not observe any change in liver TG level and steatosis (Figure 4C and D). However, plasma TG and TC levels were significantly reduced by 32.3 and 18%, respectively, in liver-specific *APOA4*-AS knockdown of *ob/ob* mice (Figure 4E and F). These data suggest that *APOA4*-AS stabilizes *APOA4* in obesity contributing to hypertriglyceridemia.

***APOA4*-AS directly binds mRNA stabilizing protein HuR**

Antisense transcript stabilizes sense mRNA through extended base-pairing or physical interaction with mRNA stabilizing protein. One of the mRNA stabilizing proteins is HuR whose expression in the liver has been reported before (32,33). *APOA4*-AS contains two HuR binding sites (Supplementary Figure S2). To determine whether *APOA4*-AS directly binds to mRNA stabilizing protein HuR, we transiently transfected human embryonic kidney 293T (HEK293T) cells with vectors expressing *APOA4*-AS transcript and flag-tagged HuR, followed with IP using anti-Flag agarose beads. Semi-quantitative RT-PCR and qRT-PCR analyses indicated that *APOA4*-AS presents in the HuR immunocomplex (Figure 5A). To further determine whether predicted HuR binding sites are essential for *APOA4*-AS and HuR interaction, one or two HuR binding sites were deleted in truncation 1 (T1) or 2 (T2) isoforms of *APOA4*-AS. Sequence (+1 to +24) and (+1 to +94) was deleted in T1 or T2, respectively. RNA immunoprecipitation (RIP) assay showed that these two sites are important for the interaction between *APOA4*-AS and HuR (Figure 5B). Furthermore, RIP-qRT-PCR assay showed that endogenous *APOA4*-AS and *APOA4* transcripts form a complex with endogenous HuR in mouse liver (Figure 5C and D). To confirm this physical interaction, we constructed three plasmids expressing *APOA4*-AS WT, T1 or T2 isoform, respectively, fused to streptavidin-binding aptamer (StA-*APOA4*-AS WT, T1 and T2), a 60-nt RNA tag that mimics biotin and binds to streptavidin with high affinity

