Functional and miRNA regulatory characteristics of *INSIG* genes highlight the key role of lipid synthesis in the liver of chicken (*Gallus gallus*)

Yaxin Yue,[*,1] Ziming Liu,[*,1] Ke Zhang,[*] Qihui Jia,[*] Dandan Wang,[*] Zhang Wang,[*] Yulong Guo,[*] Donghua Li,[*,†,‡] Ruirui Jiang,[*,†,‡] Zhuanjian Li,[*,†,‡] Yadong Tian,[*,†,‡] Xiangtao Kang,[*,†,‡] Xiaojun Liu ,[*,†,‡] and Hong Li[*,†,‡,2]

[*]*College of Animal Science and Technology, Henan Agricultural University, Zhengzhou, 450046, China;* [†]*Henan Key Laboratory for Innovation and Utilization of Chicken Germplasm Resources, Zhengzhou, 450046, China; and* [‡]*International Joint Research Laboratory for Poultry Breeding of Henan, Zhengzhou, 450046, China*

ABSTRACT The insulin-induced genes (*INSIG1* and *INSIG2*) have been demonstrated to play a vital role in regulating lipid metabolism in mammals, however the function and regulation mechanism of them remains unknown in poultry. In this study, firstly the phylogenetic trees of *INSIGs* among various species were constructed and their subcellular locations were mapped in chicken LMH. Then the spatiotemporal expression profiles, over-expression and knockdown assays of chicken *INSIGs* were conducted. Furthermore, conservation of potential miRNA binding sites in *INSIGs* among species were analyzed, and the miRNA biological function and regulatory role were verified. The results showed that chicken *INSIGs* located in cellular endoplasmic reticulum, and were originated from the common ancestors of their mammalian counterparts. The *INSIGs* were widely expressed in all detected tissues, and their expression levels in the liver of chicken at 30 wk were significantly higher than that at 20 wk ($P < 0.01$). Over-expression of *INSIGs* led no significant increase in mRNA abundance of lipid metabolism-related genes and the contents of triacylglycerol (**TG**) and cholesterol (**TC**) in LMH cells. Knockdown of *INSIG1* led to the decreased expressions of *ACSL1, MTTP-L, ApoB, ApoVLDLII* genes and TG, TC contents ($P < 0.05$). Knockdown of *INSIG*2 could significantly decrease the contents of TG and TC, and expressions of key genes related to the lipid metabolism ($P < 0.05$). Moreover, *INSIG1* was directly targeted by both miR-130b-3p and miR-218-5p, and *INSIG2* was directly targeted by miR-130b-3p. MiR-130b-3p mimic and miR-218-5p mimic treatment could significant decrease the mRNA and protein levels of *INSIG*s, mRNA levels of genes related to lipid metabolism, and the contents of TG and TC in LMH cells. The inhibition of miR-130b-3p and miR-218-5p on TG and TC contents could be restored by the overexpression of *INSIGs*, respectively. No significant alteration in expressions of sterol regulatory element binding protein (***SREBPs***) and SREBP cleavage-activating protein (***SCAP***) were observed when *INSIG*s were over-expressed. *SCAP* was down-regulated when *INSIG1* was knocked down, while *SREBP1* was down-regulated when *INSIG2* was knocked down. Taken together, these results highlight the role of *INSIG1* and *INSIG2* in lipid metabolism and their regulatory mechanism in chicken.

Key words: chicken, *INSIG*, miRNA, lipid metabolism, liver

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INTRODUCTION

In chicken, the main metabolic organ for lipids is the liver, where more than 90% of the de novo fatty acids are synthesized (Ding et al., 2015). Especially during the laying period, lipid metabolism is much more prosperous in the liver of hens. A large amount of triglyceride, cholesterol, cholesterol esters, phospholipids, and free fatty acids are synthesized and mainly assembled into very-low-density lipoprotein (**VLDL**) and vitellogenin (**VTG**), and secreted into the bloodstream to transport to the developing oocytes and eventually form egg yolk (Schneider, 2016). Although the regulatory mechanism of lipid metabolism in the liver of chicken has received great attention, no clear conclusion has been made on this issue so far.

The insulin-induced gene (***INSIG***) including *INSIG1* and *INSIG2* were discovered to be involved in

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[1]These authors contributed to this research work equally.

[2]Corresponding author: lihong19871202@163.com

cholesterol metabolism (Goldstein and Brown, 1990). Previous study demonstrated that the double knockout of *INSIG1* and *INSIG2* in mice resulted in over-accumulation of cholesterol and triglycerides in the liver (Engelking et al., 2005). As the sensors and mediators, INSIGs control the cholesterol homeostasis via binding to SCAP and HMG-CoA reductase in various tissues including adipose tissue, liver and the cultured animal cells (Dong et al., 2012). In situations where there is sufficient ER cholesterol, INSIGs bind to SCAP and prevent the SCAP-SREBPs complex from moving to the Golgi apparatus (Yabe et al., 2002; Yang et al., 2002). When cholesterol is depleted, INSIGs dissociate from SCAP, thereby allowing SCAP-SREBP compounds to move to the Golgi, and SREBP is sequentially processed in the Golgi apparatus by site-1 protein (**S1P**) and site-2 protein (**S2P**) proteases, which eventually hydrolyze to release the active transcription factor and thereby stimulate the synthesis of lipids. After dissociating from SCAP, INSIG1 is ubiquitinated and degraded (Engelking et al., 2004). It is thus clear that INSIGs play important roles in the regulation of intracellular lipid metabolism in mammals. However, functional characteristics of *INSIG* genes are still elusive in chicken.

MicroRNAs (**miRNA**) are a kind of small, endogenous, noncoding single-stranded RNA with a length of 19-25nt, that regulates gene expression at the post-transcription level (Lu et al., 2008). Many studies have shown that miRNAs are involved in lipid metabolism, especially in regulating the INSIGs-SCAP-SREBPs signaling pathway. For example, miR-145 can target *INSIG1* to affect fatty acid synthesis in goat breast cells (Wang et al., 2017). The miR-29 family members, miR-29a, -29b, and -29c can repress the expression of *SCAP* and *SREBP-1* thereby mediating a previously unrecognized negative feedback loop in SCAP/SREBP-1 signaling to control lipid metabolism (Ru and Guo, 2017). Whether *INSIGs* are regulated at post-transcription by miRNAs in chicken is yet unknown.

Our previous study on liver transcriptome showed that *INSIG1* and *INSIG2* were significantly up-regulated in liver of peak-laying (30-wk-old) hens in comparison to pre-laying (20-wk-old) hens (Li et al., 2015), implied that they might play important roles in lipid metabolism of chicken liver. To investigate the function and regulatory mechanism of *INSIGs* in chicken, we first analyzed the evolutionary relationship of INSIGs among different species, and explored the biological functions of *INSIG*s in lipid metabolism of chicken liver, and further studied their post-transcriptional regulatory mechanism mediated by miRNA. Our results for the first time provided new insights into the role of *INSIGs* for lipid metabolism in chicken.

MATERIALS AND METHODS

Ethics Statement

All the animal experiments were approved by the Animal Care Committee of the College of Animal Science and Technology, Henan Agricultural University, and were performed following the protocol approved by the Institutional Animal Care and Use Committee (IACUC) of China. All efforts were made to minimize animal suffering.

Animals and Sampling

Lushi blue-shelled-egg hens at the age of 20-wk-old (20 wk, n = 6) and 30-wk-old (30 wk, n = 6) were obtained from the Poultry Germplasm Resource Farm of Henan Agricultural University. Chickens were euthanized and tissues including liver, spleen, lung, kidney, duodenum, and heart were collected, snap-frozen in liquid nitrogen and then stored at −80°C until use.

Bioinformatics Analysis of INSIG1 and INSIG2

All amino acid sequences of INSIG1 and INSIG2 from chicken and other 23 species were retrieved from the NCBI protein database (Tables S1 & S2). The phylogenetic tree was constructed using the Maximum likelihood method (**ML**) of MEGA 7.0 software based on the alignment of INSIG1 and INSIG2 protein sequences using the Jones-Taylor-Thornton with Freqs (JTT +F) model. The reliability of the tree was assessed using 1,000 bootstrap replicates. Number at each branch represents bootstrap support value given as percentages. Meanwhile, the protein functional domains of INSIG1 and INSIG2 in chicken were predicted using SMART program (http://smart.embl.de/).

Prediction of miRNAs Interacted with INSIG1 and INSIG2

To estimate whether *INSIG*1 or *INSIG*2 was regulated by miRNA, online software TargetScan, miRDB and micro-CDS were used to predict the potential miRNA that binding in the 3'UTR of *INSIG1* and *INSIG2*. The potential miRNAs were obtained basing on the intersection of the prediction results obtained from different online softwares. Then, the potential target miRNAs which were significantly down-regulated (30 wk/20 wk) in our previous miRNA-seq database of liver were selected for further study (Li et al., 2016). The VENNY software (https://bioinfogp.cnb.csic.es/tools/venny/index.html) was used to draw the Wayne diagram.

