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RNA m^{6}A-mediated post-transcriptional repression of glucocorticoid receptor in LPS-activated Kupffer cells on broilers

Yulan Zhao,[*,†] Yidan Jiang,[*,†] Yue Feng,[*,†] and Ruqian Zhao [*,†,1]

[*]*MOE Joint International Research Laboratory of Animal Health and Food Safety, Nanjing Agricultural University, Nanjing, Jiangsu, PR China; and* [†]*Key Laboratory of Animal Physiology & Biochemistry, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, Jiangsu, PR China*

ABSTRACT Glucocorticoid receptors are distributed in various cells of the body and participate in the regulation of metabolism and immunity in response to glucocorticoids. RNA m^{6}A methylation participates in various metabolic and inflammatory responses, yet it remains elusive whether m^{6}A is involved in GR regulation during immune activation. Here, we observed uncoupled GR responses with increased mRNA yet suppressed protein levels in the LPS-challenged broilers chicken liver, in association with global elevation of RNA m^{6}A methylation, especially in the expression of METTL3 and YTHDF2. Further analysis using isolated primary hepatocytes and Kupffer cells revealed that such uncoupled GR responses and m^{6}A hypermethylation occurred specifically in KCs. The same GR and m^{6}A responses were reproduced in LPS-activated KC cell line, implying a possible role of m^{6}A in the post-transcriptional suppression of GR in KC. Indeed, m^{6}A inhibitor cycloleucine alleviated LPS-induced GR protein suppression, whilst GR antagonist RU486 had no effect on global m^{6}A methylation in KC. We observed that YTHDF2 siRNA can alleviate LPS-induced GR mRNA stability decrease. Subsequently, specific m^{6}A sites on GR were predicted and verified. Mutation m^{6}A sites and luciferase reporter assay was also applied to validate these findings. Mechanistically, m^{6}A methylation on the transcripts of GR impairs its mRNA stability in a YTHDF2-dependent manner, which leads to the decrease of its protein. Our study indicates successive roles of RNA m^{6}A modification in the down regulation of GR expression, which provides new drug targets for epigenetic therapy of liver inflammation.

Key words: GR, macrophage, RNA m^{6}A methylation, immune activation, post-transcriptional regulation

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INTRODUCTION

Glucocorticoids (**GC**) are steroid hormones produced by the adrenal cortex that have strong anti-inflammatory effects (Vandevyver et al., 2014). They can pass through cell membranes due to being lipophilic and activate the GR (Haugland et al., 1980; DeMorrow et al., 2018). In the process of chicken farming, both environmental stress, management stress, and disease stress can lead to alterations in serum glucocorticoid levels, which subsequently influence the expression of GR and participate in the body's stress response as well as maintaining immune homeostasis. Under an inflammatory state, GR binds to its ligand glucocorticoid, mediating the transcription of anti-inflammatory factors such as glucocorticoid-induced leucine zipper (**GILZ**), and inhibiting the production of inflammatory factors such as IL-6, thereby exerting anti-inflammatory effects in different tissues (Clark et al., 2003; Waage et al., 1990). Studies reveal that a deficiency in GR dimerization in GR^{dim} mice leads to exacerbated inflammation and sepsis (Kleiman et al., 2012), whereas bolstering GR expression and activation alleviates LPS-triggered endotoxic shock and inflammation (Reichardt et al., 2000; Dejager et al., 2010). These illustrative cases underscore the pivotal, anti-inflammatory role played by the GR. Nevertheless, chronic stress may induce glucocorticoid receptor resistance, hindering its ability to dampen the inflammatory response (Cohen et al., 2012). In addition, the response of different cells to GR is not consistent, and the role and expression of GR in different cells will show great differences (Chrousos and Kino, 2005). During stress-induced liver inflammation in chicken, whether different cells in the liver have different GR response patterns and the specific regulatory mechanisms have not been reported.

N6-methyladenosine (**m^{6}A**) represents a crucial chemical modification occurring at the sixth nitrogen position

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[1]Corresponding author: zhaoruqian@njau.edu.cn

of adenosine within RNA molecules (Meyer et al., 2012). This modification stands as the most prevalent and evolutionarily conserved among all modifications in mRNA, underscoring its significance in regulating diverse cellular processes (Gu et al., 2021). When mRNA undergoes m^6A modification, it is dynamically and reversibly regulated through methyltransferases, binding proteins, and demethylases (Mauer et al., 2017). RNA m^6A modifications play pivotal roles in modulating a myriad of pathobiological conditions, encompassing inflammatory responses and stress reactions. For instance, METTL3 was upregulated in KCs following the progression of liver fibrosis and in activated macrophages (Shu et al., 2021). In patients with autoimmune hepatitis, a notable upregulation of YTHDF2 expression has been observed in liver tissues, which correlates positively with the severity of inflammation (Huang et al., 2020). Our previous research has conclusively shown that GR-mediated transcriptional control over m^6A methylation metabolic genes results in modifications to the m^6A tags on lipid metabolism-related genes, which subsequently contributes to the pathogenesis of fatty liver in hens (Feng et al., 2021). However, there has been no reported evidence regarding whether m^6A directly participates in the regulation of GR activity or expression. Investigating whether m^6A is involved in the post-transcriptional regulation of GR is of paramount significance in the context of GR repression research.

In this study, GR protein expression in the liver was inhibited, and KC accompanied this phenomenon in the LPS-induced liver inflammation model. The downregulation of GR was accompanied with m^6A hypermethylation, and a significant increase in METTL3 and YTHDF2. Interestingly, m^6A modification of the GR 3′UTR was increased and combined with YTHDF2 to reduce GR mRNA stability, resulting in GR protein expression suppression.

