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Original article

Magnolol inhibits appetite and causes visceral fat loss through Growth/differentiation factor-15 (GDF-15) by activating transcription factor 4-CCAAT enhancer binding protein γ-mediated endoplasmic reticulum stress responses

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ABSTRACT

Magnolol, a compound extracted from *Magnolia officinalis*, demonstrates potential efficacy in addressing metabolic dysfunction and cardiovascular diseases. Its biological activities encompass anti-inflammatory, antioxidant, anticoagulant, and anti-diabetic effects. Growth/differentiation factor-15 (GDF-15), a member of the transforming growth factor β superfamily, is considered a potential therapeutic target for metabolic disorders. This study investigated the impact of magnolol on GDF-15 production and its underlying mechanism. The research examined the pharmacological effect of magnolol on GDF-15 expression *in vitro* and *in vivo*, and determined the involvement of endoplasmic reticulum (ER) stress signaling in this process. Luciferase reporter assays, chromatin immunoprecipitation, and *in vitro* DNA binding assays were employed to examine the regulation of GDF-15 by activating transcription factor 4 (ATF4), CCAAT enhancer binding protein γ (CEBPG), and CCCTC-binding factor (CTCF). The study also investigated the effect of magnolol and ATF4 on the activity of a putative enhancer located in the intron of the *GDF-15* gene, as well as the influence of single nucleotide polymorphisms (SNPs) on magnolol and ATF4-induced transcription activity. Results demonstrated that magnolol triggers GDF-15 production in endothelial cells (ECs), hepatoma cell line G2 (HepG2) and hepatoma cell line 3B (Hep3B) cell lines, and primary mouse hepatocytes. The cooperative binding of ATF4 and CEBPG upstream of the *GDF-15* gene or the E1944285 enhancer located in the intron led to full-power transcription of the *GDF-15* gene. SNP alleles were found to impact the magnolol and ATF4-induced transcription activity of GDF-15. In high-fat diet *ApoE*$^{-/-}$ mice, administration of magnolol induced GDF-15 production and partially suppressed appetite through GDF-15. These findings suggest that magnolol regulates GDF-15 expression through priming of promoter and enhancer activity, indicating its potential as a drug for the treatment of metabolic disorders.

1. Introduction

In traditional Chinese medicine, *Magnolia officinalis*, a widely utilized medicinal plant, plays a significant role in resolving dampness to facilitate *Qi* movement, alleviate cough, and calm respiratory distress. Clinically, magnolol is employed as a complementary therapy for various conditions, including asthma, cardiovascular diseases, anxiety, and abdominal distension. Magnolol (5,5'-diallyl-[1,1'-biphenyl]-2,2'-diol, PubChem CID: 72300), a bioactive hydroxyl biphenyl extracted from the bark of *M. officinalis* and the root and branch bark of *M. dealbata* Zucc and *M. obovate*, is recognized for its health-promoting properties.

Magnolol exhibits a broad spectrum of pharmacological activities, including anti-inflammatory, antioxidant, anticoagulant, anticancer, antibacterial, neuroprotective, and asthma-controlling properties [1]. Notably, magnolol demonstrates potential efficacy in treating metabolic dysfunction and cardiovascular diseases. It inhibits the migration, proliferation, and hyperplasia of vascular smooth muscle cells, reduces pro-inflammatory cytokine production, prevents platelet aggregation, induces vasodilation, and shows therapeutic effects on pulmonary hypertension [2]. Furthermore, magnolol displays anti-diabetic properties by enhancing glucose uptake, preventing obesity, reducing oxidative stress, inhibiting diabetic retinopathy and nephropathy, and ameliorating high-fat diet-induced body fat accumulation in mice [3, 4]. The pharmacological effects of magnolol on metabolic dysfunction and cardiovascular diseases are mediated through the activation or inhibition of various signaling pathways. For instance, magnolol mitigates mitochondrial dysfunction and oxidative stress by activating the adenosine monophosphate-activated

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protein kinase/sirtuin-1/peroxisome proliferator-activated receptor coactivator-1-pathway [5]. It improves metabolic parameters by inhibiting protein tyrosine phosphatase 1B [6], a negative regulator of the insulin signaling pathway, and by increasing the mRNA expression of peroxisome proliferator-activated receptors, glucose transporter 4, and adiponectin, as well as activating the Akt pathway [7]. Studies have also demonstrated that magnolol can prevent diabetic organ damage by increasing superoxide dismutase, catalase, and glutathione peroxidase activities, decreasing malondialdehyde levels, and reducing transforming growth factor-1 expression [8, 9]. Recently, magnolol was identified as a regulator of endoplasmic reticulum (ER) stress signaling [10], which plays a significant role in numerous disorders.

Growth/differentiation factor-15 (GDF-15), a member of the transforming growth factor β superfamily, functions as an inflammatory marker. It contributes to the pathogenesis of metabolic disorders, ischemic diseases, and neurodegenerative processes, and is considered a potential therapeutic target for metabolic disorders and cardiovascular disease [11]. GDF-15 suppresses appetite and reduces body mass through a central mechanism by binding to its receptor, glial cell-derived neurotrophic factor family receptor alpha-like (GFRAL), in the brain [11, 12]. Various stress conditions, including hypoxia, nutritional stress, heart failure, and cardiac infarction, significantly elevate circulating GDF-15 levels, which correlate closely with insulin resistance and body weight changes. Recent research has demonstrated that metformin enhances the expression of activating transcription factor 4 (ATF4) and C/EBP homologous protein (CHOP), leading to GDF-15 production [13, 14]. Furthermore, GDF-15 can be regulated by ATF4 and inhibit LPS-induced inflammation [15]. This study aims to investigate whether magnolol can regulate GDF-15 production and elucidate the underlying mechanism.

2. Material and Methods

The methods and study materials are available from the corresponding authors upon request. The online Supplemental Material and Methods section provides additional details regarding this methodology.

2.1. Animal experiments

Male C57BL/6 and *ApoE*$^{-/-}$ mice (8 weeks old) were acquired from Cyagen Biosciences (Suzhou, China). All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health, 8th Edition (2011) and approved by the Animal Care and Use Committee of Putuo Hospital, Shanghai University of Traditional Chinese Medicine (No. DWEC-A-202308005, 08/23/2023).