Recombination Vector Construction and siRNA Oligonucleotide Synthesis

To explore the biological function of chicken *INSIG*1 and *INSIG*2, the overexpression vectors were constructed. The coding sequences of *INSIG*1 and *INSIG*2 with Hind *III* and *SacII* restriction enzyme sites were obtainedby PCR. The PCR products and pcDNA3.1-

EGFP vector (Invitrogen, Carlsbad, CA) were doubled-digested by *HindIII* and *SacII*, and ligated with T4 DNA ligase (NEB, Beijing, China). The primers for pcDNA3.1-EGFP-*INSIGs* vetors construction were listed in Table S3. The two recombinant vectors were named pcDNA3.1-EGFP-*INSIG1* and pcDNA3.1-EGFP-*INSIG2*, respectively. The overexpression efficiency of the recombination plasmids were evaluated through the activity of EGFP (Figure S1 A-C). MiRNA mimics, negative control and the small interfering RNAs (**siRNA**s) targeting *INSIG1, INSIG2* and *SCAP* were synthesized by GenePharma. (Shanghai, China). All siRNA sequences are presented in Table S3.

To identify whether miR-130b-3p, miR-218-5p target *INSIG1* and *INSIG2*, the 3' UTR region of *INSIG1* gene containing the miR-130b-3p binding site was cloned by PCR and ligated into the *XhoI* and *NotI* sites of psi-CHECK2 vector (Promega, Madison, WI), named psi-CHECK2-INSIG1-miR-130b-3p-WT. Meanwhile, the 3'UTR fragment including the mutant-type binding site of *INSIG1* was amplified by overlap-PCR, and cloned into the digested psi-CHECK2 vector at *XhoI* and *NotI* sites, named psiCHECK2-INSIG1-miR-130b-3p-MUT. Similarly, the wild-type and mutant-type vectors of miR-218-5p binding site in 3'UTR of *INSIG1*, and miR-130b-3p binding sites in *INSIG*2 were constructed and named psiCHECK2-INSIG1-miR-218-5p-WT, psiCHECK2-INSIG1-miR-218-5p-MUT, psiCHECK2-INSIG2-miR-130b-3p-site1-WT, psiCHECK2-INSIG2-miR-130b-3p-site1-MUT, psiCHECK2-INSIG2-miR-130b-3p-site2-WT, psiCHECK2-INSIG2-miR-130b-3p-site2-MUT, respectively. The recombination plasmids were verified by sequencing (Figure S2 A−D). The primers for wild-type and mutant vector construction were listed in Table S4.

LMH Cell Culture and Treatment

LMH cells were cultured in Dulbecco's modified Eagle's medium F12 (DMEM-F12) (Gibco, Southfield, MI) supplemented with 10% fetal bovine serum (**FBS**) (Gibco) and 2% penicillin-streptomycin (Gibco), and were maintained at 37°C in a humidified 5% CO_2 incubator. When the cell confluence reached to 70% in 6-well culture plates, the *INSIGs* siRNAs or overexpression plasmid of pcDNA3.1-EGFP-*INSIG1* or pcDNA3.1-EGFP-*INSIG2* were transfected into the cells by using lipofectamine 2000 (Invitrogen, Carlsbad, CA), respectively. Si-NC and pcDNA3.1-EGFP were serviced as controls, respectively. The cells were collected to analyze the gene expression or detect the TG and TC contents after transfection for 24 h. MiR-130b-3p, miR-218-5p mimics and negative control (**NC**) were transfected into the cells using Lipofectamine 2000, respectively. The cells were harvested to evaluate the gene expression or the TG and TC contents after transfection for 24 h. Each transfection contained three replicates. Each experiment was repeated at least three times.

In addition, pcDNA3.1-INSIGs were co-transfected with miR-130b-3p and miR-218-5p mimics as the treatment groups, while pcDNA3.1-INSIGs with NC mimics and miR-130b-3p or miR-218-5p mimics with pcDNA3.1 were co-transfected as the control groups for WB test and phenotype rescue test, respectively. LMH cells were treated according to the above experimental methods.

Subcellular Localization of Chicken INSIG1 and INSIG2

The endoplasmic reticulum (**ER**)-tagged proteins ER-dsred-λ (Thermo Fisher Scientific, Shanghai, China) was used to locate the ER. The overexpression vectors of *INSIG1* or *INSIG2* was co-transfected with ER-dsred-λ into the LMH cells, respectively. The cell culture solution was discarded and the cells were soaked in PBS after transfection for 24 h. The cells were fixed by adding 4% paraformaldehyde for 20 min and then washed three times with PBS. The cell membrane was ruptured by adding 0.25% TritonX-100 for 5 min at room temperature and washed three times with PBS. DAPI staining was used to observe the status of cell nucleus. The cell nucleus dyestuff DAPI was added and incubated for 5 min in the dark. The cells were then soaked in PBS and washed three times. Finally, the subcellular location of INSIG1 and INSIG2 were analyzed using the laser confocal microscope (**CLSM**).

RNA Extraction, cDNA Synthesis and Quantitative Real-Time PCR (qRT-PCR)

Total RNA of tissues and cell samples were extracted using Trizol reagent (TaKaRa, Dalian, China) according to the manufacturer's protocol. The RNA concentration and integrity were determined by NanoDrop 2000 spectrophotometry (Thermo Scientific, Wilmington, DE) at a ratio of 260/280 nm and assayed by 1.5% agarose gel electrophoresis, respectively. RNA was reverse transcribed into cDNA using a Prime Script RT Reagent Kit (TaKaRa, Dalian, China), and the product was diluted to 400 ng/μL and then stored at −20°C until use. The stem-loop primers for reverse transcription of miR-130b-3p, miR-218-5p, and U6 were synthesized from GenePharma (Shanghai, China), and their sequences were listed in Table S5. The qRT-PCR was performed using an SYBR Premix Ex Taq II kit (Takara, Dalian, China) on a LightCycler 96 Real-Time PCR system (Roche, Basel, Switzerland). The reaction system was as follows: 1 μL cDNA product, 5 μL 2 × SYBR Premix Ex Taq II, 1 μL specific primer (10 μmol/L), and 3 μL deionized water. The sequences of qRT-PCR primers were listed in Table S5. The qRT-PCR amplification process for miRNA was as follows: 95°C for 3 min; 40 cycles of 95°C for 12 s, 60°C for 40 s, and 72°C for 30 s; and 10 min extension at 72°C. The qRT-PCR amplification process for mRNA was as follows: 95°C for 3 min; 35 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 20 s; and 10 min extension at 72°C. The *GAPDH* gene and

small nuclear RNA U6 were used as the internal control for mRNA and miRNAs, respectively. The relative expression levels of mRNA and miRNA were analyzed using the $2^{-\Delta\Delta CT}$ method.

Intracellular TG and TC Assay

The TG and TC contents were measured using Tissue Triglyceride Assay kit and Tissue Total Cholesterol Assay kit (Applygen, Beijing, China) respectively, according to the manufacturer's instructions. Standard curves for determination of intracellular TG and TC were generated basing on the measurements of OD550 nm of the standard at different concentrations, and standard curve for determination of total intracellular protein was generated basing on the measurements of OD595 nm of the standard at different concentrations (Figure S3 A−C).

Dual-Luciferase Reporter Assay

To verify the direct interactions of miR-130b-3p, miR-218-5p with *INSIG1*, and miR-130b-3p with *INSIG2*, the LMH cells seeded in 24-well plates were transfected with 500 ng of the wild-type or mutant-type plasmid combined with a final concentration of 80 nM miRNAs mimics or miRNAs mimics NC in serum-free medium, respectively. Transfected cells were incubated for 48 h, and collected to detect the activities of double-luciferase according to the Dual-Luciferase Reporter Assay instruction (Promega, Maddison, WI). Firefly luciferase activity served as an internal control to normalize renilla luciferase activity.

Western Blotting

LMH cells were harvested with RIPA lysis buffer (Shanghai Epizyme Biomedical Technology Co., Ltd) including the phosphatase inhibitor (Shanghai Epizyme Biomedical Technology Co., Ltd) and protease inhibitor (Shanghai Epizyme Biomedical Technology Co., Ltd) after transfection for 24 h. After centrifugation at 12,000 rpm/min for 15 min, the protein content of cell lysates was determined using a BCA protein estimation kit (Shanghai Epizyme Biomedical Technology Co., Ltd). Total protein was denatured after boiling for 5 min. Equal amounts (20 mg) of protein were loaded per lane on 10% SDS-PAGE gels, run at 120 V for 1 h. Then, the separated protein was transferred to methanol-activated polyvinylidene difluoride (**PVDF**) membranes (Millipore, Danvers, MA). The membranes were then blocked with 5% nonfat milk in 0.05% Tween-20 for 1 h and incubated with the primary antibodies of rabbit anti-INSIG1 (1:1000; 55282-1-AP, Proteintech, Wuhan, China) and mouse anti-GAPDH (1:50000; 60004-1-Ig, Proteintech, Wuhan, China) overnight at 4° C, respectively. Then, the membrane was washed with a solution of TBS supplemented with Tween-20 (TBST) 3 times (10 min/time) and incubated with secondary antibody (SA00001-1& SA00001-2, Proteintech, Wuhan, China) for 1 h at room temperature. All experiments were independently repeated three times. The band densities were quantified by densitometry (Odyssey Fc, LI-COR, Lincoln, NE). The protein level of INSIG1 was normalized to the housekeeping protein GAPDH.

Statistical Analysis

Statistical analyses of all experimental data were carried out using SPSS version 24.0 (IBM, Chicago, IL). Data was expressed as the mean ± SEM. The statistical significance of the data was determined using the t-test (unpaired, two-tailed). Values of $P < 0.05$ and $P < 0.01$ were considered as statistically significant difference and highly significant difference, respectively. Graphics were drawn using GraphPad Prism 7 software (San Diego, CA).

