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LncRNA A2ml2 inhibits fatty liver hemorrhage syndrome progression and function as ceRNA to target *LPL* by sponging miR-143-5p

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ABSTRACT

Fatty liver hemorrhage syndrome (**FLHS**) is the most common metabolic diseases in laying hens during the late-laying period, and it causes a significant economic burden on the poultry industry. The competing endogenous RNA plays crucial roles in the occurrence and development of fatty liver. Based on the previously constructed lncRNA-miRNA-mRNA networks, we selected the axis of ENSGALT00000079786-*LPL*-miR-143-5p for further study to elucidate its mechanistic role in development of fatty liver. In this study, we identified a novel highly conserved lncRNA (ENSGALT00000079786) in poultry, which we designated as lncRNA A2ml2 based on its chromosomal location. Fluorescent in situ hybridization (**FISH**) revealed that lncRNA A2ml2 was localized in both the nucleus and cytoplasm. Dual-luciferase reporter assay validated the targeted relationship between lncRNA A2ml2, miR-143-5p, and the *LPL* gene. To further analyze the lncRNA A2ml2 and miR-143-5p function, lncRNA A2ml2 overexpression vector was successfully constructed and transfected into Leghorn male hepatocellular **(LMH)** cells, which could remarkably inhibit cellular lipid deposition was detected by oil red staining ($P < 0.01$), the opposite occurred for miR-143-5p ($P < 0.01$). qPCR demonstrated an inverse correlation between miR-143-5p expression and lncRNA A2ml2 expression, and confirmed that miR-143-5p directly target lncRNA A2ml2. Similarly, we found an inverse correlation between expression of *LPL* and the expression of miR-143-5p. To further investigate the interactions among these three factors and their effects on cellular lipid metabolism, we assessed the expression levels of *LPL* by co-transfecting lncRNA A2ml2 with miR-143-5p mimic and miR-143-5p mimic binding site mutants. Co-transfection experiments showed that miR-143-5p diminished the promoting effect of lncRNA A2ml2 on *LPL*. Meanwhile, miR-143-5p has the capacity to mitigate the suppressive impact of lncRNA A2ml2 overexpression on lipid accumulation in LMH cells. The results revealed that lncRNA A2ml2 attenuated hepatic lipid accumulation through negatively regulating miR-143-5p and enhancing *LPL* expression in LMH cells. Our findings offer novel insights into ceRNA-mediated in FLHS and identify a novel lncRNA as a potential molecular biomarker.

Introduction

Fatty liver hemorrhagic syndrome (**FLHS**) is a common metabolic disease in intensively reared laying hens, particularly caged hens during the peak-laying period (Shini, et al., 2019). FLHS is characterized by hepatic lipid overaccumulation, which may lead to fatal such as hepatic rupture and sudden mortality (Miao, et al., 2021), thereby severely compromising the productivity of commercial laying flocks. Therefore, elucidating the molecular mechanisms underlying the development and progression of fatty liver in poultry is imperative for developing effective interventional strategies.

Lipoprotein lipase (**LPL**) is a pivotal regulatory enzyme of lipid metabolism, facilitating the hydrolysis of triglycerides in chylomicrons and very low-density lipoproteins within the vasculature (Wu, et al., 2021). Growing evidence links *LPL* dysfunction to dyslipidemia-associated pathophysiological conditions, including

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atherosclerosis, obesity, diabetes, and insulin resistance (Eckel, et al., 1995; Mead and Ramji, 2002). Murine models of high-fat diet-induced hepatic steatosis demonstrate that *LPL* overexpression attenuates hepatic lipid droplet accumulation and improves glucose homeostasis. Consistently, our previous studies indicate that the *LPL* is downregulated in the fatty liver of Jingxing-Huang chicken (Shimizu, et al., 2022; Xiao, et al., 2024). While *LPL*'s canonical role in hydrolyzing TG-rich lipoproteins to generate free fatty acids is well-established, its upstream regulatory mechanisms in hepatic lipid metabolism remain poorly understood.

Competing endogenous RNA (**ceRNA**) molecules encompass circRNAs, lncRNAs, mRNAs, and miRNA and it is one of the current hot topics in fatty liver research. Increasing evidence has verified that ceRNA is a very important pathway in fatty liver progress regulation. Of those, miRNAs modulate diverse physiological processes by post-transcriptionally suppressing gene expression (Ge, et al., 2022; Vishnoi and Rani, 2023). For instance, miR-375 suppresses hepatic lipid synthesis by targeting *RBPJ* and *MAP3K1* (Xie, et al., 2022; Zhang, et al., 2023). while miR-143-5p exacerbates diet-induced hepatic insulin resistance via inhibition of *MKP5* (Li, et al., 2021).

LncRNAs are involved in gene transcription and translation via epigenetic modifications or miRNA regulation. Emerging evidence supports that they play an indispensable role in lipid metabolism (Li, et al., 2014). Downregulation of lncRNA NEAT1 mitigates lipid deposition by suppressing the mTOR/*S6K1* pathway, thereby reducing free fatty acid-induced upregulation of *FAS* and *ACC* (Wang, 2018). It can regulate adipogenesis, including low-density lipoprotein oxidation, lipid uptake and breakdown. Based on the ceRNA hypothesis, lncRNAs competitively bind miRNAs to relieve miRNA-mediated repression of target mRNAs (Salmena, et al., 2011). For instance, the overexpression of lncRNA MEG3 has been shown to enhance *LRP6* expression by inhibiting miR-21, which subsequently suppresses the AKT/mTOR signaling pathway and mitigates the progression of non-alcoholic fatty liver disease (Huang, et al., 2019). Similarly, during the peak egg production period in hens, lncHLEF promotes hepatic lipid synthesis in laying hens by sequestering miR-2188-3p and enhancing *GATA6* expression (Guo, et al., 2023).

Previous studies from our laboratory constructed a triple network of differentially expressed lncRNAs, miRNAs, and mRNAs (DElncRNAs-DEmiRNAs-DEmRNA) based on the ceRNA hypothesis and identified a core subnetwork associated with lipid metabolism (Xiao, et al., 2024). Notably, it showed that *LPL* is part of the ENSGALT00000079786--miR-143-5p-*LPL* ceRNA network. Accordingly, our core objective was to reveal *LPL*-associated ceRNA network so as to support further systematic studies of fatty liver in chicken.

Materials and methods

Ethics statement

All animal experimental procedures in this study by the College of Animal Science of Jilin University Ethics Committee (SY202105020).

Plasmid construction and production of vectors

SilncRNA A2ml2 sequences, miR-143-5p mimic and inhibitor were synthesized by Guangzhou RuiBo Biological Technology Co., Ltd (Guangzhou, China). The full-length sequence of lncRNA A2ml2 was cloned into a pBI-CMV3 vector between the BamHI and NotI sites to generate pBI-CMV3-lncRNA A2ml2 by homologous recombination repair. The lncRNA A2ml2 wild-type (**WT**) sequence and mutated (**MUT**) sequence, the *LPL*-3′ untranslated region (3′UTR) WT sequence and Mut sequence, miR-143-5p MUT sequence and were synthesized by Kumei Biotech Co., Ltd. (Changchun, China), which were then inserted into a psiCHECK2 vector to establish lncRNA A2ml2-WT/MUT, *LPL*-WT/MUT, respectively.