2.2. Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM). Each experiment was independently replicated three times. Statistical significance was determined using one-way analysis of variance (ANOVA) and Tukey's multiple comparison test for three or more groups. For comparisons between two groups, an unpaired Student's *t*-test was employed. $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Magnolol activates ATF4 signaling pathway to increase GDF-15 expression

We evaluated the expression levels of GDF-15 in five distinct human cell types using quantitative polymerase chain reaction (qPCR) methodology and observed that endothelial and hepatoma cell line G2 (HepG2) cells express substantial quantities of GDF-15 (Supplementary data 1A). Subsequently, we investigated gene alterations within the GDF family in magnolol-treated endothelial cells (ECs) utilizing RNA-seq analysis. As detailed in Supplementary data 1B and 1C, GDF-15 demonstrated the highest expression level compared to six other GDF family members and was upregulated by magnolol. Employing qPCR and western blotting techniques, we verified that the *GDF-15* gene induction was both dose- and time-dependent (Figs. 1A–1C). Additionally, an ELISA assay was employed to quantify GDF-15 levels secreted into culture media. As illustrated in Supplementary data 1D, the ECs exhibited an enhanced capacity to secrete GDF-15 following magnolol exposure. The expression of *GDF-15* in ECs was also found to be induced by honokiol, a structural isomer of magnolol containing biphenolic groups (Fig. 1D). Furthermore, magnolol increased the mRNA expression of *GDF-15* in hepatocellular carcinoma cell lines (Fig. 1E and Supplementary data 1E) and primary mouse hepatocytes (Supplementary data 1F).

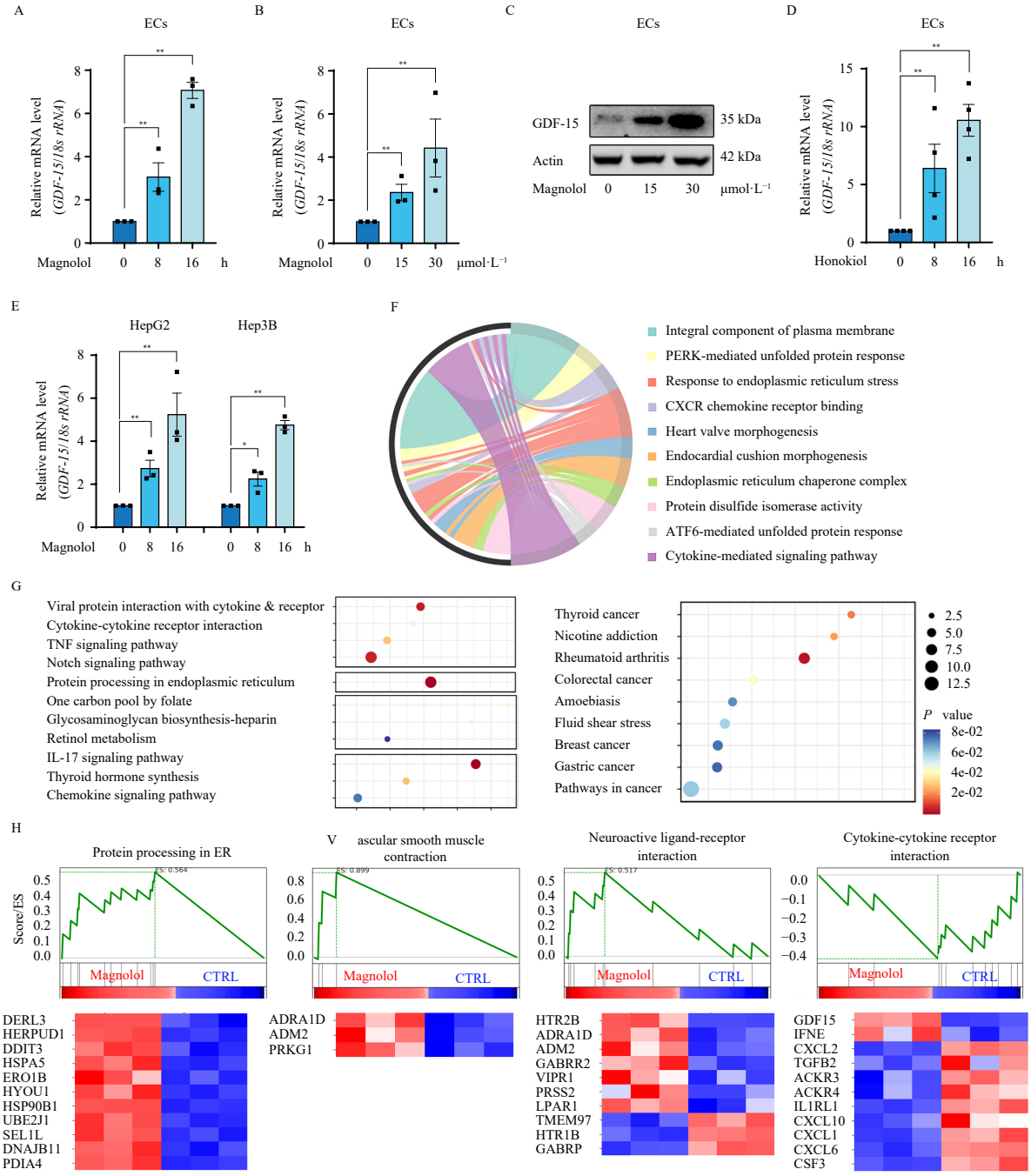
Subsequently, the potential biological functions of magnolol were investigated through GO, KEGG, and gene set enrichment analysis (GSEA) analyses. GO and KEGG pathway analyses revealed that differentially expressed genes (DEGs) were enriched in protein kinase R-like endoplasmic reticulum kinase (PERK)-mediated unfolded protein response, ATF6-mediated unfolded protein response, response to ER stress, CXCR chemokine receptor binding, and cytokine-mediated signaling pathway (Figs. 1F and 1G). The GSEA analysis indicated that magnolol was positively correlated with protein processing in ER, vascular smooth muscle contraction, and neuroactive ligand-receptor interaction, but negatively correlated with cytokine-cytokine receptor interaction (Fig. 1H). The expression of ER stress genes, including ATF4, CHOP, derlin-3 (DERL3), and homocysteine-inducible ER protein with ubiquitin-like domain 1 (HERPUD1), was confirmed by qPCR or western blotting assays (Supplementary data 1G–1I). Selected candidates from RNA-seq, as shown in Fig. 1H, were also validated at the transcript level by qPCR method (Supplementary data 1J–1M). To determine whether CHOP and ATF4 were essential for magnolol-induced GDF-15 expression, CHOP and ATF4 siRNA were employed. The expression of GDF-15 decreased with ATF4 knockdown, whereas CHOP knockdown had only a minor effect on it in response to magnolol treatment (Figs. 1I and 1J). These findings suggest that magnolol primarily increases GDF-15 expression through an ATF4-mediated signaling mechanism.