MATERIALS AND METHODS

Animals and Experimental Design

The animal experiment was approved by the Animal Ethics Committee of Nanjing Agricultural University. The sampling procedure followed the "Guidelines on Ethical Treatment of Experimental Animals" (2006) No. 398 set by the Ministry of Science and Technology, China.

Chicken and Treatment

The feeding experiment was conducted at the Gaolaozhuang breeding base in Nanjing, Jiangsu. Chickens were allowed free access to feed and water throughout the experiment. Eighteen 27-day-old male AA broilers were randomly divided into control group (CON group) and lipopolysaccharide (LPS group) and injected with saline or LPS (C2505, Sigma Aldrich, USA) at 40 days of age. LPS was injected at a dose of 1 mg/kg. Six hours later, 6 chickens in each group were selected to collect wing venous blood to separate serum, and molecular and fixed liver samples were collected. The other 3 were perfused to isolate primary hepatocytes and primary Kupffer cells (Aparicio et al., 2017).

Histological Analysis of Liver Tissue

Fresh liver tissue was fixed with 4% paraformaldehyde and paraffin-embedded, and then the sections of liver were stained with hematoxylin eosin (**HE**).

Corticosterone Measurement

Corticosterone measurement was performed using the Corticosterone EIisa kit (Elabscience, E-EL-0160, China) according to the manufacturer's instructions. The intra and interassay coefficients of variations the kits were less than 10%. Hormonal data were analyzed with 1-way ANOVA with Bonferroni correction.

Cell Culture and Treatment

Mouse KC lines (BeNa Culture Collection, Beijing, China; BNCC340733) were cultured in RPMI 1640 (Wisent, SaintJean-Baptiste, Canada) containing 10% fetal bovine serum (TransGen Biotech, China), and 1% penicillin/streptomycin (TransGen Biotech, China) at 37°C under 5% CO2. Cells were cultured to 60% confluence and then treated with 500 ng/mL LPS (L2880, Sigma-Aldrich) for 24 hours.

RNA Isolation, Real-Time Quantitative PCR (qPCR)

Total RNA was isolated from 30 mg liver samples by using TRIzol Reagent (TSP401; Beijing Tsingke Biotech Co., Ltd., China). One μg of RNA was reverse-transcribed into complementary DNA by using a TransScript Uni All-in-One First-Strand cDNA Synthesis SuperMix for qPCR kit (AU341; TransGen Biotech, China). The complementary DNA (1:20, v:v) was used for real-time qPCR by using a Mx3000P Real-Time PCR System (Stratagene, San Diego). All primers (Table 1) were synthesized by Tsingke Biotech (China). GAPDH, which was not affected by treatment, was chosen as a reference gene. Data were analyzed by using the method of $2^{-\triangle\triangle CT}$.

Protein extraction and Western blot analysis

KCs were harvested using RIPA lysis buffer (BD0032, Bioworld) containing protease inhibitor cocktail (b14001, Selleckchem) and incubated on ice for 10 min. The protein concentrations were measured by BCA Protein Assay kit (TransGen Biotech, Beijing). Protein samples (30 μg) were used for electrophoresis on a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred onto a nitrocellulose membrane. The primary antibodies used for Western blot analysis are listed in Table 2. β-actin was selected as internal control. Images were captured by VersaDoc 4000MP system

Table 1. Nucleotide sequences of primers.

Target	Primer sequences (5′−3′)		Used for
IL-1β	F: AATGCCACCTTTTGACAGTGATG	R: GGAAGGTCCACGGGAAAGAC	Real-time PCR
IL-6	F: GGAAGGTCCACGGGAAAGAC	R: GGAAGGTCCACGGGAAAGAC	Real-time PCR
METTL3	F: GCTCCATCCAGGCCCATAAG	R: CCCACTCACCGTATCGATGG	Real-time PCR
METTL14	F: GTGATTCTCCTGGAGCCACC	R: TGGGGTCCAGAGTCTTCGTT	Real-time PCR
FTO	F: TGAAGGTAGCGTGGGACATAGA	R: TGAAGGTAGCGTGGGACATAGA	Real-time PCR
YTHDF1	F: ACAAGCGTTGACCCTCAGAGA	R: TGTTCCCCAAGCTGAGAAGG	Real-time PCR
YTHDF2	F: TCCTACTCTCTGGGTGAGGC	R: GCGTAATTGCTGCTGTAGCC	Real-time PCR
YTHDF3	F: CCACCAACACTGGTGCAAAG	R: GCCCACACCCCTATTACGAG	Real-time PCR
GAPDH	F: TCTCCTGCGACTTCAACA	R: TGTAGCCGTATTCATTGTCA	Real-time PCR

Table 2. The primary antibodies used for western blot analysis.

Primary Antibody	Company	Catalogue NO.	Dilution
METTL3	Abcam	AB98009	1:3000
METTL14	Abcam	AB98116	1:1000
FTO	Abcam	AB77547	1:1000
YTHDF1	Proteintech	17479-1-AP	1:2000
YTHDF2	Proteintech	24744-1-AP	1:2000
YTHDF3	Proteintech	25537-1-AP	1:1000
β-actin	Bioworld	AP0060	1:10000

(Bio-Rad, Hercules) and the band density was analyzed with Quantity One software (Bio-Rad, Hercules).