Cell culture and cell transfection

LMH cells were purchased from the BeNa Culture Collection (BNCC, Beijing, China) Co., Ltd. The cells were cultured in a DMEM/F12 medium (BI, Kibbutz, Beit Haemek, Israel) containing 1 % penicillin (100 U/mL), 10 % fetal bovine serum (ThermoFisher, Massachusetts, USA) and 1 % streptomycin (Pricella, Wuhan, China) and seeded into a 6-well-plate at about 70 % confluence and cultured. cells were transfected with miR-143-5p mimic/inhibitor, pBI-CMV3-lncRNA A2ml2, silncRNA A2ml, respectively. In addition, psiCHECK-2-lncRNA A2ml2-WT, psiCHECK-2-lncRNA-A2ml2-MUT, *LPL*-3′UTR-WT and *LPL*-3′UTR-MUT were co-transfected with miR-143-5p mimic into LMH cells using the Lipofectamine 3000 reagent (Invitrogen, USA).

Total RNA extraction and qRT-PCR analysis

Liver tissues were provided by the China Agricultural University. Total RNA was extracted from each tissue sample and LMH cells separately using FastPure Cell/Tissue Total RNA Isolation Kit (Vazyme, Nanjing, China). RNA concentrations were measured with NanoDrop® ND-2000 Spectrophotometer and RNA integrity was assessed through agarose gel electrophoresis. *U6* was utilized as an internal reference for miR-143-5p and *β-actin* was the internal reference for lncRNA A2ml2 and *LPL*. All primers for qRT-PCR were designed using Primer 5.0 software and primer sequences are listed in Table 1. Total RNA was reverse transcribed to cDNA using FastKing RT Kit (With gDNase; TIANGEN, Beijing, China) with referring to the kit instructions. The reverse transcription program was 42°C for 15 minutes and 95°C for 3 minutes. Quantitative real-time PCR (**qRT-PCR**) was conducted according to the BlasTaq™ 2X qPCR MasterMix kit instructions (abm, Canada). The reaction program was as follows: 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 s and 55°C for 30 s. qRT-PCR was performed on an

Table 1
qRT-PCR genes and related primers

Name of gene	sequences
lncRNA A2ml2	F: 5'-AGCCCAGAAAGCCACTCAT-3' R: 5'-TCCAACAGGTCCACGTCTC-3'
LPL	F: 5'-GCAGAGCCTGATGAAGATG-3' R: 5'-AAGCAGCAGACACTGGGTA-3'
miRNA-143-5p	F: 5'-ACACTCCAGCTGGGGGTGCAGTGCTGCAT-3' R: 5'-CTCAAGTGTGGAGTCGGCAA-3'
U6	F: 5'-GCTTCGGCACATATACTAAAAT-3' R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'
β-actin	F: 5'-GAGAAATTGTGCGTGACATCA-3' R: 5'-CCTGAACCTCTCATTGCCA-3'
miR-143-5p-RT	5'-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGCCAGAGAT-3'
U6-RT	5'-CGCTTCACGAATTTGCGTGTCAT-3'
pBI-CMV3-lncRNA A2ml2	F:5′-CGTCAGATCCGCTAGGGATCCGTTTCTTCTCTTGCTGTTATCCACA-3′ R:5′-GACAAGCTTATCGATGCGGCCGCTGAAAAGTCTCCGGGAACAGG-3′
psiCHECK-2-lncRNA A2ml2-WT	F:5′-AATTCTAGGCGATCGCTCGAGAGCCCAGAAAGCCACTCAT-3′ R:5′-ATTTTATTGCGGCCAGCGGCCGCTCCAACAGGTCCACGTCTC-3′
psiCHECK-2-lncRNA A2ml2-MUT	F:5′-AATTCTAGGCGATCGCTCGAGAGCCCAGAAAGCCACTCAT-3′ R:5′-ATTTTATTGCGGCCAGCGGCCGCTCCAACAGGTCCACGTCTC-3′
psiCHECK-2-*LPL*-3'UTR-WT	F:5′-TAATTCTAGGCGATCGCTCGAGGTTCCTCTTTAGCAGTTT-3 R:5′-TAAAGATATTTTATTGCGGCCAGCGGCCGCTACTCACTTG-3′
psiCHECK-2-*LPL*-3'UTR-MUT	F:5′-TAATTCTAGGCGATCGCTCGAGGTTCCTCTTTAGCAGTTT-3 R:5′-TAAAGATATTTTATTGCGGCCAGCGGCCGCTACTCACTTG-3′

Eppendorf PCR system (Hamburg, Germany) and data were analyzed by the $2^{-\triangle\triangle CT}$ method.

RNA fluorescence in situ hybridization

Fluorescent in situ hybridization (**FISH**) for lncRNA A2ml in LMH cells using the RiboTM Fluorescent In situ Hybridization Kit (RiboBio, Guangzhou, China). All cells were grown to 70 % confluence and washed thrice with ice-cold 1X phosphate buffer saline (**PBS**) for 5 minutes each, followed by fixation with 4 % paraformaldehyde for 10 minutes. Next, cells were permeabilized in 0.5 % Triton X-100 in PBS for 5 minutes. Then, the supernatant was discarded and the cells were washed. The cells were blocked with pri-hybridization buffer for 30 minutes at 37°C. lncRNA A2ml2 FISH Probe Mix Storage solution was added to 100 μL hybridization buffer and incubated at 37°C overnight in a humidified chamber in the dark. The cells were subsequently rinsed with hybridization buffer under conditions void of light in order to limit background signal and DAPI was used to stain the nuclei for 10 minutes. PBS was used to wash the LMH cells before being photographed with confocal microscopy (Leica, Germany).

Dual luciferase reporter assay

These cells were harvested after transfection for 48h, and dual luciferase reporter assay kit (Promega, Madison, WI, USA) was performed to detect the luciferase activity in strict accordance with the manufacturer's instructions.

Oil Red O (ORO) staining

Staining of intracellular lipid accumulation was performed using Oil Red O (Sigma, MO, USA). Briefly, LMH cells were harvested and fixed in 10 % buffered formalin solution for 10 minutes. PBS was then used to wash the sections 3 times, with each wash lasting 5 minutes and rinsed in 60 % isopropanol for 15 s to facilitate the staining of neutral lipids. Cells were stained with filtered ORO working solution keep out of light for 30 minutes at 37°C. After fat droplets in adipocytes were stained, the samples were rinsed with 60 % isopropyl alcohol again. The cells were observed and photographed under fluorescent microscopy.

Triglyceride assay

Triglycerides level was measured using Triglyceride Assay Kit (Nanjing, China) following manufacturer's protocols and normalized to total protein concentration.

Statistical analysis

Validation data for the quantitative results were calculated, using the $2^{-\triangle\triangle CT}$ method for statistical analysis. Analyses were carried out using GraphPad Prism 9.0 (GraphPad Prism Software Inc.). Students *t*-tests were applied to evaluate the difference between groups, while the comparison among multiple groups was conducted by one-way ANOVA. $P < 0.05$ was considered to indicate a statistically significant difference. * Indicated $P < 0.05$; ** indicated $P < 0.01$; *** indicated $P < 0.001$.

Results

Bioinformatics annotation and Localization of lncRNA A2ml2

The ENSGALT00000079786 sequences were aligned using the UCSC genome browser. Sequence comparison indicated that its located on chromosome 1 (76,115,087-76,118,440) spanning 3.31 kb in chicken genome through genetic mapping and comprised exons 10, 11 and 12 of the parental gene *A2ML2* (Fig. 1 A). Following current noncoding RNA nomenclature guidelines, this lncRNA is hereafter designated as lncRNA A2ML2 (Gschwendt, et al., 1989). Further analysis of the DNA sequence alignment of lncRNA A2ml2 showed that it is highly conserved in most of the known avian genomes (97 % sequence similarity), including *Aquila chrysaetos, Haliaeetus albicilla, Accipiter gentilis, Caprimulgus europaeus* and *Falco punctatus* (Fig. 1 B).

