HuR/miR-124-3p/VDR complex bridges lipid metabolism and tumor development in colorectal cancer

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Research Article

Abstract

Background: Maintaining lipid homeostasis to prevent lipotoxicity is crucial for various tumors, including colorectal cancer (CRC). Hu-antigen R (HuR) is a member of the RNA binding protein family and overexpressed in many cancer types, which implicted that in regulating cell proleferation, migration, invasion, and lipid metabolism. However, the role of HuR in regulating abnormal lipid metabolism of CRC is unknown.

Methods: Western blot was performed to screen differentially expressed HuR between CRC tissues and adjacent normal tissues. Lipidomic profiling, RNA sequencing (RNA-seq), Cell Counting Kit-8 (CCK-8), total cholesterol and triglycerides assays testified the critical role of HuR/miR-124-3p/VDR complex in CRC cells. RNA pull-down and luciferase reporter Assays were performed to verify the interaction between HuR protein and the VDR mRNA. We also conducted a mouse xenograft model to elucidate the effect of HuR on lipid homeostasis and proliferation in vivo.

Results: Our study identified that HuR promotes the expression of VDR, then modulates lipid homeostasis by enhancing TG and TC levels in CRC. Here, our study demonstrated that overexpressing HuR enhanced the expression of VDR through directly binding to its CDS and 3'-UTR. Simultaneously, HuR also indirectly affecting VDR by inhibiting miR-124-3p. We identified that HuR can suppress the expression of miR-124-3p, while miR-124-3p can bind to 3'-UTR of VDR to inhibit the expression of VDR. Moreover, xenograft models showed that targeting HuR suppressed the expression of VDR, blocked TG and TC formation, then suppressed CRC growth.

Conclusion: Our findings propose a regulatory connection between HuR, miR-124-3p and VDR in CRC cells. We suggested that HuR/miR-124-3p/VDR complex modulates lipid homeostasis by influencing TG and TC formation in CRC, and may provide a potential target for CRC treatment and prevention.

Introduction

Colorectal cancer (CRC) is an aggressive primary intestinal malignancy with the third leading incidence and second-highest mortality of all types of cancers worldwide[1]. Despite significant progress in surgical techniques and treatment for CRC, the 5-year relative survival rate for CRC patients has not changed significantly in recent decades[2,3]. Metabolic reprogramming is an important hallmark of CRC[4−6], but the mechanisms by which tumor cells maintain lipid homeostasis to prevent lipotoxicity are not well understood. Therefore, it is important to study the potential oncogenic molecular mechanisms of abnormal lipid metabolism in order to develop new therapies targeting CRC.

RNA-binding proteins (RBPs) play crucial roles in maintaining cellular homeostasis by regulating numerous essential cellular processes, including RNA splicing, modification, transport, localization, stability, degradation, and translation[7,8]. Dysregulation of some RBPs has been linked to the development of diseases such as CRC[9,10]. Hu-antigen R (HuR) is a well-known RBP that plays a vital role in post-

transcriptional regulation in various diseases[11–14]. HuR mainly binds to AU-rich elements (AREs) and stabilizes its target mRNAs, which are involved in human malignancies. For example, HuR promotes tumor cell growth by stabilizing Bcl-2 in glioblastoma[15]. In CRC, the roles of HuR have also been studied extensively. HuR has been found to be upregulated in CRC[16,17] and to stabilize many oncogenes, such as COX-2[16], VEGF[18], and IL-8[18], leading to enhanced CRC cell growth and tumorigenicity. One study found a strong correlation between increased cytoplasmic HuR levels and COX-2 expression and colon cancer stage[19]. High levels of cytoplasmic HuR have been associated with worse clinical outcomes, increased incidence of lymphatic spread, and distant metastasis[11,14]. Overall, these studies support the oncogenic role of HuR in CRC. However, the underlying mechanism for the abnormal expression of HuR in CRC is not well understood.

Recently, studies have focused on the role of HuR in lipid metabolism. It was reported that hepatic HuR regulates lipid homeostasis in response to a high-fat diet[20]. And HuR post-transcriptionally modulates ATP-binding cassette transporter A1 (ABCA1), which plays an important role in cholesterol metabolism of liver cancer cells and monocytic cells[21]. All the data demonstrated that HuR makes an important role in regulating lipid metabolism. However, the expression pattern and function of HuR have rarely been studied in the lipid metabolism of cancers. Recent studies illustrate that tumor development is closely dependent on the interaction of target protein with tumor lipid metabolism, which is a dynamic, changing network.

A previous study showed that HuR regulated the expression of VDR in the normal intestinal epithelium, and HuR modulated rapid epithelial restitution after wounding by VDR[22]. VDR is a transcription factor that regulates lipid metabolism. VDR was found to regulate glycerolipid and phospholipid metabolism in human hepatocytes[23]. And the involvement of VDR in lipid metabolism has been established in vitro and in animal models[24,25]. The results suggest that VDR may play a critical role in lipid metabolism in cancer.

RBPs can interact with various classes of RNAs, including mRNAs, tRNAs, snRNAs, snoRNAs, and ncRNAs[26]. Some studies have found that multiple RBPs beyond Drosha/Dicer are involved in the processing of microRNAs (miRNAs)[27,28]. miRNAs are important regulators of gene expression that bind to complementary target mRNAs and repress their expression[28]. HuR has been shown to influence the expression of certain miRNAs, such as miR-7[29]. In human liver cells under stress, HuR binds to the cationic amino acid transporter 1 (CAT1) mRNA and inhibits the recruitment of the miR-122/AGO complex to target sites in the 3' UTR, thus relieving CAT1 mRNA repression[30]. Similarly, in colorectal cancer, HuR binds to AREs (AU-rich elements) in target mRNAs and hinders miR-16-guided repression of COX2, promoting the tumorigenesis of intestinal tumors[31,32]. Therefore, there may be multiple pathways by which HuR regulates target genes and is involved in the modulation of lipid metabolism. Given the broad effects of HuR on post-transcription, it is worth studying whether HuR increases VDR expression and modulates lipid metabolism in CRC.

In this study, we investigated the expression and function of HuR in lipid metabolism in CRC. We found that HuR promotes the expression of VDR in CRC cells and modulates lipid homeostasis by enhancing TG and TC levels. Overexpression of HuR enhances the expression of VDR by directly binding to its CDS and 3'UTR, and indirectly by inhibiting miR-124-3p. We also found that HuR can suppress the expression of miR-124-3p, while miR-124-3p can bind to the 3'UTR of VDR to inhibit its expression. Inhibiting HuR/miR-124-3p/VDR axis induces a reduction of tumor growth in vitro and in vivo. Xenograft models showed that targeting HuR suppressed the expression of VDR, blocked TG and TC formation, and suppressed CRC growth. Our findings propose a regulatory connection between HuR, miR-124-3p, and VDR in CRC cells, and suggest that the HuR/miR-124-3p/VDR complex may be a potential target for CRC treatment and prevention by modulating lipid homeostasis and influencing TG and TC formation.

Materials And Methods

Bioinformatics analysis

The expression levels of HuR and VDR and their correlation in the tissues of patients with CRC were downloaded using Gene Expression Profiling Interactive Analysis (GEPIA; http://gepia.cancer-pku.cn/). Three mRNA expression profiles (GSE39582, GSE41258, GSE20916) are publicly available in the Gene Expression Omnibus (GEO) database.

Samples from Individuals with CRC

Tumor samples from individuals with CRC were obtained from the surgical specimens at the Zhongnan Hospital of Wuhan University. Each sample was snap-frozen in liquid nitrogen and stored at – 80°C. The use of CRC and normal colon tissues was approved by the WHU Institutional Review Board.

Mice

Female BALB/c nude mice (4 weeks old) were purchased from GemPharmatech (Jiangsu, China). The mice were housed 5 per cage in a conventional barrier facility on a 12-hour light/dark cycle at 22°C with free access to water and food. Mice health status was checked by following the protocols. All animal experiments were conducted under a protocol approved by the Zhongnan Hospital of Wuhan University Institutional Animal Care Animal Welfare Committee.

Cell Cultures And Cell Transfection

The human CRC (HCT116 and HT29) cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HCT116 was cultured in McCoy's 5A (Procell, Wuhan, China). HT29 cells were cultured in RPMI-1640 (Procell, Wuhan, China). All cells were incubated in a 37°C incubator with 5%

CO2 and maintained in a basic medium supplemented with 10% fetal bovine serum (FBS) (hyclone, USA) and 1% antibiotics (100 U/ml penicillin and 100 mg/ml streptomycin; Biosharp, Wuhan).

Chemicals

Palmitic acid (P5585) was purchased from Sigma Aldrich.

Cell Viability Assay

Cells were seeded at 5,000 cells per well in 96-well plates. 24 hours after seeding, the cells were transfected with siRNA. Ultimately, the cell viability was measured using cell counting Kit-8 (CCK-8) (Dojindo, Japan), according to the manufacturer's instructions.

RNA Extraction and Quantitative Polymerase Chain Reaction

Total RNAs of siNC or siHuR-transfected cells were isolated and extracted using Trizol reagent (15596026, Invitrogen), and cDNA was synthesized using TOYOBO ReverTra Ace Kit (TOYOBO, Japan). The cDNA was then used as a template for the qRT-PCR experiments using UltraSYBR Mixture (CWbio, China). The primers were synthesized by TSINGKE Biological Technology (Wuhan, China). All qPCR data were normalized to the internal control gene expression of GAPDH to ensure accurate gene quantification. The relative expression was calculated based on the $2^{-\Delta\Delta CT}$ method. The genes and their respective parameters are listed in Supplementary Table S1.