RNA m^{6}A Dot Blot Assay

500 ng RNA sample was denatured at 95°C for 5 min and transferred onto a Hybond-N+membrane. The RNA dot blots were stained with 0.02% methylene blue (in 0.3 mol/L sodium acetate, pH = 5.2) as loading controls. After UV cross-linking, the membranes were washed with TBST buffer, blocked with 5% nonfat milk, and incubated with anti-m^{6}A antibody (AB151230, Abcam, diluted 1:1,000) overnight at 4°C. Then, the membrane was incubated with Goat Anti-Mouse IgG (BL001A, Biosharp, China, diluted 1:200,000) at room temperature for 2 h. The signals were visualized by a chemiluminescence system (Bio-Rad), and the dot density was analyzed with Quantity One software (Bio-Rad).

SELECT for the Detection of m^{6}A

Sequences with m^{6}A peaks were retrieved for GR mRNAs and subjected to specific m^{6}A site prediction with SRAMP (http://www.cuilab.cn/sramp). Five very high/high confidence m^{6}A sites were predicted. Among these 5 sites, site 1 and site 4 were significantly affected by LPS stimulation and thus selected for site-specific m^{6}A quantification by using a single-base elongation and ligation-based qPCR amplification method (SELECT). Probes and primers used in the SELECT assay are listed in Table 3.

Luciferase Reporter Assay

The indicated cells were seeded in 12-well plates and transfected with the pMIR-REPORT luciferase vector (AM5795, Thermo, China) fused with or without the wild-type or mutated GR (A to T). KCs were transfected with respective plasmids for 12 h and treated with or without LPS for another 12 h. Cells were subjected to luciferase activity analyses following the instructions of the dual luciferase reporter assay kit (DL101-1, Vazyme, China).

siRNA Transfection and Inhibitor Treatment

Kupffer cells at 60% confluence were transfected with siRNAs specifically designed and verified to knockdown the expression of YTHDF2. The confirmed siRNA targeting sequences used in this study is GCAAACTTGCAGTTTATGTAT. Transfection of cells was performed using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions.

RNA Decay Assay

Actinomycin D (HY-17559, MCE) was added to each well to a final concentration of 5 μg/mL to inhibit *de novo* transcription. Cells were collected at 0, 3, 6 h after the administration of actinomycin D. Total RNA was isolated and subjected to RT-qPCR to quantify the relative abundance of GR mRNAs (relative to 0 h).

Statistical Analysis

All data are presented as mean ± SE. All experiments were repeated at least twice. The differences between groups were analyzed using Student's t-test or 2-way analysis of variance followed by Tukey's test for multiple comparisons with SPSS 20.0 (IBM). The differences were considered statistically significant when $P < 0.05$.

RESULTS

GR Expression Was Accompanied by an Increase in m^{6}A Modification in the Liver of LPS-Injected Broiler Chickens

It is shown that serum ALT, AST and AST/ALT were elevated in the LPS group (Figure 1A). We also observed significant increase in the mRNA levels of proinflammatory cytokines IL-1β, IL-6, and TNF-α in the livers of LPS-treated chickens over control group (Figures 1B, 1C and 1D). Microscopic evaluation of HE-stained liver section revealed that the LPS-treated exhibited higher

Table 3. Nucleotide sequences of SELECT method.

Target	Sequences (5′– 3′)	
GR 3′UTR Motif 1 X site	Up probe: tagccagtaccgtagtgcgtgTTTTAATGTCAGATTGTTCATG	Down probe: TTTCTGTGAGCCTGGGGGcagaggctgagtcgctgcat
GR 3′UTR Motif 2 X site	Up probe: tagccagtaccgtagtgcgtgCACACATTTTAATGTCAGATTG	Down probe: TCATGTTTTCTGTGAGCCTGGcagaggctgagtcgctgcat
GR 3′UTR Motif 3 X site	Up probe: tagccagtaccgtagtgcgtgCCAACACTCACACATTTTAATG	Down probe: CAGATTGTTCATGTTTTCTGTGcagaggctgagtcgctgcat
GR 3′UTR Motif 4 X site	Up probe: tagccagtaccgtagtgcgtgTGAACACTCACACATTTTAATG	Down probe: CAGATTGTTCATGTTTTCTCTCcagaggctgagtcgctgcat
GR 3′UTR Motif 5 X site	Up probe: tagccagtaccgtagtgcgtgCAAACACTCACACATTTTAATT	Down probe: CAAATTGTTCATGTTTTCTGAAcagaggctgagtcgctgcat
GR 3′UTR Motif N site	Up probe: tagccagtaccgtagtgcgtgTGTCAGATTGTTCATGTTTTCT	Down probe: TGAGCCTGGGGGTTCTCTcagaggctgagtcgctgcat
qPCR	Forward Primer: ATGCAGCGACTCAGCCTCTG	Reverse Primer: TAGCCAGTACCGTAGTGCGTG

infiltration cells compared with untreated (Figure 1E). Serum CORT content was increasing expression (Figure 1F), CBG mRNA was increasing expression (Figure 1G). Interestingly, GR mRNA expression was significantly up-regulated (Figure 1H), but GR protein expression was significantly down-regulated in liver (Figure 1I). Meanwhile, the levels of m^{6}A modification were measured using m^{6}A mRNA dot blot. It was observed that the levels of m^{6}A modification were significantly increased in LPS-treated chickens (Figure 1J). RNA methyltransferase METTL3 was significantly increased, while the protein levels of METTL3 showed no significant alterations (Supplementary Figure S1A). There was no difference on the expression of m^{6}A demethylase FTO in neither mRNA (Figure 1K) or protein levels (Supplementary Figure S1B). Both mRNA (Figure 1K) and protein levels (Figure 1L) of the reader protein YTHDF2 was significantly increased in the liver of chickens injected with LPS. No significant alterations were detected for the expression of the reader proteins YTHDF1 and YTHDF3 (Figure 1K).