Given the functional relevance of lncRNA subcellular localization, we first assessed lncRNA A2ML2 distribution in LMH cells. The prediction results of the lncLocator website (http://www.csbio.sjtu.edu.cn/bioinf/lncLocator/) showed that lncRNA A2ml2 was mainly located in cytoplasm, accounting for about 91 % (Fig. 1 C). The distribution of lncRNA A2ml2 in LMH cells was determined using an RNA-FISH assay, and we observed that lncRNA A2ml was located in both nucleus and cytoplasm (Fig. 1 D), suggesting that lncRNA A2ml2 might possess a functional role.

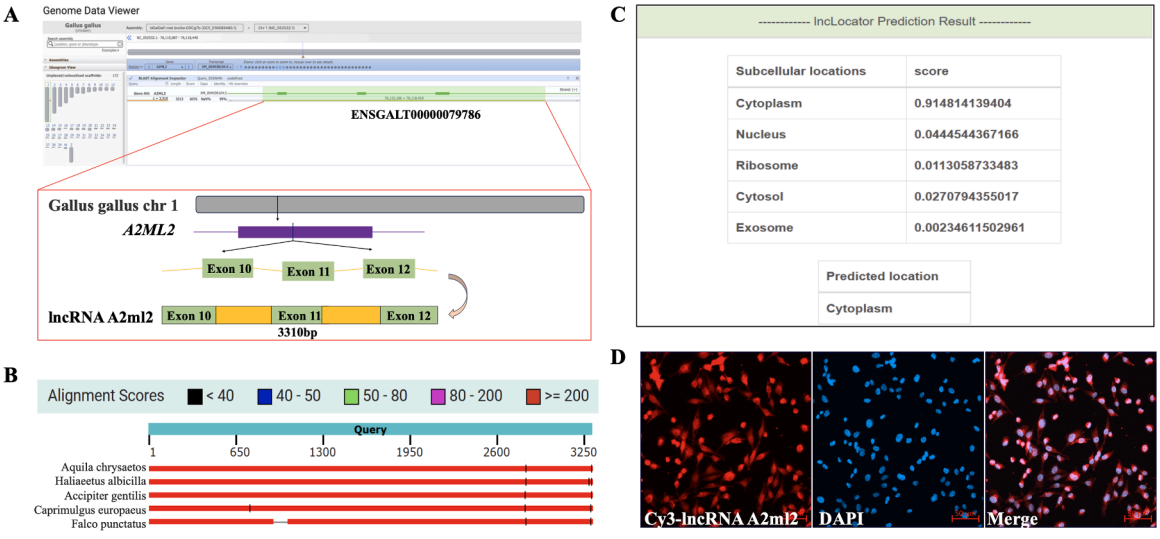

Fig. 1. Bioinformation analysis of lncRNA A2ml2. (**A**) lncRNA A2ml2 sequence from the chicken genome. (**B**) Sequence alignment of lncRNA A2ml2. (**C**) Subcellular localization of lncRNA A2ml2 predicted by the lncLocator website. (http://www.csbio.sjtu.edu.cn/bioinf/lncLocato). (**D**) Subcellular localization of lncRNA A2ml2 in cells analyzed by FISH assay (Scale bars = 50 μm).

Effects of lncRNA A2ml on intracellular lipid accumulation in LMH cells

To elucidate a potential role of lncRNA A2ml2 in lipid accumulation, experiments were performed in cultured LMH cells. The full-length sequence of lncRNA A2ml2 was cloned into the pBI-CMV3 plasmid vector and verified by sequencing (Fig. 2 A-C). Efficient transfection was confirmed by co-expression of a green fluorescent protein reporter gene visualized by fluorescence microscopy (Fig. 3 A). Next, we synthesized three distinct siRNAs targeting lncRNA A2ml2 and identified the most effective construct (silncRNA A2ml2-2, $P < 0.01$) by PCR (Fig. 3 B). Quantitative analysis showed that the relative abundance of the transcript of lncRNA A2ml2 increased significantly ($P < 0.001$) post-transfection with pBI-CMV3-lncRNA A2ml2, while silncRNA A2ml2-2 transfection resulted in marked reduction ($P < 0.01$) (Fig. 3 C). Subsequent evaluation of intracellular triglycerides revealed that both triglyceride deposition and lipid droplet formation were significantly decreased ($P < 0.05$) upon lncRNA A2ml2 overexpression (Fig. 3 D-E).

RT-qPCR validation of lncRNA A2ml2-LPL-miR-143-5p base on transcriptome sequencing

Following preliminary bioinformatics analysis (Xiao, et al., 2024), we established a functionally annotated lncRNA-miRNA-mRNA ceRNA network. To predict the lncRNA A2ml2-*LPL*-miR-143-5p interactions of the differentially expressed data. Transcriptome analysis revealed miR-143-5p was highly expressed ($P < 0.05$), while lncRNA A2ml2 and *LPL* were downgraded ($P < 0.01$) in fatty liver group compared with controls (Fig. 4 A-C). In order to verify the transcriptome data, we carried out the quantitative real-time PCR experiments, the result show that the trend in expression of miR-143-5p was in contrast to that of lncRNA A2ml2 and *LPL* ($P < 0.001$) (Fig. 4 D-F). Collectively, these findings implied the association of miR-143-5p and lncRNA A2ml2 with *LPL*.

Regulatory associations between LncRNA A2ml2 and miR-143-5p

To investigate the interaction between lncRNA A2ml2 and miR-143-5p, the miRanda website predicted potential binding sites between them (Fig. 5 B). Consequently, we constructed luciferase reporter vectors containing either wild-type or mutant sequences of lncRNA A2ml2, with successful vector construction confirmed by sequencing (Fig. 5 A). The lncRNA A2ml2-WT sequence or lncRNA A2ml2-MUT containing vector was co-transfected into LMH cells with miR-143-5p mimic. Dual-luciferase reporter gene assay revealed that transfection of miR-143-5p mimic decreased the luciferase activity of the lncRNA A2ml2-WT reporter ($P < 0.001$), while miR-143-5p mimic had no impact on the lncRNA A2ml2-MUT reporter activity (Fig. 5 C). These results demonstrated that miR-143-5p was able to directly bind to lncRNA A2ml2 at specific recognition sites.