Western Blot Protein Analysis

The radioimmunoprecipitation (RIPA) buffer in the presence of a proteinase and phosphatase inhibitor cocktail was used to extract protein from tissues and cells. Protein concentrations were quantified using a BCA Protein assay kit (Beyotime, China). Cell lysates (25 ug protein/line) were separated by a 10% SDS-PAGE gel, which was then transferred onto nitrocellulose membranes (Millipore Corp, Billerica, MA, United States). The membrane was then sealed in 5% bovine serum albumin (BSA) for 1 h. Antibodies against HuR (1:1000 Santa Cruz), VDR (1:1000 Santa Cruz), vinculin (1:1000 Santa Cruz), and FASN (1:1000 CST) were added to the membrane and incubated at 4 °C overnight. After washing the membranes with fresh TBST buffer, secondary antibodies of peroxidase affinipure goat anti-rabbit or mouse IgG (1:5000; Promoter; China) were added and incubated at room temperature for 2 h. Protein detection was performed using the enhanced chemiluminescence (ECL) method and visualized using a Tanon-5200 Chemiluminescent Imaging System (Tanon Science and Technology, Shanghai, China). Protein bands were quantified using ImageJ.

Cell Transfection

HuR and VDR siRNAs were synthesized by RiboBio (Guangzhou, China) and transiently transfected into cells using the Lipofectamine 2000 transfection reagent (Invitrogen, Green Island, CA). After 6–8 h, the medium was refreshed with a complete medium, and cells were cultivated overnight to prepare for the following treatment. The siRNA sequence data of HuR and VDR are listed in Supplementary Table S2.

HuR and VDR plasmids were synthesized by Sino Biological (Beijing, China) and transiently transfected into cells using the Lipo8000™ Transfection Reagent (Beyotime, China). After 6–8 h, the medium was refreshed with a complete medium, and cells were cultivated overnight to prepare for the following treatment.

TG and TC Detection

HCT116 and HT29 cells were cultured in a 6-well plate. After transfection with siHuR or siNC for 48 h, cells were harvested for measuring TG and TC. The intracellular TG and TC were measured using the TG Assay kit (APPLYGEN, Beijing, China) and the TC Assay Kit (Jianchengbio, Nanjing, China) according to the manufacturer's instructions.

RNA Stability Assay

Actinomycin D (5 μg/mL) was used to treat CRC cells. The cells were collected after culture for 0 h, 2 h, 4 h, and 6 h. The Trizol reagent was used to extract the RNA. The levels of GAPDH and VDR were measured by Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR).

Dual-Luciferase Assay

HCT116 cells were cultured in 96-well plates and co-transfected with plasmid and miR-mimic or miR-NC using Lipofectamine 2000 (Invitrogen). The VDR 3' UTRs (1428 to 4616bp downstream of transcription start sites) were modified (S1, S2, S3, S4, S5, and S6) and cloned downstream of a luciferase reporter gene in the pGL3 vector. Cells were co-transfected with the reporter, oeNC, and oeHuR. Forty-eight hours post-transfection, luciferase activity was measured using the luciferase assay system (Promega). Cells were lysed 48 hours after transfection. Luciferase activity was measured using a dual-luciferase reporter assay system (Promega, Madison, Wisconsin). Luciferase activity was averaged with five replicates for each transfection.

RNA Pull-Down Assay

The Pierce™ RNA 3' End Desthiobiotinylation Kit was utilized to obtain biotinylated VDR 3 UTR probe (Bio-VDR-3 UTR), biotinylated HuR probe (Bio-HuR), and negative control probes (Bio-NC). The supplier's instructions were strictly followed. Then, the biotinylated RNAs were incubated with streptavidin beads.

After obtaining cell lysates, streptavidin-coated magnetic beads were added for incubation overnight. Western blot was subsequently performed to detect the expressions of relevant proteins.

Animal Experiments

siRNAs were synthesized and purchased from RiboBio (Guangzhou, China) and chemically modified in the form of 2' Ome + 5' Chol (Guangzhou, China). Chemically modified siRNAs are very stable and can be used in vivo assays. Adenovirus oeNC and oeVDR were bought from Heyuan Bio (Shanghai, China). Four-week-old female BALB/C nude mice were purchased from GemPharmatech (Jiangsu, China) and bred in pathogen-free conditions. HCT116 cells were inoculated subcutaneously into the right flank of BALB/c nude mice (3×10^6 cells per mouse). 7 days later, tumors of comparable size were established. Mice with tumor formation were randomly divided into three groups (control, siHuR, siHuR + oeVDR) of five mice each, and then the mice received intra-tumoral administration of (2.5 nmol siRNA and adenovirus 3×10^8 per mouse each time), respectively, for six times. Tumor dimension was monitored every other day, and their volumes were calculated using the formula: volume = length×width×width/2. Tumor tissues were collected and processed for TG and TC analysis, RT-qPCR, protein expression, and immunohistochemical analysis.

Histology and Immunohistochemistry

The freshly isolated tumor tissues were fixed in 4% paraformaldehyde overnight, embedded in paraffin, sectioned, and mounted on slides. Hematoxylin and eosin (H&E) staining or immunohistochemistry (IHC) was performed using routine methods. In brief, the primary antibodies, including HuR (1:200 Santa Cruz), VDR (1:200 Santa Cruz), and vinculin (1:200 Santa Cruz), were incubated at 4°C overnight. Staining was performed using the Vectastain Elite ABC kit and DAB peroxidase substrate kit (Vector Laboratories). Images were randomly taken from five samples per group at ×200 magnification using an Olympus BX43 microscope. Afterwards, the image analysis was performed using IHC Profiler of ImageJ, which calculates the percentage of positive cells automatically.

Statistical Analysis

In this study, all experiments were performed three times, and data are expressed as the mean ± s.e.m. For analysis, the GraphPad Prism software (GraphPad Software 9.0, USA) was used, unless otherwise stated in the Methods. Significance was set at $P < 0.05$. Statistical analysis for lipidomics and RNA sequencing is described in the respective sections (in Supplemental Material). When the variance between the two groups was similar, a student's t-test was used to analyze the data difference between the two groups.

Results

HuR is significantly upregulated and modulates lipid metabolism in CRC.

To analyze the expression of HuR in colon and rectal adenocarcinoma, we used the TCGA, GTEx, GEO, and CPTAC databases. The results showed that the mRNA and protein levels of HuR are upregulated in tumor tissues compared to adjacent normal tissues (Fig. 1A, B, C, and Figure S1A). To further confirm the change of HuR in CRC, we also analyzed tumor tissues from Zhongnan Hospital of Wuhan University. Western blotting showed that the protein levels of HuR were upregulated in tumor tissues (Fig. 1D). These results indicate that HuR is upregulated in CRC tissues.

To explore the downstream effects of HuR, we applied small interfering RNA (siRNA) specific to HuR. We used RNA sequencing to compare the two groups (siNC vs. siHuR) in HCT116 cells, and performed Kyoto Encyclopedia of Genes and Genomes (KEGG) and Disease Ontology (DO) analysis. This analysis revealed that HuR is related to lipid metabolism in CRC (Fig. 1E and F). Based on these results, we used palmitate (PA), a lipid cytotoxic agent that changes lipid homeostasis in cells, to culture RKO cells. DGAT1, a key enzyme in the synthesis of triglycerides, drastically reduced protein levels upon PA co-culture in CRC cells (Fig. 1G). SOAT1 converts elevated cholesterol into oxysterols and stores them as cholesteryl esters in lipid droplets[33]. SOAT1 was upregulated when PA was added (Fig. 1G). To verify whether PA disturbs lipid homeostasis in CRC cells, we measured intracellular triglyceride and total cholesterol levels. The results showed that TG and TC levels were increased in the PA group (Fig. 1F), indicating that lipid metabolism was altered in CRC cells. Furthermore, the protein level of HuR was significantly reduced in the PA group (Fig. 1G), suggesting that HuR is related to lipid homeostasis. Overall, these data strongly suggest that HuR expression is upregulated and that HuR may play a critical role in regulating lipid homeostasis in CRC.

HuR significantly promotes TG and TC formation and reduces CRC cell death.

The protein and mRNA levels of HuR that were overexpressed or silenced are shown in Fig. 2A and B. To investigate the biological function of HuR, we overexpressed HuR and applied untargeted lipidomics to analyze the lipidome in HCT116 cells. The results revealed distinct differences in TG between the oeNC and oeHuR groups. The classification of the lipidome was analyzed using a pie chart, and the overall change of the lipidome was demonstrated with a volcano plot (Figure S1B and C). As shown in the heat map in Fig. 2C, the TAG level of HCT116 cells increased significantly after overexpressing HuR. Meanwhile, acylcarnitines (ACar), which are converted from FAs and shuttled into mitochondria for oxidation and energy production, were significantly decreased (Fig. 2C), along with the increase in TG. In addition, the lipids associated with DAG, such as PC, PE, LPC, and LPE, were significantly decreased upon overexpressing HuR (Fig. 2D). DGs are precursor molecules for the synthesis of TGs, PC, and PE[34,35], and two diacylglycerol-acyltransferases (DGAT1 and DGAT2) catalyze the esterification of FA-CoA and DGs into TGs[36,37]. We analyzed the change in fold of TAG and DAG (Fig. 2D), ACar (Fig. 2E), PC, and PE (Fig. 2F) to show the difference in the lipidome between oeNC and oeHuR. Altogether, the lipidomics analysis showed that overexpressing HuR reduced DAG, PC, PE, and ACar formation, and induced TAG accumulation. HuR may balance lipid homeostasis and oxidative stress in CRC.

To verify whether HuR regulates lipid metabolism in CRC cells, we measured intracellular triglyceride and total cholesterol levels. Overexpression of HuR increased the TC and TG levels in HCT116 and HT29 cells (Fig. 2G and H). On the contrary, knockdown of HuR decreased the intracellular levels of triglycerides and cholesterol in CRC cells (Fig. 2G and H). We also found that knockdown of HuR decreased the survival rate of HCT116 and HT29 cells (Fig. 4I). These data suggest that upregulation of HuR promotes TG and TC formation and mediates the levels of other lipids in CRC cells. Simultaneously, blockade of HuR induced CRC cell death. These data strongly indicate that HuR may play a critical role in lipid homeostasis in CRC.

