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RESEARCH ARTICLE

Gga-miR-213 Promotes the Differentiation of Intramuscular Preadipocytes by Targeting the KLF10 Gene in Chicken

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ABSTRACT

Intramuscular fat (IMF) is one of the crucial factors determining chicken quality. IMF deposition depends on the hyperplasia and hypertrophy of intramuscular preadipocytes, in which microRNAs (miRNAs) play an important regulatory role. This study aimed to elucidate how gga-miR-213 and Kruppel-like factor 10 (*KLF10*) regulate IMF deposition through intramuscular preadipocyte development. RNA-seq profiling revealed that *KLF10* and gga-miR-213 were identified as likely involved in the adipogenic differentiation of chicken embryonic breast muscle. Experimental evidence demonstrated that gga-miR-213 mimics significantly suppressed mitotic activity while accelerating lipid accumulation in developing adipocytes. Conversely, inhibition of gga-miR-213 through specific antagonists enhanced cellular proliferation capacity and effectively inhibited adipogenic differentiation. Gga-miR-213 was found to directly bind to the 3′ untranslated region of *KLF10* by dual-luciferase reporter assay. The regulatory effect of *KLF10* was opposite to that of gga-miR-213. Loss-of-function experiments suggested that gga-miR-213 inhibitor eliminated the impact of *KLF10* siRNA on intramuscular preadipocytes. Collectively, gga-miR-213 inhibits proliferation but promotes differentiation of intramuscular preadipocytes by targeting *KLF10*, thereby controlling embryonic IMF deposition. The miR-213/*KLF10* axis provides molecular targets for precision breeding to modulate poultry meat quality.

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INTRODUCTION

Consumers regard chicken meat as one of the primary sources of high-quality protein owing to its low fat, calories, and cholesterol content. However, the nutritional value and taste of different chicken varieties substantially vary. The meat of high-quality local chickens is fresh and juicy, rich in fatty acids, and more fragrant. Skeletal muscles represent a vital component of avian organisms and primarily comprise a large number of myofibers (Mo *et al.,* 2023). IMF, defined as lipid reserves localized among muscle fascicles and within intracellular lipid droplets. These deposits serve as critical determinants of organoleptic characteristics, modulating multiple palatability attributes including textural softness, aromatic profile, visual appearance, and moisture retention in poultry products (Velleman, 2007). However, high-density breeding and high-intensity growth selection resulted in a decrease in IMF content and a change in aroma compounds in chickens, and poorer meat quality, resulting in diminished consumer acceptability and preference (Gai *et al.,* 2023). This underscores the necessity for a comprehensive elucidation of the molecular pathways governing IMF accretion.

The process of adipocyte differentiation includes proliferative and hypertrophic phases. The number of adipocytes increases rapidly in the proliferative phase until contact inhibition occurs, whereas the number of cells remains constant. Following this developmental phase, adipocytes enter a hypertrophic expansion period. This stage is characterized by cellular volumetric enlargement and sustained accumulation of cytoplasmic lipid vesicles. Subsequently, pre-adipocytes mature into adipocytes capable of secreting lipid droplets (Wier e*t al.,* 1986; Gregoire e*t al.,* 1998). The deposition of IMF is governed by the proliferation and differentiation of intramuscular preadipocytes, which is a complex dynamic process regulated by multiple functional factors. It includes peroxisome proliferator-activated receptors (PPARs), sterol regulatory element binding proteins (SREBPs),

CCAAT/enhancer binding-proteins (C/EBPs), and Krüppel-like factors (KLFs) (Nobre *et al.,* 2018). KLFs form a conserved family of zinc-dependent transcription factors. They exhibit broad tissue distribution across multiple organ systems, with particularly high prevalence in musculoskeletal tissue, hepatic parenchyma, and adipose compartments. In these tissues, KLFs demonstrate significant functional roles (Dang *et al.,* 2000). KLFs dynamically form complexes with transcriptional co-regulators. These complexes mediate epigenetic modifications that regulate key cellular homeostatic processes, including mitogenesis, lineage specification, programmed cell death, and cellular senescence (Pearson *et al.,* 2008). These molecular interactions undergo reciprocal modulation, mediated through crosstalk with multiple signaling cascades. This orchestration establishes coordinated molecular circuitry that maintains equilibrium in intramuscular adipocyte lipid dynamics and metabolic homeostasis.

The intricate modulatory networks governing intramuscular adipogenesis suggest probable involvement of microRNAs (miRNAs), as epigenetic modulators in lipid deposition control. These noncoding elements exert post-transcriptional control through sequence-specific interactions with mRNA 3' untranslated regions (3'UTRs), thereby fine-tuning gene expression dynamics in adipocyte regulation (Bartel, 2004; Victor, 2004). Accumulating research data show that miRNAs participate in the differentiation of preadipocytes and the deposition of IMF. Studies on chicken IMF have shown that miR-128-3p functions as potent suppressors of intramuscular adipocyte differentiation (Zhang *et al.,* 2019), miR-140-5p promotes the differentiation of intramuscular adipocytes (Zhang *et al.,* 2018), and miR-24-3p enhances adipogenic commitment in intramuscular adipocytes through targeted suppression of ANXA6-mediated inhibitory signaling (Lin *et al.,* 2022). MiR-15a and miR-124-3p have been identified in the intramuscular fat of Gushi chicken breast muscle and are related to intramuscular fat deposition. MiR-15a directly targets *ACAA1*, *ACOX1* and *SCP2*, thus promoting the differentiation of preadipocytes. MiR-124a-3p directly targets *ACAA2*, functioning as a master regulator of lipid homeostasis and adipose tissue accumulation (Li *et al.,* 2019).

Rose-crown chicken (RC) is a local chicken breed cultivated in Xinjiang, China (Supplementary Fig. S1-S4) (Chen *et al.,* 2023). Cobb broiler (CB) is a large, fast-growing commercial broiler breed introduced from abroad (Collins *et al.,* 2014). It is vital to study the genetic differences in the mechanisms of IMF in the two breeds of chickens to improve meat quality. In our preliminary study, we performed high-throughput sequencing of breast muscle tissue samples from two chicken breeds on the 8 days of the embryonic stage, identifying the key genes involved in IMF deposition (Zhang *et al.,* 2021). The novel miRNA gga-miR-213 is highly differentially expressed between the two breeds. Bioinformatics analysis revealed that the target gene of gga-miR-213 is *KLF10*, a key regulatory transcription factor involved in adipogenesis. These findings implied that gga-miR-213 may have a key effect on IMF deposition. Subsequently, we verified the main function of gga-miR-213 in intramuscular preadipocytes. The results advance our mechanistic understanding of intramuscular adipogenesis in Gallus gallus and identify a promising molecular target for precision breeding strategies to enhance meat quality traits. This study, therefore, aimed to elucidate gga-miR-213's role in avian intramuscular adipogenesis, determine its regulatory relationship with *KLF10*, and assess the axis' potential for precision breeding of meat quality traits.

MATERIALS AND METHODS

Ethical statement: All experimental animals were handled according to a protocol approved by the Medical Ethics Committee of the First Affiliated Hospital, Medical College, Shihezi University (A2016-095-09). All animal experiments were in line with the Guide for the Care and Use of Laboratory Animals by the International Committee.

Culture of intramuscular preadipocyte: Chicken embryo pectoral muscle tissue was digested with Collagenase I/II, sequentially filtered through 100, 70, and 40μm cell strainers, centrifuged, and cultured in DMEM medium containing 10% FBS. The intramuscular preadipocytes were purified by differential adhesion method (Lin *et al.,* 2022).

qRT-PCR: Total RNA isolation was performed with TransZol reagent, assessed for integrity by electrophoresis, and quantified by NanoDrop 2000, then reverse transcribed into cDNA. qRT-PCR was conducted using SYBR green in triplicate on a LightCycler® 480 system. The primer sequences used for qRT-PCR are shown in Table S1.