To further demonstrate the regulatory relation between lncRNA A2ml2 and miR-143-5p, RT-qPCR was performed to examine miR-143-5p levels in LMH cells transfected with pBI-CMV3-lncRNA A2ml2 or si-lncRNA A2ml2, showing obviously amplified miR-143-5p expression after the decrement of lncRNA A2ml2 ($P < 0.05$) (Fig. 5 D). Similarly, miR-143-5p mimic treated cells showed significantly lower lncRNA A2ml2 expression while miR-143-5p inhibitor treated cells showed significantly higher lncRNA A2ml2 expression ($P < 0.01$) (Fig. 5 E).

miR-143-5p promotes accumulation of lipids in LMC cells by repressing LPL expression

Due to the associations between lncRNA A2ml2 and miR-143-5p, the regulatory effects of miR-143-5p were further evaluated. Firstly, the miR-143-5p mimic and miR-143-5p inhibitor into LMH cells, respectively. As shown by RT-qPCR, the miR-143-5p level was significantly enhanced after transfection with miR-143-5p ($P < 0.001$) (Fig. 6 A). To obtain additional insight into the functional roles of the miR-143-5p, Oil

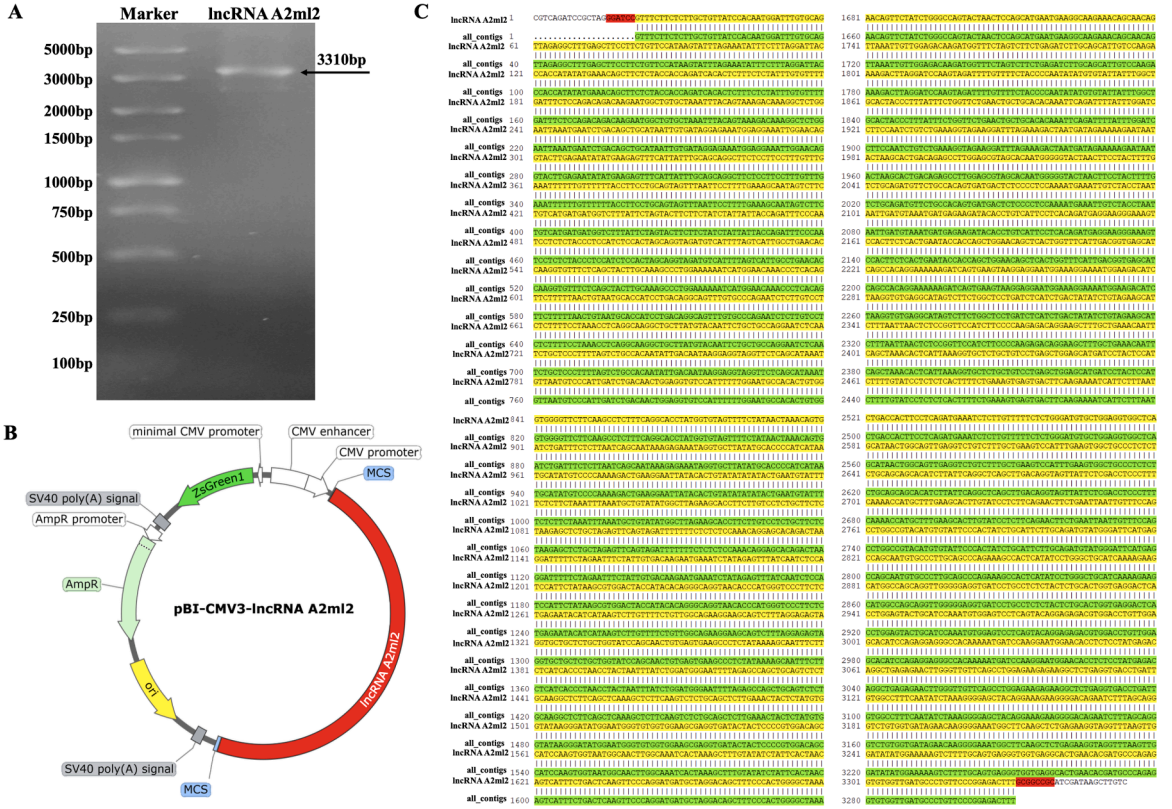

Fig. 2. Construction of lncRNA A2ml2 overexpression vector. (**A**) Agarose gel electrophoresis of PCR. (**B**) Construction map of the lncRNA A2ml2 overexpression vector (pBI-CMV3-lncRNA A2ml2). (**C**) vectors were sequenced and analyzed.

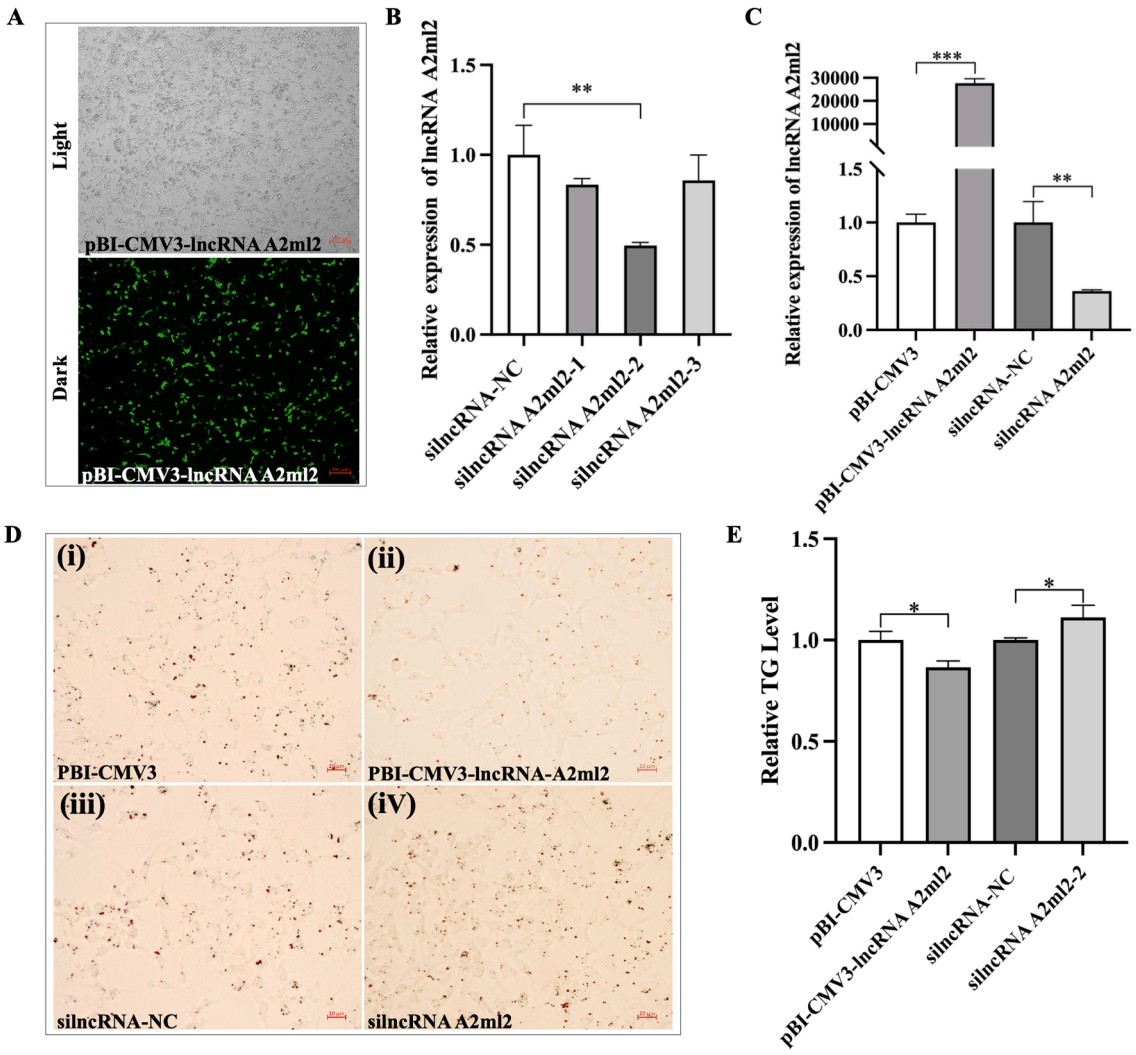

Fig. 3. lncRNA A2ml2 inhibited the lipid deposition in LMH cells. (**A**) Evaluation of transfection efficiency of pBI-CMV3-lncRNA A2ml2, which expressed a green fluorescent protein. (**B**) Screening of si-lncRNA A2ml2. (**C**) Overexpression efficiency of lncRNA A2ml2 in LMH cells was detected by quantitative PCR. (**D**) Oil red O staining of LMH cells treated with pBI-CMV3-lncRNA A2ml2 and silncRNA A2ml2. Scale bars = 50 μm. (i: Oil Red O staining of LMH cells treated with pBI-CMV3. ii: Oil Red O staining of LMH cells treated with pBI-CMV3-lncRNA A2ml2. iii: Oil Red O staining of LMH cells treated with silncRNA NC. iv: Oil Red O staining of LMH cells treated with silncRNA A2ml2.). (**E**) Triglyceride content of LMH cells treated with pBI-CMV3-lncRNA A2ml2 and silncRNA A2ml2. * Represent $P < 0.05$; ** represent $P < 0.01$; *** represent $P < 0.001$.