RESULTS

Phylogenetic Tree and Functional Domains of Chicken INSIGs

To verify the evolutionary origin of INSIG1 and INSIG2 in chicken, the amino acid sequences of INSIG1 and INSIG2 from 23 species were retrieved from the NCBI database and used to construct phylogenetic trees. The results showed that chicken INSIG1 and INSIG2 are conserved with domestic geese and turkeys in the evolution. The divergent evolutionary relationship between INSIG1 and INSIG2 among species implied that the two genes could be functionally distinct (Figure 1 A, B).

To explore the potential biological functions, the protein functional domains of INSIG1 and INSIG2 were predicted. The results showed that both chicken INSIG1 and INSIG2 have a typical INSIG functional domain. In addition, INSIG1 has one low-complexity domain and five transmembrane domains (Figure S4 A), whereas INSIG2 contains five transmembrane domains (Figure S4 B).

Subcellular Localization of Chicken INSIGs

Subcellular localizations of INSIG1 and INSIG2 were performed in LMH cells. The results indicated that both the green fluorescence spectrums emissed by fusion proteins of INSIG1 and INSIG2 were completely overlapped with the red fluorescence spectrum emissed by the endoplasmic reticulum tag protein, and formed the yellow fluorescence, suggesting that both INSIG1 and INSIG2 proteins are located in the cell endoplasmic reticulum (Figure 2).

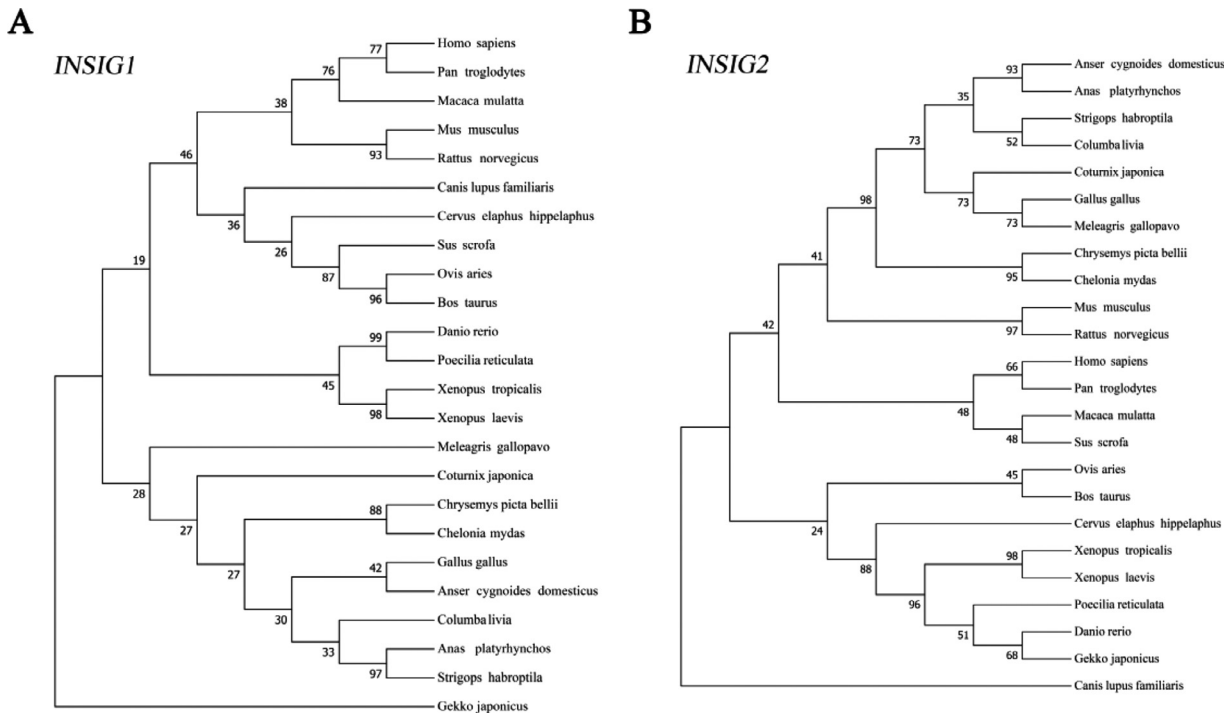

Figure 1. Phylogenetic trees of INSIG1 and INSIG2 among species. (A) The phylogenetic tree of INSIG1; (B) The phylogenetic tree of INSIG2.

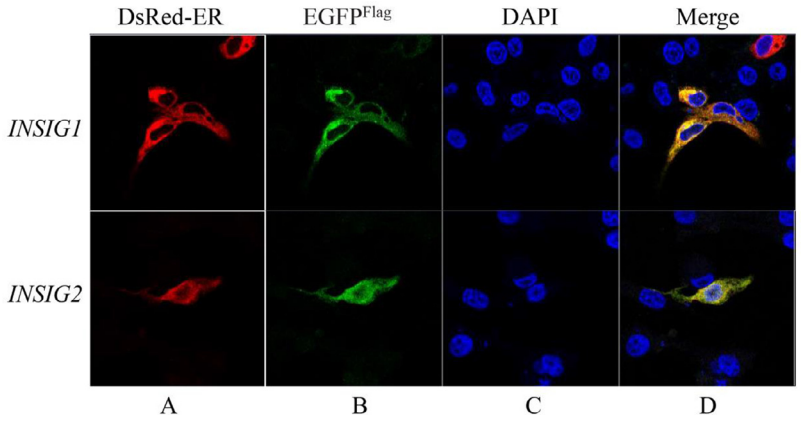

Figure 2. Subcellular localization of INSIG1 and INSIG2 protein in chicken LMH cell. (A) The endoplasmic reticulum of cell labled by dsred; (B) The locations of INSIG1 and INSIG2 proteins labeled by EGFP; (C) The cell nucleus labeled by DAPI; (D) The co-localization of the INSIGs protein, endoplasmic reticulum and nucleus.

Expression Profiles of INSIGs in Chicken

The expression patterns of *INSIG1* and *INSIG2* mRNAs in the liver, heart, spleen, lung, kidney and duodenum of chickens at 30-wk-old were analyzed using qRT-PCR. The results showed that *INSIG1* was highly expressed in liver tissue, while *INSIG2* was highly expressed in lung tissue (Figure 3 A, B). The expression levels of both *INSIG1* and *INSIG2* were significantly higher in the liver of hens at the peak-laying stage than that at the pre-laying stage ($P < 0.01$; Figure 3 C, D).

Functional Characteristics of INSIGs in TG and TC Synthesis

To explore the biology functions of INSIGs in the synthesis of TG and TC in liver of chicken, overexpression and knockdown of *INSIGs* were carried out in LMH cells, respectively. The results showed that, compared with the control group, the mRNA levels of both *INSIG1* and *INSIG2* were highly significantly increased ($P < 0.01$; Figure 4 A, B and Figure S1 A-C), but the TG and TC contents were not significantly changed in the overexpression groups ($P > 0.05$; Figure 4 C, D). The *INSIG1* overexpression significantly increased the expression levels of *SCD* and *APOB,* and repressed the expression level *SQLE*, but not significant alteration to the the expression levels of other genes related to TG syntheses, TC syntheses, and the lipid transportation ($P < 0.05$ or 0.01; Figure 4 E). Similar to the above, only the expression level of *MVD* was significantly increased in *INSIG2* overexpression group ($P < 0.01$, Figure 4 F).

The expression levels of *INSIG1* and *INSIG2* were reduced by approximately 60% when transfected with corresponding siRNAs ($P < 0.01$), respectively (Figure 5 A, B). Compared with the control group, *INSIG1*