Transfection of gga-miR-213 mimic/inhibitor, pcDNA3.1(+)-KLF10, and si-KLF10: *KLF10* overexpression vector (pcDNA3.1(+)-KLF10) and control vector (pcDNA3.1(+)) were obtained from Youkang (Wulumuqi, China). Gga-miR-213 mimics, mimics negative control (MNC), gga-miR-213 inhibitor, inhibitor negative control (INC), *KLF10* siRNA (si-KLF10), and si-NC were obtained from Gene Pharma (Shanghai, China). Chicken intramuscular preadipocytes were transfected at 2.5-3.0 × 10^4/cm² using Lipofectamine 2000 (Invitrogen, Grand Island, USA), with medium replacement after 6h, and harvested at 48h for proliferation assays. For differentiation studies, cells were transfected at 3.5-4.0 × 10^4/cm² and induced with 120μmol/L oleic acid upon reaching 5.0-6.0 × 10^4/cm² (Sun *et al.,* 2025).

Target prediction and luciferase Activity Assay: TargetScan 7.0 predicted gga-miR-213's target genes, and dual-luciferase reporter assays were performed by co-transfecting HEK293T cells with wild-type/mutant psiCHECK-2-KLF10-3'UTR vectors and miR-213 mimics or NC. After 48h, relative Renilla/firefly luciferase activity was measured to validate *KLF10* as a direct target.

Cell counting Kit-8 (CCK-8) assay: Cell viability was assessed at 12, 24, 48, 72, and 96h post-transfection using the CCK-8 kit, following the manufacturer's instructions.

5-Ethynyl-2-Deoxyuridine (EdU) assay: Cell proliferation was evaluated using the BeyoClick™ EdU Kit (TransGen Biotech, Beijing, China), and ImageJ (v1.8.0) quantified EdU-positive cells across multiple fields.

Lipid droplet staining: Cells were stained with Oil Red O (Solarbio, Beijing, China) to visualize lipid droplets, and quantified by measuring absorbance at 490 nm after extraction with 100% isopropanol. For fluorescence imaging, cells were stained with BODIPY (1μg/mL; Invitrogen, Grand Island, USA) and DAPI, then observed under a fluorescence microscope.

Western Blots (WB): After 72h of transfection and differentiation induction, total protein was extracted for SDS-PAGE separation and PVDF membrane transfer. Blocking was performed with 5% skimmed milk, after which the membranes were exposed to the primary antibodies overnight (4°C) and the secondary antibodies for 1.5 hours (25°C). Protein bands were exposed with ODYSSEY CLx (LI-COR, Nebraska, USA) and quantified using Image J 1.8.0. Following primary antibodies were used: KLF10 (1:1000; Affinity, State of New Jersey, USA), PPARγ (1:2000; Bioss, Beijing, China), C/EBPα (1:2000; Bioss, Beijing, China), GAPDH (1:10000; Affinity, State of New Jersey, USA). The secondary antibodies were anti-rabbit and anti-mouse antibodies.

Detection of lipid metabolism index content: The contents of lipid metabolism indexes of intramuscular preadipocytes were detected by Triglyceride (TG) Content Assay Kit (Boxbio, Beijing, China), Total Cholesterol (TC) Content Assay Kit (Solarbio, Beijing, China) and Tissue Glycerol (GL) Content Assay Kit (Applygen, Beijing, China) detection kits, respectively.

Statistical analysis: All data represent mean±SEM of three biological replicates (n=3). SPSS 26.0 was used to conduct comparative assessments between experimental cohorts via Student's t-test, with predefined significance thresholds ($P<0.05$ denoted by * and $P<0.01$ by **). Visual representations were generated using GraphPad Prism 8.0 for data visualization and graphical interpretation.

RESULTS

Gga-miR-213 is a potential regulator of chicken IMF adipogenesis: Gga-miR-213 exhibited significantly elevated expression in abdominal fat and muscle compared to other tissues ($P<0.05$) (Fig. 1A). It is speculated that gga-miR-213 may promote fat deposition. Chicken intramuscular preadipocytes were isolated and cultured in the laboratory. Through oil red staining, it was found that larges number of lipid droplets were generated after 3 days of induction and differentiation (Fig. 1B). The OD value of oil red was detected by spectrophotometer, which showed a positive correlation with lipid content ($P<0.01$) (Fig. 1C). The expression levels of gga-miR-213 were detected at 12, 24, 48, 72 and 96h during cell proliferation and differentiation. The results showed that gga-miR-213

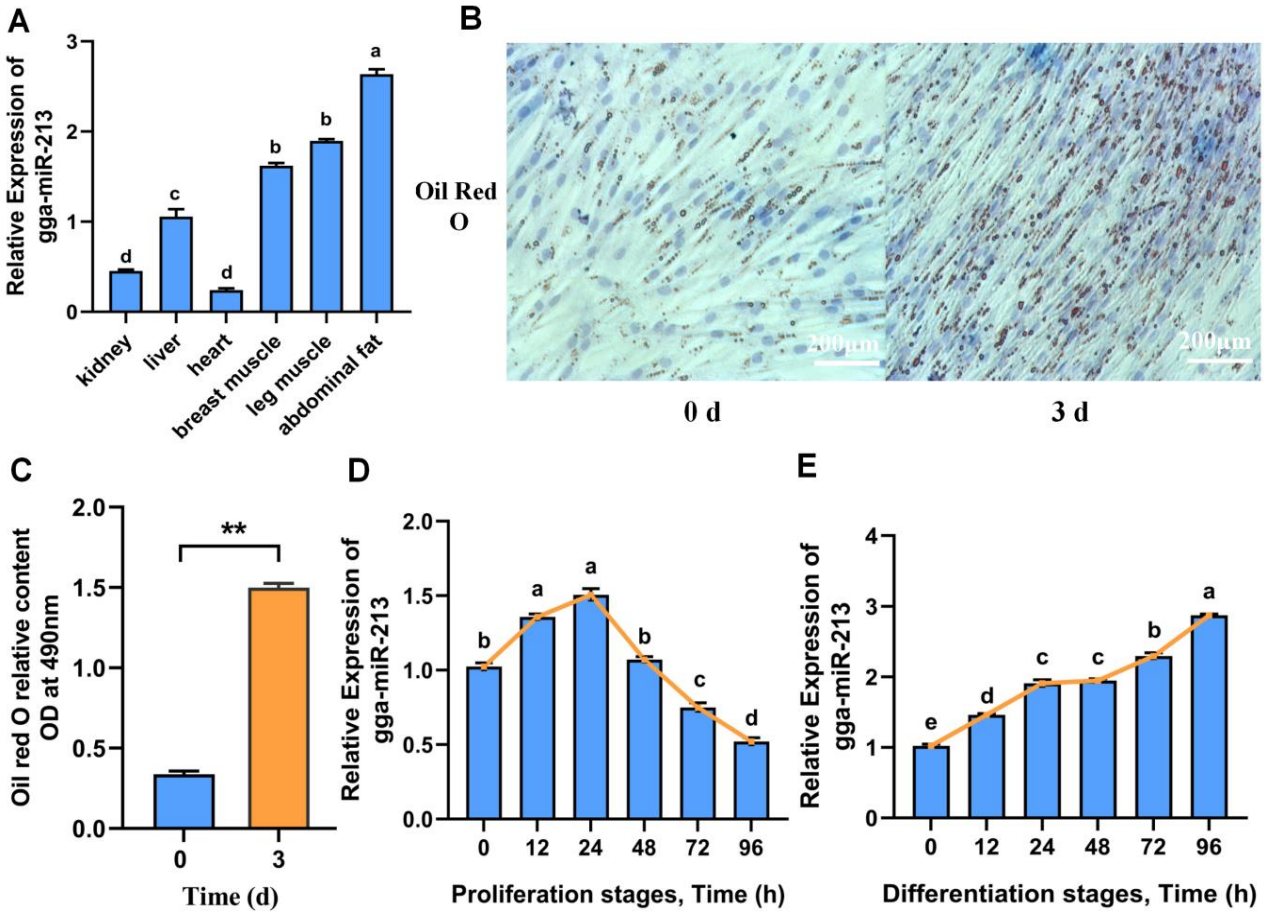

Fig. 1: Gga-miR-213 is a potential regulator of chicken IMF adipogenesis. (A) Expression pattern of gga-miR-213 in different tissues of chicken embryo. (B) In vitro culture and induced differentiation of chicken intramuscular preadipocytes. (C) The content of lipid droplets was analyzed by oil red O staining 0d and 3d after cell differentiation. (D) Expression pattern of gga-miR-213 during intramuscular preadipocyte proliferation in chickens. (E) Expression pattern of gga-miR-213 during intramuscular preadipocyte differentiation in chickens. Distinct superscript letters (a, b) denote significant differences ($P<0.05$) between treatment groups (columns). Data are presented as the mean±SD (n = 3 biological replicates). * $P<0.05$, ** $P<0.01$, same below.

increased first and then decreased in the proliferative phase and gradually increased in the differentiation phase (Fig. 1D, E). These results suggest that gga-miR-213 may promote intramuscular fat deposition.