Global m^{6}A Methylation and GR Expression Have Shown Cell-Specific Characteristics in the Liver of LPS-Injected Broiler Chickens

The same pattern was observed for the expression of GR in Kupffer cells (Figures 2E and 2F) while both mRNA and protein of GR were both increasing in hepatocytes

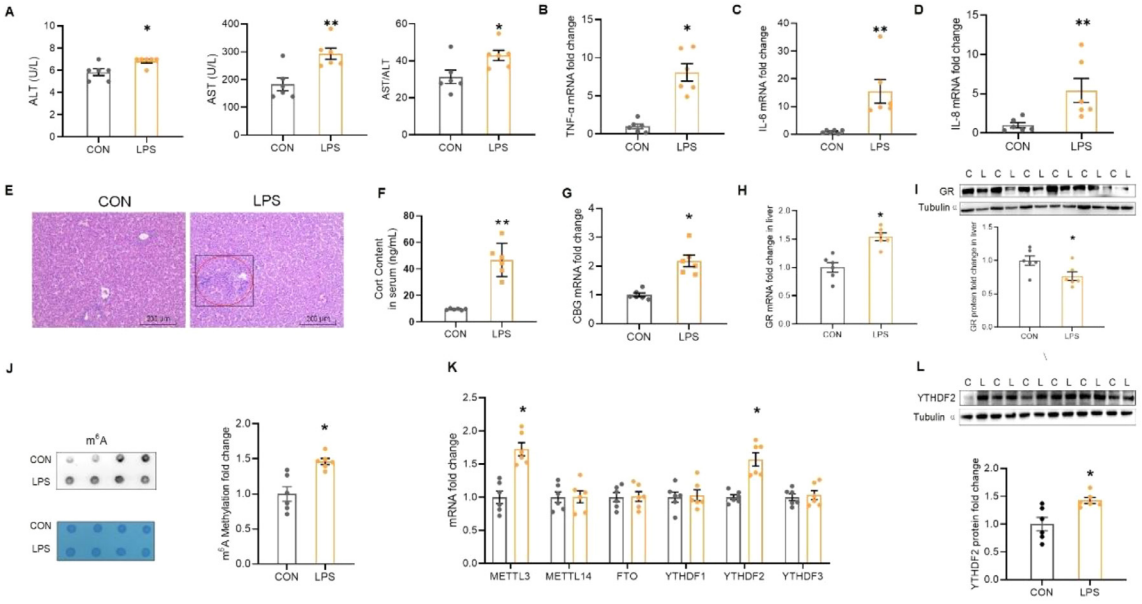

Figure 1. m^{6}A modification increases in the liver of LPS-injected chickens. (A) AST and ALT changes in serum (n = 6). (B) TNF-α mRNA expression (n = 6). (C) IL-6 mRNA expression (n = 6). (D) IL-8 mRNA expression (n = 6). (E) HE staining of hepatic sections. Scale Bar=200μm. (F) Cort content in serum using Corticosterone (n = 6). (G) CBG mRNA expression in liver (n = 6). (H, I) mRNA and protein levels of GR in liver (n = 6). (J) Total RNA m^{6}A modification in liver (n = 6). (K) Hepatic mRNA expression of m^{6}A (n = 6). (L) Hepatic protein expression of YTHDF2 (n = 6). Values are means ± SEM, $^*P < 0.05$.