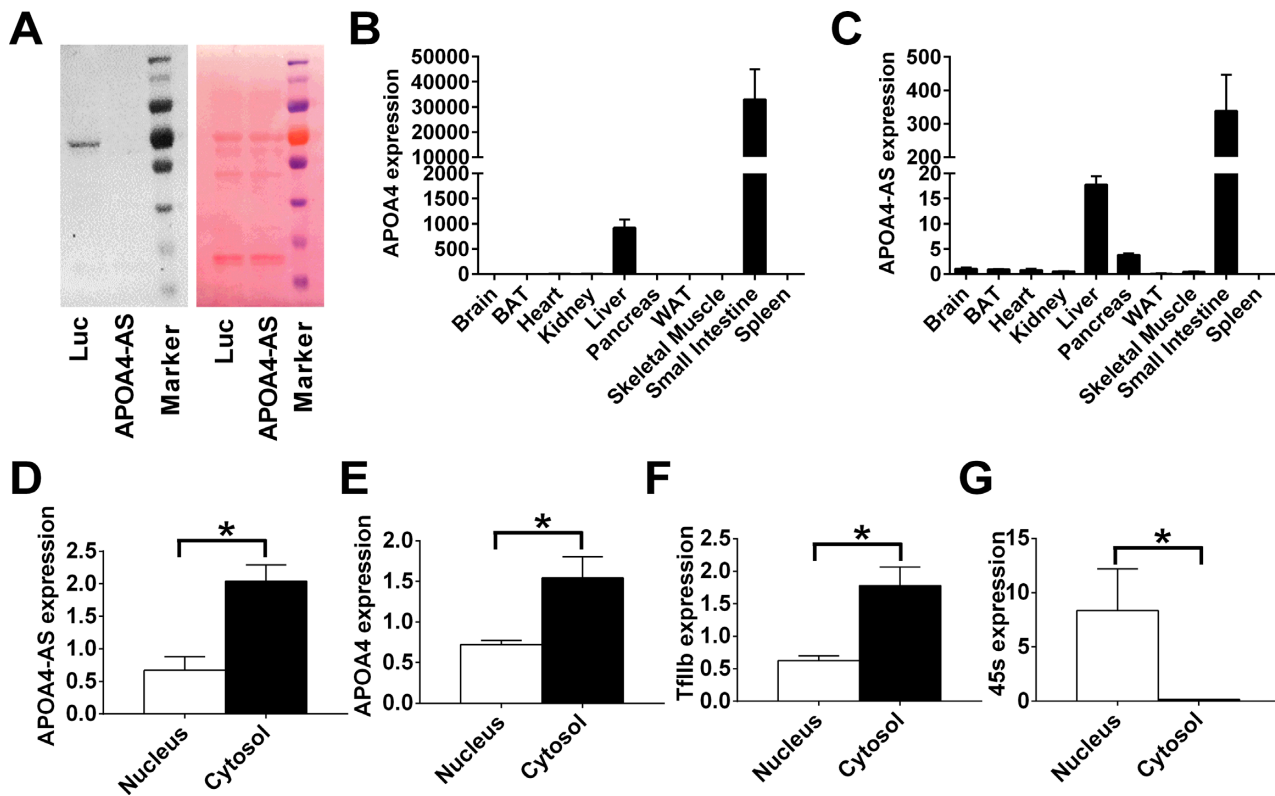


Figure 2. Characterization of the non-coding property, expression profile and cellular location of *APOA4-AS*. (A) *In vitro* translation assay using luciferase (Luc) and *APOA4-AS* RNA. Strep-HRP detection of translational incorporation of biotinylated lysine (left). The same membrane was stained by Ponceau S (right). (B) and (C) Expression analysis of *APOA4* and *APOA4-AS* in 10 mouse tissues. (D–G) Expression of *APOA4-AS*, *APOA4*, *TfIIB* and 45s in isolated cytosol and nucleus from primary hepatocytes. $n = 4–6$. * $P < 0.05$.

(34). Streptavidin precipitation of lysates from HEK293T cells transiently transfected with HuR alone or in combination with StA-*APOA4-AS* (WT, T1 or T2) revealed that HuR was only detected in the *APOA4-AS* (WT) RNA complexes (Figure 5E). The amount of HuR in *APOA4-AS* T1 or T2 RNA complexes was dramatically reduced (Figure 5E). These data demonstrate that *APOA4-AS* directly binds to HuR, which may contribute to *APOA4* mRNA stabilization.

Deletion of HuR decreases both *APOA4-AS* and *APOA4* expression

To further determine whether HuR affects the stability of *APOA4-AS* and *APOA4* expression, CRISPR/Cas9 mediated deletion of HuR was performed. As shown in Figure 6A, HuR was dramatically reduced by CRISPR/Cas9 mediated gene editing. The expression of *APOA4-AS* and *APOA4* was reduced by 89.6 and 90.5%, respectively (Figure 6B). Overexpression of *APOA4-AS* in HuR deleted hepatocytes could barely increase *APOA4* expression (Figure 6C and D). These data indicate that HuR is an essential component to stabilize both *APOA4-AS* and *APOA4* transcripts, and *APOA4-AS* regulates *APOA4* expression depending on HuR.

DISCUSSION

APOA4 controls multiple metabolic pathways including fat absorption (35), food intake (36), energy expenditure (37), TG secretion (16), insulin secretion (17) and hepatic glucose production (18), and its expression is also tightly regulated by metabolic state. High fat diet or steatosis increases *APOA4* level in the liver, while the deletion of *APOA4* leads to a reduction of the plasma level of TG (16,38). At molecular level, CREBH, CREB3, ERRa and PPARa drive the expression of *APOA4* (21,39,40). In current investigation, we demonstrate that *APOA4-AS*, a long non-coding antisense transcript for *APOA4*, functions as a concordant regulator of *APOA4* gene expression and plays a role in regulating plasma TG and TC levels. We present data showing that *APOA4-AS* is co-expressed with *APOA4* in the liver, and HuR-*APOA4-AS* complex specifically regulates *APOA4* expression both *in vitro* and *in vivo*.

lncRNAs are involved in lipid homeostasis (41). We initially discovered a subset of lncRNAs dysregulated in the liver of *db/db* mice, an obese mice model with leptin receptor deficiency. The GO analysis of dysregulated lncRNAs-neighboring mRNAs indicates that these mRNAs are majorly involved in TG and cholesterol metabolism, especially the TG catabolism, suggesting that these dysregulated lncRNAs may be related to TG and cholesterol metabolism.