Gga-miR-213 inhibits the proliferation of chicken intramuscular preadipocyte: The gga-miR-213 mimics, MNC, gga-miR-213 inhibitor, and INC were transfected into cells. It was demonstrated that mimics could significantly increase the expression of gga-miR-213 (P<0.01), while the inhibitor significantly reduced the expression of gga-miR-213 (P<0.01) (Fig. 2A). Relative to Control (untransfected cells), neither MNC nor INC significantly altered gga-miR-213 expression (Fig. 2A). Gga-miR-213 mimic transfection significantly decreased viability at both 48 and 72h, and gga-miR-213 inhibitor significantly increased cell viability at 72h (P<0.01) (Fig. 2B, C). The gga-miR-213 mimics suppressed EdU+ cells (P<0.05), while gga-miR-213 inhibitor increased them (P<0.01) (Fig. 2D, E). The results showed that gga-miR-213 mimics significantly reduced the expression of *PCNA* and *CCND1* (P<0.01) (Fig. 2F). Transfection of gga-miR-213 inhibitor significantly upregulated *PCNA* and *CDK6* expression levels (P<0.01) (Fig. 2F). These findings demonstrate that gga-miR-213 inhibits the proliferation of chicken intramuscular preadipocytes.

Gga-miR-213 promotes the differentiation of chicken intramuscular preadipocytes: After 72h of oleic acid induction, the effect of gga-miR-213 on the differentiation of intramuscular preadipocytes was analyzed. The results of oil red O and BODIPY staining showed that the volume of lipid droplets in the gga-miR-213 mimic group was larger and the lipid droplets were more aggregated (P<0.01) (Fig. 3A, B). The distribution range and volume of lipid droplets in the gga-miR-213 inhibitor group were smaller than INC (P<0.05) (Fig. 3A, B). Gga-miR-213 mimics significantly increased the levels of TC, GL, and TG in cells (P<0.01), and gga-miR-213 inhibitor significantly decreased the levels of them (Fig. 3C, D, E). Gga-miR-213 mimics transfection markedly reduced both KLF10 mRNA and protein expression (P<0.01), while gga-miR-213 inhibitor significantly increased the mRNA and protein expression levels of KLF10 (P<0.05) (Fig. 3F, G, H). The mRNA *PPARγ* and *C/EBPα* in the gga-miR-213 mimics group were significantly increased, which was opposite in the gga-miR-213 inhibitor group (P<0.01) (Fig. 3F). And gga-miR-213 inhibitor significantly reduced the expression of *FABP4* (P<0.05) (Fig. 3F). Protein levels of differentiation marker genes were consistent with mRNA (Fig. 3G, H). These results indicate that gga-miR-213 can promote the differentiation of chicken intramuscular preadipocytes.

***KLF10* promotes the proliferation of chicken intramuscular preadipocyte:** The expression of *KLF10* increased gradually during proliferation period (Fig. 4A). PcDNA3.1(+)-KLF10 transfection significantly

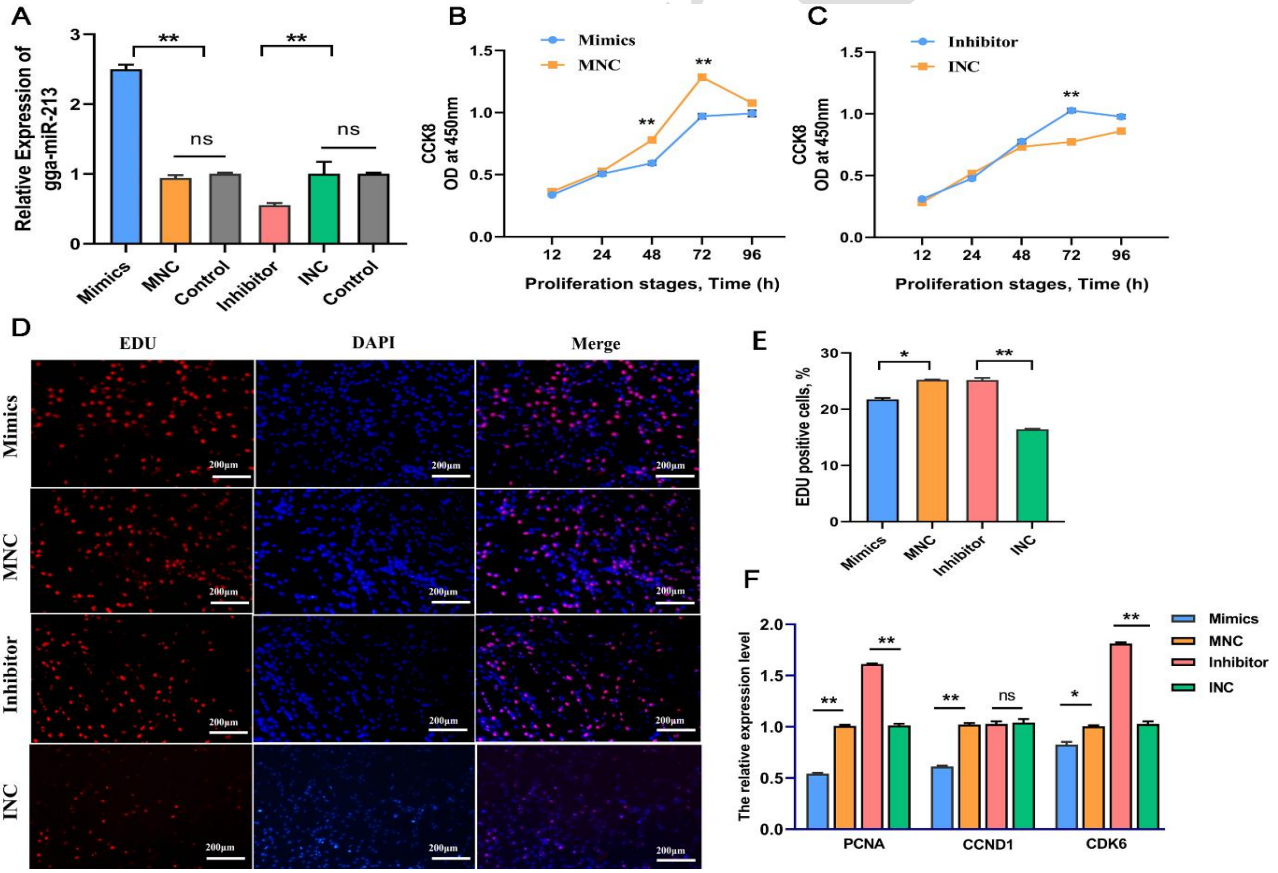

Fig. 2: Effects of gga-miR-213 on intramuscular preadipocyte proliferation in chickens. (A) Detection of transfection efficiency of gga-miR-213 mimics and inhibitors. (B, C) CCK8 assay of chicken intramuscular preadipocytes transfected with gga-miR-213 mimics and inhibitors. (D) Representative images from the EdU assay of chicken intramuscular preadipocytes transfected with gga-miR-213 mimics, MNC, gga-miR-213 inhibitor, and INC for 48h. (E) Quantification ratio of EdU-positive cells/total cells. (F) The effect of gga-miR-213 on the expression of cell proliferation marker genes *PCNA*, *CCND1*, and *CDK6*.