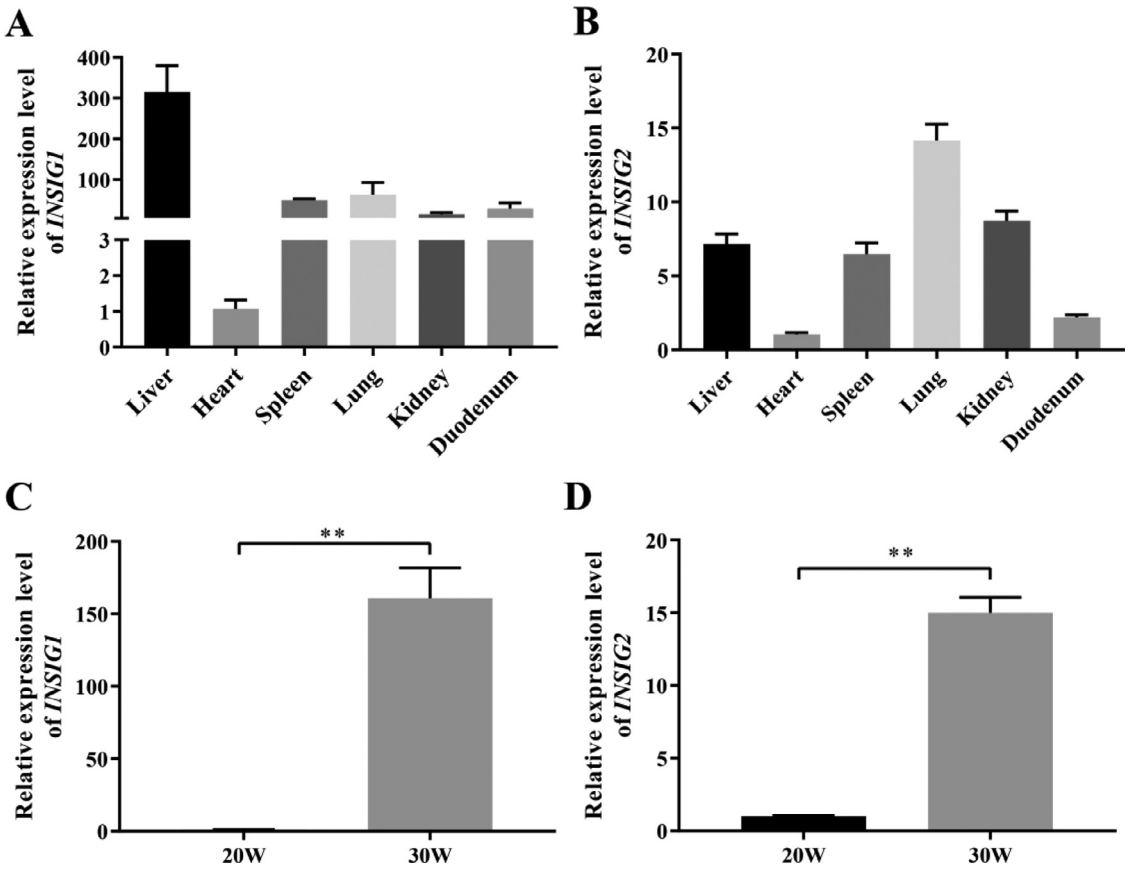

Figure 3. The spatial-expressions of *INSIG1* and *INSIG2* in different tissues and ages. (A, B) Tissue expression profiles of *INSIG1* and *INSIG2* in chicken at the age of 30wk (W); (C, D) Expression patterns of chicken *INSIG1* and *INSIG2* in chicken liver at different laying periods. β-Actin was used as the referenced gene to estimate mRNA. Data are presented as Mean± SEM (n= 6 for each group), $^{*}P< 0.05$, $^{**}P< 0.01$.

knockdown could significantly reduce the contents of TG and TC ($P < 0.01$; Figure 5 C, D), but the expression levels of genes related to TG and TC synthesis were not significantly changed except *ACSL1* ($P < 0.05$), and the expression levels of lipid transport-related genes including *MTTP-L, APOB,* and *APOVLDLII* were highly significantly reduced ($P < 0.01$; Figure 5 E). Interestingly, after *INSIG2* knockdown, the expression levels of genes related to lipid transport, TG and TC synthesis were all significantly down-regulated ($P < 0.01$; Figure 5 F).

Identification and Validation of Target miRNAs for INSIGs

Five and eleven miRNAs which potentially interact with the 3′ UTR of *INSIG1* and *INSIG2* were predicted by three online softwares, respectively (Figure 6 A, B). Integrated analysis of the predicted miRNAs with our previous mRNA (Li et al., 2015) and miRNA transcriptome databases (Li et al., 2016) showed that only the expression patterns of miR-130b-3p and miR-218-5p were opposite to that of *INSIG1* and *INSIG2* in liver of chicken at the pre- (20 wk) and peak-laying (30 wk) stages (Figure 6 C−G). The miR-130b-3p binds with *INSIG1* at one site, and with *INSIG2* at two sites. MiR-218-5p binds with *INSIG1* at one site. The binding sites of miR-130b-3p and miR-218-5p in the 3′ UTRs of *INSIG1* are highly conserved among various species (Figure 7 A−D).

To validate the interactive relationship, the plasmids that contained the wild-type or mutated binding site of miR-130b-3p and miR-218-5p in the 3′ UTRs of *INSIGs* were co-transfected with the corresponding miRNA mimics or mimics NC into LMH cells, respectively. The luciferase assays results revealed that, compared with the psiCHECK2-INSIG1-miR-130b-3p-WT and mimics NC co-transfection group, the luciferase activity in psiCHECK2-INSIG1-miR-130b-3p-WT and miR-130b-3p mimics co-transfection group was significantly decreased ($P < 0.01$), but not significantly altered in the psiCHECK2-INSIG1-miR-130b-3p-MUT and miR-130b-3p mimics co-transfection group ($P < 0.01$; Figure 7E). Compared with the psiCHECK2-INSIG1-miR-218b-5p-WT and mimics NC co-transfection group, the luciferase activity in psiCHECK2-INSIG1-miR-218b-5p-WT and miR-218b-5p mimics co-transfection group was significantly decreased ($P < 0.01$), but not significantly altered in the psiCHECK2-INSIG1-miR-218b-5p-MUT and miR-218b-5p mimics co-transfection group ($P < 0.01$; Figure 7F). Compared with the psiCHECK2-INSIG2-miR-130b-3p-site1-WT and mimics NC co-transfection group, the luciferase activity in psiCHECK2-INSIG2-miR-130b-3p-site1-WT and miR-130b-3p mimics co-transfection group was significantly decreased ($P < 0.01$), but not significantly altered in the psiCHECK2-INSIG2-miR-130b-3p-site1-MUT and miR-130b-3p

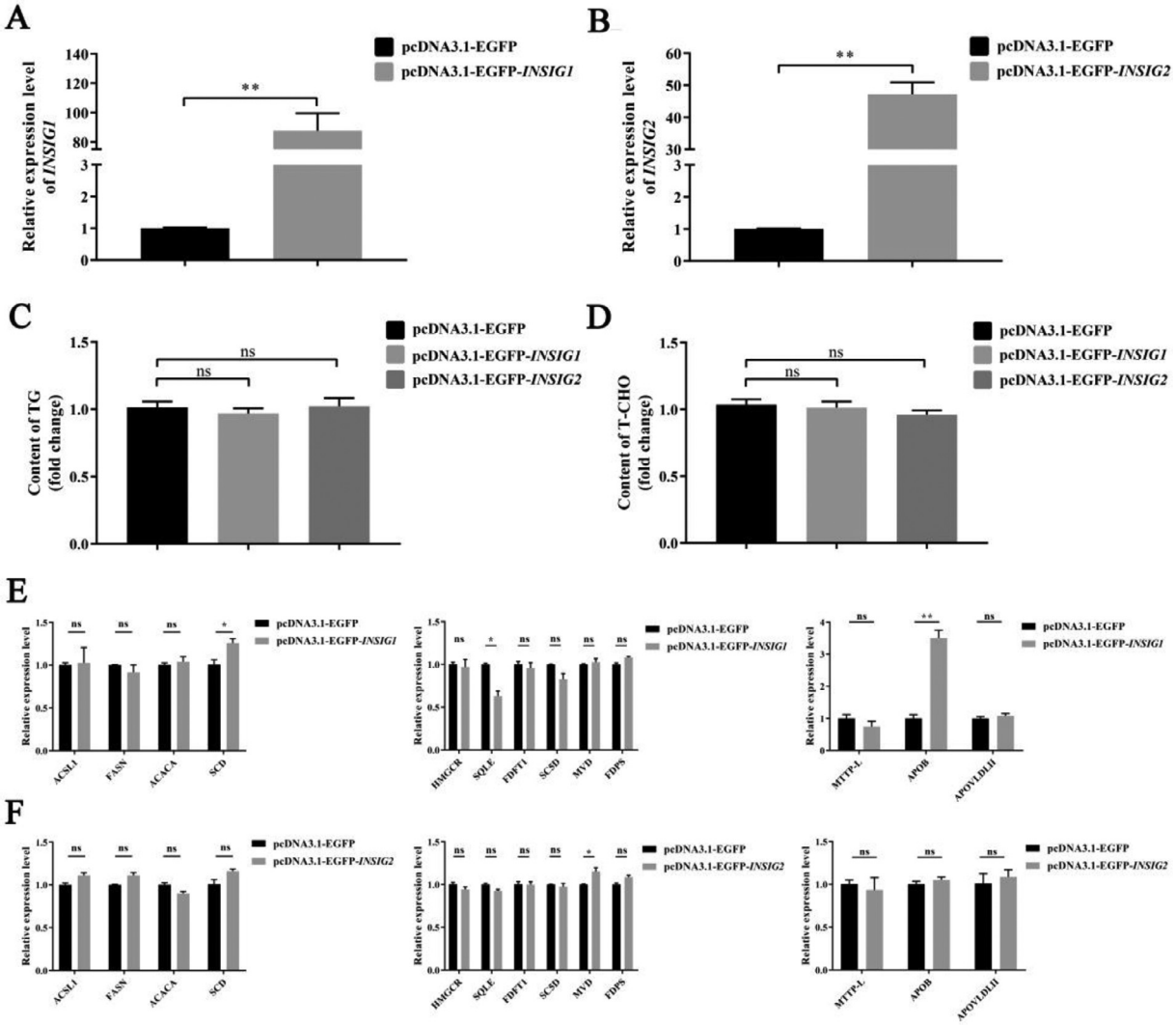

Figure 4. Effects of *INSIG1* and *INSIG2* overexpression on intracellular TG, TC contnts and relevant genes expressions in LMH cells. (A, B) Overexpression efficiencies of *INSIG1* and *INSIG2*; (C, D) Effects of *INSIGs* overexpression on intracellular TG and TC contents; (E, F) Effects of *INSIG1* and *INSIG2* overexpression on expression of genes related to triglyceride synthesis, cholesterol synthesis and lipid transport. NC: pcDNA3.1-EGFP; *INSIG1*: pcDNA3.1-EGFP-*INSIG1*; *INSIG2*: pcDNA3.1- EGFP-*INISG2*. *β-Actin* was used as the reference gene. Data are expressed as mean ± SEM (n = 3); $^{*}P < 0.05$, $^{**}P < 0.01$.and ns means $P > 0.05$.

mimics co-transfection group ($P < 0.01$; Figure 7G). The result for miR-130b-3p binding in the site 2 in 3'UTR of *INSIG2* was the same as in site 1 (Figure 7H). It demonstrated that miR-130b-3p and miR-218-5p could directly bind in 3'UTR of *INSIG1*, and miR-130b-3p could directly bind in 3'UTR of *INSIG2* at two sites.