red O staining and triglyceride content analysis was conducted. The result revealed that the miR-143-5p mimic significantly increased lipid droplet numbers and TG, while the miR-143-5p inhibitor decreased them (Fig. 6 B-C).

Furthermore, we explored how miR-143-5p exerts its functional effects. Previous studies show that transcription factor *LPL* level in fatty liver group is considerably reduced, but its upstream regulatory factor is still unclear. The potential binding sites between *LPL* and miR-143-5p were predicted by TargetScan software (Fig. 6 E). We constructed *LPL*-WT and *LPL*-MUT reporter vectors and verified by sequencing (Fig. 6 D). These vectors were co-transfected into LMH cells with miR-143-5p mimic, and the luciferase activity was determined. The analysis showed that the luciferase activity decreased by about 60 % after co-transfection of *LPL*-WT and miR-143-5p mimic ($P < 0.001$), and insignificantly after transfection of *LPL*-MUT and miR-143-5p mimic (Fig. 6 F). On the basis of the interaction between miR-143-5p and *LPL*, we further studied the effect of miR-143-5p on the expression of *LPL*. A qRT-PCR detection of *LPL* expression in LMH cells transfected with miR-143-5p mimic, mimic NC, miR-143-5p inhibitor and inhibitor NC. The results showed that miR-143-5p mimic led to the downregulation of *LPL* compared with the mimic NC ($P < 0.01$), whereas the miR-143-5p inhibitor resulted in the upregulation of *LPL* ($P < 0.05$) (Fig. 6 G). These findings collectively indicated that miR-143-5p directly inhibits *LPL* gene expression by targeting its 3′UTR.

LncRNA A2ml2 affected LMH cells lipid deposition through the miR-143-5p/LPL axis

As a known mechanism, lncRNA-miRNA and miRNA-mRNA interactions can form lncRNA-miRNA-mRNA triplets. TargetScan analysis confirmed the predicted binding sites among lncRNA A2ml2, miR-143-5p and *LPL* (Fig. 7 A). Quantitative analysis in Fig. 7 B indicated that miR-143-5p overexpression inhibited the expression of *LPL* ($P < 0.05$), whereas lncRNA A2ml2 overexpression significantly upregulated *LPL* level ($P < 0.001$). This interesting phenomenon still needs further explanation. T Rescue assays were performed using both miR-143-5p mimic and miR-143-5p mimic-mut constructs. Quantification analysis revealed that co-transfection of miR-143-5p mimic with pBI-CMV3-lncRNA A2ml2, *LPL* expression was suppressed compared with pBI-CMV3-lncRNA A2ml2 group ($P < 0.001$). Conversely, co-transfection

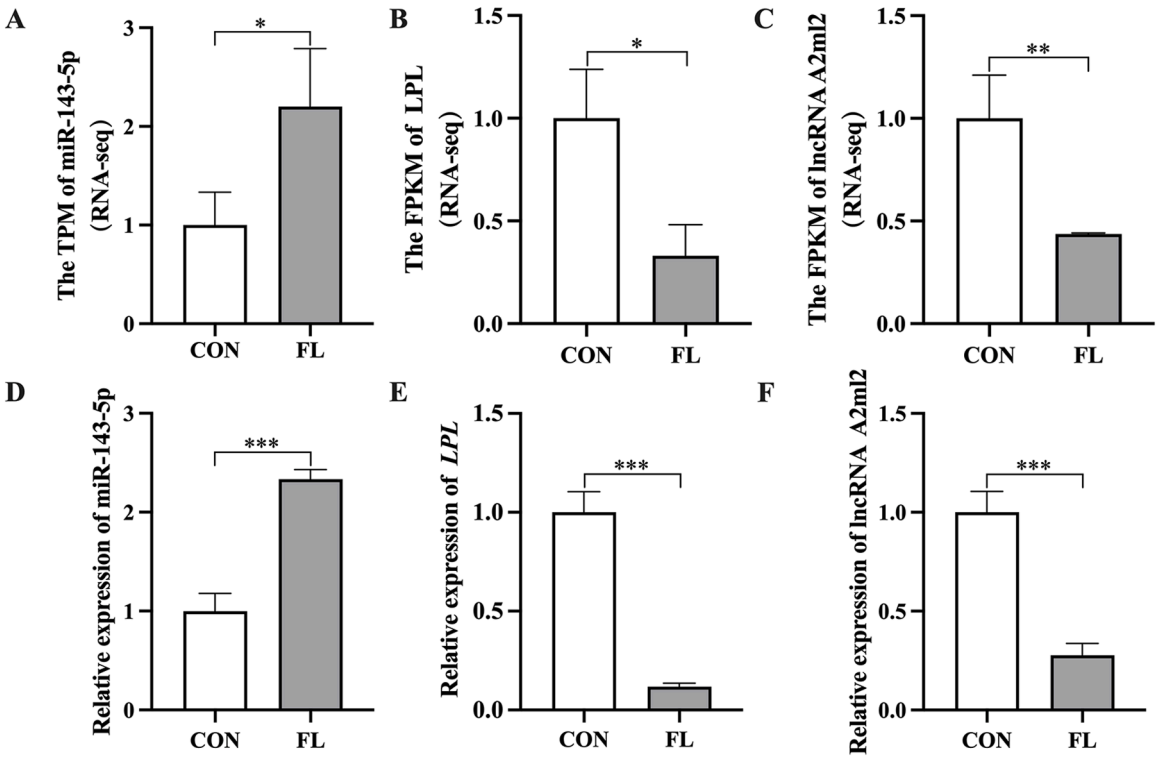

Fig. 4. verity the reliability of the sequencing data. (CON: control group, FL: fatty liver group). (**A**) The expression of miR-143-3p in CON and FL was analyzed by miRNA-seq. (**B**) The expression of *LPL* in CON and FL was analyzed by RNA-seq. (**C**) The expression of lncRNA A2ml2 in CON and FL was analyzed by RNA-seq. (**D**) miR-143-5p expression was examined via reverse transcription quantitative PCR. (**E**) *LPL* expression was examined via reverse transcription quantitative PCR. (**F**) lncRNA A2ml2 expression was examined via reverse transcription quantitative PCR. * Represent $P < 0.05$; ** represent $P < 0.01$; *** represent $P < 0.001$.

miR-143-5p mimic-mut and pBI-CMV3-lncRNA A2ml2 group dramatically enriched *LPL* compared with miR-143-5p mimic+pBI-CMV3-lncRNA A2ml2 group. These findings suggest that overexpression of miR-143-5p attenuates the promotive effect of lncRNA A2ml2 on *LPL* expression in LMH cells. Moreover, to assess lipid accumulation in LMH cells, Oil Red O staining and triglyceride assays were conducted. Both Oil Red O staining (Fig. 7 C) and triglyceride quantification (Fig. 7 D) results correlated with the qPCR findings, demonstrating concordant patterns of lipid metabolism regulation. Taken together, lncRNA A2ml2, which reduces intracellular lipid content in LMH cells, act as a ceRNA by sponging miR-143-5p and indirectly regulated *LPL* expression.