Among the 15 dysregulated lncRNAs, we found that *APOA4-AS* is co-elevated with *APOA4* in the liver of *ob/ob*

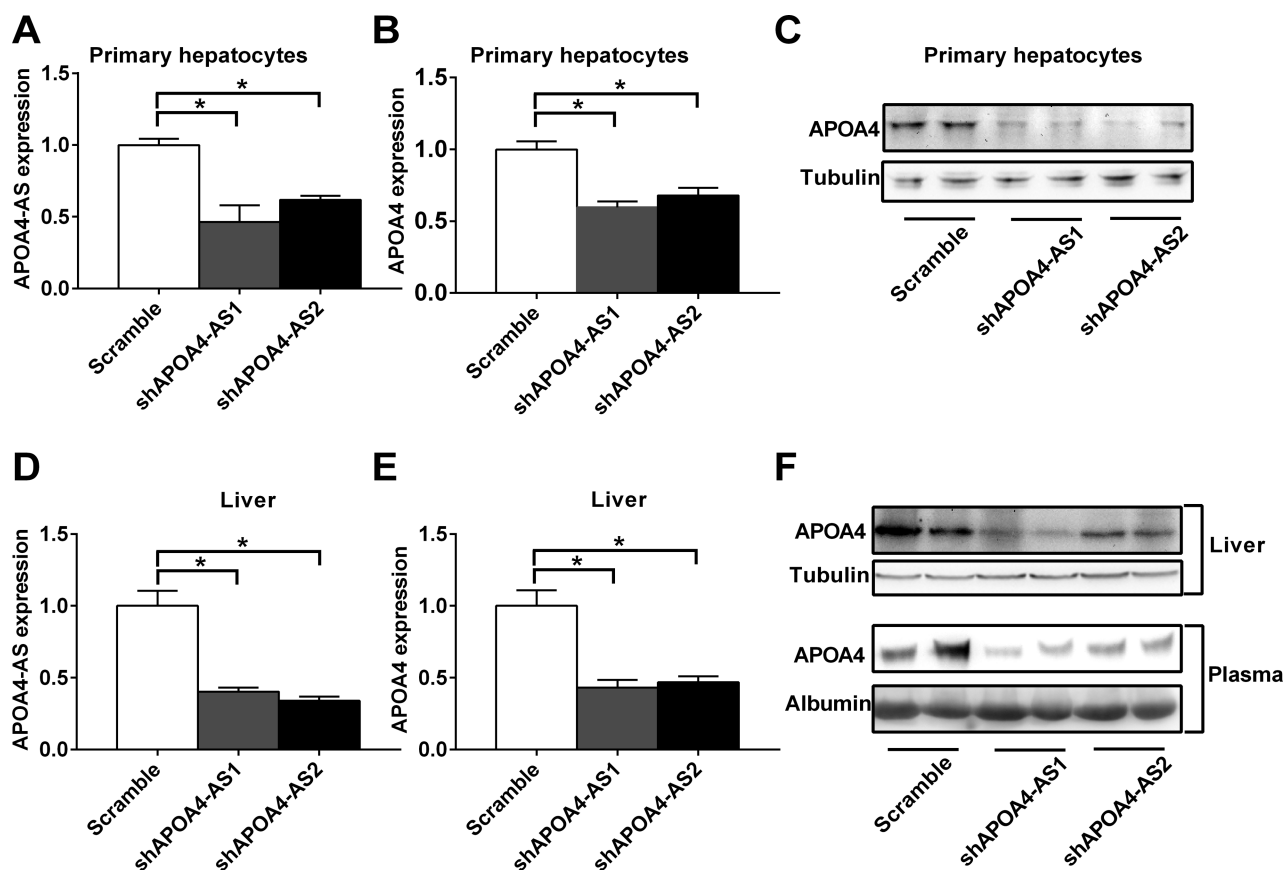


Figure 3. Knockdown of *APOA4-AS* transcript decreases *APOA4* mRNA and protein levels both *in vitro* and *in vivo*. (A–C) Primary hepatocytes were isolated from C57BL/6 mice. Cells were infected with ad-scramble or two different ad-shRNAs (sh*APOA4-AS1* or sh*APOA4-AS2*) adenovirus targeting to *APOA4-AS* transcript. *APOA4-AS* (A) and *APOA4* (B) transcripts were measured by qRT-PCR. *APOA4* protein level was measured by western blot (C). (D–F) C57BL/6 mice (8 weeks old) were tail-vein injected with ad-scramble or two different ad-shRNAs (sh*APOA4-AS1* or sh*APOA4-AS2*) adenovirus targeting *APOA4-AS* transcript. *APOA4-AS* (D) and *APOA4* transcripts (E) in the liver were measured by qRT-PCR. *APOA4* protein levels in the liver and plasma were measured by western blot (F). $n = 3-6$. * $P < 0.05$.