3.2. ATF4 binding to the promoter and intron regions of the *GDF-15* gene is altered by single nucleotide polymorphisms (SNPs)

Previous research demonstrated ATF4's ability to bind to the *GDF-15* promoter [15]. However, another study found ATF4 binding to the intron rather than the *GDF-15* promoter [16]. Our analysis of the Gene Expression Omnibus (GEO) profile database (GSE127432) revealed two potential ATF4-binding regions in the promoter and intron of *GDF-15*, respectively. Examination using the JASPAR dataset identified three ATF4 binding sites (BS): two (BS1 and BS2) within the promoter and one (BS3) within the intron of the *GDF-15* gene. BS1 and BS2 are not conserved among species, whereas BS3 is (Supplementary data 2A). To elucidate ATF4's role in GDF-15 transcription regulation, we conducted a luciferase assay using pGL3-luciferase reporter constructs containing the BS1 (−1775 ~ −1448 bp), BS2 (−1391 ~ −1 bp), BS3 (+625 ~ +1263 bp) fragments of the *GDF-15* gene, and BS1 or BS3 deletion mutants (BS1-mut and BS3-mut) (Fig. 2A). Luciferase reporter assays showed increased activity in wild-type BS1 construction following ATF4 overexpression. BS1 mutation resulted in decreased luciferase activity (Supplementary data 2B). Corres-

pondingly, luciferase activity of the wild-type BS1 construct was induced by magnolol and restored by siATF4 (Fig. 2B). ATF4 overexpression and magnolol or tunicamycin treatment did not affect the luciferase activity of the BS2 construct (Supplementary data 2C, D), but induced strong luciferase activity in the wild-type BS3 construct (Figs. 2C and 2D). Deletion of the BS3 binding element or ATF4 knockdown reversed the luciferase activity (Figs. 2C–2E). The ATF4 mutant, lacking the DNA binding domain, partially restored the luciferase activity of the BS3 construct caused by magnolol or wt-ATF4 (Supplementary data 2E and 2F). We then examined ATF4's association with BS1 and BS3. ChIP analyses indicated ATF4 binding to both BS1 in the promoter region (Supplementary data 2G) and BS3 in the intron region (Supplementary data 2H) of the *GDF-15* gene. Additionally, an *in vitro* DNA binding affinity assay to detect DNA–ATF4 protein interactions revealed ATF4's association with wild-type but not mutant BS1 and BS3 oligonucleotides (Fig. 2F).

SNPs have the potential to influence disease pathogenesis and drug efficacy. The UCSC Genome database indicates that the BS1 region contains rs1971807704, rs1355135929, rs1265550883, and rs1246294864 SNPs, while the BS3 region contains rs536224215, rs926183545, rs934399180, rs1052840861, and rs1971838159 SNPs (Fig. 2G and 2H). To investigate these SNP variations, we synthesized these alleles and subcloned them into a promoter-less firefly luciferase expression vector. Our findings revealed that T/G of rs1971807704, C/T of rs1355135929, and C/A/T of rs1265550883 were associated with low ATF4 and magnolol-induced BS1 construction activities. Similarly, G/A of rs926183545, AAA/A of rs934399180, and C/T of rs1052840861 were associated with low ATF4 and magnolol-induced BS3 construction activities (Figs. 2I–2L). Notably, T/C of rs1246294864 increased BS1 construct activity induced by ATF4 and magnolol. ATF4 knockdown reversed rs1246294864-linked BS1 construct activity (Supplementary data 2I), suggesting that rs1246294864 contributes to GDF-15 expression up-regulation. Furthermore, our *in vitro* DNA binding affinity assay demon-



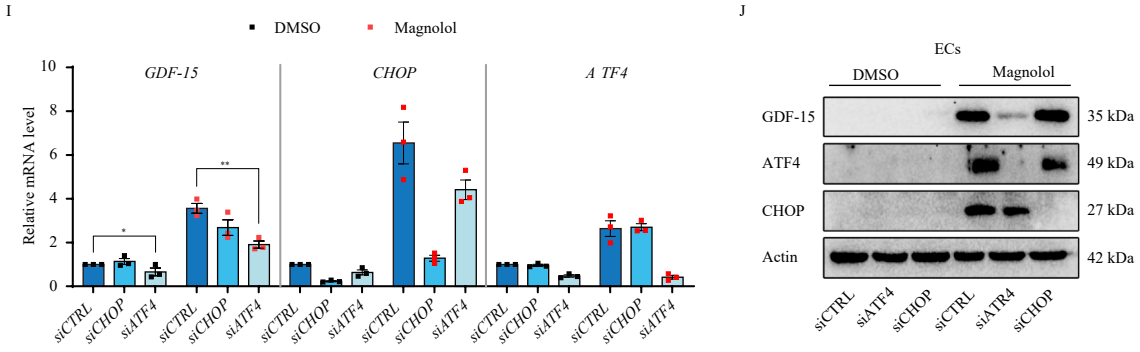


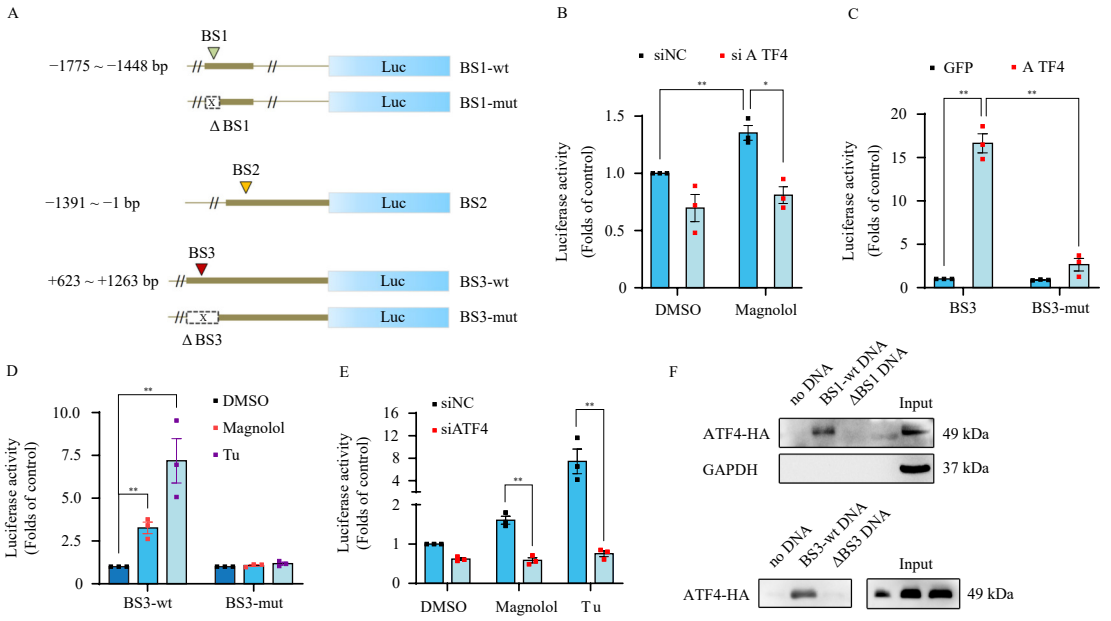
Fig. 1 Magnolol induces expression of GDF-15. (A–B) ECs were exposed to magnolol at a dose of 30 μmol·L^{-1} for 8 and 16 h (A), or at doses of 0, 15, and 30 μmol·L^{-1} for 16 h (B). The mRNA expression of GDF-15 was analyzed using the quantitative reverse transcription polymerase chain reaction (qRT-PCR) method (n = 3). (C) ECs were treated with dimethyl sulfoxide (DMSO) or magnolol (15 and 30 μmol·L^{-1}) for 16 h. The protein expressions of GDF-15 and actin were examined using western blotting assay (n = 3). Representative images are shown. (D) ECs were treated with honokiol (20 μmol·L^{-1}) for the indicated time. qRT-PCR analysis was performed to examine GDF-15 expression (n = 4). (E) HepG2 and hepatoma cell line 3B (Hep3B) cells were incubated with DMSO or magnolol (30 μmol·L^{-1}) for 8 and 16 h. Expression of GDF-15 was assessed using qRT-PCR assay (n = 3). (F) Gene ontology (GO) enrichment chord plot. Lines connect genes and their corresponding GO terms. (G) Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis results of DEGs. (H) Pathway-based gene set enrichment analysis of mRNAs. (I) ATF4 or CHOP silenced ECs were treated with DMSO or magnolol (30 μmol·L^{-1}) for 16 h. The mRNA expressions of GDF-15, ATF4, and CHOP was analyzed using qPCR method (n = 3). (J) ECs were transfected with scrambled ATF4 or CHOP siRNA for 32 h and then exposed to magnolol for 16 h. The protein expressions of GDF-15, ATF4, and CHOP were examined using western blotting (n = 3). Representative images are shown. Data are presented as mean ± SEM. *P < 0.05, **P < 0.01. Statistical significance was calculated by Tukey multiple comparison test.