Discussion

FLHS is a metabolic disease caused by nutritional, hormonal, environmental and genetic factors. It is characterized by excessive fat accumulation in hepatocytes (You, et al., 2023), which can lead to decrease performance and increase mortality in laying hens. Fatty liver can further progress to steatohepatitis (Feng, et al., 2024), cirrhosis (Zheng, et al., 2024), and even liver cancer (Mantovani, et al., 2020), Additionally, it may lead to various complications, including cardiovascular disease, diabetes, hypertension and other metabolic diseases, adversely affecting both human health and animal production. Accumulating evidence suggests that aberrant lncRNAs expression is associated with lipid metabolism disorder of human and animals (Muret, et al., 2019). According to ceRNA hypothesis, lncRNAs function act as "sponges" to bind and absorb miRNA, thereby attenuating their ability to mRNA and regulating gene expression (Chen, et al., 2024). In our previous research, we predicted the ceRNA network involved in chicken fatty liver lipogenesis. through biometric analysis and found that ENSGALT00000079786-miR-143-5p-*LPL*. To elucidate underlying mechanisms, we employed LMH cells, which have been shown to effectively simulate hepatic steatosis in poultry (Zhuang, et al., 2024). Compared with primary chicken hepatocytes, LMH cells exhibit superior proliferative capacity and phenotypic stability during culture, enhancing experimental reproducibility in hepatic steatosis research (Song, et al., 2023). This research was the first evidence that ENSGALT0000079786 expression significantly downregulated in fatty liver tissues of chickens. Bioinformatic analysis revealed its genomic localization on chicken chromosome 1, where it is transcribed from three exons of the *A2ML2* gene. Following HUGO Gene Nomenclature Committee guidelines for lncRNAs, we designated this transcript as lncRNA A2ML2. No previous studies have reported a link between lncRNA A2ml2 and FLHS. Notably, our findings demonstrate that lncRNA A2ml2 attenuates lipid accumulation and suppressed FLHS progression by promoting *LPL* expression.

Fatty acid and lipid synthesis are tightly regulated through transcriptional control of adipogenic genes (Wang, et al., 2015). *LPL* is a central enzyme in lipid metabolism and adipose biology of animals and mainly expressed in liver tissue and adipose tissue. It is primarily responsible for the breakdown of triglycerides in very low-density lipoproteins and chylomicrons (Augustus, et al., 2004; Kersten, 2021; Mead, et al., 2002). Research has shown that inhibiting the *LPL/FABP4/CPT1* axis can effectively delay tumor growth in mice and decrease liver cancer stem cell proliferation (Yang, et al., 2021). Emerging evidence positions *LPL* as a promising therapeutic target for non-alcoholic fatty liver disease (Li, et al., 2023). This study found a positive correlation between *LPL* and lncRNA A2ml2 expression in the Jingxing-Huang chicken fatty liver model, suggesting that lncRNA A2ml2 may inhibit FLHS pathological processes. The functional mechanisms of lncRNAs depend on their cell type-specific expression and subcellular localization (nuclear or cytosolic), as previously characterized (Bridges, et al., 2021). FISH revealed dual nuclear and cytoplasmic localization of A2ml2 in LMH cells, implying its involvement in post-transcriptional regulation. Dual luciferase assay revealed that miR-143-5p binds to both lncRNA A2ml2 and *LPL*. Moreover, expression of lncRNA A2ml2 and miR-143-5p was negatively correlated in fatty