mice, another obese mice model with leptin deficiency. Both hepatic *APOA4-AS* and *APOA4* are also abnormally elevated in *ob/ob* mice compared with that in WT mice. This observation is consistent with previous reports that hepatic *APOA4* expression is positively associated with liver TG accumulation (16) indicating that *APOA4-AS* may be associated with *APOA4* expression and involved in TG metabolism. *APOA4* could enhance hepatic TG secretion and increase plasma TG level in mice (16). We also found that in *ob/ob* mice, knockdown of *APOA4-AS* not only reduced *APOA4* expression but also significantly decreased plasma TG and TC levels. However, knockdown of *APOA4-AS* in *ob/ob* mice did not affect liver TG level, which indicates that the primary role of *APOA4* in the liver is to assist hepatic TG secretion, but not TG accumulation (16).

To delve into the regulatory mechanism of *APOA4-AS* on *APOA4*, we manipulated the expression level of *APOA4-AS* both *in vitro* and *in vivo*, demonstrating that *APOA4-AS* is closely associated with the *APOA4* mRNA stability, possible through the extended overlap region between lncRNA and mRNA to further assist the mRNA stability. Further mechanistic investigation revealed HuR (an mRNA stabilizing protein) involved in this process. Our study first determined that both *APOA4-AS* and *APOA4*

transcripts are present in cytosol of primary hepatocytes. shRNA-mediated *APOA4-AS* knockdown decreases *APOA4* mRNA level, suggesting that *APOA4-AS* may stabilize sense *APOA4* mRNA through extended base-pairing with sense mRNA at *APOA4* 3'-end to prevent mRNA from 3' exonuclease-mediated decay (42). Previously, a study performing 3' region extraction and deep sequencing (3' READS) identified that in mammalian cells about 66% long non-coding RNA genes are polyadenylated (43). In current investigation, we noted that cDNA synthesis of *APOA4-AS* by oligo (dT) was failed unless polyadenylation reaction is performed or random hexamer is utilized (Supplementary Figure S3B), suggesting that *APOA4-AS* transcript is non-polyadenylated and requires additional RNA stabilization protein to prevent exonuclease-mediated RNA decay. Here, through bioinformatics analysis, we identified two putative HuR binding sites in the sequence of 5' end of *APOA4-AS*. Truncated *APOA4-AS* with deletion of HuR binding site abolished the HuR-binding to *APOA4-AS*. Deletion of HuR by CRISPR/Cas9 mediated gene editing dramatically reduced both *APOA4-AS* and *APOA4* transcripts. We also showed that in *in vitro* cultured mouse hepatocyte treated with Actinomycin D (ActD), a transcriptional inhibitor, shows that *APOA4-AS* is more stable than *APOA4*