strated that these SNPs can alter ATF4 binding affinity to BS1 and BS3 (Figs. 2M and 2N). These findings indicate that inter-individual differences in GDF-15 expression induced by magnolol and stress conditions correlate with these ATF4 binding–disrupting SNPs.

3.3. The expression of GDF-15 is regulated by CCAAT enhancer binding protein γ (CEBPG)

CEBPG can heterodimerize with ATF4 and interact with common genome binding sites to regulate stress-induced genes. This study aimed to investigate if CEBPG interacts with the ATF4 signal to regulate magnolol-induced GDF-15 expression. Analysis of ChIP-seq data from the GEO database (GSE105532) revealed that CEBPG binds to the promoter and intron regions of the *GDF-15* gene. The JASPAR database indicated that CEBPG and ATF4 interact with identical binding sites (BS1 and BS3) on the *GDF-15* gene and showed that CEBPG's binding to these sites could be disrupted by SNPs (Fig. 3A). ChIP and *in vitro* DNA binding affinity assays were conducted to confirm CEBPG binding to the *GDF-15* promoter or intron. ChIP-qPCR analysis demonstrated CEBPG enrichment in both regions (Fig. 3B). The *in vitro* binding assay confirmed CEBPG interaction with BS1 and BS3 and identified that rs1971807704, rs1355135929, rs926183545, and rs934399180 disrupted CEBPG binding to regulatory elements (Fig. 3C). Further investigation examined CEBPG's role in stress-induced GDF-15 expression. Magnolol increased CEBPG mRNA levels in ECs, an effect reversed by ATF4 silencing (Fig. 3D). CEBPG depletion reversed magnolol's effect on GDF-15 expression (Fig. 3E). Additionally, CEBPG knockdown decreased ATF4 and magnolol-induced luciferase activity of BS1 and BS3 constructs (Figs. 3F and 3G). These findings suggest that magnolol induces CEBPG expression through ATF4 activation, and CEBPG and ATF4 may cooperatively increase GDF-15 expression.

Furthermore, we evaluated the influence of CEBPG on GDF-15 expression using adenovirus-mediated CEBPG overexpression. Notably, CEBPG overexpression inhibited magnolol-induced mRNA expression of *GDF-15* in ECs (Figs. 3H and 3I). At 24 hours post-transfection, CEBPG overexpression also suppressed the luciferase activity induced by ATF4 and magnolol from BS1 and BS3 constructs (Figs. 3J and 3K). Given that CEBPG lacks the transcriptional activation domain and may function as a dominant negative repressor of gene expression, potentially acting as a general inhibitor of other transcription factors, we constructed a