HuR is significantly upregulated and modulates lipid metabolism in CRC.

We have previously found that HuR is highly expressed in CRC and regulates lipid metabolism in colorectal cancer. Meanwhile, the TCGA database revealed that VDR in both COAD and READ showed increased expression levels compared to normal colon or rectum tissue (Fig. 3A). Since HuR and VDR are highly expressed in CRC, and based on our results and previous reports, we hypothesized that VDR is a potential target gene of HuR, and that HuR regulates VDR expression. We analyzed TCGA data and tissue samples from Zhongnan Hospital of Wuhan University, and found that there is a positive correlation between HuR and VDR expression in CRC tissues (Fig. 3B, C). These results indicate that VDR is upregulated and HuR is high correlation with VDR in CRC tissues.

According to our results and previous report, we hypothesized that VDR is a potential target gene of HuR, and HuR plays a role via regulating the expression of VDR. To further test this hypothesis, we silenced or overexpressed HuR expression using siRNA or an oeHuR plasmid, respectively. We then measured VDR mRNA expression in HT29 cells, and found that HuR can upregulate VDR mRNA expression (Fig. 3D). We also performed Western blotting to confirm the regulation of VDR expression by HuR. VDR protein expression was significantly decreased in HCT116 cells by siRNA-mediated knockdown of HuR (Fig. 3E, F). Similarly, overexpression of HuR promoted VDR protein expression, while downregulation of HuR significantly decreased VDR protein expression in CRC cells (Fig. 3E, F). In summary, these observations strongly support that HuR promotes VDR expression in CRC.

HuR significantly promotes TG and TC formation and reduces CRC cell death.

The mRNA and protein levels of VDR after silencing HuR expression are shown in Fig. 4A and B, respectively. To further explore the molecular mechanism, we first show a schematic representation of the VDR mRNA, highlighting the predicted target binding sites of HuR in the CDS and 3'UTR (see Fig. 4C). We then measured VDR mRNA levels in HCT116 cells expressing normal or reduced levels of HuR after treatment with actinomycin D, a transcription inhibitor (see Fig. 4D). The results showed that the reduced VDR expression was due to decreased mRNA stability. The coding sequence (CDS) of VDR consists of

1,284 bases, and the 3'UTR is 3,209 bases. We used the AREsite2 web database (http://rna.tbi.univie.ac.at/) to predict possible binding sites of HuR in the VDR mRNA. As shown in Fig. 4E, there were eight predicted binding sites of HuR in the VDR mRNA. We divided the 3'UTR region containing six predicted binding sites into three segments, each containing two binding sites. To confirm the direct interaction between HuR and VDR mRNA, we performed an RNA pull-down assay and found that HuR is directly bound to the CDS and 3'UTR of VDR mRNA in CRC cells (see Fig. 4F). We then used a luciferase reporter assay to determine whether HuR binds to the VDR CDS or 3'UTR. As shown in Fig. 4G and 4H, overexpression of HuR led to increased VDR CDS and 3'UTR reporter expression, but not Ctrl reporter expression, indicating that HuR can bind to VDR and upregulate its expression.

Finally, we compared the expression of a luciferase reporter gene (primGlo) in HCT116 cells transfected with different mutations of the VDR 3'UTR primGlo vector. We found that cells transfected with some mutations (S1, S2, S3, and S6) showed significantly lower expression of primGlo compared to cells transfected with the full-length 3'UTR vector, while others (S4 and S5) showed no difference (see Fig. 4I and J). Together, these observations strongly support the idea that HuR enhances VDR mRNA stability and promotes VDR expression by directly binding to the CDS and 3'UTR of VDR mRNA in CRC.

HuR may promote VDR expression through the regulation of miR-124-3p.

Previous research has shown that HuR can interact with non-coding RNAs, such as miRNAs, to regulate the expression of its target genes. miRNAs are small (19–23 nucleotides) non-coding RNAs that bind to the 3' untranslated regions (3'UTRs) of target mRNAs, leading to their degradation or translational repression through incomplete base pairing with the mRNA[38,39]. To study the potential role of miRNAs in regulating VDR expression, we performed small RNA sequencing and identified two miRNAs (miR-124-3p and miR-125a-5p) that are regulated by HuR and potentially target VDR (see Fig. 5A). We then measured the expression of miR-124-3p after silencing or overexpressing HuR in HCT116 and HT29 cells. As shown in Fig. 5B, HuR inhibited the expression of miR-124-3p in these cells. Additionally, to confirm the role of miR-124-3p in regulating VDR expression, we overexpressed miR-124-3p in CRC cells and performed western blot analysis to measure VDR protein levels. As shown in Fig. 5C, overexpression of miR-124-3p resulted in a significant decrease in VDR protein expression in these cells. We also measured the expression of VDR mRNA after overexpressing miR-124-3p in HCT116 and HT29 cells and found that miR-124-3p reduced VDR mRNA levels (see Fig. 5D).To confirm that miR-124-3p directly binds to VDR, we used the online website TargetScan (http://www.targetscan.org) to predict the miR-124-3p binding sites in the VDR 3'UTR. As shown in Fig. 5E, there is a potential binding site for miR-124-3p in the VDR 3'UTR. We then performed a luciferase reporter assay to measure the effect of miR-124-3p on the activity of the VDR 3'UTR reporter. As shown in Fig. 5F, miR-124-3p significantly reduced the activity of the reporter with the wild-type 3'UTR of VDR, while no effect was observed with the control reporter. In summary, our results suggest that HuR can suppress the expression of miR-124-3p, which in turn directly binds to VDR and downregulates its expression in CRC cells.

Targeting the HuR/miR-124-3p/VDR complex can downregulate TG and TC levels in CRC

We previously found that deleting HuR inhibited VDR expression and decreased TG and TC levels in CRC cells. To further explore this, we performed untargeted lipidomics and rescue experiments. The results showed distinct differences in TG between the oeHuR and oeHuR + siVDR groups. The overall change of oeHuR and oeHuR + siVDR groups was shown using pie charts and a volcano plot (figure S2 A and B). As shown in the heat map (Fig. 6A), the TAG level of HCT116 cells decreased significantly after VDR was knocked down. We then analyzed the change in TAG, DAG, ACar, PC, and PE levels to show the differences in the lipidome between oeHuR and oeHuR + siVDR groups (Fig. 6B, 6C). Contrary to the decreased TAG levels, DAG, ACar, and PC levels increased after VDR was inhibited. ACar is converted from FAs, and it can partially respond to changes in FA oxidation. PC is one of the major structural lipids. These results showed that the upregulation of lipid metabolism by HuR in CRC can be reversed by inhibiting VDR.

To verify whether HuR regulates lipid metabolism through VDR in CRC cells, we performed intracellular TG and TC detection. Compared to oeHuR, knocking down VDR after oeHuR reduced TC and TG levels in HCT116 and HT29 cells (Fig. 6D, 6E; figure S2 C, D). Additionally, to verify the biological function of miR-124-3p in CRC cells, intracellular TG and TC detection was performed. miR-124-3p decreased TC and TG levels in HCT116 and HT29 cells (Fig. 6F, 6G). Results also showed that inhibiting miR-124-3p increased TG levels in CRC cells (figure S2 F). CCK8 results indicated that overexpressing VDR can reverse the reduction in survival rate due to inhibiting HuR in HCT116 and HT29 cells (figure S2 G).

Based on the lipidomics analysis, the decrease in TAG levels did not result in free FA accumulation, which suggests that it may be caused by FAs being shifted to major structural lipids PC/PE and mitochondria (ACar and PC levels increased). We therefore hypothesize that the effect of the HuR/miR-124-3p/VDR complex on lipid homeostasis may be achieved by regulating the balance of triglyceride synthesis and fatty acid beta-oxidation, rather than by affecting total fatty acid content. Together, these data suggest that inhibiting HuR/miR-124-3p/VDR regulates lipid metabolism through VDR in CRC cells (Fig. 6H).

HuR promotes TG and TC formation and tumor growth via VDR in vivo.

In vitro, we found that the levels of HuR promoted VDR expression and regulated lipid metabolism. To confirm this regulation effect in vivo, a xenograft model in nude mice was built. HCT116 cells were injected subcutaneously in BALB/c nude mice.

When subcutaneous tumors were observed 7 days later, siHuR or siNC were injected intratumorally every three days and adenovirus oeVDR or oeNC were injected 3times in a week (Fig. 7A). All mice were sacrificed and tumors were removed 28 days after HCT116 cells injection (Fig. 7B). Tumor volume was measured and the growth curves were plotted (Fig. 7C). The results exhibited that tumors size in the siHuR group was significantly smaller than that in the siNC group (Fig. 7B), while the size of tumors in the siHuR + oeVDR group was bigger than in the siHuR group. Similarly, the tumor growth rate of siHuR was

slower compared with the control group (Fig. 7C). And we observed that the siHuR + oeVDR had a faster growth rate than the siHuR group.

The tumor weight also consists of previous results. IHC and WB experiment results showed that the expression of HuR was reduced in the siHuR group, and VDR expression was downregulated in the siHuR group compared with control (Fig. 7E). Intracellular triglyceride and total cholesterol detection were performed. It was shown that siHuR decreased the TC and TG level in vivo and siHuR + oeVDR group increased TG and TC level compared with siHuR group (Fig. 7F). The oil red o-stained tumor sections results indicated that the neutral lipids were decreased in siHuR group, and were recovered in the siHuR + oeVDR group (Fig. 7G). Collectively, our results suggested that Knockdown of HuR inhibited CRC tumor growth and lipid metabolism by regulating VDR expression in vivo.

In conclusion, the RNA-binding protein HuR modulates lipid homeostasis in CRC by regulating VDR, playing an important role in CRC development. In CRC, HuR promotes VDR expression not only by directly binding with the CDS and 3'UTR of VDR mRNA and enhancing its mRNA stability, but also by inhibiting miR-124-3p which targets VDR (Fig. 8).