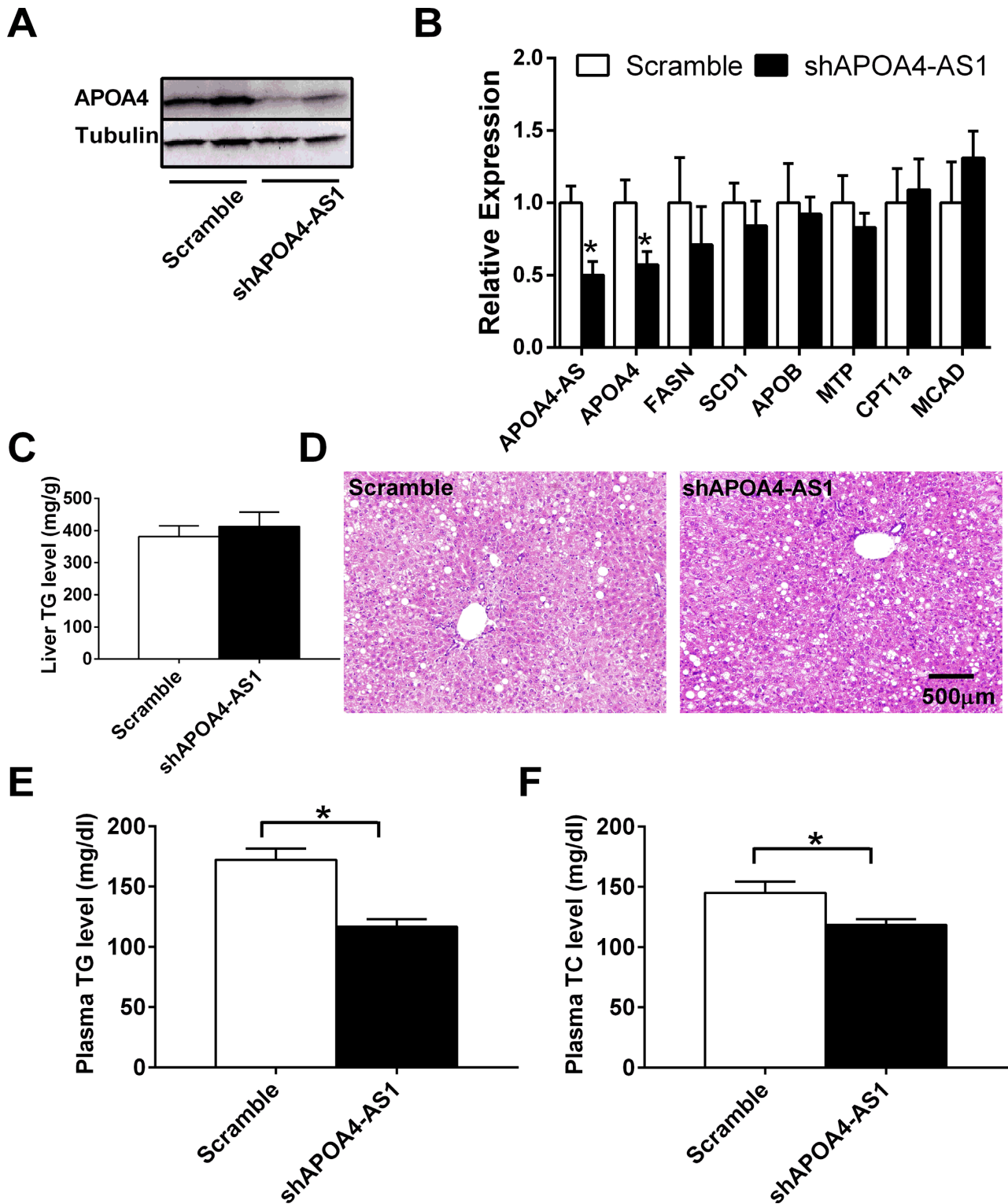


Figure 4. Knockdown of *APOA4-AS* in *ob/ob* mice decreases *APOA4* expression and plasma TG and TC levels without affecting steatosis. *ob/ob* mice (8 weeks old) were tail-vein injected ad-scramble or ad-sh*APOA4-AS1* purified adenovirus. Mice were sacrificed at 10–12 days after adenovirus injection. (A) *APOA4* and tubulin were measured by western blot. (B) *APOA4-AS*, *APOA4*, *FASN*, *SCD1*, *APOB*, *MTP*, *CPT1a* and *MCAD* transcripts in the liver were measured by qRT-PCR. (C) Liver TG level. (D) H&E staining of liver sections. (E) Plasma TG levels. (F) Plasma TC levels. $n = 5-6$. * $P < 0.05$.