Effect of miR-130b-3p and miR-218-5p on the Expression of INSIG1 *and* INSIG2

To explore the regulation role of miRNAs in the expression of *INSIGs*, the LMH cells were transfected with miR-130b-3p mimics and miR-218-5p mimics, respectively. Compared with the NC group, the miR-130b-3p and miR-218-5p levels were significantly increased ($P < 0.01$; Figure 8 A, B), and the expression levels of *INSIG1* and *INSIG2* were significantly down-regulated ($P < 0.01$), respectively (Figure 8 C, D). It suggested that miR-130b-3p and miR-218-5p could negatively regulate the expression of the *INSIG1* and *INSIG2* genes. Meanwhile, a marked reduction of TC and TG contents were observed in the LMH cells transfected with miR-130b-3p mimics or miR-218-5p mimics ($P < 0.05$ or 0.01; Figure 8 E, F). In addition, the expression levels of the genes related to TG biosynthesis including *ACSL1, FASN, ACACA* and *SCD*, genes related to TC biosynthesis including *HMGCR, SQLE, FDFT1, SC5D, MVD,* and *FDPS*, and genes related to lipid transport including *MTTP-L* and *APOB* were significantly repressed ($P < 0.05$ or 0.01). The expression level of *APOVLDLII* was significantly down-regulated by transfection with miR-130b-5p, but was not affected by miR-218-5p (Figure 8 G).

INSIGs Rescue the Inhibitory Effect of miR-130b-3p and miR-218-5p on TC and TG Synthesis

To validate the interactions between INSIGs and target miRNAs, rescue experiments were conducted.

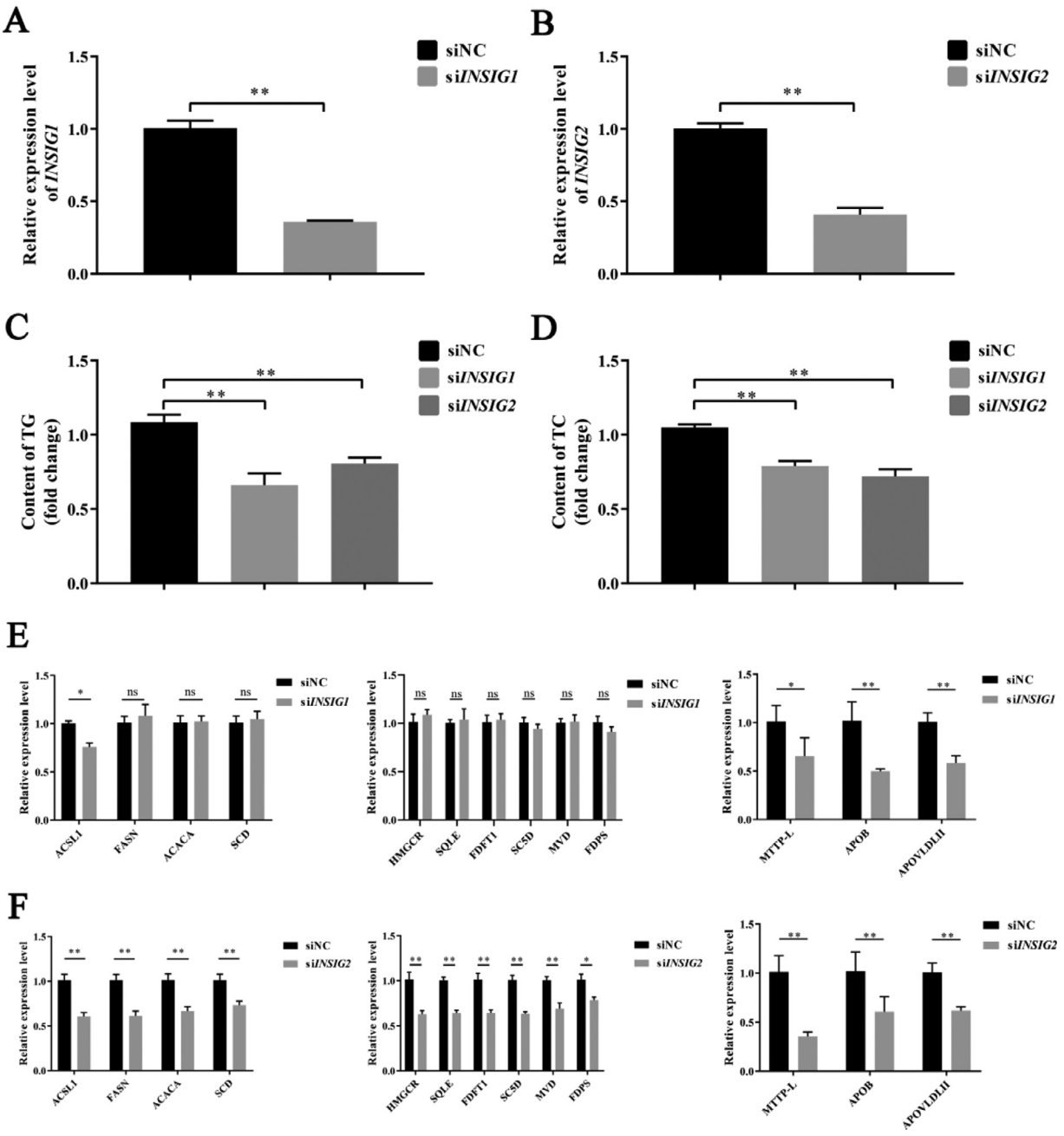

Figure 5. Effects of *INSIG1* and *INSIG2* knockdown on intracellular TG, TC contents and relevant genes expressions in LMH cells. (A, B) Knockdown efficiencies of *INSIG1* and *INSIG2*; (C, D) Effects of *INSIGs* knockdown on intracellular TG and TC contents; (E, F) Effects of *INSIG1* and *INSIG2* knockdown on expression of genes related to triglyceride synthesis, cholesterol synthesis and lipid transport. NC: siNC; *INSIG1*: si*INSIG1*; *INSIG2*: si*INISG2*. *β-Actin* was used as the reference gene. Data are expressed as mean ± SEM (n = 3);$^{*}P < 0.05$, $^{**}P < 0.01$.and ns means $P > 0.05$.

Western blot analyses showed that, compared to the INSIG1 overexpression group, the protein levels of INSIG1 in LMH cells co-transfected with either miR-130b-3p mimic or miR-218-5p mimic and overexpression plasmid of INSIG1 were significantly decreased ($P < 0.01$; Figure 9 A). Compared to the miR-130b-3p mimics or miR-218-5p mimics treated group, the contents of TG and TC in cells co-transfected with miR-130b-3p mimics or miR-218-5p mimics and corresponding *INSIGs* overexpression vector were significantly increased ($P < 0.05$ or 0.01; Figure 9 B, C).

Effects of INSIGs *on expression of* SCAP *and* SREBPs

To verify the effects of *INSIGs* on the expression of *SCAP, SREBP1* and *SREBP2* in chicken, *INSIG1* and *INSIG2* overexpression and knockdown experiments were performed. The results showed that, compared with the control group, the expression levels of *SREBPs* and *SCAP* were not significantly changed in *INSIG1* or *INSIG2* overexpression group, respectively (Figure 10 A, B). Knockdown of *INSIG1* led no significant