Discussion

Over the last decade, lipid metabolism disorders have attracted more attention, and their effects on carcinogenesis have been studied intensively[40]. However, how tumor cells regulate lipid homeostasis to prevent potential lipotoxicity has been rarely investigated. In this study, we demonstrate that the HuR/miR-124-3p/VDR complex modulates lipid metabolism to store excess fatty acids (FAs) into lipid droplets (LDs), thereby maintaining the homeostasis of structural lipid synthesis and FA oxidation. We also investigated the oncogenic roles of HuR in colorectal cancer (CRC) and found that it promotes CRC cell proliferation in vitro and accelerates CRC tumor growth in vivo. Interestingly, HuR increases the mRNA and protein levels of VDR in two ways. First, it binds directly to the coding sequence (CDS) and 3'-untranslated region (UTR) of VDR. Second, it modulates miR-124-3p, which has also been shown to be an upstream repressor of VDR by binding directly to its 3'-UTR.

The data generated by large cancer genomics consortia have become a critical tool for developing new therapeutic approaches against aggressive tumors like colorectal cancer (CRC). For example, The Cancer Genome Atlas (TCGA) has produced an extensive transcriptomic map, identified prevalent chromosomal alterations, and defined important CRC driver mutations[41]. This knowledge has improved molecular classification, but so far, therapeutic strategies based on these findings have not led to a breakthrough. Post-transcriptional processes such as splicing, polyadenylation, decay, and translation are often linked to tumorigenesis, and many of their regulators have been shown to have tumor-suppressive or oncogenic activity[42,43]. Accumulating evidence suggests that HuR, a regulator of these processes, is a promising drug target for cancer clinical treatment[44−48]. It is especially upregulated in many cancers, including papillary, follicular, and anaplastic thyroid cancers[49,50]. HuR has also been shown to promote carcinogenesis and the development of digestive system tumors[51]. In addition to its many biological

functions, such as cell differentiation, cell growth, DNA repair, cell activation, and inflammatory response, growing evidence suggests that HuR and other RNA-binding proteins (RBPs) can also impact metabolic homeostasis by affecting mRNAs involved in glucose and lipid metabolism[52]. RBPs have also been shown to participate in brown adipogenesis[53].

Cancer cells capture and use nutrients more efficiently, repurposing metabolic pathways for alternative energy and biomass production. Many metabolic pathways are involved in CRC, such as lipid synthesis[54]. Clinical and preclinical data strongly suggest that targeting metabolism is a realistic strategy for treating aggressive tumors like CRC. Therefore, to target dysregulated tumor metabolism, novel preventive and therapeutic applications are needed[55]. However, the functional roles of HuR in the regulation of lipid metabolism remain unexplored in CRC.

Our data suggest that the HuR/miR-124-3p/VDR axis contributes to modulating the formation of total cholesterol (TC) and triglycerides (TGs), promoting the growth of colon cancer. Previous studies have shown that TGs are the main disturbed lipid markers of CRC[56] and that their accumulation is linked directly to invasion in prostate cancer[57]. Inhibiting triglyceride synthesis has also been shown to cause dysregulation of lipid homeostasis and cell death in glioblastoma[58,59], and similar mechanisms may exist in other cancers, including CRC. This finding highlights the role of lipid metabolism disorder in cancer and provides new insight into CRC tumor growth, suggesting that HuR serves as a critical regulator of lipid metabolism in the development of CRC.

HuR contains RNA recognition motifs (RRMs), which are important for binding AU- and U-rich sequences on RNAs and mediating their stabilization. In this study, we revealed the specific mechanism by which HuR modulates VDR and the molecular and cellular effects of this interaction in colorectal cancer (CRC). Our data suggest that HuR enhances the stability of VDR mRNA in CRC cells, and overexpression of HuR is responsible for altered cell growth. Furthermore, our results indicate that HuR stimulates VDR translation in CRC by directly interacting with the VDR mRNA via its 3'-untranslated region (3'-UTR) and coding sequence (CDS), rather than the 5'-untranslated region (5'-UTR). Previous studies have shown that HuR increases the stability of cyclin A, c-fos, and cyclin E1 by binding to their 3'-UTRs. For example, HuR increases the mRNA stability of cyclin E1, leading to its overexpression in breast cancer and directly affecting cell proliferation[60,61]. These findings indicate that HuR plays a critical role in regulating the expression of various genes involved in cell growth and proliferation.

MicroRNAs (miRNAs) are essential in cellular processes, including proliferation, differentiation, apoptosis, and metabolism[62,63]. Increasing evidence suggests a strong connection between HuR and miRNAs, which regulate downstream target molecules in different ways[64–70]. For instance, HuR has been shown to inhibit miRNA-16 targeting of cyclooxygenase-2 (COX-2)[31], leading to increased expression of COX-2. Previous reports have also shown that miR-124-3p is one of the most well-known major regulators of metabolism, including the Warburg effect, TG metabolism, and the pentose phosphate pathway[71–74]. The level of miR-124-3p has been found to decrease significantly in CRC tissues from patients with shorter

survival times[73]. In our current study, we found that HuR inhibits miR-124-3p in CRC cells, consistent with previous research showing that miR-124-3p is suppressed by the RNA-binding protein HuR in ovarian cancer cells[75]. Additionally, our data demonstrate that miR-124-3p directly binds to VDR and downregulates its expression. These findings provide new insight into the relationship between HuR, miRNAs, and the regulation of gene expression in CRC.

Recent studies have shown that cancer cells' survival depends on lipids from the microenvironment and lipids that can be reused from their own structural and storage pools[76–78]. By regulating core metabolism pathways such as lipid synthesis and mitochondrial oxidation, aggressive CRC cells can sustain their high energy demand[79–81]. Fatty acids (FAs) are crucial mediators that regulate the tricarboxylic acid (TCA) cycle catabolism and energy oxidation[82,83]. In aggressive CRC cells, FAs are elevated[84], which are either stored as triglycerides or oxidized to generate ATP to fulfill immediate cellular needs. However, excess energy metabolism can cause cell damage due to cytotoxicity[58,59]. A study showed that FA-induced lipotoxicity is mediated by ER stress[85]. Moreover, another study demonstrated that excessive levels of fatty acid β-oxidation can lead to increased ROS production[86].

Our study revealed that inhibition of HuR decreased the levels of TG and TC in CRC cells. This finding is supported by a previous study that found elevated levels of triglycerides in the plasma of advanced-stage CRC patients[56], but not early-stage CRC patients. In addition, the decreasing levels of TG and TC due to inhibition of HuR were partly recovered by overexpression of VDR. Furthermore, our study showed that overexpression of HuR significantly downregulated the levels of acylcarnitines, which are converted from FAs and then oxidized for energy in the mitochondria. Inhibition of VDR based on overexpression of HuR significantly increased the levels of acylcarnitines, PC, and LPC metabolites. More fatty acids shuttle into mitochondria may lead to a rise in ROS levels. And elevated ROS impaired the oxidative capacity of mitochondria then increased oxidative stress and cell death. PC is the major membrane structural phospholipid. Previous reports revealed that VDR downregulates respiratory chain activity, reduces lipid catabolism, and slows β-oxidation[87]. Moreover, it was reported that several cancer cell lines silenced for VDR are inhibited in their growth[88].

These results suggest that HuR plays a role in regulating lipotoxicity, in part by regulating VDR-induced respiratory chain braking and leading to acetyl-CoA biosynthesis. It demonstrates that inhibition of the HuR/miR-124-3p/VDR complex altered the levels of TG and TC, resulting in excessive FA redistribution into structural phospholipids and shuttling into the mitochondria. However, the target genes and pathways that can influence lipid synthesis and lipid homeostasis still need to be explored. The adverse consequences of lipid metabolism disorders in cancer have prompted researchers to focus on the roles of oncogenic genes in promoting abnormal lipid metabolism in human cancers[89]. Therefore, understanding how tumor cells mediate lipid homeostasis to protect cells from potential lipotoxicity is critical.

RNA-binding proteins (RBPs) modulate gene expression from RNA processing to translation. Studies using TCGA data have shown that many mutations in RBPs and alterations in their expression levels are

present across tumor types. HuR, in particular, can be targeted by small-molecule inhibitors or by RNA aptamers or modified RNA oligos bearing the consensus binding motif of the RBP to be targeted. Targeting RBPs provides an opportunity to disrupt multiple cancer-relevant pathways at once. Our study suggests that disrupt of complex of HuR/VDR/miRNA can depresses cancer metabolism homeostasis and proliferation regulation, which may be the potiential target in CRC therapy.

This study has some limitations. Firstly, the downstream lipid metabolism-related genes affected by HuR are unknown, and this will be the focus of future research. Another limitation of our study is that we have not yet confirmed the exact effects of specific lipid changes, particularly for structural lipids caused by inhibition of HuR on CRC cells. In addition, future studies should include a larger clinical sample to investigate the effects of the HuR/miR124-3p/VDR complex on CRC lipid metabolism and progression.

In summary, our study has confirmed that the RNA-binding protein HuR plays an important role in CRC development. Mechanistically, HuR promotes VDR expression by directly binding to the CDS and 3'UTR of VDR mRNA and enhancing its mRNA stability, as well as by inhibiting miR-124-3p, which targets VDR. We also demonstrated that the HuR/miR124-3p/VDR complex modulates lipid homeostasis by enhancing TG and TC levels and inhibiting fatty acid beta-oxidation in CRC cells, providing a novel insight into the molecular mechanisms implicated in CRC.