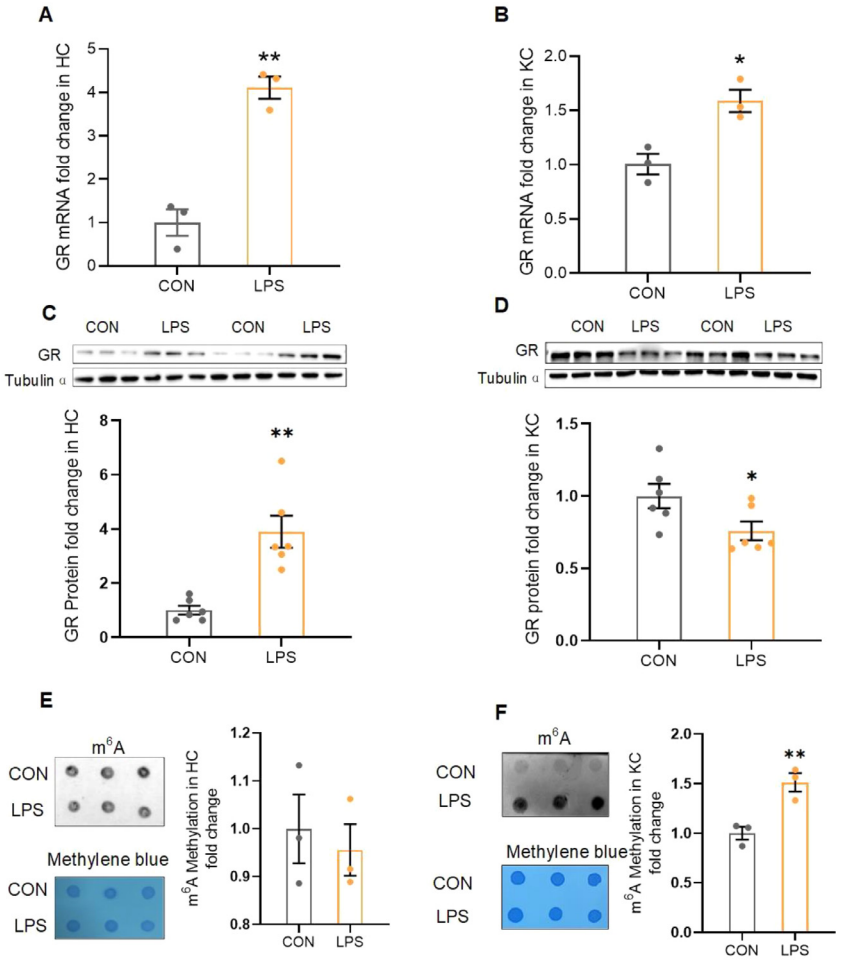

Figure 2. Hepatic methylation level and GR expression in primary cells in chicken show cell-type specificity. (A, B) mRNA and protein levels of GR in primary hepatocytes (n = 3). (C, D) mRNA and protein levels of GR in primary Kupffer cells (n = 3). (E) Total RNA m^{6}A modification in primary hepatocytes (n = 3). (F) Total RNA m^{6}A modification in primary Kupffer cells. (n = 3). Values are means ± SEM, $^*P < 0.05$.

cells (Figures 2C and 2D). The elevation on total m^{6}A modification in liver indicates that a post-transcriptional regulation on GR might take place. To determine the specific cells where epigenetic regulation took place, total RNA m^{6}A levels were determined in hepatic cells and Kupffer cells. Identification of primary hepatocytes and primary Kupffer cells were detected using each mRNA markers respectively (Supplementary Figures S1C and S1D). Global m^{6}A modification in hepatocytes was no significant change (Figure 2E). Global m^{6}A modification increased in Kupffer cells (Figure 2F). Thus, RNA m^{6}A hypermethylation and the uncoupled responses of GR expression specifically took place in Kupffer cells.

RNA m^{6}A Hypermethylation Regulates GR Expression Upon KCs Activation

LPS-treated KCs resulted in high mRNA expression of IL-6 and IL-1β in *vitro* (Supplementary Figure S2A). Not only overall upgradation of m^{6}A level was reappeared (Figure 3A), the reverse between GR mRNA and its protein was repeated (Figures 3B and 3C). We designed primers to specifically amplify total, unspliced, and spliced GR mRNA. The total RNA was significantly up-regulated, while there was no significant change in unspliced and spliced RNA (Supplementary Figure S2B). Combining this finding with the down-regulation of GR protein, it further strengthens the notion that there exists post-transcriptional regulation of GR. We can observe that METTL3 was significantly increased at both mRNA and protein level (Figures 3D and 3E). Still, no significant alterations were detected for the expression of METTL14 and FTO (Supplementary Figures S2C and D). To explore the mechanism of m^{6}A regulating GR, the expression of m^{6}A reader proteins YTHDF1, 2 and 3 were detected. Among the 3 reading proteins, only YTHDF2 was significantly increased at both mRNA and protein levels (Figures 3H and 3I), while YTHDF1 and YTHDF2 showed no change (Supplementary Figures S2E and F).

YTHDF2 Decreases GR mRNA Stability by Catalyzing its m^{6}A Methylation in LPS-Treated KC

To find out if m^{6}A regulates GR first or GR regulates m^{6}A, both specific m^{6}A inhibitor Cycloleucine (**CYC**) and specific GR inhibitor RU486 were used. CYC, but