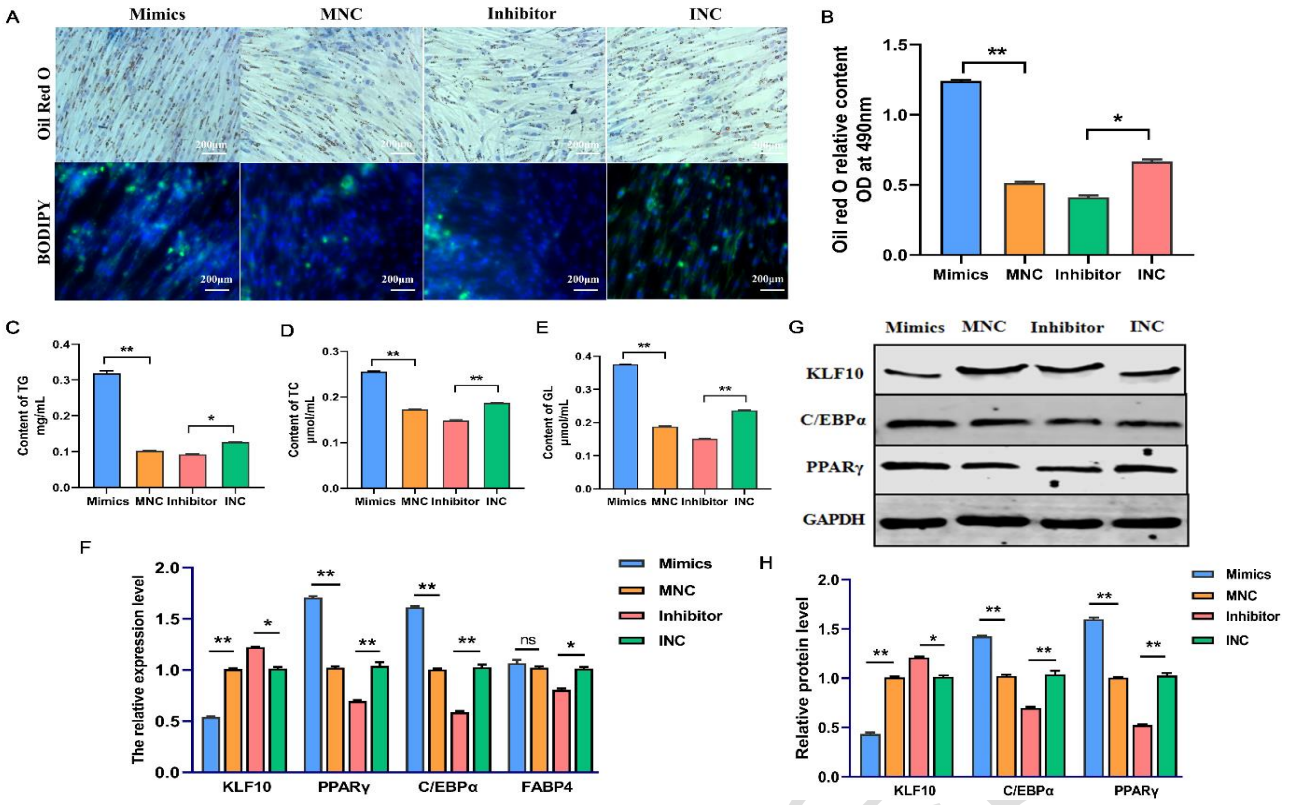

Fig. 3: Effects of gga-miR-213 on intramuscular preadipocyte differentiation in chickens. (A) Oil red O and BODIPY staining of the differentiated chicken intramuscular preadipocytes transfected with gga-miR-213 mimics and inhibitor. (B) After extracting oil red O with isopropanol, the OD value was detected, 490 nm. (C, D, E) The levels of TG, TC and GL were detected after transfection with gga-miR-213 mimics and inhibitor. (F) qRT-PCR was used to detect *KLF10* and the adipogenesis genes *C/EBPα*, *PPARγ*, and *FABP4*. (G) WB analysis of KLF10, C/EBPα and PPARγ after transfection with gga-miR-213 mimics and inhibitor. (H) Protein quantitative analysis of KLF10, C/EBPα and PPARγ.

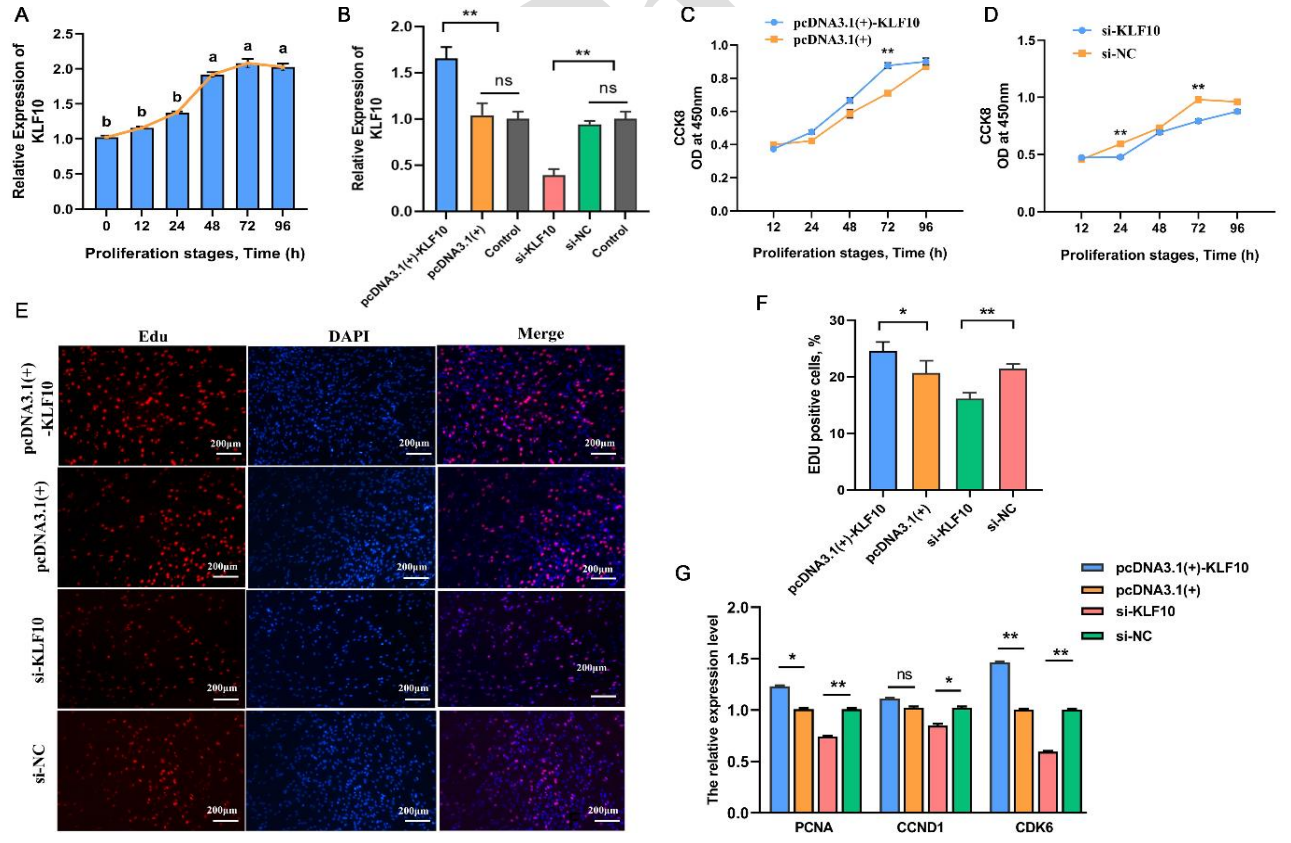

Fig. 4: Effects of *KLF10* on intramuscular preadipocyte proliferation in chickens. (A) Expression pattern of *KLF10* during intramuscular preadipocyte proliferation in chickens. (B) Detection of transfection efficiency of pcDNA3.1(+)-KLF10 and si-KLF10. (C, D) CCK8 assay of chicken intramuscular preadipocytes transfected with pcDNA3.1(+)-KLF10 and si-KLF10. (E) Representative images from the EdU assay of chicken intramuscular preadipocytes transfected with pcDNA3.1(+)-KLF10 and si-KLF10. (F) Quantification ratio of EdU-positive cells/total cells. **(G)** The effect of *KLF10* on the expression of cell proliferation marker genes *PCNA*, *CCND1*, and *CDK6*.