Abbreviations

ACar Acylcarnitine

AREs AU-rich elements

CER Ceramide

CRC Colorectal cancer

DAG Diacylglycerol

FFA Free fatty acid

IHC Immunohistochemistry

RBP RNA binding protein

PA Phosphatidic acid

PC Phosphatidylcholine

PE Phosphatidylethanolamine

PG Phosphatidylglycerol

PS Phosphatidylserine

SM Sphingomyelin

TAG Triglycerides

TC Total cholesterol

TCGA The Cancer Genome Atlas

Declarations

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Not applicable

Authors' contributions

HFX, BLP, and LMT designed the study; HFX, BLP, and LMT performed animal experiments and related bioinformatics analysis;. HFX and BLP contributed to patient samples and clinical information. WYW, ZRN, and SY performed bioinformatic analysis. YH and LK conducted the statistical analysis. HFX and BLP wrote the manuscript. HFX, BLP, LMT, ZQ and LL revised the paper. All authors read and approved the final manuscript.

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Availability of data and materials

the UCSC Xena browser (https://xenabrowser.net/datapages/)

Gene Expression Omnibus database (http://wwwncbinlmnih.gov/geo/)

AREsite2 Web (http://nibiru.tbi.univie.ac.at/AREsite2)

Declarations

Ethics approval and consent to participate

This study was authorized by the Ethical Committee of Zhongnan Hospital of Wuhan University. Animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals of Wuhan University.

Consent for publication

All of the authors consent for publication.

Competing interests

All authors declare that they have no conflict of interest.

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Figures
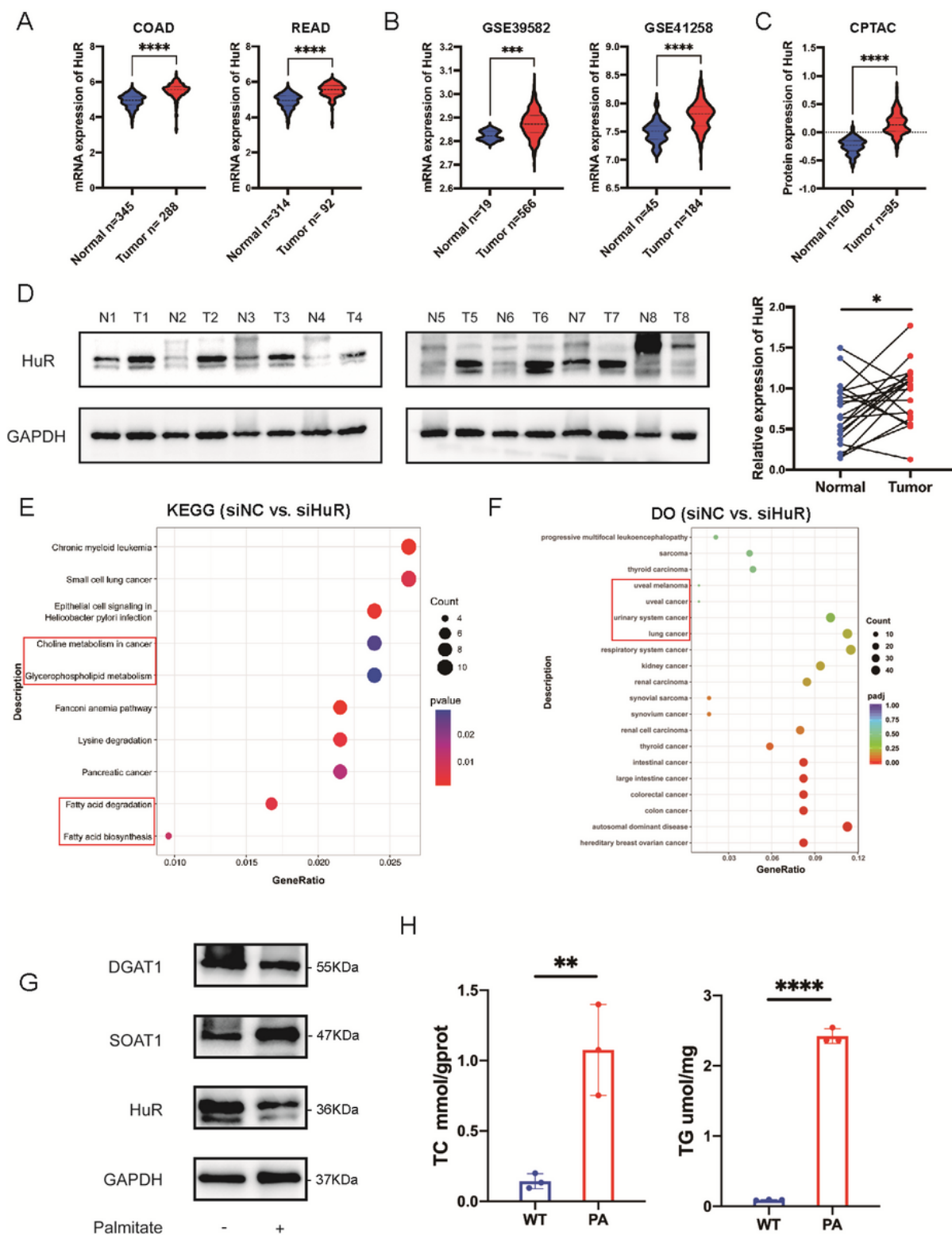
Figure 1

HuR is significantly upregulated and modulates lipid metabolism in CRC

(A) Boxplot analysis of HuR gene expression in samples from individuals with CRC cancer in the COAD (n = 633) and READ (n = 406) of TCGA. *** <0.001; **** <0.0001.

(B) Boxplot analysis of HuR gene expression in GSE39582 (n = 585) and GSE41258 (n = 229) RNA-seq databases. *** <0.001; **** <0.0001.

(C)Boxplot analysis of HuR protein expression in samples from individuals with CRC cancer in the CPTAC (n = 195) database. **** <0.0001.

(D) A representative Western blot of Patients with CRC cancer (n=20). * <0.05.

(E) KEGG analysis of two groups (siNC vs. siHuR) in HCT116 cells.

(F) DO analysis of two groups (siNC vs. siHuR) in HCT116 cells.

(G) HuR protein level and lipid metabolism-related genes (SOAT1 and DGAT1) were measured in RKO cells treated with Palmitate for 48 h.

(H) Levels of triglycerides and total cholesterol in RKO cell coculture with Palmitate. ** <0.01; **** <0.0001.
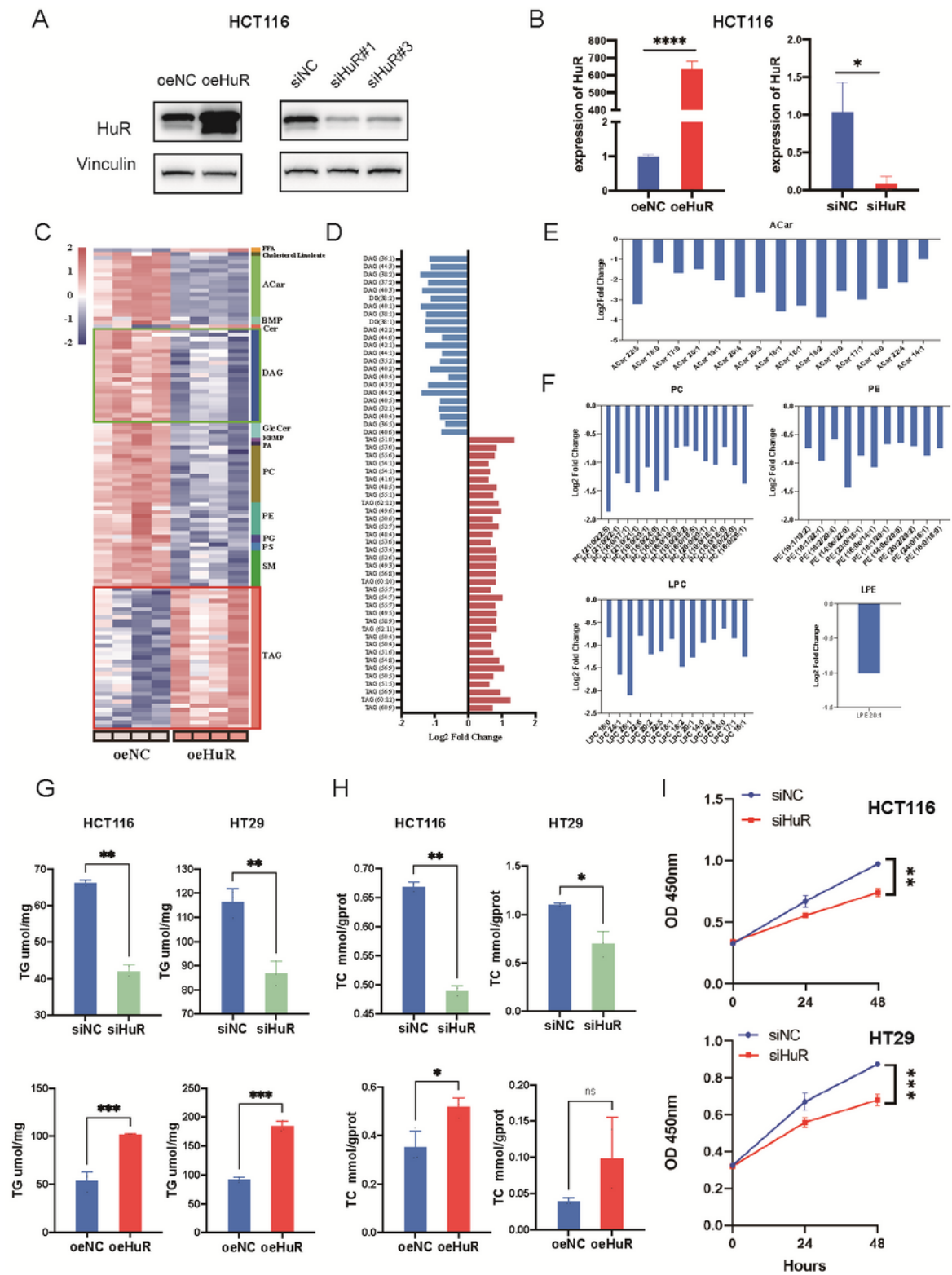
Figure 2

HuR significantly promotes TG and TC formation and reduces CRC cell death.