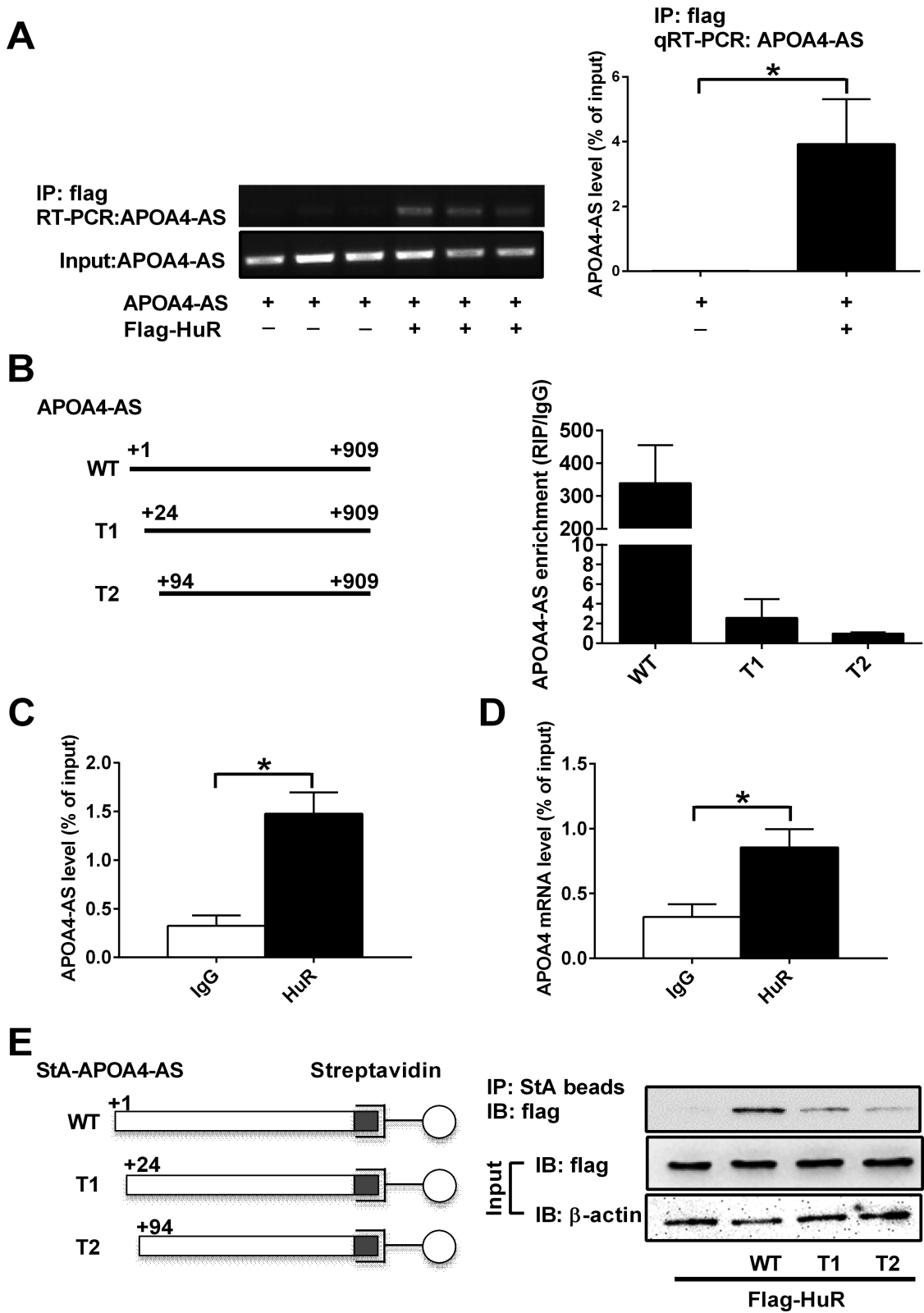


Figure 5. *APOA4-AS* transcript interacts with mRNA stabilizing protein HuR. (A) Semi-quantitative RT-PCR (left) analyses of *APOA4-AS* in RNA isolated from a-flag immunocomplex (top) or input (bottom) from transiently transfected HEK293T cells. qRT-PCR analyses (right) were also performed by using the same samples and normalized by input. (B) Flag-HuR was co-transfected with *APOA4-AS* WT or two truncated *APOA4-AS* (T1 or T2) in HEK293T cells. *APOA4-AS* enrichment in flag immunocomplex was present by RIP/IgG. (C) and (D) qRT-PCR analyses of endogenous *APOA4-AS* and *APOA4* transcripts in the HuR immunocomplex from mouse liver. (E) Immunoblots of Flag-HuR after streptavidin-APOA4-AS (StA-APOA4-AS) precipitation from transfected HEK293T cell lysates. Schematic diagrams of StA-APOA4-AS are shown on the right. $n = 3-5$. * $P < 0.05$.