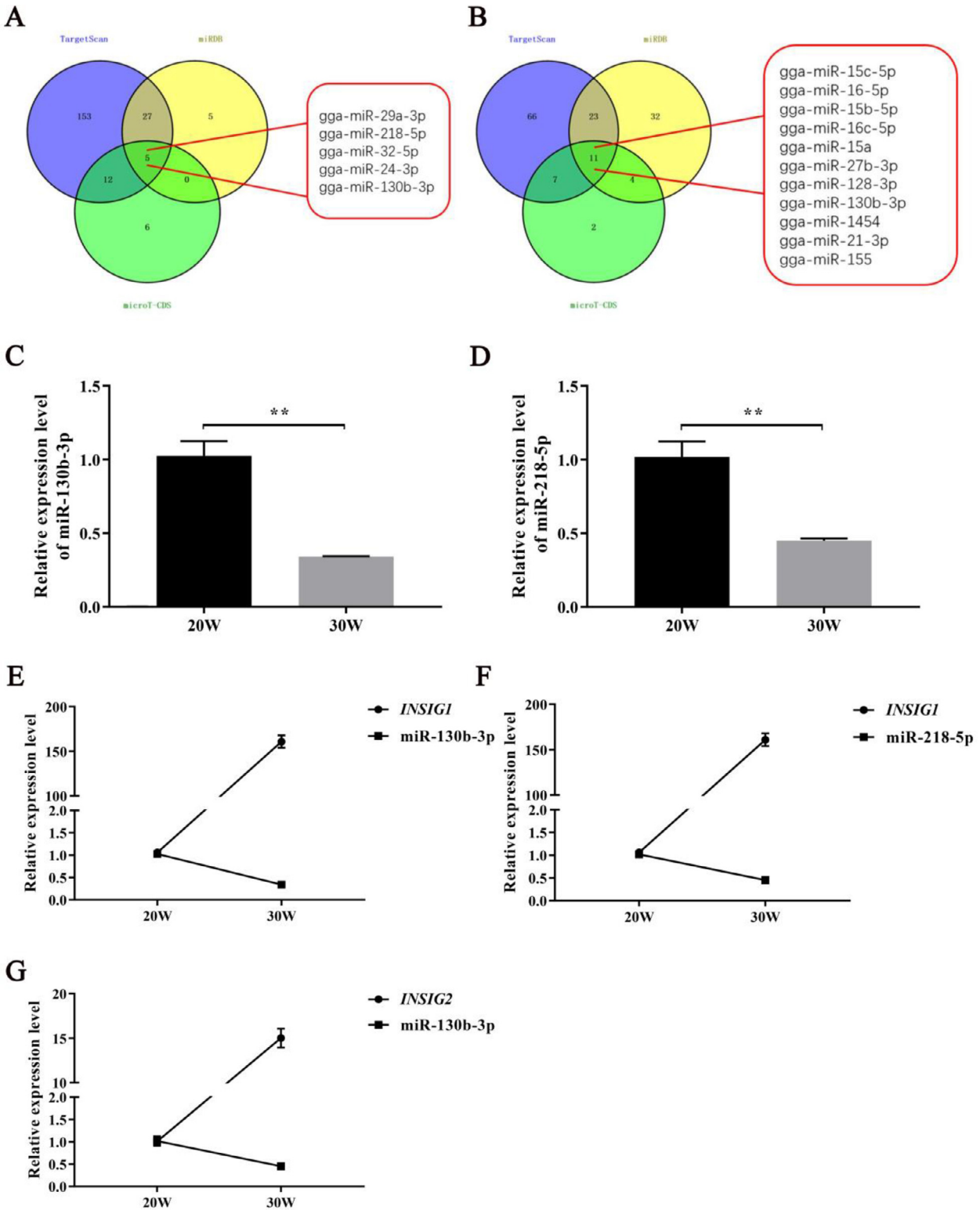

Figure 6. Screening of miRNAs bind with *INSIG1* and *INSIG2*. (A) Prediction results of *INSIG1* target miRNAs; (B) Prediction results of *INSIG2* target miRNAs; (C) The relative expression level of miR-130b-3p in laying hens at 20 wk (W) and 30 wk (W); (D) The relative expression level of miR-218-5p in laying hens at 20 wk (W) and 30 wk (W); (E, F) Comparisons of miR-130b-3p expression with *INSIG1* and *INSIG2* expression; (G) Comparison of miR-218b-5p expression and *INSIG1* expression. *β-Actin* used as the reference gene of mRNA, and U6 as the reference of miRNA. Data are expressed as mean ± SEM (n = 6); $^{**}P < 0.01$.

alteration to the expression levels of *SREBP1* and *SREBP2*, but a significant decrease in *SCAP* expression ($P < 0.05$; Figure 10 C). Knockdown of *INSIG2* led significant decrease in the expression levels of *SREBP1* and *SCAP* ($P < 0.01$), but no significant alteration to *SREBP2* (Figure 10 D).

Furthermore, we found that, compared with the NC group, the expression levels of *SCAP*, *SREBP1* and *SREBP2* genes were significantly down-regulated in miR-130b-3p mimics and in miR-218-5p mimics treated groups ($P < 0.01$; Figure 10 E–G). Meanwhile, knockdown of *SCAP* could significantly down-regulate the

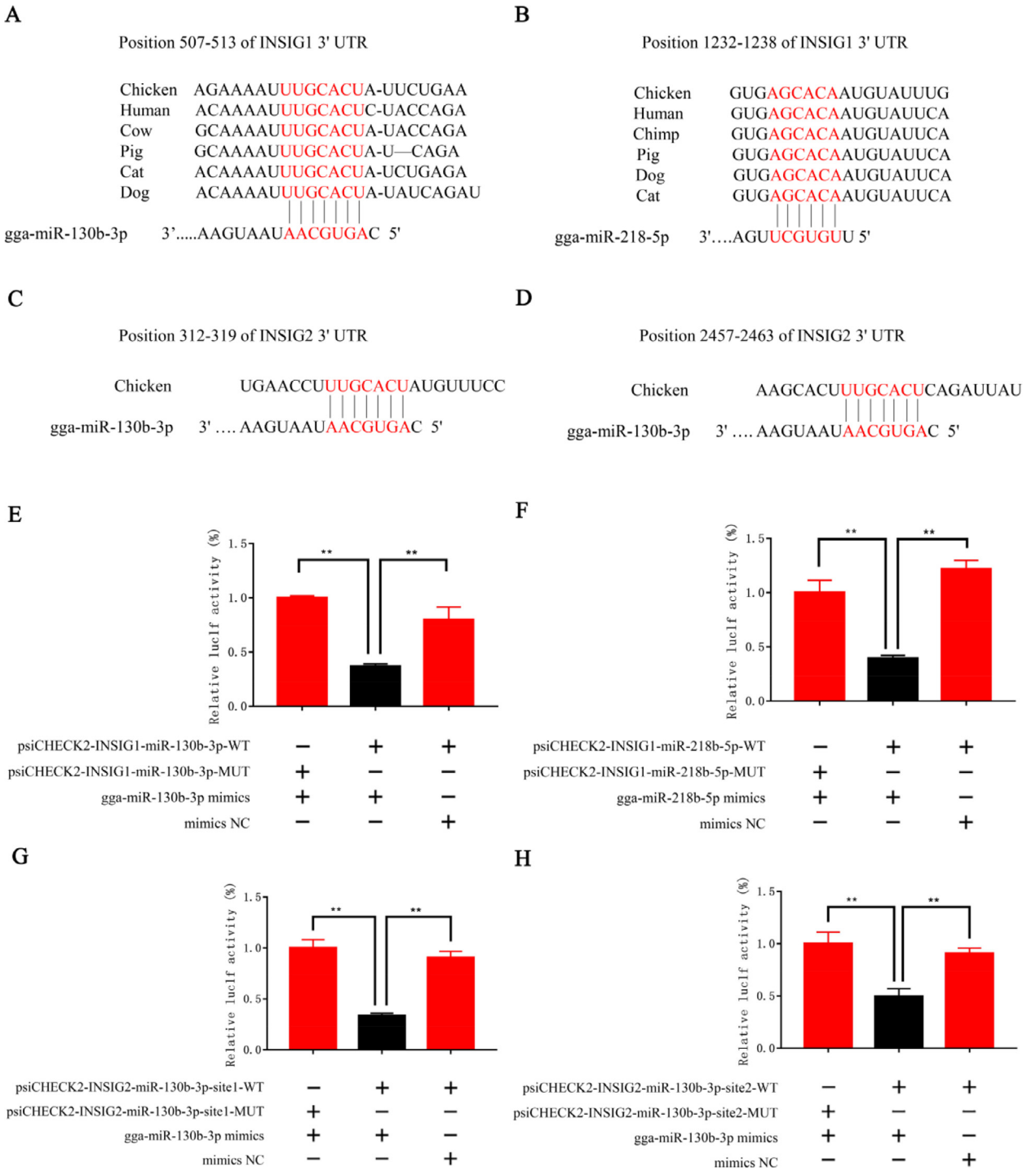

Figure 7. Validation of targeting relationship between miR-130b-3p, miR-218-5p and *INSIG1*, *INSIG2*. (A, B) Conservative analysis of miR-130b-3p and miR-218-5p binding sites in the 3'UTR of *INISG1* among species; (C, D) The potential binding site of miR-130b-3p in the 3'UTR of *INISG2*; (E) Validation of the interaction between miR-130b-3p and *INISG1* by dual-luciferase reporter system; (F) Validation of the interaction between miR-218-5p and *INISG1* by dual-luciferase reporter system; (G, H) Validation of the two interaction sites between miR-130b-3p and *INISG2* by dual-luciferase reporter system. $^{**}P < 0.01$.

expression of *INSIG1* ($P < 0.05$), but no significant difference in other genes (Figure S6).

DISCUSSION

Numerous studies on INSIGs in hepatic lipid metabolism have been reported in mammals. However, few such researches were done in chicken until now. In this study, for the first time, we systematically looked into the functional and regulatory characteristics of INSIGs, and highlighted their roles in lipid metabolism in chicken.