(A) Western blot analysis of HuR and GAPDH in HCT116 cell coculture with HuR overexpression or coculture with HuR inhibition for 48 h.

(B) RT-qPCR analysis of HuR mRNA expression with HuR overexpression or HuR inhibition (mean ± SD, n = 3) in HCT116 cell. *P < 0.01; ns, not significant. * <0.05; **** <0.0001.

(C) Heatmap of representative lipids in HCT116 cells with/without overexpression of HuR (48 h) analyzed by lipidomics. DAG, diacylglycerol; TAG, triacylglycerol; ACar, acylcarnitine; FFA, free fatty acid; CER, ceramide; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG phosphatidylglycerol; PA, phosphatidic acid.

(D-F) Levels of representative individual lipid species in HCT116 cells with/without HuR overexpression. LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine.

(G,) Levels of triglycerides in HCT116 and HT29 cells with HuR overexpression or with HuR knockdown. ** <0.01; *** <0.001.

(H) Levels of total cholesterol in HCT116 and HT29 cells with HuR overexpression or with HuR knockdown. * < 0.05; ** <0.01; ns, not significant.

(I) Cells proliferation assay of HCT116 and HT29 cells after HuR knockdown for 0, 24, 48h by CCK-8 assay. Data are presented as the mean ± SD of three independent experiments. ** <0.01; *** <0.001.
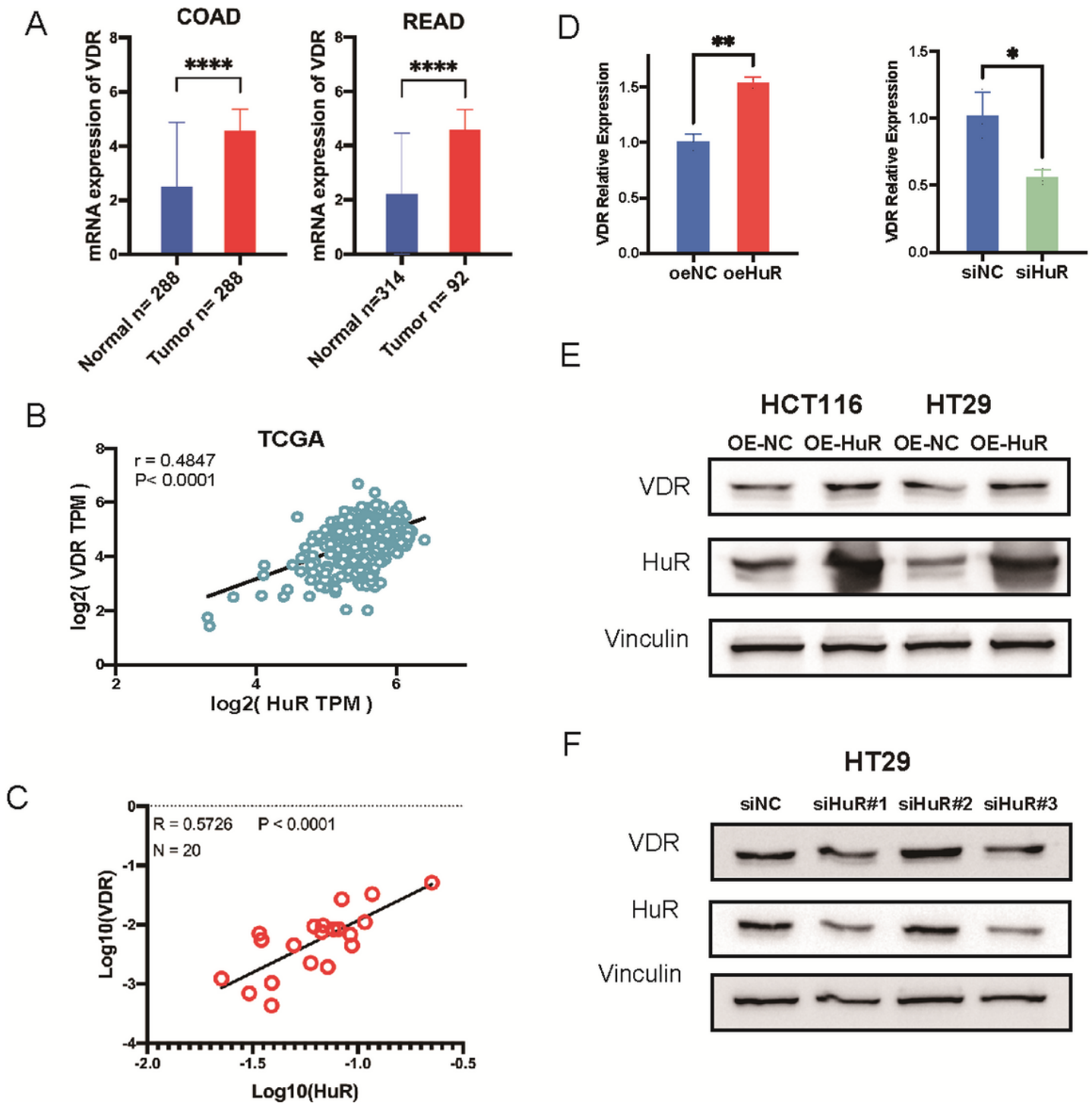
Figure 3

HuR is related to VDR and promote VDR expression

(A) Boxplot analysis of VDR gene expression in samples from individuals with CRC cancer in the COAD (n = 576) and READ (n = 406) of TCGA. **** <0.0001.

(B) Expression correlation analysis of HuR and VDR in the TCGA dataset (n = 114) using Broad portal analysis of RNA sequencing-based gene expression data. Pearson correlation coefficients r and the corresponding P value are shown.

(C) Expression correlation analysis of HuR and VDR in the Wuhan university Zhongnan hospital cohort (n = 20) using Broad portal analysis of RNA sequencing-based gene expression data. Pearson correlation coefficients R and the corresponding P value are shown.

(D) RT-qPCR analysis of VDR mRNA expression with HuR overexpression or with HuR inhibition (mean ± SD, n = 3) in HCT116 cell. * < 0.05; ** <0.01.

(E,F) Western blot analysis of HuR, VDR and GAPDH in HCT116 and HT29 cells coculture with HuR overexpression or coculture with HuR inhibition for 48 h.
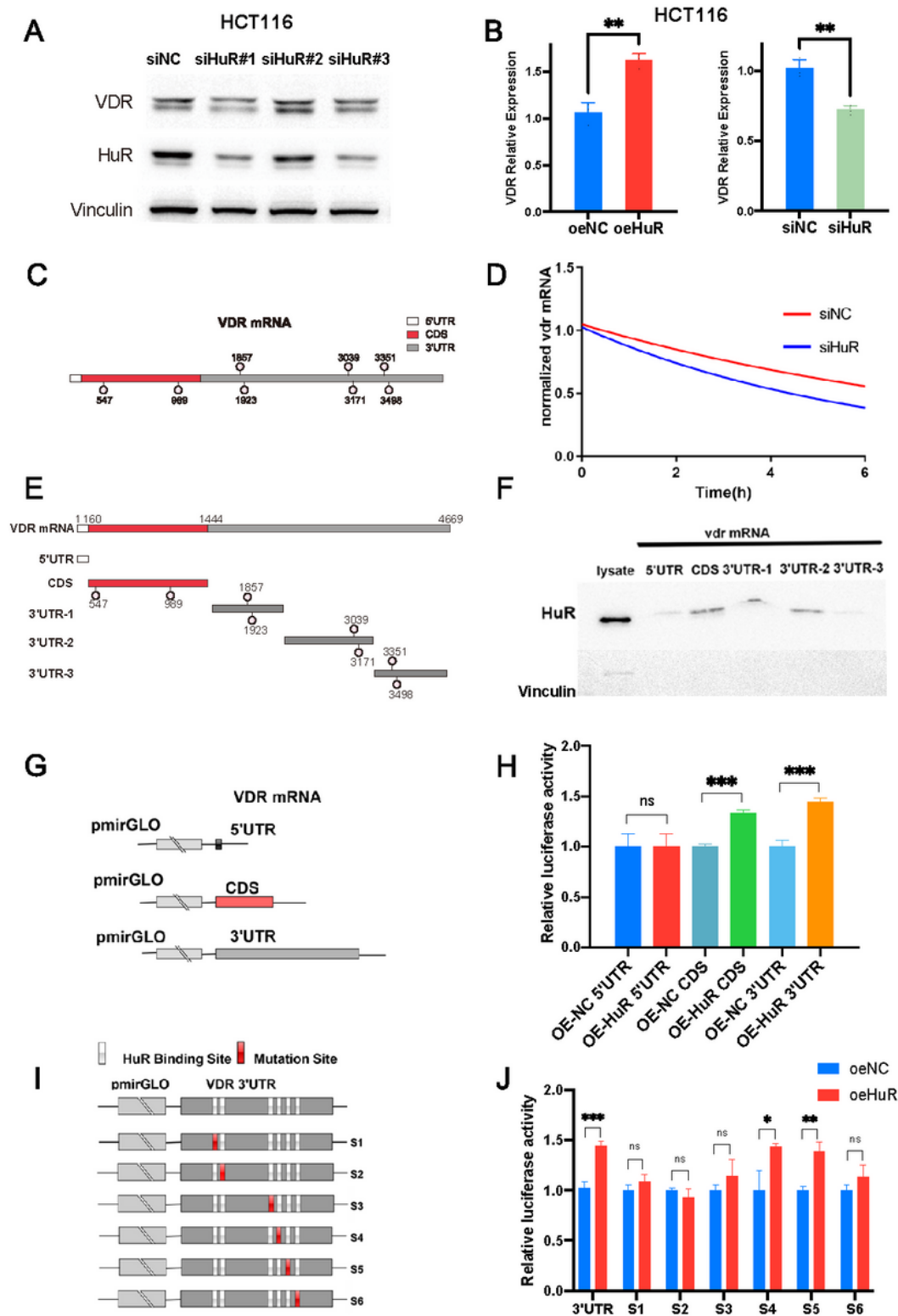
Figure 4

HuR enhances VDR mRNA stability by binding to its CDS and 3'UTR in CRC

(A) Western blot analysis of HCT116 cells culture with HuR inhibition for 48 h.