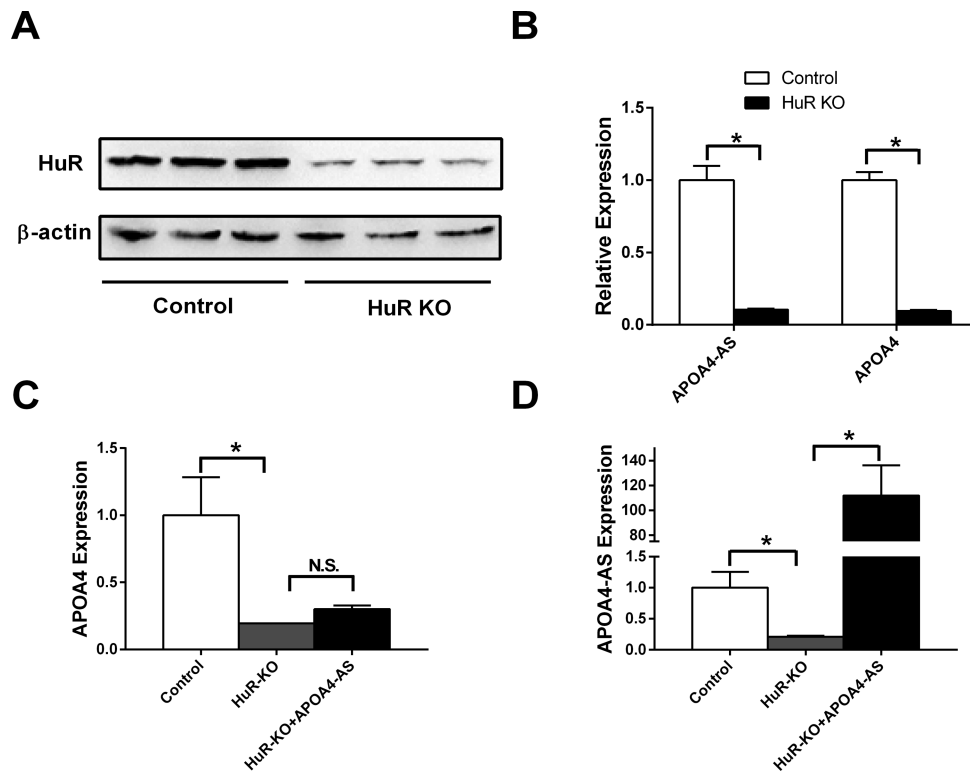


Figure 6. Deletion of HuR decreases both *APOA4-AS* and *APOA4* mRNA in primary hepatocytes. Primary hepatocytes were isolated from C57BL/6 mice. Cells were infected with ad-Cas9 and two different ad-sgRNAs adenovirus targeting to *HuR*. (A) HuR and β -actin protein levels were measured by western blot. (B) *APOA4-AS* and *APOA4* transcripts were measured by qRT-PCR. (C) and (D) Hepatocytes with HuR deletion were infected with *APOA4-AS* adenovirus. *APOA4* (C) and *APOA4-AS* (D) transcripts were measured by qRT-PCR. $n = 4-6$. * $P < 0.05$.

transcript (Supplementary Figure S3C). These data all together demonstrate that HuR is essential for the stability of *APOA4-AS* and *APOA4* transcripts, and *APOA4-AS* RNA may recruit HuR to form a complex with *APOA4* mRNA.

In summary, our work reveals a novel mechanism by which HuR–*APOA4-AS* complex stabilizes *APOA4* mRNA. *APOA4-AS* could be a potential therapeutic target for metabolic diseases.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We thank Dr. Liangyou Rui (University of Michigan) for kindly providing ob⁺/– mice, Dr. Yanjing Zhang for vector cloning and Dr. Li Liu (Nanfeng Hospital, Southern Medical University, China) for nicely providing flag-HuR construct. Liwei Xie together with all authors thank Dr. Hang Yin (University of Georgia) for support of the studies proposed in this manuscript.

FUNDING

Changbai Mountain Scholars Program of The People's Government of Jilin Province [2013046 to Z.C.]; Jilin Talent Development Foundation [111860000 to Z.C.]; Jilin Science and Technology Development Program [20160101204JC

to Z.C.]; Fok Ying Tong Education Foundation [151022 to Z.C.]; National Natural Science Foundation of China [31500957 to Z.C., 31470798 to Y.Z.]; American Heart Association [16POST26400005 to L.X.]; Northeast Normal University [120401204 to Z.C.]. Funding for open access charge: Changbai Mountain Scholars Program of The People's Government of Jilin Province [2013046].

Conflict of interest statement. None declared.

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