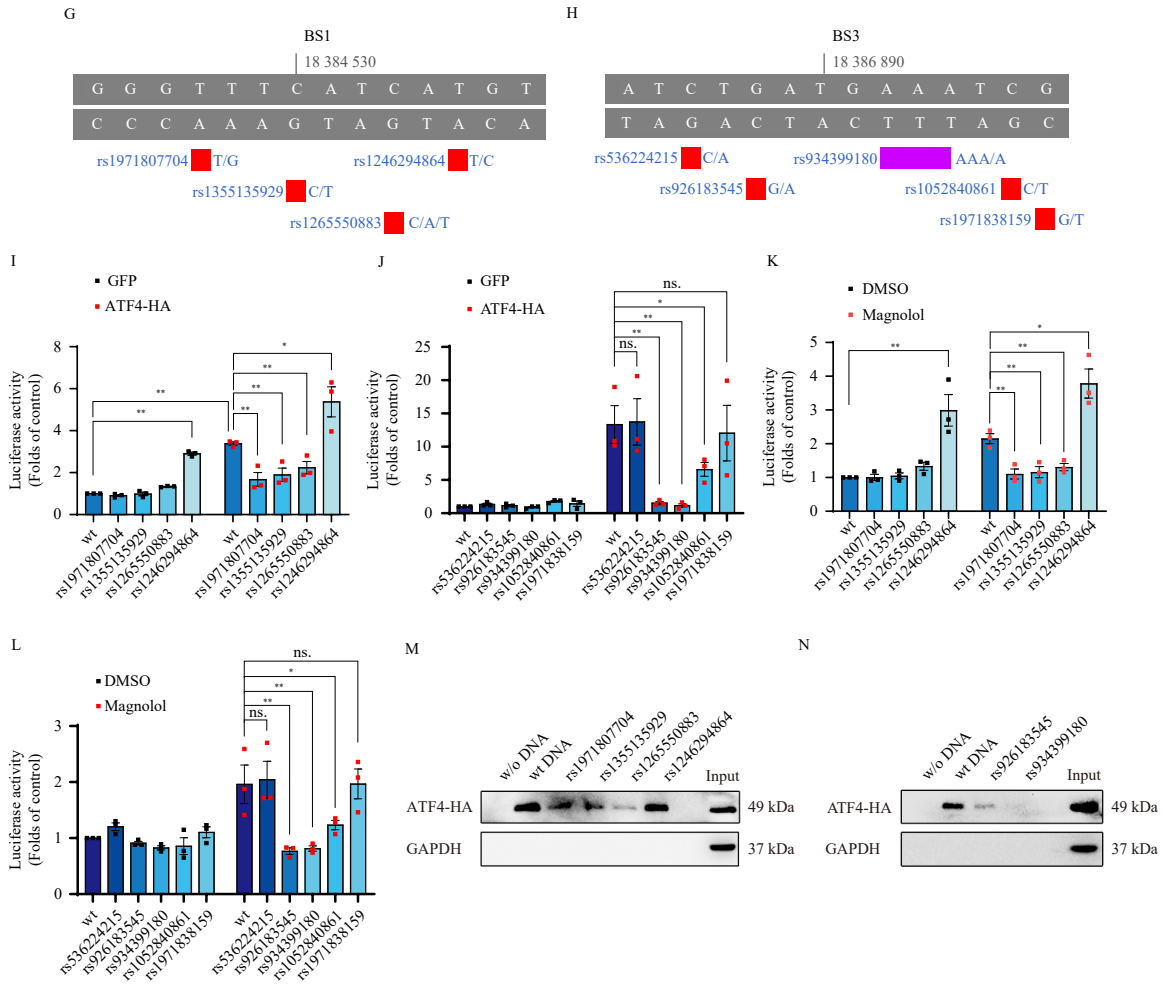


Fig. 2 The ATF4 signaling pathway is crucial for mediating magnolol's effect on GDF-15 expression. (A) Schematic representation of the luciferase reporter construction. DNA fragments containing BS1, BS2, or BS3 were cloned into the pGL3-basic vector. (B) Control and ATF4 knockdown HEK-293 cells were transfected with the reporter bearing BS1, stimulated with magnolol, and subsequently analyzed for reporter activity. (C) HEK-293 cells were transfected with reporters bearing BS3 and BS3 deletion mutant alone or co-transfected with ATF4. Cells were harvested 24 h post-transfection and subjected to luciferase assay. (D) HEK-293 cells, transfected with reporters bearing BS3 and BS3 deletion mutant, were treated with magnolol (30 μmol·L^{-1}) and tunicamycin (1 μg·mL^{-1}) for 16 h before luciferase activity analysis. (E) Control or ATF4 knockdown HEK-293 cells were transfected with reporters bearing BS3, treated with magnolol (30 μmol·L^{-1}) and tunicamycin (1 μg·mL^{-1}) for 16 h, then lysed and analyzed using the luciferase activity assay. (F) Nuclear extracts from ATF4-HA-expressing HEK-293 cells were incubated with oligonucleotides containing BS1 and BS3 or not. Precipitations were analyzed by western blotting using HA and GAPDH antibodies. Results represent three independent experiments. Representative images are shown. (G, H) Genotypes and alleles of the SNPs in BS1 (G) and BS3 (H). (I, J) Four BS1 (I) or five BS3 (J) reporter constructs containing various polymorphisms and constructs encoding GFP or ATF4 were co-transfected into HEK-293 cells. Luciferase activity in cell lysates was measured. (K, L) Four BS1 (K) or five BS3 (L) reporter constructs containing various polymorphisms were transfected into HEK-293 cells. Transfected cells were treated with DMSO or magnolol and lysed for luciferase activity assay. (M, N) Nuclear extracts from ATF4-HA-expressing cells were incubated with oligonucleotides containing various polymorphisms. Oligonucleotide-binding proteins were analyzed by western blotting using HA and GAPDH antibodies. Data are presented as mean ± SEM (n = 3). ns. no significance; $^{*}P < 0.05$, $^{**}P < 0.01$. Representative images are shown. Statistical significance was calculated using Tukey multiple comparison test.

constitutively active CEBPG (VP16-CEBPG) by fusing the activation domain of herpes virus VP16 to wild-type CEBPG. We observed that VP16-CEBPG overexpression enhanced ATF4-induced activity in wild-type BS3 constructs (Fig. 3L) according to the luciferase reporter assay. Our findings suggest that CEBPG serves as a key regulator controlling GDF-15 transcription.

3.4. Magnolol activates E1944285 enhancer of the GDF-15 gene

The regulation of gene expression is significantly influenced by enhancers located in introns. The Search Candidate *cis*-Regulatory Elements (SCREEN) web tool indicates that the *GDF-15* intron contains numerous candidate enhancers. We observed that a candidate enhancer, E1944285, includes ATF4-BS3 binding sites (Fig. 4A). Our chromatin immunoprecipitation (ChIP)-qPCR results demonstrate that ATF4 and magnolol enhance the enrichment of promoter and intron in H3K27ac, a marker for active enhancers (Figs. 4B and 4C). To validate whether ATF4 and magnolol regulate the enhancer activity of E1944285, DNA fragments containing BS1 (−1775 ~ −1448 bp), BS3 (+625 ~ +1262 bp), and rs1246294864 were cloned into a pGL3-promoter vector with an SV40 promoter (Fig. 4D). The enhancer assay revealed that BS3, but not BS1 and rs1246294864 constructs, can be activated by ATF4 or magnolol, confirming that magnolol induces GDF-15 expression by modulating the E1944285 enhancer activity (Figs. 4E and 4F). Additionally, we examined the impact of CEBPG on E1944285 enhancer activity and found that both CEBPG knockdown and overexpression decrease ATF4- and magnolol-induced E1944285 enhancer activity in cells (Figs. 4G–4J). However, VP16-CEBPG overexpression increases ATF4-induced E1944285 enhancer activity in cells (Fig. 4K).

3.5. CCCTC-binding factor (CTCF) binding is not necessary for sustaining GDF-15 gene expression

CTCF possesses two transcription repressor domains and functions as a transcriptional repressor by modulating the enhancer-promoter interactions of target genes. Given that the

E1944285 enhancer can regulate GDF-15 transcription, we conducted a re-analysis of the SCREEN dataset. The online ChIP-sequencing (seq) data revealed that CTCF can bind to the exons of the *GDF-15* gene (Fig. 5A). Additionally, a CTCF binding site (CBS) was identified between the E1944285 enhancer and GDF-15 promoter, exhibiting conservation across species (Fig. 5B). The sequenced genomic data indicates that this CTCF-binding motif contains a series of SNPs. JASPAR analysis predicts that rs1250971253, rs577008845, rs779393592, rs1971826696, and rs1971826744 are reduced-function variant alleles (Fig. 5C). Consequently, we examined CTCF binding to CBS and the impact of SNPs on this interaction. An *in vitro* DNA binding affinity assay demonstrated that wild-type CBS can bind CTCF-FLAG, while rs1250971253, rs577008845, rs779393592, and rs1971826696 SNPs reduce CTCF-FLAG DNA binding (Fig. 5D). A ChIP assay further confirmed the direct binding of CTCF to this site (Fig. 5E).