(B) RT-qPCR analysis of VDR mRNA expression with HuR inhibition or with HuR overexpression (mean ± SD, n = 3) in HCT116 cell. ** < 0.01.

(C) Schematic representation of the Vdr mRNA depicting predicted target sites for HuR in CDS and 3'UTR.

(D) HCT116 cells were transfected with HuR siRNA or scrambled control, then stimulated for 3 hours, at which time Actinomycin D was added. At 0, 2 and 4 hours of Actinomycin D exposure, RNA was isolated and target mRNA accumulation was assessed by quantitative RT-PCR normalized to GAPDH. All values are normalized as % of time 0.

(E) Three schematic segmentsrepresentation of the Vdr mRNA depicting predicted target sites for HuR in CDS and 3'-UTR.

(F) RNA Pull Down assay by HuR and its antisense RNA followed by Western blot from HCT116 cells.

(G-H) Luciferase reporter assay was performed to determine whether HuR bind with VDR CDS or 3 -UTR.

(I-J) Luciferase reporter assay was performed that HCT116 transfected with some mutation of VDR 3'UTR primGlo vector (S1,S2,S3,S4,S5 and S6).
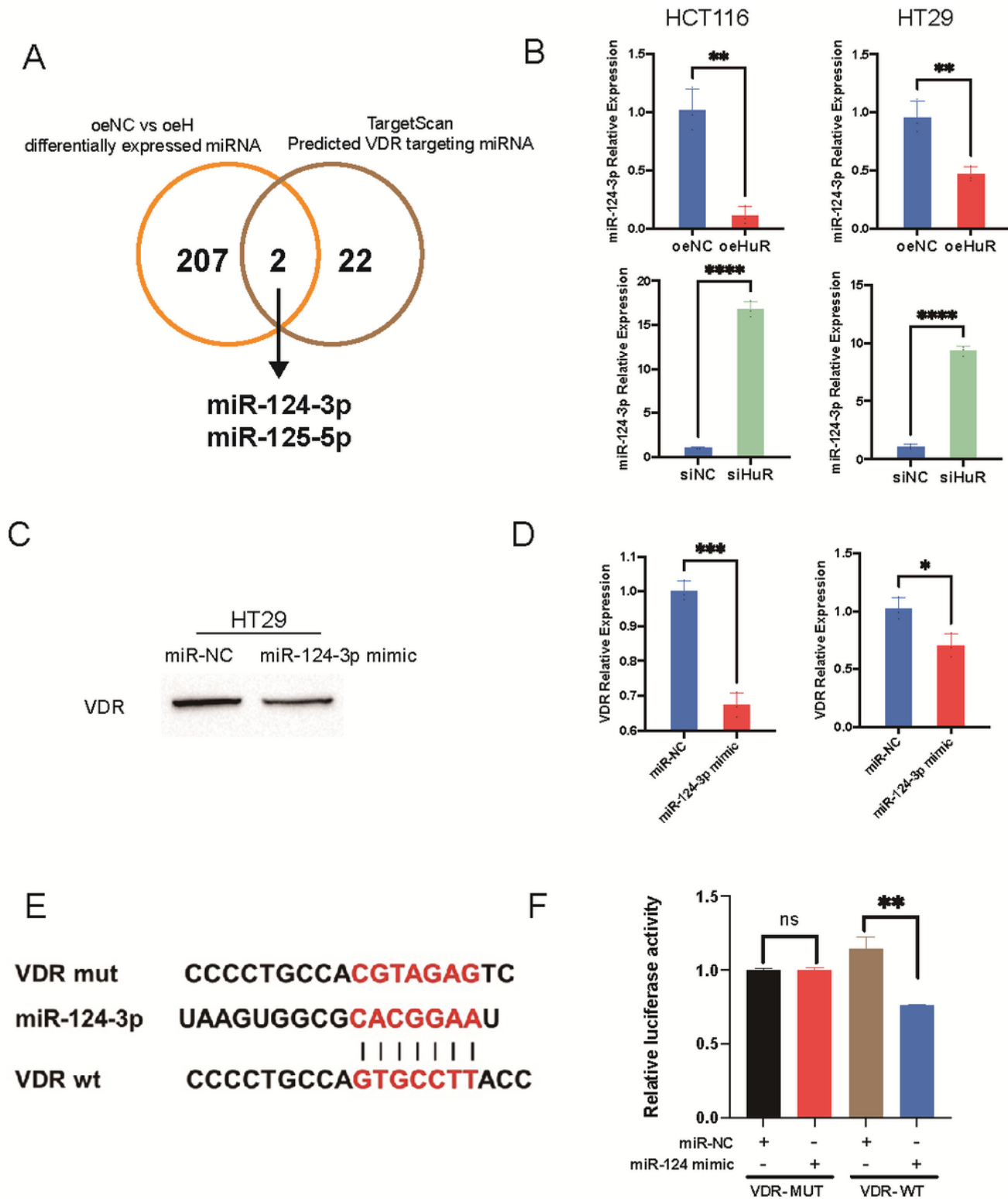
Figure 5

HuR may promote VDR expression through the regulation of miR-124-3p.

(A) Overlapping analysis (Venn diagram) with HuR-related differentially expressed miRNA and predicted VDR targeting miRNA.

(B) RT-qPCR analysis of miR-124-3p expression with HuR overexpression or HuR inhibiton (mean ± SD, n = 3) in HCT116 and HT29 cells. **< 0.01; ****< 0.0001.

(C) Western blot analysis of HT29 cells cultrue with miR-124-3p mimic for 48 h.

(D) RT-qPCR analysis of VDR expression with miR-124-3p mimic (mean ± SD, n = 3) in HCT116 and HT29 cells. *< 0.05; ***< 0.001.

(E) The predicted wild-type and mutated type of miR-124 binding sites in the 3′ UTR of VDR were exhibited.

(F) Luciferase reporter assay was performed to determine whether miR-124-3p bind with VDR.
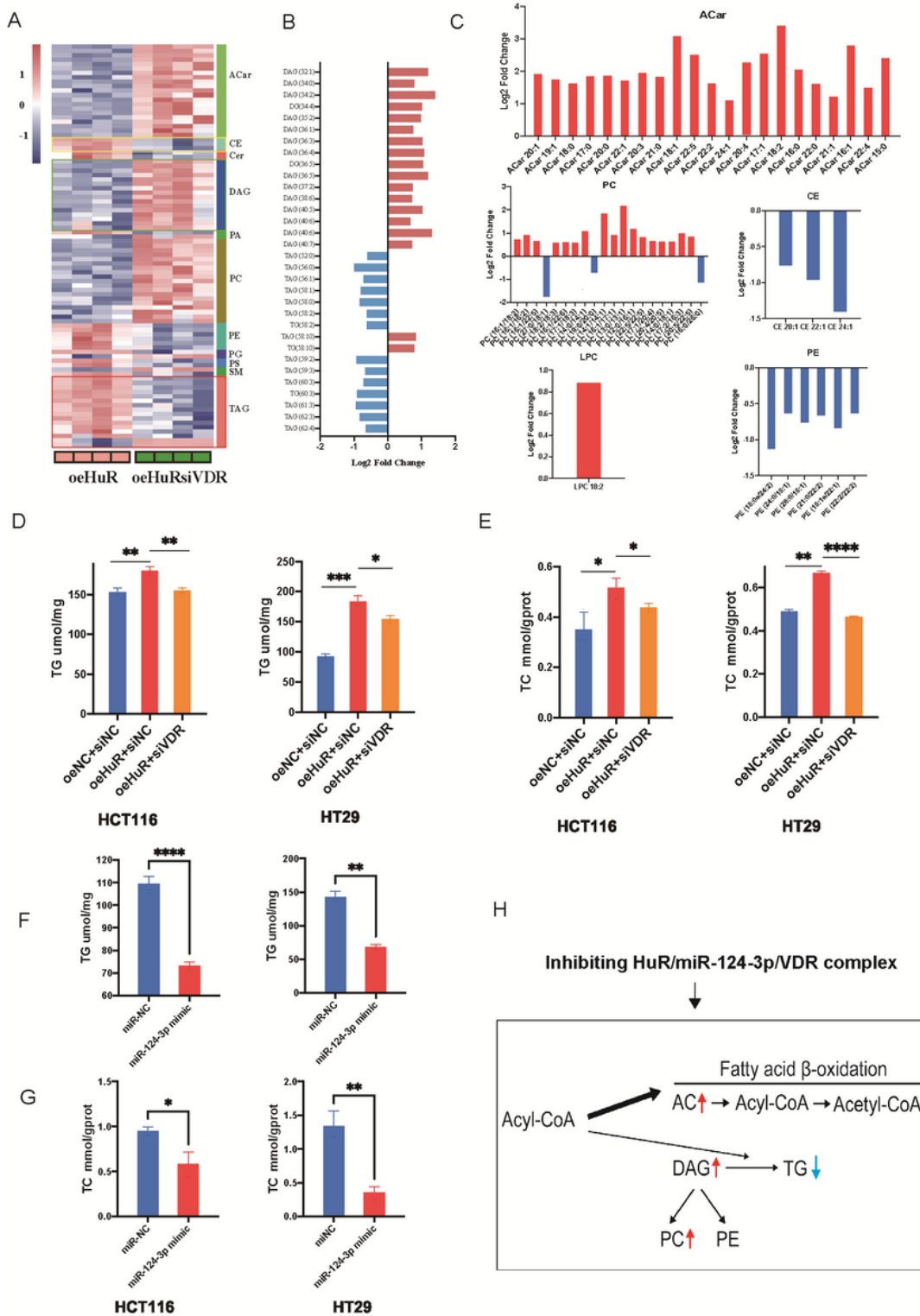
Figure 6

Targeting the HuR/miR-124-3p/VDR complex can downregulate TG and TC levels in CRC

(A) Heatmap of representative lipids in HCT116 cells with overexpression of HuR (24 h) then with/without VDR inhibition (24 h) analyzed by lipidomics. DAG, diacylglycerol; TAG, triacylglycerol; ACar, acylcarnitine;

FFA, free fatty acid; CE, cholesteryl esters; CER, ceramide; SM, sphingomyelin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PG phosphatidylglycerol; PA, phosphatidic acid.