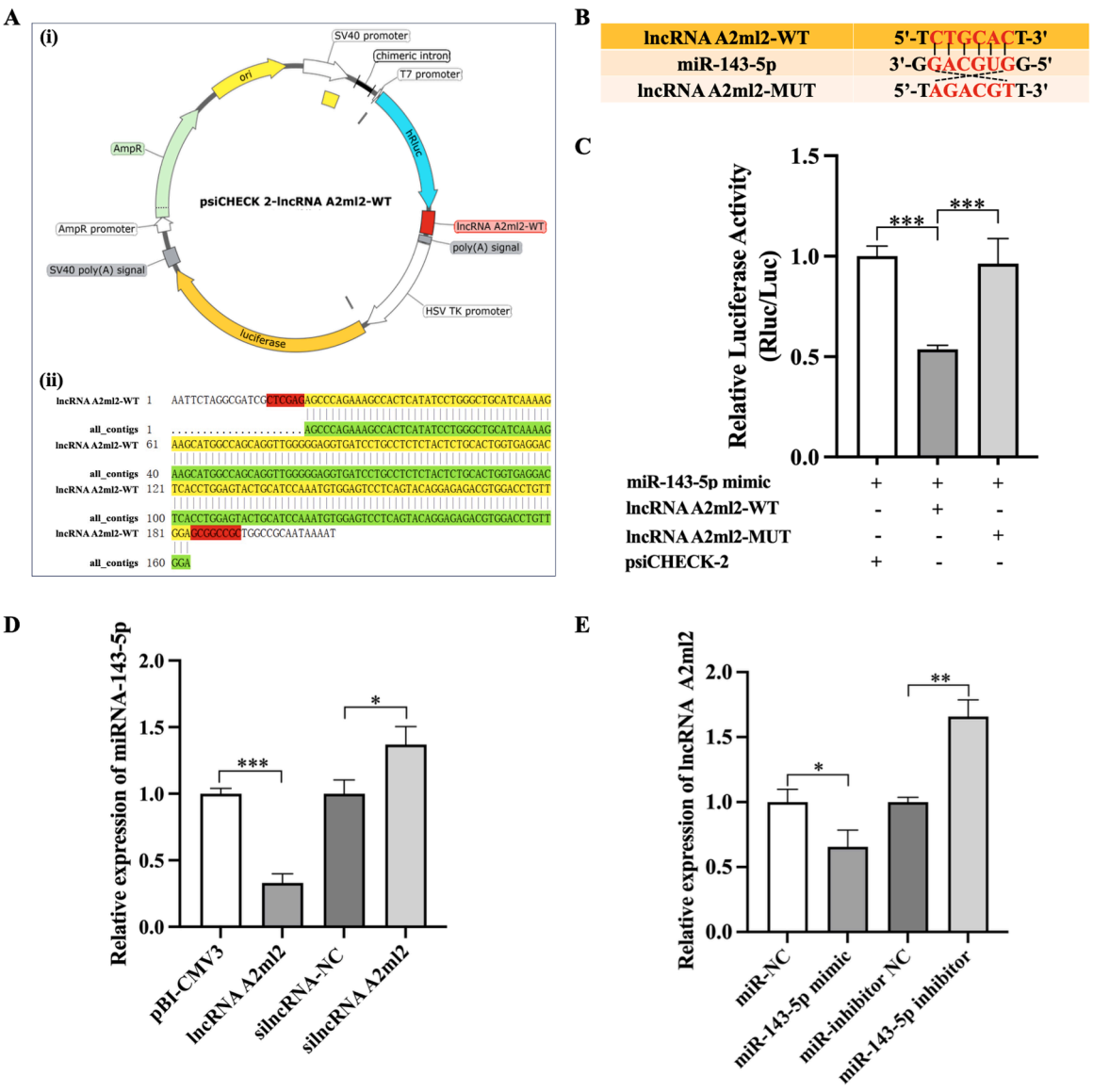

Fig. 5. lncRNA A2ml2 is targeted and negatively mediated by miR-143-5p. (**A**) The plasmid profile of the constructed dual-luciferase reporter vector psiCHECK-2-lncRNA A2ml2. (i: Schematic of lncRNA A2ml2-WT inserted into psiCHEC-2 vector. ii: Verification of the psiCHECK-2-lncRNA A2ml2-WT vector by sequencing.). (**B**) The binding site between lncRNA A2ml2 and miR-143-5p. (**C**) A Dual luciferase reporter assay validated the binding of miR-143-5p with lncRNA A2ml2-WT. ("+" means added, "−" means not added.) (**D**) The expression level of miR-143-5p in lncRNA A2ml2-knockdown and overexpression was validated by RT-qPCR. (**E**) The expression level of lncRNA A2ml2 in miR-143-5p mimic and miR-143-5p inhibitor was validated by RT-qPCR.

liver tissues, and positively correlated with *LPL*. Some lncRNAs (such as lncRNA-TCONS_00026907, ZEB2-AS1, and LINC01207) enhance the invasiveness of cancer cells by inhibiting the expression of miR-143-5p (Sanada, et al., 2019). This expression pattern largely mimicked that of the conclusion of the study.

Recently studies have shown that miR-143 is involved in lipid metabolism (Wang, et al., 2011). It has been reported that miR-143 affected adipogenesis by regulating MAP2K5-ERK pathways. Notably, miR-143-5p (a member of the miR-143 family), was significantly upregulated in fatty liver tissue within this study, suggesting a potential association between FLHS and miR-143-5p. Mechanistically, miR-143-5p exerts its functions by modulating downstream effector genes. Importantly, this study is the first to identify direct binding between miR-143-5p and the 3′-UTR of *LPL* in LMH cells. It has been confirmed that *LPL* plays an important role in maintaining glycolipid metabolism and energy balance. For example, overexpression of *LPL* in the liver of high-fat diet-fed mice reduced lipid droplet accumulation in the liver and improved glucose metabolism (Shimizu, et al., 2022). We further demonstrated that miR-143-5p mimic and inhibitor not only engage in target binding but also negatively regulate the expression of *LPL*. Moreover, *LPL* expression level was inversely correlated with intracellular triglyceride content and lipid droplet accumulation in LMH cells. Consequently, these findings suggest that miR-143-5p promotes lipid deposition in LMH cells by repressing *LPL* expression.

With the in-depth study of lncRNA and miRNA, the role of lncRNA as a molecular sponge has been revealed, they alleviate the inhibitory effect of miRNA on target genes by adsorbing miRNA. For instance, lncRNA Tug1 acts as a ceRNA, releasing *SELENOF* and activating the IRS/AKT pathway by sequestering miR-1934-3p, which decreases hepatic steatosis and glycolysis (Wang, et al., 2023). Moreover, studies have demonstrated that lncRNA Gm15232 is significantly upregulated in the epididymal white adipose tissue of aging mice. This upregulation suppresses miR-192-3p, resulting in increased glucocorticoid receptor gene expression and enhanced fat synthesis (Hu, et al., 2024). In this study, co-transfection experiments revealed that overexpression of lncRNA-A2ml2 led to the upregulation of *LPL* expression, accompanied