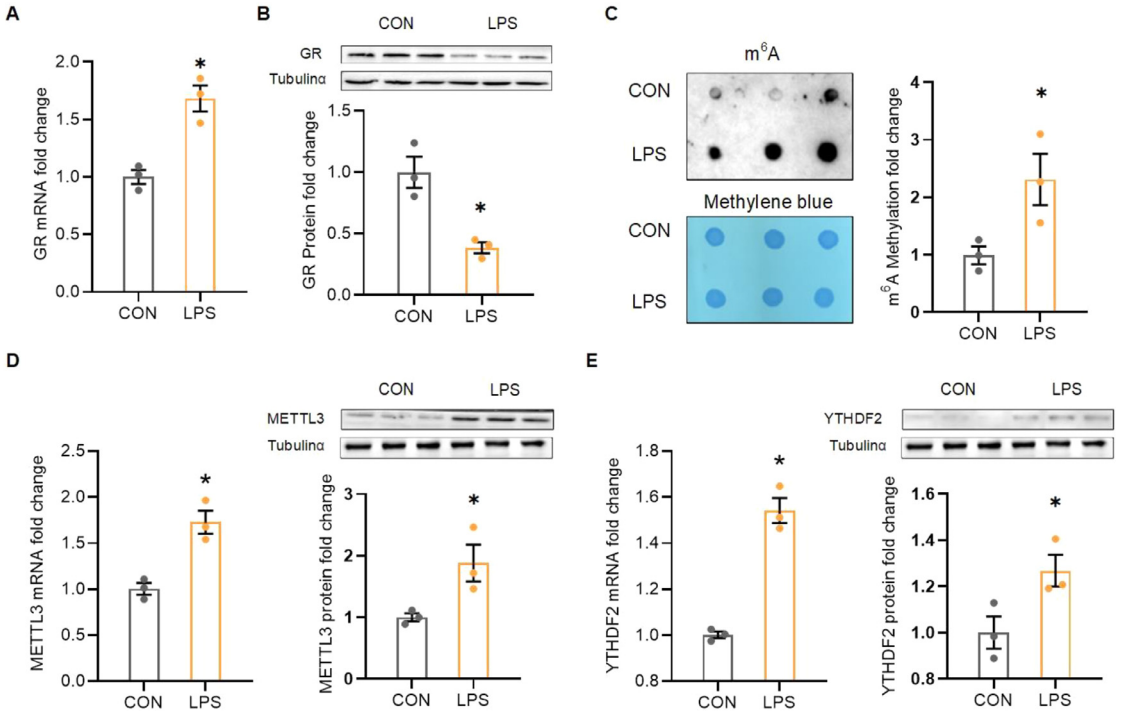

Figure 3. m^{6}A participates in the post-transcriptional regulation of GR upon Kupffer cell activation. (A) Total RNA m^{6}A modification in Kupffer cells (n = 3). (B, C) GR mRNA and protein levels (n = 3). (D) Protein expression of GR, METTL3, METTL14, FTO, YTHDF2 (n = 3). (E, F) METTL3 mRNA and protein levels (n = 3). (D, G) GR mRNA and protein levels (n = 3). (H, I) YTHDF2 mRNA and protein levels (n = 3). (J) Detection of mRNA stability of GR (n = 3). Values are means ± SEM, $^*P < 0.05$.

not RU486, reduced the increased level of m^{6}A modification caused by LPS (Figure 4A). RU486 alleviated the LPS-induced increase in GR mRNA, but the addition of CYC could further increase the mRNA level of GR (Figure 4B). In addition, CYC restored the LPS-induced decrease in GR protein (Figure 4C). The above proved that m^{6}A is upstream of GR, and to test this conjecture, we examined mRNA stability experiments after the addition of CYC. The results showed that CYC administered 6 h before LPS stimulation alleviated the LPS-induced decrease in GR mRNA stability (Figure 4D). These findings indicate that m^{6}A can regulate the expression of GR by impairing its mRNA stability. We wanted to make sure whether m^{6}A can impair the stability of GR through its reader YTHDF2, we practice upon activated KC while knocking down YTHDF2 (Figure 4E). YTHDF2 knockdown significantly increased the LPS-induced stability of GR mRNA (Figure 4F).

Mutant Specific m^{6}A Sites Alleviates GR mRNA Stability

Five high-potential m6A sites on GR 3′UTR were predicted with SRAMP, and referred as X1, X2, X3, X4, and X5, respectively, a non-m^{6}A modification site of this mRNA was selected as the N Site, which was used as an internal reference in SELECT assay for quantification of m^{6}A level on predicted high-potential modification sites (Figure 5A). No significant changes in cycle of threshold (**Ct**) were determined at N, X2, X3, or X5 sites (Figure 5A), while significant increase at X1 and X4 sites of GR 3′UTR were detected in LPS-treated KCs (Figures 5B−5G). Reporter plasmids that contained the mutation of specific m^{6}A X1ang X4 sites on GR 3′UTR were transiently transfected into KCs (Figure 5H), followed by measurement of luciferase activity. It was observed that mutation of specific m^{6}A sites significantly rescued luciferase activity in KCs (Figure 5H). m^{6}A on mRNA of GR was significantly increased in the activated KCs. We observed that mutations at specific m6A sites significantly restored the LPS-induced decrease in luciferase activity (Figure 5I).

DISCUSSION

GR plays an important role in the anti-inflammatory process of the body, as it can control the inflammatory process by influencing the expression of downstream anti-inflammatory genes such as Gilz (Rao et al., 2011). GR are affected by potential post-translational modifications, including phosphorylation, ubiquitination, crotylation, acetylation, and nitrosylation in localization, stability, and DNA binding and ligand reaction (Timmermans et al., 2019). Our study shows that GR is under post-transcriptional regulation by mainly m^{6}A of the GR 3′UTR binding to YTHDF2, reducing GR mRNA stability and reducing GR translation in LPS-activated KCs.

The metabolic effects of GC are mediated by intracellular GR, which translocate to the nucleus and binds to the GREs to regulate the transcription of its target genes (Schmid et al., 1989). GR can control the