upregulated *KLF10* expression levels (P<0.01), while the si-KLF10 significantly reduced the expression of *KLF10* (P<0.01) (Fig. 4B). Relative to Control, neither pcDNA3.1(+) nor si-NC significantly altered *KLF10* expression (Fig. 4B). PcDNA3.1(+)-KLF10 significantly increased cell viability at 72h and si-KLF10 decreased it at at 24 and 72h (P<0.01) (Fig. 4C, D). The pcDNA3.1(+)-KLF10 significantly increased the proportion of Edu positive cells (P<0.05), while si-KLF10 significantly decreased the proportion of Edu positive cells (P<0.01) (Fig. E, F). The results of the expression of proliferation marker genes showed that pcDNA3.1(+)-KLF10 increased the expression of *PCNA* (P<0.05) and *CDK6* (P<0.01), and si-KLF10 significantly reduced the expression of *PCNA* (P<0.01), *CCND1* (P<0.05) and *CDK6* (P<0.01) (Fig. 4G). These findings demonstrate *KLF10*'s proliferative effect on chicken intramuscular preadipocytes.

***KLF10* inhibits the differentiation of chicken intramuscular preadipocyte:** *KLF10* expression progressively declined throughout chicken intramuscular preadipocyte differentiation (Fig. 5A). Oil Red O and BODIPY staining revealed that the distribution range and volume of lipid droplets in the pcDNA3.1(+)-KLF10 group were significantly smaller than pcDNA3.1(+) (P<0.05), and the area of red lipid droplets in si-KLF10 group was significantly larger and the content of lipid droplets was significantly increased (P<0.01) (Fig. 5B, C). PcDNA3.1(+)-KLF10 significantly decreased the levels of TC, GL and TG in cells (P<0.05), and si-KLF10 significantly increased the levels of GL (P<0.01), TG (P<0.01) and TC (P<0.01) (Fig. 5D, E, F). The mRNA expression levels of *C/EBPα* and *FABP4* in the pcDNA3.1(+)-KLF10 group were significantly decreased (P<0.01), while the levels of *PPARγ*, *C/EBPα* and *FABP4* in the si-KLF10 group were significantly increased (P<0.01) (Fig. 5G). Similarly, *KLF10* upregulated the protein levels of KLF10 (P<0.01), while downregulating the protein levels of PPARγ (P<0.01) and C/EBPα (P<0.05) (Fig. 5H, I). These findings demonstrate *KLF10*'s ability to enhance the differentiation of chicken intramuscular preadipocytes.

Functional prediction and network interaction of gga-miR-213 and target genes: Bioinformatic analysis identified *KLF10* as a putative gga-miR-213 target gene (Fig. 6A). The *KLF10*-3'UTR was amplified to obtain a 480bp sequence fragment (Fig. 6B). The *KLF10*-3'UTR was ligated to the psiCHECK-2 vector, and the recombinant plasmid was named KLF10-WT (Fig. 6C, D). The KLF10 mutant plasmid was amplified and constructed by overlap PCR and named KLF10-Mut (Fig. 6C, D). KLF10-WT/KLF10-Mut and gga-miR-213 mimics/MNC were co-transfected into HEK-293T cells. Dual-luciferase assays demonstrated significantly reduced activity in KLF10-WT/gga-miR-213 mimic co-transfections

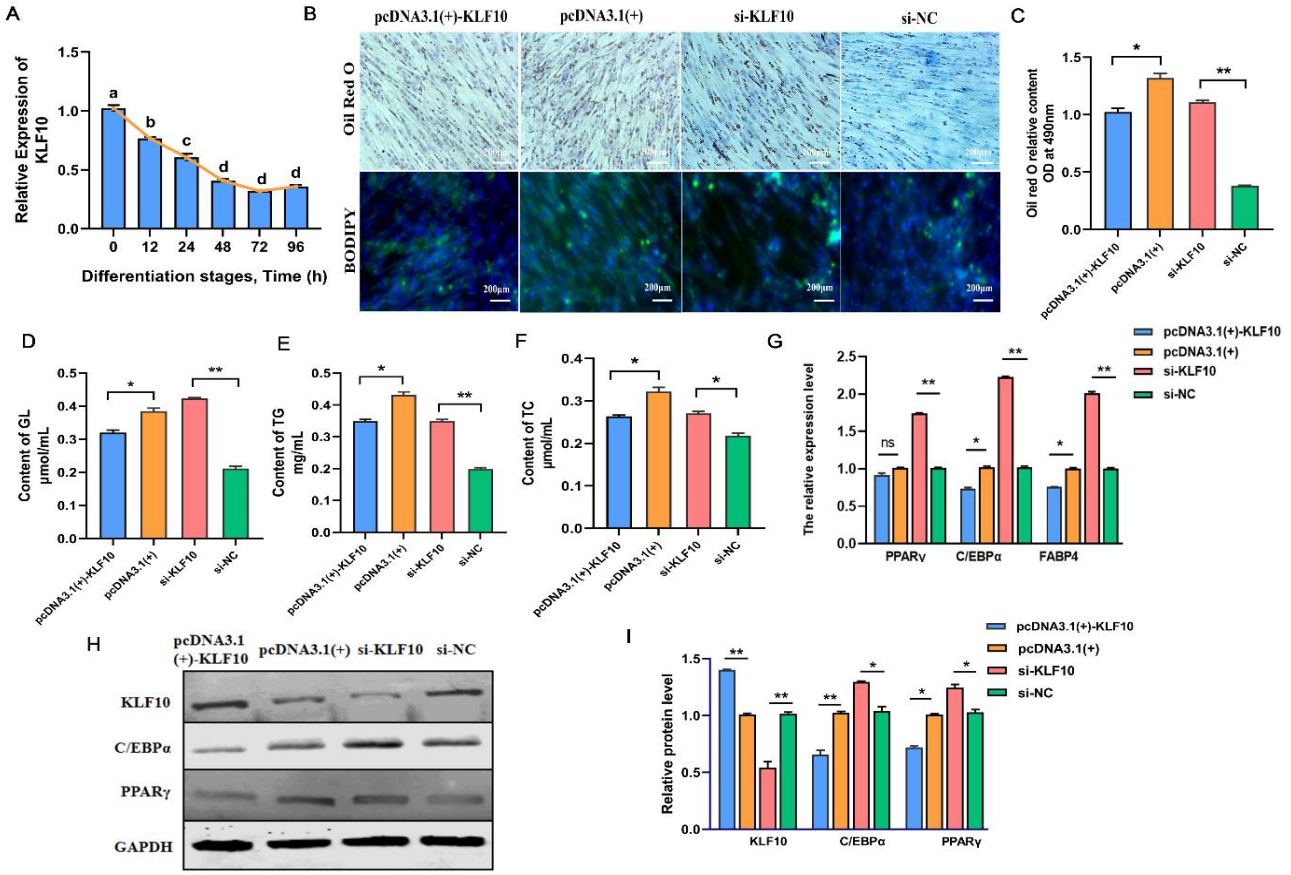

Fig. 5: Effects of *KLF10* on intramuscular preadipocyte differentiation in chickens. (A) Expression pattern of *KLF10* during intramuscular preadipocyte differentiation in chickens. (B, C) Oil red O and BODIPY staining of the differentiated chicken intramuscular preadipocytes transfected with pcDNA3.1(+)-KLF10 and si-KLF10. (D, E, F) The levels of GL, TG, and TC were detected after transfection with pcDNA3.1(+)-KLF10 and si-KLF10. (G) qRT-PCR was used to detect the adipogenesis genes *C/EBPα*, *PPARγ*, and *FABP4*. (H) WB analysis of KLF10, C/EBPα and PPARγ after transfection with pcDNA3.1(+)-KLF10 and si-KLF10. (I) Protein quantitative analysis of KLF10, C/EBPα, and PPARγ.