To investigate the functional impact of CTCF on E1944285 enhancer activity, a DNA fragment (+70 ~ +138 bp) containing the CBS (+87 ~ +120 bp) was inserted between the BS3 and SV40 promoter in the pGL3-promoter vector (Fig. 5F). Subsequent luciferase reporter assays revealed that CTCF overexpression did not alter ATF4 and magnolol-induced transcriptional activity (Figs. 5G and 5H). Further assessment of GDF-15 expression in CTCF-silenced ECs using qPCR demonstrated no significant change (Fig. 5I). These findings suggest that CTCF binding is not essential for maintaining *GDF-15* gene expression.

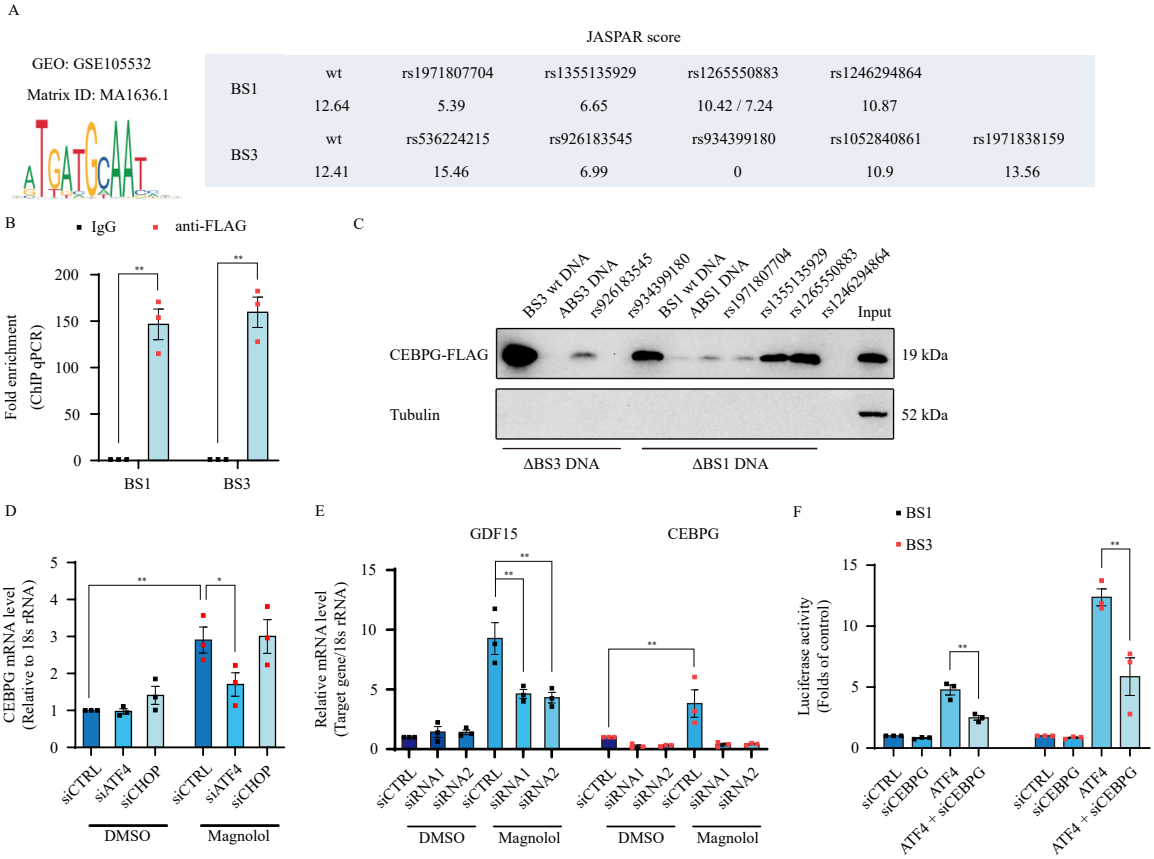
3.6. Magnolol reduces food intake in ApoE$^{-/-}$ mice partly through GDF-15

To investigate the impact of magnolol on GDF-15 expression *in vivo*, C57BL/6 mice were administered magnolol *via* oral gavage. Subsequently, GDF-15 levels in plasma and tissues were assessed. Oral administration of magnolol at a dosage of 24 mg·kg^{-1} increased GDF-15 mRNA expression in the liver (Fig. 6A) but not in the kidney (Fig. 6B), eWAT (Supplementary data 3A), or brain (Data not shown). Plasma GDF-15 content in mice increased 16 h after magnolol gavage at a dose of 24 mg·kg^{-1} (Fig. 6C). After 30 h, GDF-15 expression levels in the liver and blood returned to baseline (Supplementary data 3B and 3C). Correspondingly, the expressions of ATF4 and CHOP in the liver were elevated following magnolol administration (Fig. 6D). These findings suggest that magnolol enhances GDF-15 production *in vivo*.

Finally, we investigated the effect of magnolol on the expression of GDF-15 in *ApoE*$^{-/-}$ mice, which are known to develop obesity and hyperinsulinemia when fed a high-fat diet. In this study, the *ApoE*$^{-/-}$ mice received magnolol *via* gavage three times per week for two weeks, with an oral glucose tolerance test (OGTT) performed on day 15 (Supplementary data 3D). The results indicated that administration of 8 mg·kg^{-1} magnolol did not alter body weight. However, administration of 24 mg·kg^{-1} of magnolol resulted in a slight decrease in weight gain in *ApoE*$^{-/-}$ mice on a high-fat diet during the second week (Fig. 6E). Additionally, magnolol reduced food intake, suggesting an effect on appetite in *ApoE*$^{-/-}$ mice (Fig. 6F). The magnolol-treated *ApoE*$^{-/-}$ mice demonstrated improved glucose tolerance (Fig. 6G) and a downward trend in fasting plasma triglyceride and cholesterol levels (Figs. 6H and 6I). Magnolol administration also significantly reduced the ratio of epididymal visceral fat (Fig. 6J) to body weight but not that of the liver (Data not shown). To further examine changes in visceral fat, morphological analysis of mouse epididymal adipose tissue was conducted using hematoxylin and eosin (H&E) staining. The histological analysis revealed that magnolol-treated mice had smaller epididymal adipocyte sizes compared to the control mice (Figs. 6K and 6L).