(B-C) Levels of representative individual lipid species in HCT116 cells with HuR overexpression then with/without VDR inhibition. LPC, lysophosphatidylcholine.

(D, F) Levels of triglycerides in HCT116 and HT29 cells compared with three groups (control, overexpression HuR, overexpression HuR and VDR knockdown). * < 0.05; ** <0.01.

(E) Levels of total cholesterol in HCT116 and HT29 cells compared with three groups (control, overexpression HuR, overexpression HuR and VDR knockdown). * < 0.05; ** <0.01; *** <0.001; **** <0.0001; ns, not significant.

(H) Summary of the lipid profiling changes in HCT116 cell after knockdown of HuR/miR-124-3p/VDR complex.
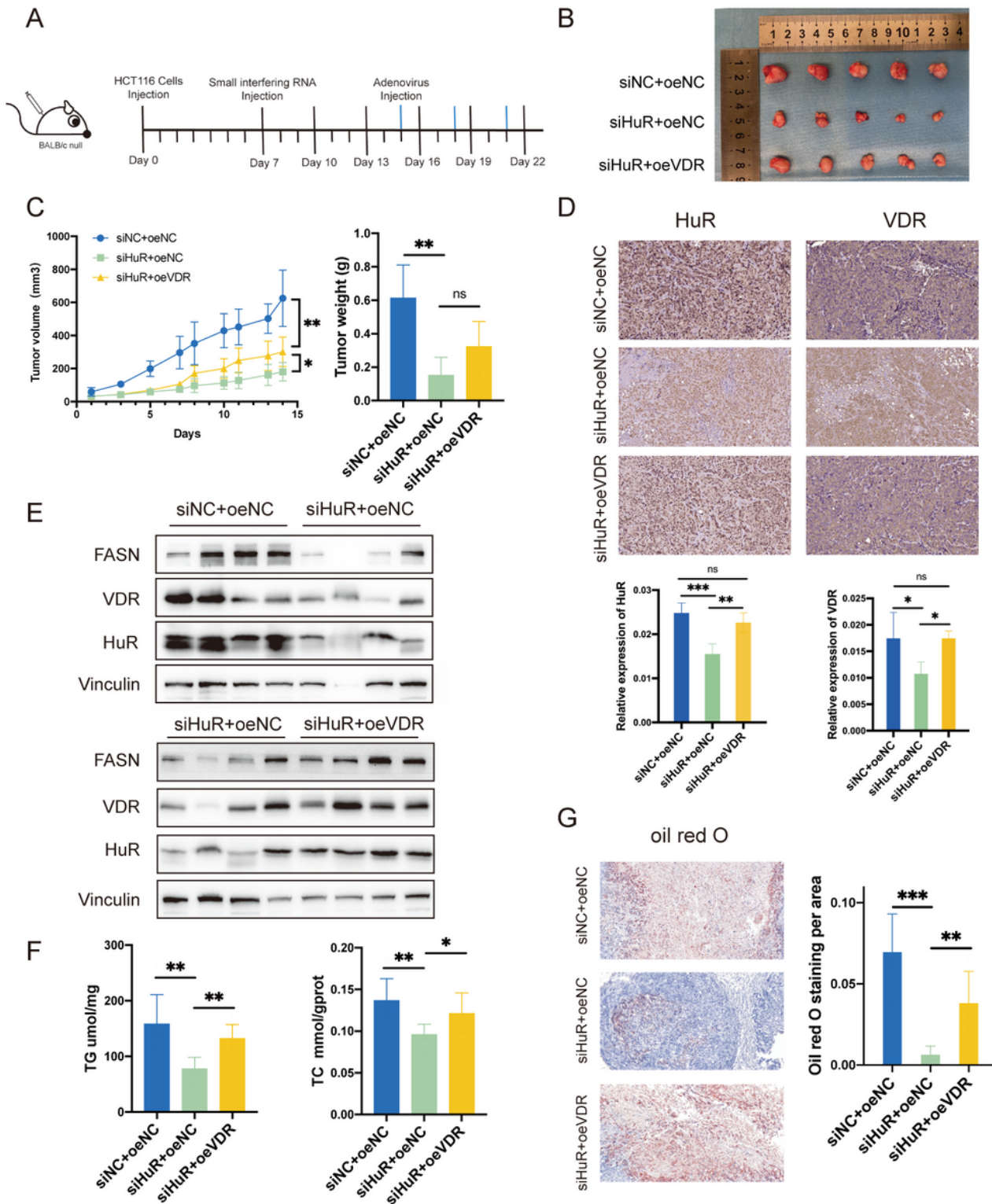
Figure 7

HuR promotes TG and TC formation and tumor growth via VDR in vivo.

(A) Schematic diagram of mouse modeling.

(B-C) Xenograft weight (g) and size (mm) were measured.

(D) Immunohistochemistry staining of HuR, VDR and Ki-67 in xenograft tissues and Quantitative analysis (bottom).

(E) WB analysis of the expression of HuR, VDR and GAPDH expression from control, siHuR and siHuRoeVDR groups xenograft tumor.

(F) Levels of triglycerides and total cholesterol in xenograft tumor compared with three groups (siNC+oeNC, siHuR+oeNC and siHuR+oeVDR). *< 0.05; **< 0.01.

(G) Oil Red O-stained tumor sections from mice. Scale bar, 20 μm. Quantitative analysis (bottom) of the mean Oil-red O-stained area (n = 5). **< 0.01; ***< 0.001.
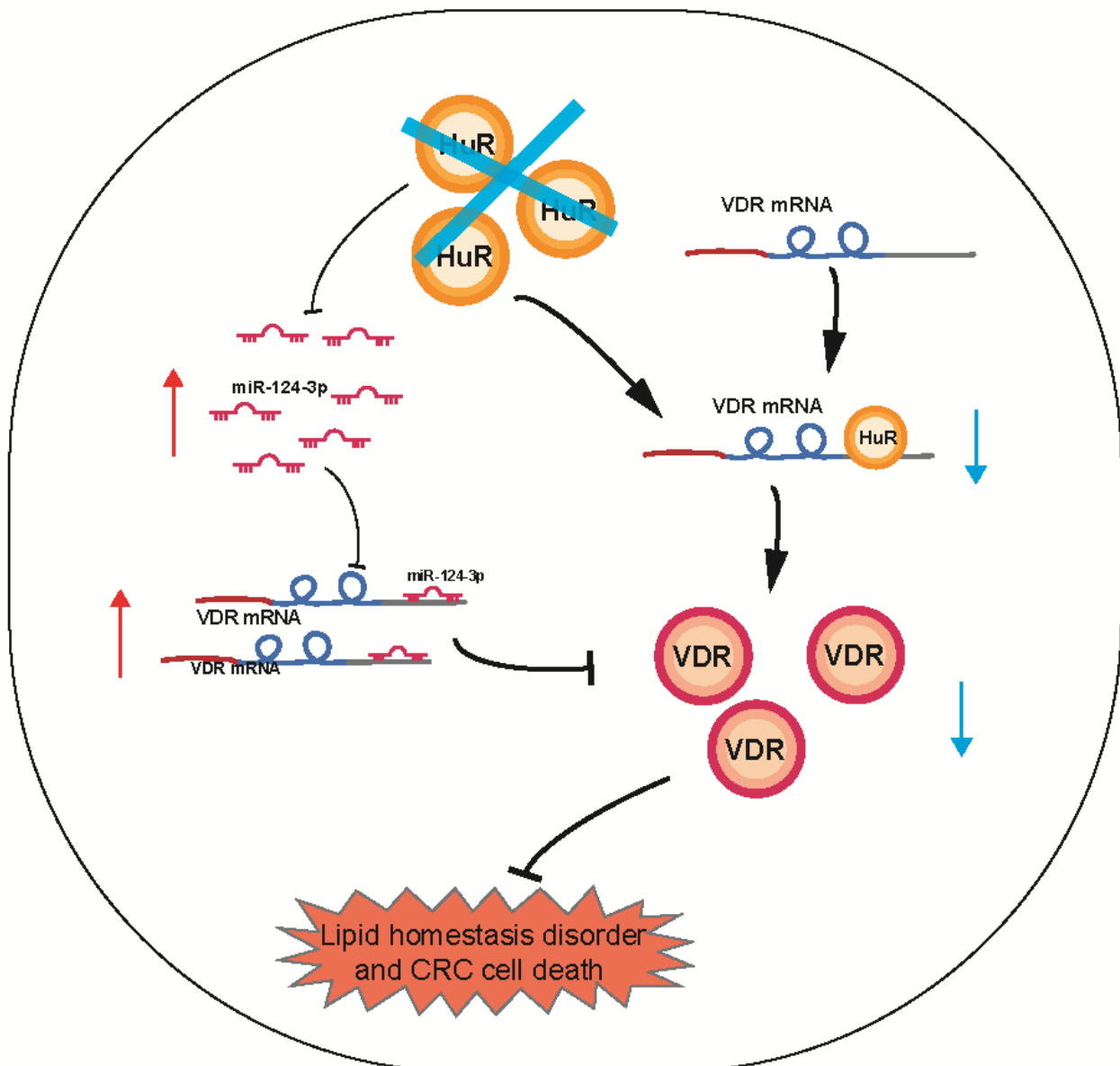
Figure 8

Schematic model illustrating the function of HuR/miR-124-3p/VDR complex in regulating lipid homeostasis resulting from its inhibition in CRC cells.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementarydata.pdf