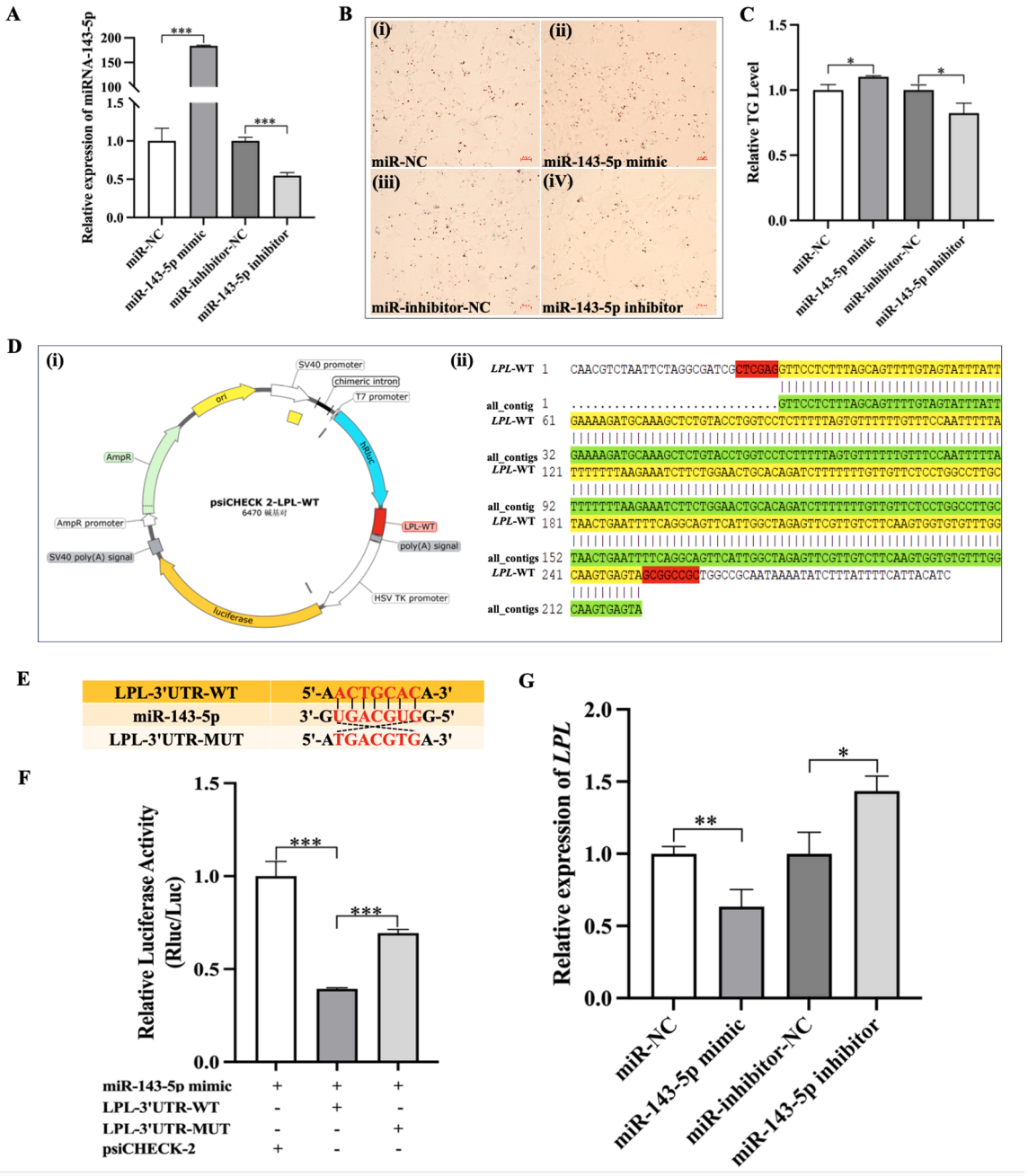

Fig. 6. miR-143-5p promotes TG and lipid droplet contents in LMH cells by suppressing *LPL* expression. (**A**) Evaluation of miR-143-5p expression in LMH cells transfected with miR-143-5p mimic and miR-143-5p inhibitor by RT-qPCR. (**B**) representative Oil red O staining (Scale bars = 50 μm). (i: representative Oil red O staining from miR-NC groups; ii: representative Oil red O staining from miR-143-5p mimic groups; iii: representative Oil red O staining from miR inhibitor NC groups; iv: representative Oil red O staining from miR-143-5p inhibitor groups.) (**C**) Triglycerides level in LMH cells transfected with miR-143-5p mimic, miR-143-5p inhibitor and miR-NC, miR-inhibitor. (**D**) The plasmid profile of the constructed dual-luciferase reporter vector psiCHECK-2-*LPL*. (i: Schematic of *LPL*-3′UTR -WT inserted into psiCHEC-2 vector. ii: Verification of the psiCHECK-2-*LPL*-WT vector by sequencing.). (**E**) The binding site between miR-143-5p and *LPL* 3′UTR. (**F**) A Dual luciferase reporter assay validated the binding of *LPL* WT with miR-143-5p. ("+" means added, "−" means not added.). (**G**) The expression level of *LPL* in miR-143-5p mimic and miR-143-5p inhibitor was validated by RT-qPCR. * Represent $P < 0.05$; ** represent $P < 0.01$; *** represent $P < 0.001$.

by reductions in lipid droplet content and triglyceride levels. Conversely, miR-143-5p was found to counteract the effects of lncRNA-A2ml2, leading to a decrease in *LPL* expression and an increase in both lipid droplet content and triglyceride production. Notably, mutation of miR-143-5p binding site abolished, its antagonistic effect, resulting in decreased *LPL* expression and subsequent decreases in lipid droplet content and triglyceride levels. Furthermore, prior studies have shown that lncRNA DAPK1-IT1 enhances *LPL* expression by modulating miR-590-3p, thereby modulating cholesterol metabolism and inflammation in macrophages in vitro (Zhen, et al., 2019). These findings are consistent with our own results. The findings conclusively illustrate that lncRNA A2ml2 as a molecular sponge by binding to miR-143-5p, thereby attenuating its inhibitory impact on *LPL* and ultimately modulating lipid accumulation in LMH cells.

Conclusion

In conclusion, this study identified the lncRNA A2ml2 as a novel

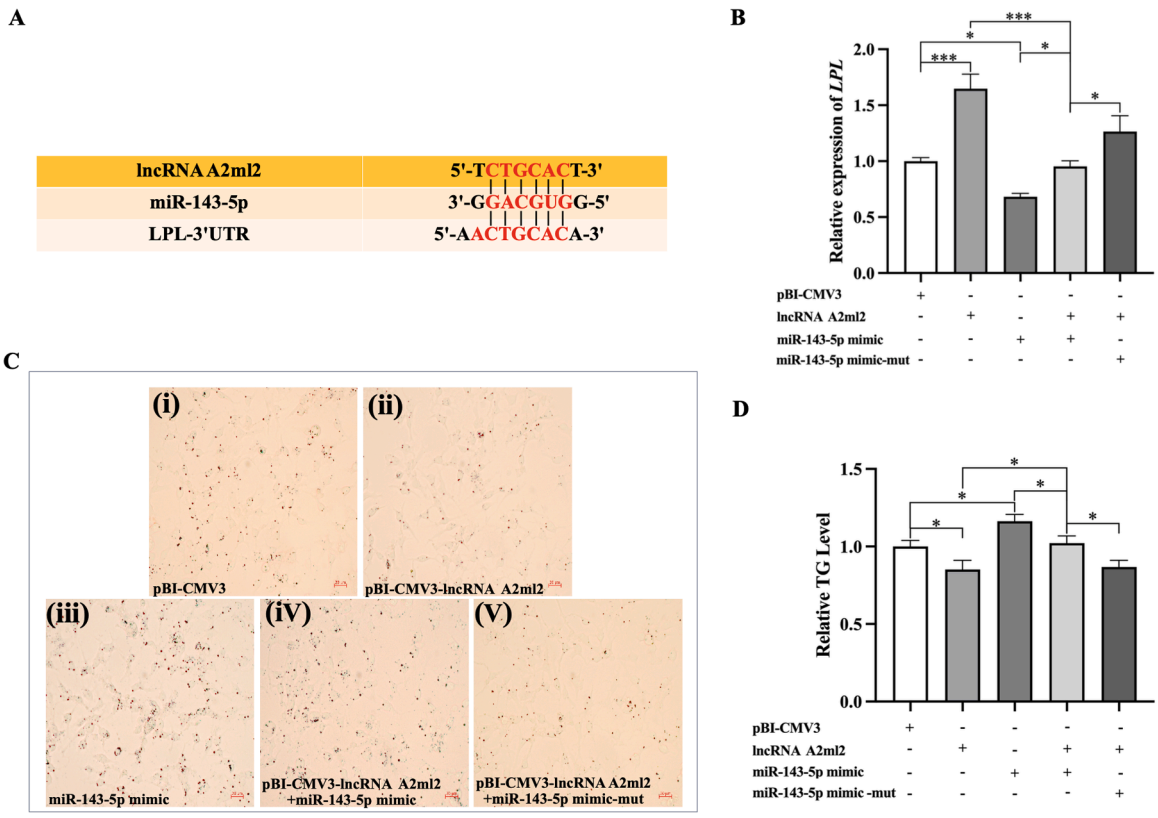

Fig. 7. lncRNA A2ml2 regulates *LPL* through binding with miR-143-5p in LMH cells. (**A**) The binding site among lncRNA A2ml2, miR-143-5p and *LPL* 3′UTR. (**B**) The expression levels of *LPL* in LMH cells co-transfected with the lncRNA A2ml2 and miR-143-5p by RT-qPCR. (**C**) Representative Oil red O staining in LMH cells co-transfected with the lncRNA A2ml2 and miR-143-5p. (Scale bars = 50 μm). (i: representative Oil red O staining from pBI-CMV3 group; ii: representative Oil red O staining from pBI-CMV3-lncRNA A2ml2 group; iii: representative Oil red O staining from miR-143-5p mimic group; iv: representative Oil red O staining from miR-143-5p mimic and pBI-CMV3-lncRNA A2ml2 group; v: representative Oil red O staining from miR-143-5p mimic mut and pBI-CMV3-lncRNA A2ml2 group.). (**D**) Triglycerides level in LMH cells transfected with miR-143-5p mimic, miR-143-5p mimic mut and pBI-CMV3-lncRNA A2ml2, respectively. * Represent $P < 0.05$; ** represent $P < 0.01$; *** represent $P < 0.001$.

regulator modulating hepatic lipid metabolism in chickens, demonstrating its inhibitory effect on lipid accumulation in LMH cells. Our findings provide definitive evidence for the regulatory mechanism of ceRNA in chicken liver cells and elucidate the pivotal role of the lncRNA A2ml2/miR-143-5p/*LPL* axis in lipid accumulation within LMH cells. Epigenetically, this work advances mechanistic understanding of fatty liver pathogenesis by delineating lncRNA-mediated regulatory circuitry, providing unprecedented resolution of lipid metabolic networks in poultry hepatic models. More importantly, our discovery positions lncRNA A2ml2 and miR-143-5p as dual therapeutic targets for fatty liver intervention, while simultaneously providing molecular markers for precision breeding strategies to augment hepatic disease resilience in poultry.

Disclosures

The authors declare that they have no competing interests.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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