Subsequently, we examined magnolol's potential to modulate GDF-15 expression in *ApoE*$^{-/-}$ mice. Administration of magnolol led to an elevation in plasma GDF-15 protein levels (Fig. 6M). To evaluate GDF-15's role in mediating magnolol's effect on food intake, an antibody targeting GDF-15 was administered. In

Figure 3



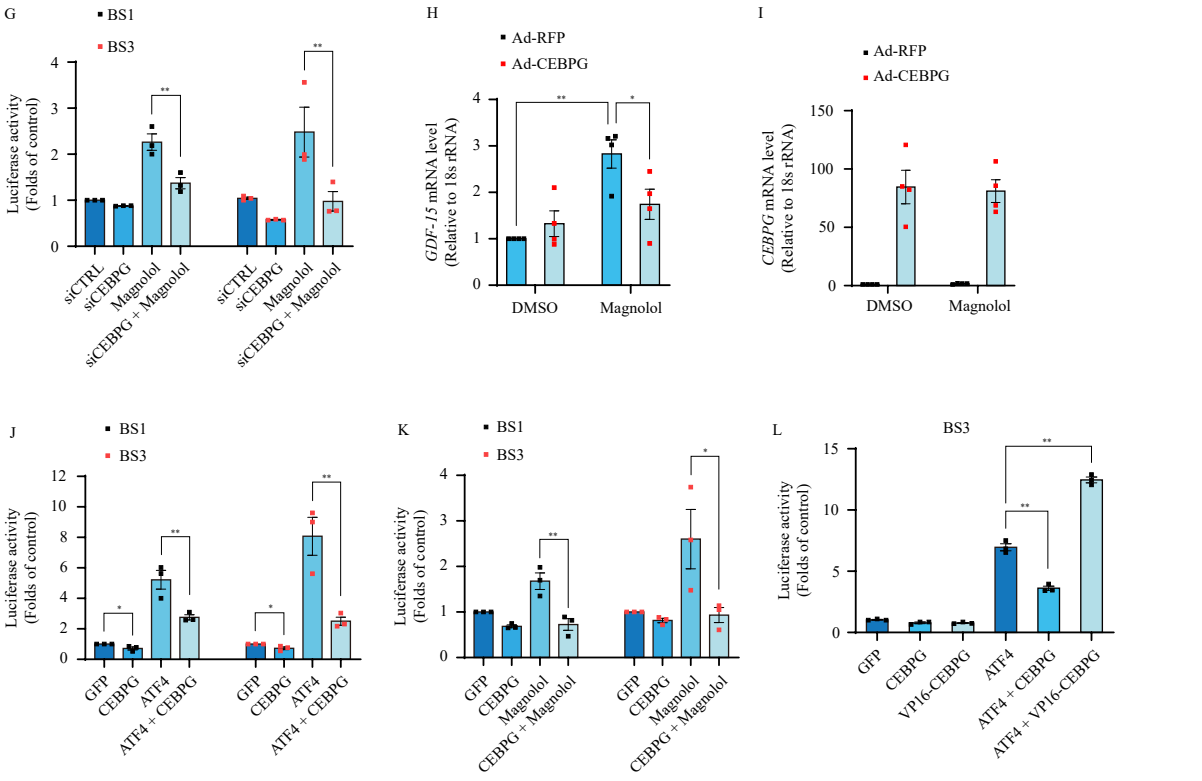


Fig. 3 The regulatory effect of CEBPG on GDF-15 expression. (A) The enrichment scores for each polymorphism of the CEBPG binding site set from JASPAR. (B) HEK-293 cells were transfected with the expression vector for CEBPG-Flag for 24 h. ChIP assays were performed with anti-FLAG antibodies. Primers amplified regions near BS1 and BS3 (n = 3). (C) Biotin assay of oligonucleotides containing various polymorphisms bound to CEBPG-FLAG (n = 3). Representative images are shown. (D) ECs, transiently transfected with scrambled ATF4 or CHOP siRNA were treated with DMSO or 30 μmol·L^{-1} magnolol for 16 h. CEBPG mRNA levels were determined using qRT-PCR (n = 3). (E) ECs were transfected with scrambled or two sets of siRNAs targeting different CEBPG regions, then treated with DMSO or 30 μmol·L^{-1} magnolol for 16 h. *GDF-15* and *CEBPG* mRNA levels were evaluated by qRT-PCR (n = 3). (F, G) Effect of CEBPG siRNA on ATF4- (F) and magnolol- (G) induced BS1 and BS3 reporter activity (n = 3). (H, I) ECs were infected with Ad-RFP and Ad-CEBPG for 32 h, then exposed to magnolol (30 μmol·L^{-1}) for 16 h. *GDF-15* (H) and *CEBPG* (I) mRNA levels were evaluated by qRT-PCR (n = 4). (J, K) Effects of CEBPG overexpression on BS1 and BS3 reporter activity induced by ATF4 (J) and magnolol (K) (n = 3). (L) Effects of VP16-CEBPG overexpression on BS3 reporter activity induced by ATF4 (n = 3). Results are representative of three independent experiments. Data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$. Statistical significance was calculated by Tukey multiple comparison test.

ApoE$^{-/-}$ mice maintained on a high-fat diet, the anti-GDF-15 antibody partially reversed magnolol-induced suppression of food intake, suggesting that magnolol's impact on appetite is partly mediated by GDF-15 (Fig. 6N).

Epididymal fat pads were surgically extracted from *ApoE*$^{-/-}$ mice, and gene expressions related to lipid metabolism pathways were evaluated using qPCR analysis. Notably, the results revealed that magnolol administration reduced the mRNA expression of lipin 2 (LPIN2) (Fig. 6O), which regulates fatty acid metabolism, and fatty acid synthase (FASN) (Fig. 6P), which catalyzes the *de novo* synthesis of long-chain fatty acids. Additionally, magnolol decreased the mRNA expression of transmembrane protein 97 (TMEM97) and C-X-C motif chemokine ligand 3 (CXCL3) (Fig. 6R), both of which positively regulate adipogenic differentiation [17, 18]. However, magnolol did not significantly affect the mRNA expressions of fourteen other genes involved in lipid metabolism (Figs. 6O–6R).