Evolutionary analysis showed that chicken *INSIGs* genes were originated from the early common ancestors. Spatiotemporal expression analyses indicated that the chicken *INSIG1* was highly expressed in liver, while chicken *INSIG2* was highly expressed in the lung and kidney, consistent with previous reports in mammals

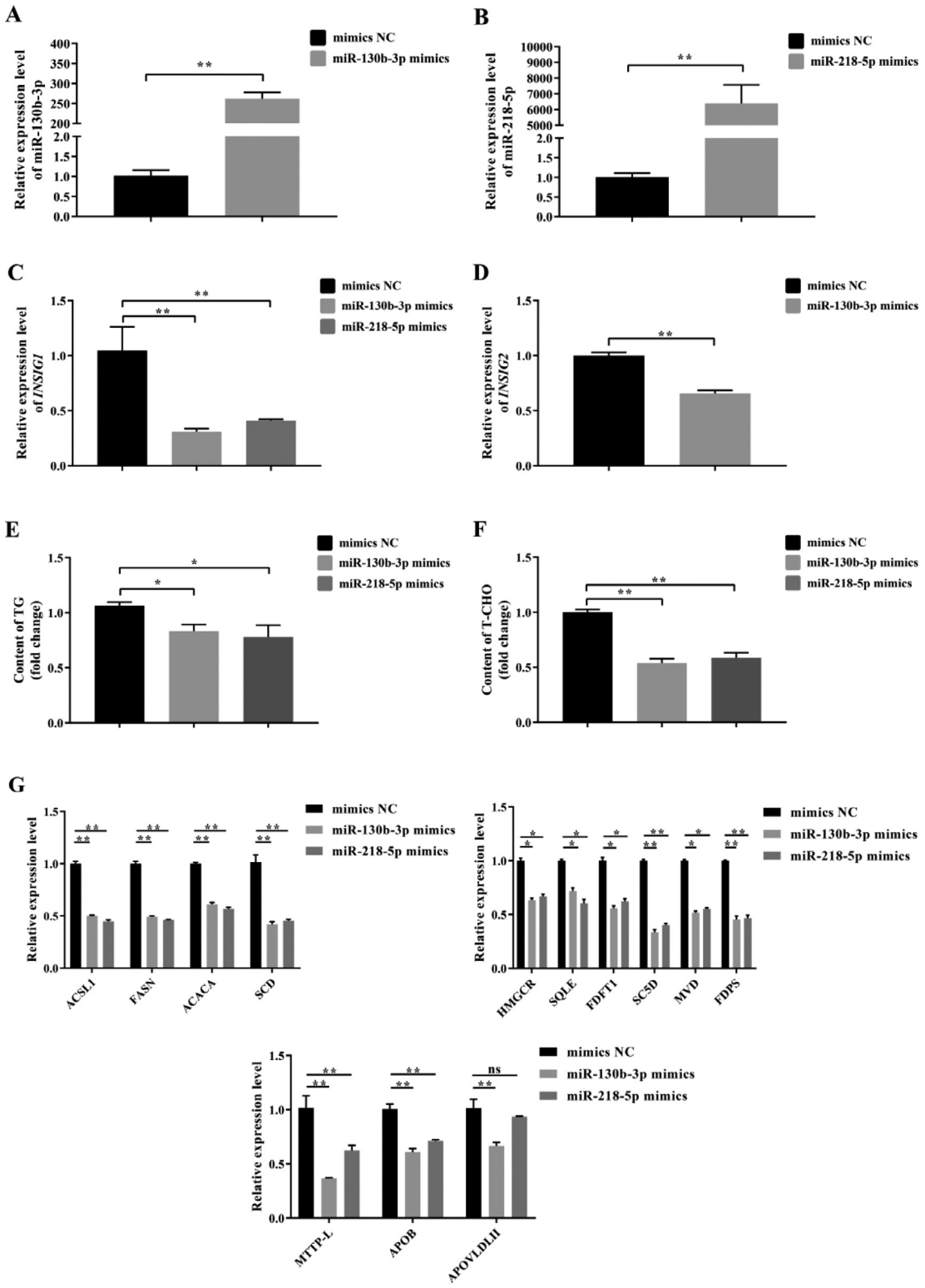

Figure 8. Effects of miR-130b-3p and miR-218-5p on expression of *INSIG1*, *INSIG2* and other relevent genes, and intracellular contents of TG and TC. (A, B) Overexpression efficiencies of miR-130b-3p and miR-218-5p in LMH; (C) Effects of miR-130b-3p and miR-218-5p overexpression on *INSIG1* gene expression; (D) Effect of miR-130b-3p overexpression on *INSIG2* gene expression; (E, F) Effects of miR-130b-3p and miR-218-5p overexpression on intracellular TG and TC contents; (G) Effects of miR-130b-3p and miR-218-5p overexpression on gene related with triglyceride, cholesterol synthesis and lipid transport. *β-Actin* used as the reference gene of mRNA, and U6 as the reference of miRNA. Data are expressed as mean ± SEM (n = 6); $^{*}P < 0.05$, $^{**}P < 0.01$.and ns means $P > 0.05$.

(Peng et al., 1997; Li et al., 2002; Krapivner et al., 2008; Hu et al., 2012; Wu et al., 2014; Wang et al., 2016a). Protein structure predication revealed that both the INSIG1 and INSIG2 proteins contain five transmembrane domains, which are the same to structures of INSIGs in buffalo (Wu et al., 2014) and goat (Wang et al., 2016b), but different to human's that contain six transmembrane domains (Feramisco et al., 2004). In addition, INSIG1 and INSIG2 have been localized to the endoplasmic reticulum (ER) of cells in both

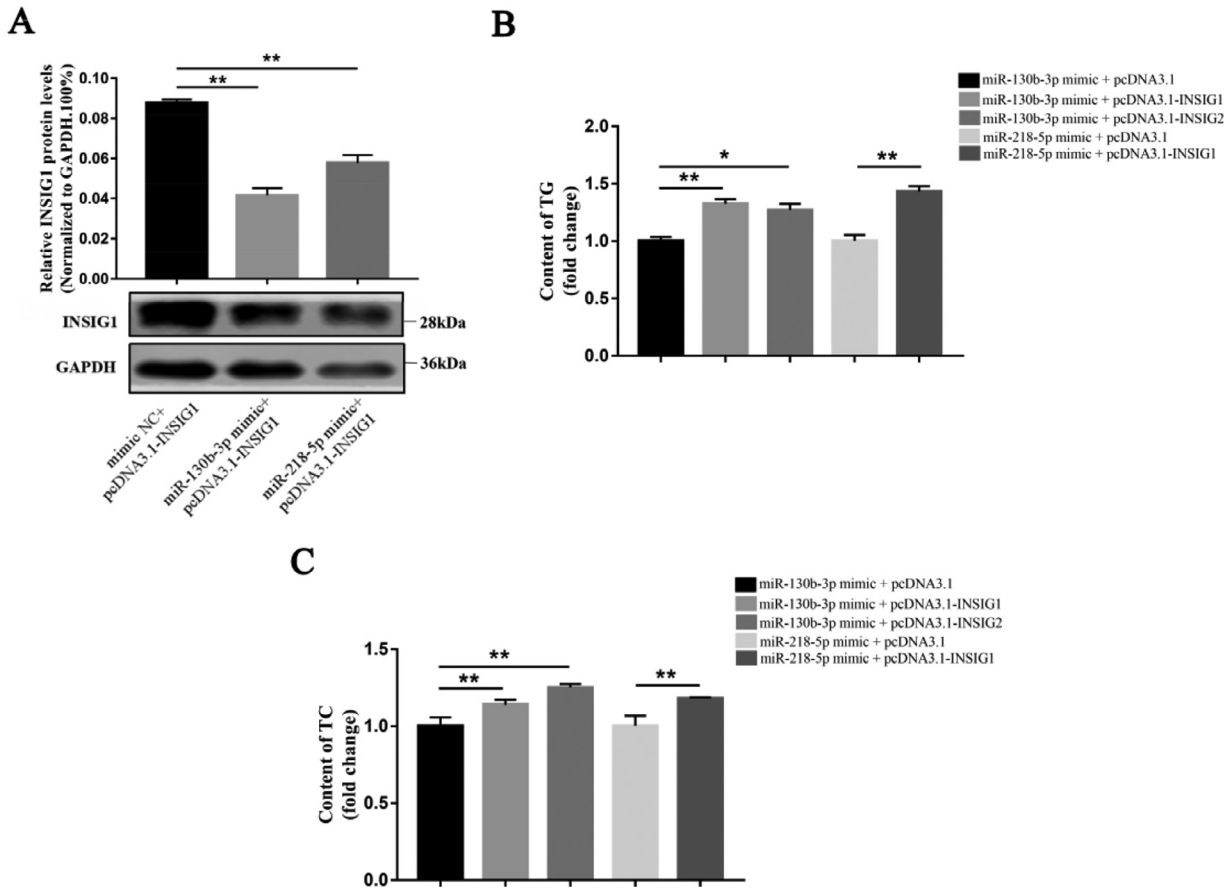

Figure 9. INSIGs mediated the inhibitory effect of miR-130b-3p and miR-218-5p on TC and TG synthesis in LMH cells. (A) Western blot shows the effect of co-expression of miR-130b-3p or miR-218-5p and *INSIG1* onprotein levels of INSIG1 in LMH cells; (B) Effects on intracellular TG content after co-transfection of miR-130b-3p mimic and pcDNA3.1-INSIG1, and miR-218-5p mimic and pcDNA3.1-INSIG1, respectively; (C) Effects on intracellular TC content after co-transfection of miR-130b-3p mimic and pcDNA3.1-INSIG1, and miR-218-5p mimic and pcDNA3.1-INSIG1, respectively. Data represent mean ± SEM. (n = 3); $^{*}P < 0.05$, $^{**}P < 0.01$.

chickens and mammals (Feramisco et al., 2004). It seems that the biological role of INSIG1 and INSIG2 in chicken might be similarity to that in mammals.