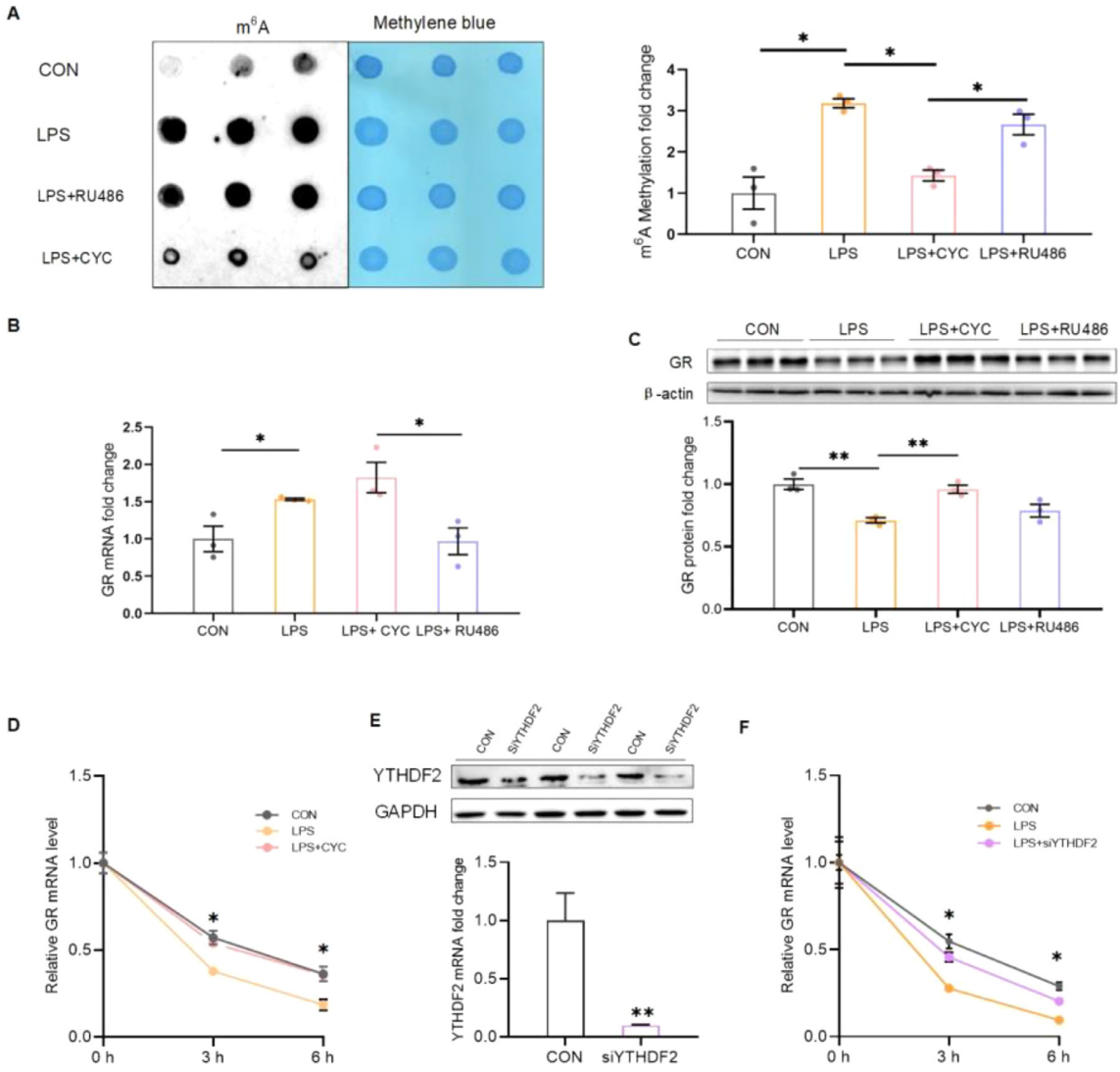

Figure 4. YTHDF2-mediated m^{6}A mechanism impairs GR mRNA stability in LPS-treated Kupffer cells. (A) m^{6}A modification of GR mRNA in the effect of RU486, CYC. (n = 3). (B, C) IL-6 and IL-1β mRNA expression in LPS-treated Kupffer cells (n = 3). (D, E) Effect of RU486 and CYC on GR mRNA and protein content (n = 3). (F)Effect of YTHDF2 siRNA on YTHDF2 mRNA and protein (n = 3). (G) GR stability by siYTHDF2 (n = 3). Values are means ± SEM, $^*P < 0.05$.

inflammatory process by influencing the expression of downstream anti-inflammatory genes in the anti-inflammatory process of the body (Jenniskens et al., 2018). Although GR plays a positive role in the anti-inflammatory process, the occurrence of some inflammation is often accompanied by the reduction of GR protein expression. For example, hepatic GR protein levels are reduced in patients with sepsis, and hepatic GR-deficient mice have increased liver injury and mortality (Jenniskens et al., 2018). Our results showed that GR mRNA expression was upregulated and protein expression was downregulated in LPS-treated chicken livers. Further analysis using isolated primary hepatocytes and Kupffer cells revealed that such uncoupled GR responses occurred specifically in Kupffer cells. This suggests post-transcriptional regulation of GR. Several mechanisms responsible for the decrease of GR protein specifically include transcriptional repression, post-translational protein degradation, and stability of mRNA levels (Schmid et al.,1989).

RNA m^{6}A as a way of post-transcriptional regulation of gene expression levels participates in various processes of mRNA metabolism, such as mRNA transcription, splicing and stability, and realizes the regulation of genes. As the most common RNA methylation modification of RNA, m^{6}A has been reported to be involved in the occurrence of inflammatory responses (Gan et al., 2022). Previous research in our lab found that p65 activates METTL3/METTL14, which significantly increases the level of total m^{6}A modification of mRNA during LPS induced KC activation (Feng et al., 2021). In our study, KCS activation was also accompanied by increased m^{6}A modification and reading proteins YTHDF2. YTHDF2 has also been reported to be involved in the production of proinflammatory cytokines in THP1 and RAW (Wu et al., 2020; Yu et al., 2019). In addition, the main function of YTHDF2 is to recognize and bind m^{6}A and influence mRNA stability and translation (Du et al., 2016). In our study, we found that the downregulation of GR was accompanied by the