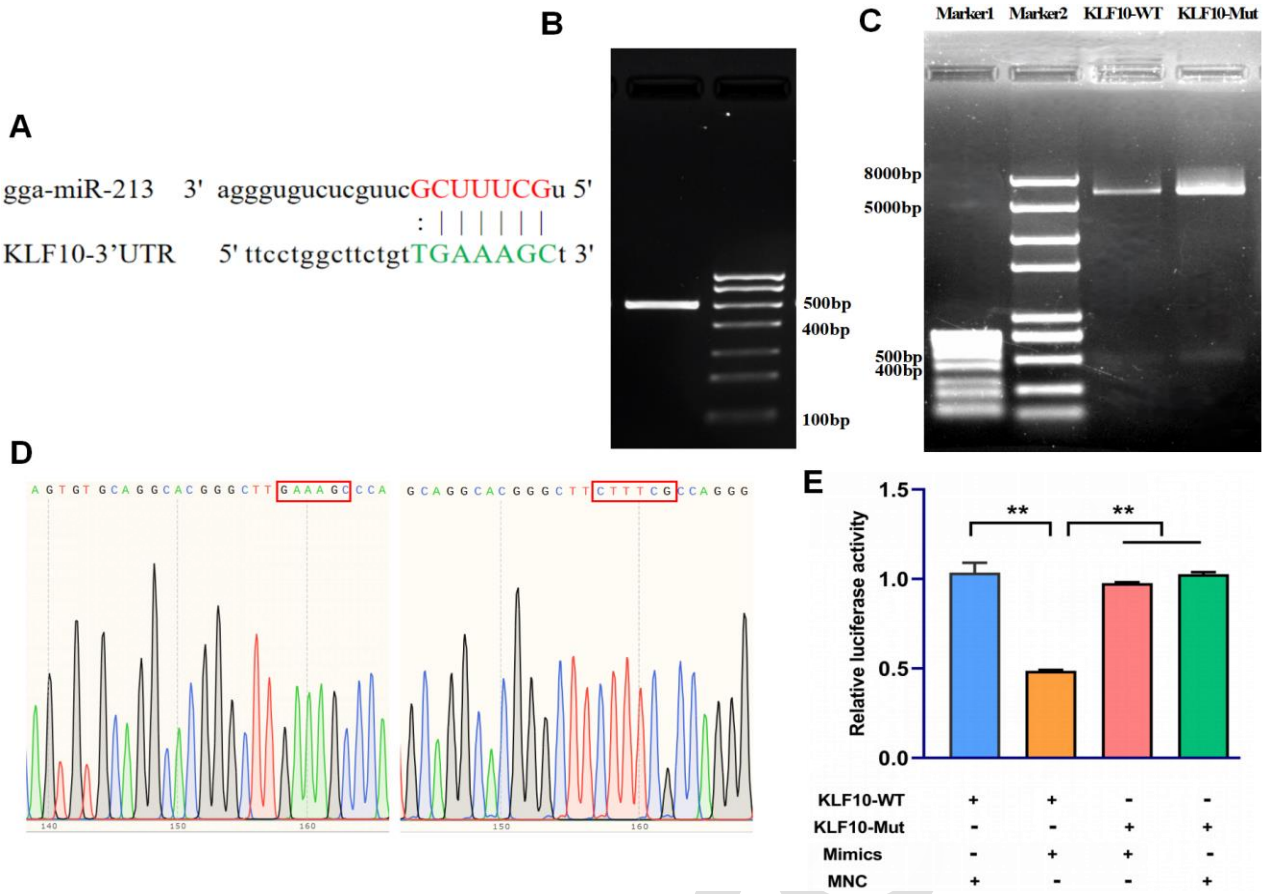

Fig. 6: The *KLF10* gene is a direct target of gga-miR-213. (A) *KLF10* was predicted to be a target of gga-miR-213 by TargetScan software. (B) Sequence amplification of *KLF10*-3'UTR region. (C) Double digestion of WT and MUT psiCHECK-2.0-KLF10 vectors. (D) Sequencing of WT and MUT psiCHECK-2.0- *KLF10* vector. (E) Relative luciferase activity of *KLF10* responding to gga-miR-213 mimics.

(P<0.01), indicating that gga-miR-213 could inhibit the luciferase activity of wild-type KLF10 plasmid (Fig. 6E). While gga-miR-213 did not affect the relative luciferase activity of the KLF10-Mut reporter vector in 293T cells (Fig. 6E). Collectively, *KLF10* represents a direct gga-miR-213 target gene.

Gga-miR-213 regulates intramuscular preadipocyte proliferation and differentiation by targeting *KLF10*: Compared with the Control group, si-NC+INC had no significant effect on the expression of *KLF10* (Fig. 7A). The expression level of *KLF10* in the si-KLF10+INC group was significantly decreased (P<0.05), whereas the si-KLF10+Inhibitor group exhibited significant upregulation (P<0.05) (Fig. 7A). CCK-8 assays revealed markedly reduced absorbance in the si-KLF10+INC group at 48, 72 and 96h after transfection (P<0.05), and the absorbance in si-KLF10+Inhibitor group was significantly higher than that in si-KLF10+INC group (P<0.05) (Fig. 7B). *PCNA*, *CCND1* and *CDK6* in si-KLF10+INC group were significantly lower than that in the si-NC+INC and si-KLF10+Inhibitor group (P<0.05) (Fig. 7C). EdU assays showed significantly fewer positive cells in si-KLF10+INC versus si-NC+INC and si-KLF10+Inhibitor groups. EdU assays showed significantly fewer positive cells in si-KLF10+INC versus si-NC+INC and si-KLF10+Inhibitor groups (P<0.05) (Fig. 7D, E). Oil Red O and BODIPY staining revealed significantly enhanced lipid accumulation in the si-KLF10+INC group (P<0.05), but it was significantly lower in the si-KLF10+Inhibitor group than in the si-KLF10+INC after 72h of transfection (P<0.05) (Fig. 8A, B). The levels of GL and TG showed the same results (Fig. 8C, D, E). si-KLF10+INC group showed significantly higher *PPARγ/C/EBPα/FABP4* mRNA expression than si-NC+INC and si-KLF10+Inhibitor groups (P<0.05) (Fig. 8F). Protein levels by WB correlated with mRNA trends (Fig. 8G, H). These results suggest that gga-miR-213 inhibitor can restore the role of si-KLF10 in promoting lipid droplet differentiation, and gga-miR-213 may inhibit the proliferation of chicken intramuscular preadipocytes by down-regulating *KLF10* and promote cell differentiation (Fig. 9).

DISCUSSION

The generation of the IMF mainly involves the proliferation and differentiation of adipocytes, which is strictly regulated by the interaction of various differentiation factors and cell cycle regulators (Lefterova *et al.,* 2009; Fajas, 2013). As a conserved member of the SP/KLF transcription factor superfamily, *KLF10* functions as a critical modulator of cellular mitogenesis and lineage commitment. Initially identified as a TGF-β-inducible early gene product, this transcriptional regulator exerts its regulatory influence through sequence-specific recognition of GC-rich promoter elements and strategic protein-protein crosstalk with chromatin remodeling complexes. Its pleiotropic nature enables coordinated activation of diverse genetic targets across multiple cellular lineages (Memon and Lee, 2018).