In this study, *INSIGs* overexpression led no significant changes in intracellular TG and TC contents, and no significant alteration in the expression levels of genes related to TG and TC synthesis and lipid transport. Only the expression levels of *SCD* and *APOB* were significantly increased, and *SQLE* was significant decreased when *INSIG1* was overexpressed, and the expression level of *MVD* was significantly increased when *INSIG2* was overexpressed. Previous study found that overexpression of *INSIG*s in Zucker diabetic fatty (**ZDF**) (fa/fa) rats significantly downregulated the TG levels in plasma and liver, and the expression levels of *ACCα, FASN, SCD1* and other genes involved in TG synthesis, and suggested that *INSIG2* plays a greater role in hepatic lipid metabolism than *INSIG1* (Takaishi et al., 2004). Interestingly, it was found that knockdown of chicken *INSIG1* led significant decrease in TG and TC contents, followed by the significant down regulation of *ACSL1* and lipid assembly related genes *MTTP-L, APOB* and *APOVLDLII*, while knockdown of *INSIG2* caused significant decrease in the TG and TC contents and expression levels of all genes related to lipid synthesis and assembly. Knockdown of *INSIGs* in mice caused a significant rise in total TC in plasma and liver but no significant change in TG content (McFarlane et al., 2014). Double knockout of *INSIG1* and *INSIG2* in mice resulted in over-accumulation of cholesterol and triglycerides in the liver (Engelking et al., 2005). Unexpectedly, the biological functions of *INSIGs* in chicken and mammals are obviously opposite.

INSIGs were reported to be regulated by miRNA in the post-transcription level in mammals. In rat, miR-24 could inhibit *INSIG1* gene expression, thereby promote lipid synthesis (Ng et al., 2014). In *SREBP-1a* and *SREBP-2* transgenic mice, overexpression of miR-96 could significantly reduce the expression level of INSIG2, and increase the nuclear forms of SREBP-1 and -2, resulting in increased mRNA levels of *SREBP-1* and *-2* target genes such as *HMGCR, SCD*1, *FAS*, and *ELOVL*6, thereby affecting endogenous lipids synthesis (Jeon et al., 2013; Jo et al., 2017). In the present study, miR-130b-3p could repress the expressions of *INSIG1* and *INSIG2*, and miR-218-5p could also repress the expressions of *INSIG1*, reduce the TG and TC contents. It was consistent with the results of *INSIG1* and *INSIG2* knockdown. Meawhile, our results demonstrated that miR-130b-3p and miR-218-5p could also regulate the protein levels of INSIG1. While the effect of miR-130b-3p on the INSIG2 expression in protein level was fail to

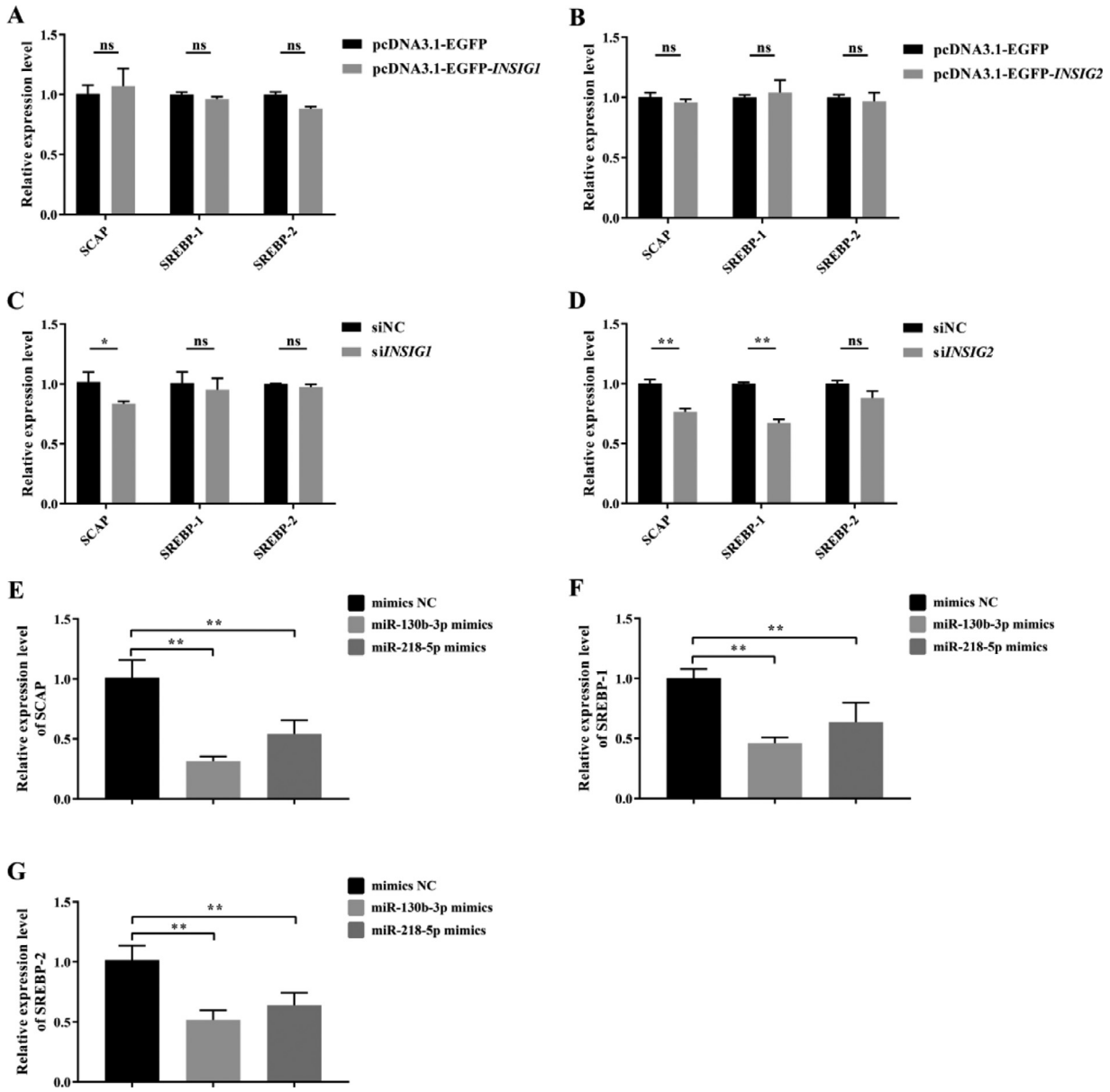

Figure 10. Effect of *INSIGs* on the expression levels of *SCAP* and *SREBP*1, *SREBP*2. (A) Effects of *INSIG1* overexpression on expression of *SCAP* and *SREBP1*, *SREBP2*; (B) Effects of *INSIG2* overexpression on expression of *SCAP* and *SREBP1*, *SREBP2*; (C) Effects of *INSIG1* knockdown on expression of *SCAP* and *SREBP1*, *SREBP2*; (D) Effects of *INSIG2* knockdown on expression of *SCAP* and *SREBP1*, *SREBP2*; (E) Effects of miR-130b-3p and miR-218-5p overexpression on expression of *SCAP*, respectively; (F) Effects of miR-130b-3p and miR-218-5p overexpression on expression of *SREBP1*, respectively; (G) Effects of miR-130b-3p and miR-218-5p overexpression on expression of *SREBP2*, respectively. *β-Actin* used as the reference gene of mRNA, and U6 as the reference of miRNA. Data are expressed as mean ± SEM (n = 6); $^{*}P < 0.05$, $^{**}P < 0.01$.and ns means $P > 0.05$.

prove, due to no useful antibody could be obtained. The inhibitory effects of miR-130b-3p and miR-218-5p on the cellular TG and TC synthesis could be recovered by overexpression *INSIG1* and *INSIG2*, respectively. It proved that miR-130b-3p and miR-218-5p control the lipid metabolism via target INSIG1 and INSIG2 in chicken.

It is well-established that the INSIGs-SCAP-SREBPs pathway plays an essential role in control of triglyceride and cholesterol biosynthesis (Desvergne et al., 2006). Studies in mice have shown that INSIGs can prevent the synthesis of lipids by inhibiting the maturation of SREBPs, but the increased endogenous INSIGs are not enough to completely prevent the expression of SREBPs and the synthesis of lipids (Ye and DeBose-Boyd, 2011). Some studies on the role of INSIG1 in the INSIGs-SCAP-SREBPs signaling pathway have found that INSIG1 overexpression in the liver of transgenic mice inhibits the proteolytic process of SREBPs, the mRNA level of enzymes needed to synthesize cholesterol, fatty acids and triglycerides were decreased significantly, and the plasma cholesterol level was decreased significantly as well (Gong et al., 2006). In this study, the overexpression of *INSIG1* did not significantly affect the expression levels of *SCAP* and *SREBPs*, and TG and TC contents in LMH. It further indicated that the *INSIGs* play different role in chicken than their counterparts in mammals.

In conclusion, chicken *INSIGs* were evolutionarily conserved, located in cell endoplasmic reticulum and widely expressed in various tissues. Overexpression of

INSIGs led no significant changes in mRNA abundance of lipid metabolism-related genes and the contents of TG and TC in LMH. *INSIG1* knockdown could cause the significant decrease in expression levels of *ACSL1, MTTPL, ApoB* and *ApoVLDLII* and the contents of TG and TC ($P < 0.05$ or 0.01), while *INSIG2* knockdown could significantly reduce the expression levels of genes involved in lipid synthesis and transport, and the TG and TC contents ($P < 0.05$ or 0.01). Moreover, miR-130b-3p and miR-218-5p regulate INSIG1 in both mRNA and protein levels. INSIG2 was directly targeted by miR-130b-3p. MiR-130b-3p and miR-218-5p regulated the lipid metabolism through targeting *INSIG1* and *INSIG2*. No significant alteration in the expression of *SCAP* and *SREBPs* was found when *INSIG*s were overexpressed, while *SCAP* and *SREBP1* were downregulated when *INSIG*2 was knocked down. Taken together, our results highlight the essential role and miRNA post-transcriptional control of *INSIG1* and *INSIG2* in lipid metabolism in chicken.

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DISCLOSURES

The authors declare no conflict of interest.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.psj.2022.102380.

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