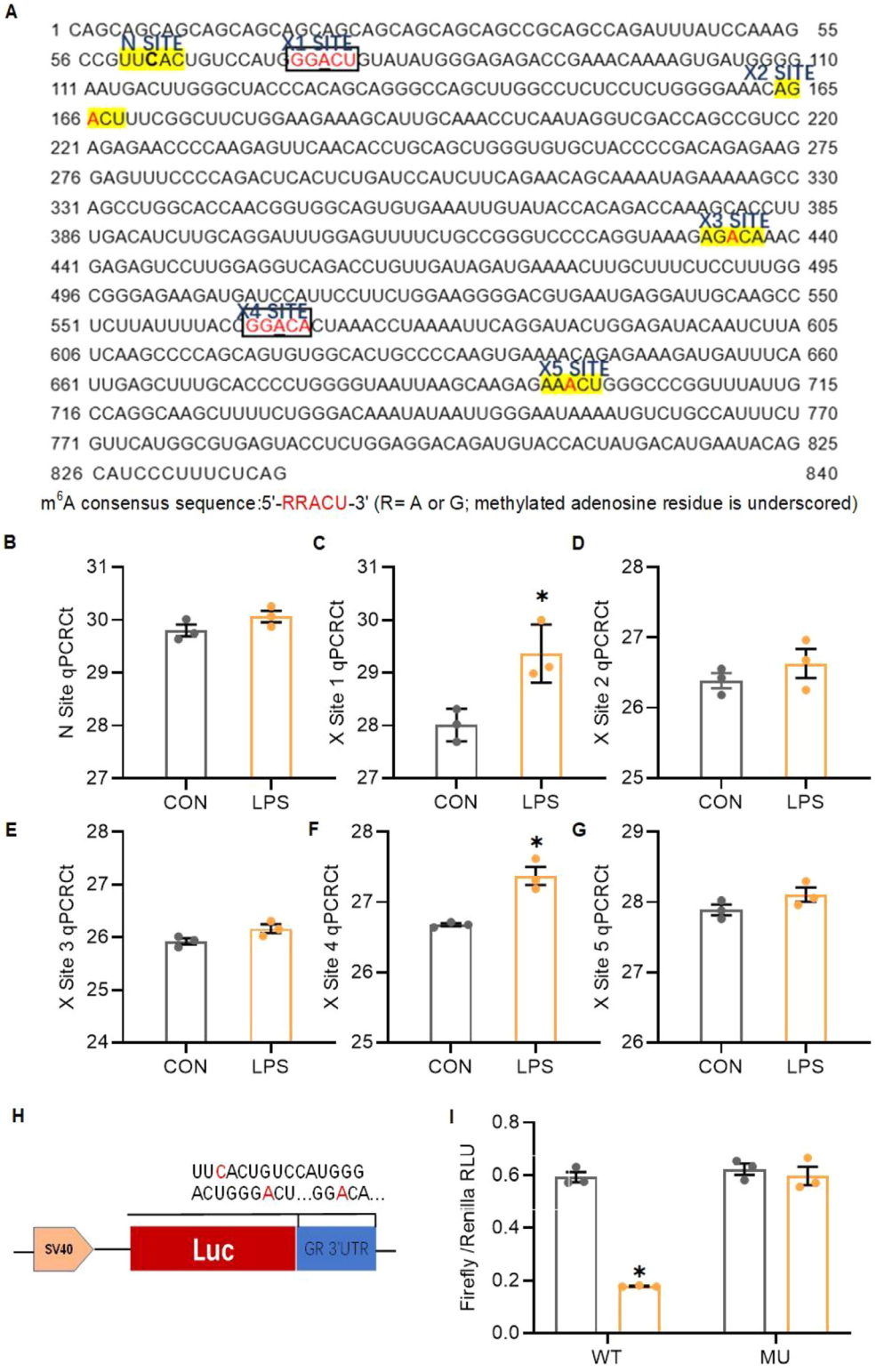

Figure 5. Mutation on specific m^{6}A modification sites alleviates decrease of GR mRNA stability. (A) Sequence analysis of GR 3′UTR revealed 2 matches to the 5′-RRACU-3′ (methylated adenosine residue is underscored) m^{6}A consensus. The m^{6}A site was predicted in a sequence-based m^{6}A modification site predictor for GR sequence. The RRACU-compliant motif was named motif 1−5. A high confidence site A was obtained, and this motif named motif 1 and 4, and this A was named the X1 site. The nonmodification A was named the N site. Validation of m^{6}A modification in GR using SELECT. X site was predicted by SRAMP. (B) N site was negative control. (C−G) X1 and X4 sites showed m^{6}A hypermethylation while other possible showed no significant alteration. (H) Mutation of X1 and X4 sites on a reporter plasmid. (I)Luciferase assays were performed in Kupffer cells transfected with WT or mutant (MUT) luciferase-GR 3′UTR reporter. The ratio of luciferase activity in cells transfected with mutant GR 3′-UTR relative to empty vector was determined. (Mean ± SEM); n = 3; $^*P < 0.05$. *P*-values were calculated with student's t-test.

elevation of m^{6}A methylation and YTHDF2 expression. Therefore, we speculated that m^{6}A could affect the expression of GR mRNA by affecting its stability. By examining the stability of GR mRNA, we determined that m^{6}A impairs the stability of GR mRNA by targeting GR through YTHDF2, which was further supported

by the restoration of GR mRNA stability after YTHDF2 knockdown.

CONCLUSION

LPS treatment can aggravate inflammation by reducing the stability of GR mRNA and inhibiting its protein expression in KC through m^6A-YTHDF2. Our results will enrich the understanding of the mechanism of GR action and provide new targets for the regulation of GR in specific cells under inflammatory conditions. These findings for the development of prevention and treatment of liver inflammatory disease chicken effective treatment strategy provides a new revelation.

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DISCLOSURES

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2024.104393.

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