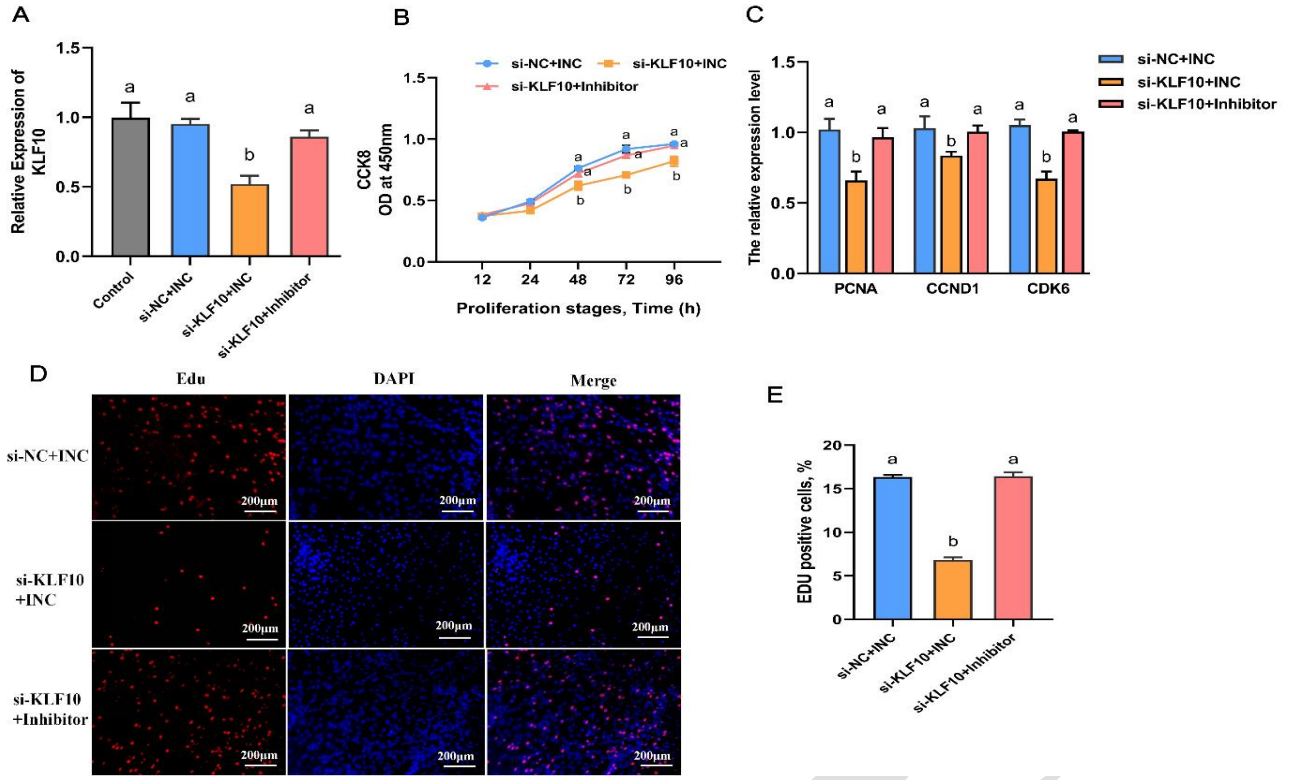

Fig. 7: Gga-miR-213 regulates intramuscular preadipocyte proliferation by targeting *KLF10*. (A) Expression level of *KLF10* transfected with si-NC + INC, si-KLF10 + INC and si-KLF10 + Inhibitor. (B) CCK8 assay of chicken intramuscular preadipocytes transfected with si-NC + INC, si-KLF10 + INC and si-KLF10 + Inhibitor. (C) qRT-PCR was used to detect cell proliferation marker genes *PCNA*, *CCND1*, and *CDK6*. (D) Representative images from the EdU assay. (E) Quantification ratio of EdU-positive cells/total cells.

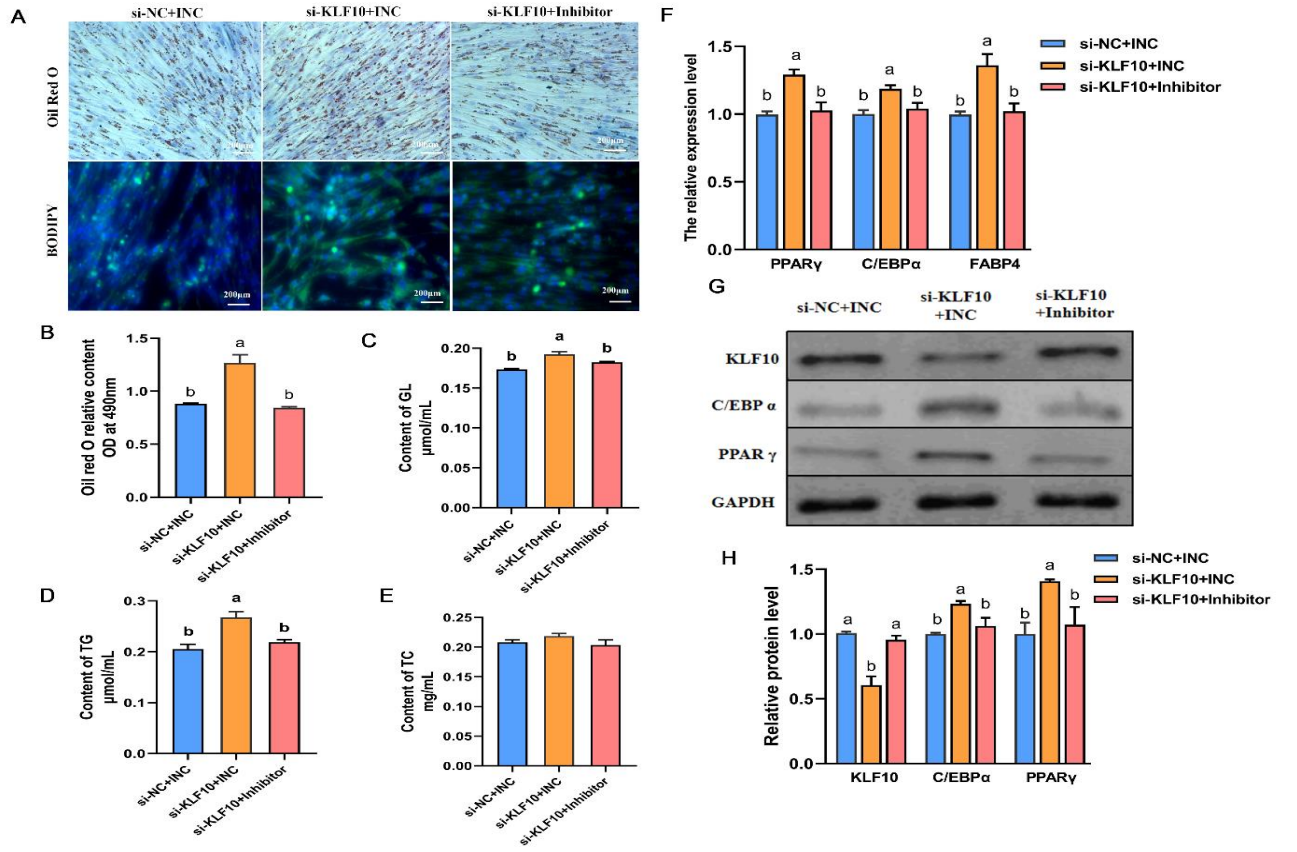

Fig. 8: Gga-miR-213 regulates intramuscular preadipocyte differentiation by targeting *KLF10*. (A) Oil red O and BODIPY staining of the differentiated chicken intramuscular preadipocytes transfected with si-NC + INC, si-KLF10 + INC and si-KLF10 + Inhibitor. (B) After extracting oil red O with isopropanol, the OD value was detected, 490 nm. (C-E) The levels of GL, TC and TG were detected after transfection with si-NC + INC, si-KLF10 + INC and si-KLF10 + Inhibitor. (F) qRT-PCR was used to detect the adipogenesis genes *C/EBPα*, *PPARγ*, and *FABP4*. (G) WB analysis of KLF10, C/EBPα and PPARγ after transfection with si-NC + INC, si-KLF10 + INC and si-KLF10 + Inhibitor. (H) Protein quantitative analysis of KLF10, C/EBPα and PPARγ.

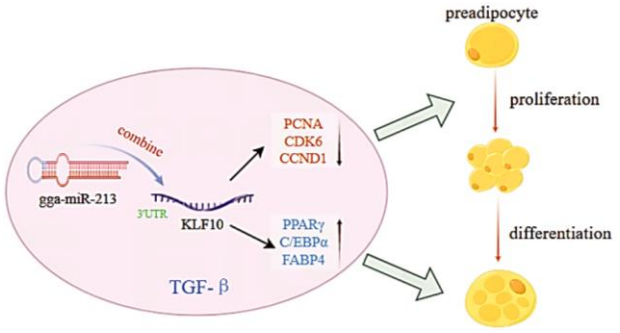

Fig. 9: Gga-miR-213 inhibits the proliferation of chicken intramuscular preadipocytes by down-regulating the expression of *KLF10* and promotes cell differentiation.

KLF10-mediated potentiation of TGF-β signaling occurs through TβRI kinase-dependent post-translational modification of C-terminal serine residues in regulatory Smad proteins (Smad2/3). Subsequent nuclear translocation of Smad4-containing complexes establishes a feedforward mechanism, wherein *KLF10* orchestrates transcriptional activation by directing chromatin occupancy at the regulatory regions of TGF-β pathway components, including Smad2, Smad7, and TGF-β1 loci (Johnsen *et al.,* 2002). Notably, *KLF10* transcriptional activity is subject to modulation by TGF-β superfamily ligands including bone morphogenetic proteins (BMPs) and activins, suggesting its functional integration within a multi-tiered regulatory architecture. We hypothesize this ligand-specific crosstalk implies *KLF10* serves as a signal integration node coordinating pleiotropic regulation of TGF-β pathway effectors across developmental and metabolic contexts.

The *PCNA*-encoded polypeptide functions as a key regulator of DNA replication initiation, serving as an established biomarker for mitotic activity assessment (González-Magaña *et al.,* 2020). As a member of evolutionary conserved cell cycle regulators, Cyclin D1 principally modulates the catalytic competency of cyclin-dependent kinases (CDKs) through stoichiometric complex formation (O'Connor *et al.,* 2021). *CDK6,* a serine/threonine kinase within this regulatory axis, serves as a pivotal mediator governing progression through the G1 restriction point and subsequent mitotic commitment (Kohoutek *et al.,* 2004). *CCND1* serves as a regulatory partner for *CDK6* kinase activation, with coordinated elevation of both factors shown to promote G1/S phase transition (Li *et al.,* 2018). In our system, knockdown of *KLF10* induced marked suppression of proliferative markers *PCNA*, *CCND1*, and *CDK6*, culminating in cell cycle arrest and impaired mitotic entry in chicken intramuscular preadipocyte. The effect of gga-miR-213 is opposite to that of *KLF10*.

The TGF-β superfamily mediates pleiotropic regulation of cellular homeostasis, predominantly manifesting growth suppressive or apoptosis-inducing activities across multiple cellular systems. Notably, *KLF10* emerges as a functional analog of TGF-β signaling, acting as a molecular surrogate capable of recapitulating its canonical pathways—inducing programmed cell death through caspase-dependent mechanisms while suppressing mitogenic signaling cascades (Fernandez-Zapico *et al.,* 2003; Buck *et al.,* 2006; Jin *et al.,* 2007; Gohla *et al.,* 2008). Jin et al demonstrated that *KLF10* upregulates the pro-apoptotic Bcl-2 family members, subsequently initiating mitochondrial apoptosis pathways in K562 leukemic cells (Jin *et al.,* 2007). However, our data indicates *KLF10's* regulatory effect appears cell-type dependent, as evidenced by Hep3B apoptosis enhancement through elevated oxidative activity, and there is no significant change in the expression level of Bcl-2 family members (Ribeiro *et al.,* 1999). This may explain why our results suggest that *KLF10* promotes rather than inhibits the proliferation of chicken intramuscular preadipocytes.

The induced differentiation of poultry adipocytes differs from that of mammals. Therefore, it is necessary to add a certain amount of exogenous fatty acids. Oleic acid is usually selected as an external stimulator to alter the expression patterns of genes in preadipocytes (Mou *et al.,* 2013). In our experimental system, 120 μmol oleic acid optimally induced differentiation. We observed that gga-miR-213 mimics significantly accelerated adipogenic differentiation, manifested as increased intracellular lipid droplet synthesis and increased TG, TC and GL accumulation. These findings suggest gga-miR-213 coordinates lipid homeostasis as a key regulator of intramuscular adipocyte differentiation. *KLF10* exerts opposing effects to gga-miR-213 on chicken intramuscular preadipocyte adipogenesis, indicating a potential targeting relationship between them.

The study in Dalian purple sea urchin demonstrated that *KLF10* inhibited the activity of *C/EBPα* by targeted regulation, thereby inhibiting adipocyte differentiation (Wu *et al.,* 2017). In 3T3-L1 cells, *KLF10* binds *C/EBPα* promoters to inhibit transcription and adipogenesis (Liu *et al.,* 2018). These conserved mechanisms align with our data, suggesting *KLF10* controls adipocyte differentiation by modulating *C/EBPα* transcriptional activation. Mechanistically, TGF-β signaling during adipogenic differentiation inhibits the differentiation of mesenchymal stem cells into adipocytes by epigenetically silencing *PPARγ* and *C / EBPα*. As a TGF-β-responsive transcriptional modulator, *KLF10* participates in this cascade by binding promoter regions of TGF-β/Smad elements, regulating signaling duration and amplitude, and ultimately affecting adipocyte differentiation (Spittau and Krieglstein, 2012).

Our results revealed a model in which gga-miR-213 regulates the KLF10-TGF-β pathway during early intramuscular adipogenesis. Mechanistically, the miRNA attenuates the expression of *KLF10* by post-transcriptional silencing and enhances lipid droplet formation in intramuscular preadipocytes. We postulate this accumulation amplifies downstream TGF-β pathway gene expression, thereby affecting the deposition of IMF. Our observations indicate that gga-miR-213 partially regulates IMF formation in chickens by downregulating *KLF10*. However, *KLF10* may be regulated by multiple miRNAs. Due to limitations in culture duration and microenvironmental complexity, in vitro systems fail to fully recapitulate avian developmental physiology or elucidate gga-miR-213's long-term regulatory effects on IMF deposition. Therefore, systematic in vivo validation is imperative to investigate the underlying mechanisms. This should integrate multi-omics analyses and functional validation approaches to comprehensively dissect the

molecular pathways, epigenetic regulatory mechanisms, and spatiotemporal integration of gga-miR-213 within the muscle development-metabolic network.

From an applied perspective, future work should assess whether enhanced IMF deposition via this axis improves meat palatability without compromising feed efficiency, critical for determining its translational value in poultry production systems. Our findings demonstrate that gga-miR-213 directly targets *KLF10* to suppress proliferation while promoting differentiation of intramuscular preadipocytes, thereby governing embryonic IMF deposition in chickens. The miR-213/*KLF10* axis operates bidirectionally-miR-213 inhibition reverses KLF10-knockdown phenotypes, establishing a regulatory circuit critical for adipogenesis. This mechanistic insight identifies actionable targets for precision breeding strategies to modulate poultry meat quality through IMF optimization.

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Data available: Sequence of novel miRNA gga-miR-213 have been uploaded to Sequence Read Archive Nos. BankIt2625210 gga-miR-213 OP490726.

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