4. Discussion

Magnolol and its isomer, honokiol, have been identified as significant modulators of ER stress signaling. Research indicates that ATF4 and CHOP-mediated ER stress signaling contribute to the anticancer effects of magnolol and honokiol. For instance, magnolol demonstrates anticancer activity by modulating CHOP signaling in hepatocellular carcinoma cells and enhances the therapeutic effects of TNF-related apoptosis-inducing ligand through ATF4-mediated death receptor expression in clear cell carcinoma [19]. Honokiol has been shown to induce ER stress-mediated apoptosis or paraptosis in human lung cancer cells and acute promyelocytic leukemia cells [20]. Conversely, studies have also observed the beneficial effects of magnolol and honokiol in mitigating ER stress and treating various diseases. For example, magnolol protects neurons against ischemia injury by downregulating CHOP [21]. Honokiol prevents chronic restraint stress-induced cognitive impairment by inhibiting ER stress in the hippocampus of mice, and ameliorates high glucose and high fat-induced dysfunction by suppressing the expression of ER stress markers in ECs [22]. Additionally, honokiol attenuates ER stress and inhibits LPS-induced inflammatory response and apoptosis in bovine endometrial epithelial cells [23]. These varied effects may be attributed to differential cellular responses under diverse stress conditions.

The CEBP family proteins comprise six members: C/EBP-α, β, γ, δ, ε, and ζ. ATF4 consistently binds to DNA-regulatory sequences, known as C/EBP:ATF response elements, by associating with CEBP family members to regulate various gene expressions. For example, ATF4 binds with CEBPB to promote adipogenic differentiation of mesenchymal stem cells by inducing adipogenic gene expression, regulate muscle atrophy by inducing Gadd45a expression, and alter cellular metabolism by driving the expression of 3-phosphoglycerate dehydrogenase. CEBPD and ATF4 collaborate with FOXO1 to regulate myofiber formation [24]. Previous research has demonstrated that transcription of CEBP family members can be enhanced by ER stress. ATF4 has been shown to drive the expression of CEBPB, which subsequently induces the expression of musclin [25]. Additionally, ATF4 can regulate myeloid gene expression by potentiating CEBPE while inhibiting CEBPA-mediated transcriptional activation.

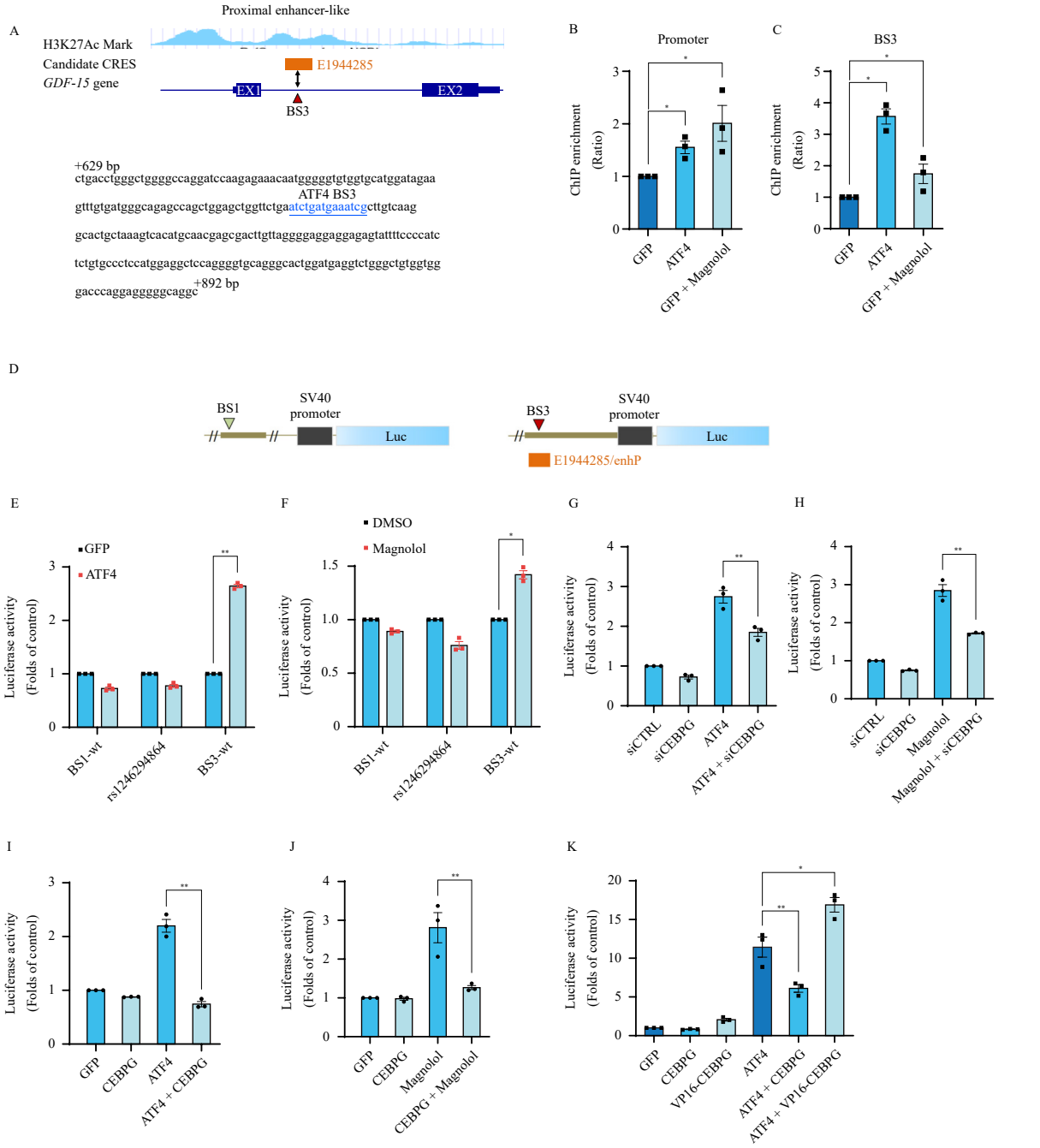


Fig. 4 The enhancer-mediated transcription of GDF-15. (A) Map of the E1944285 enhancer region. (B, C) H3K27ac ChIP-qPCR of HEK-293 cells transfected with ATF4 or treated with magnolol. ChIP-qPCR analysis confirmed the presence of acetylated H3K27 at the *GDF-15* promoter (B) and intron (C). (D) Schematic representation of luciferase reporter constructs. DNA fragments containing BS1 (−1775 ~ −1448 bp) and BS3 (+ 625 ~ +1262 bp) were cloned into the pGL3-SV40 promoter-containing vector. (E) pGL3-BS1 wt-SV40 promoter, pGL3-rs1246294864-SV40 promoter, and pGL3-BS3 wt-SV40 promoter were co-transfected with pGFP or pATF4-HA into HEK-293 cells. Luciferase activity was measured 24 h post-transfection. (F) Impact of magnolol on the activities of BS1-SV40 promoter, rs1246294864-SV40 promoter, and BS3-SV40 promoter. (G, H) The effect of CEBPG siRNA on BS3-SV40 promoter activity induced by ATF4 (G) and magnolol (H). (I, J) The effect of CEBPG overexpression on BS3-SV40 promoter activity induced by ATF4 (I) and magnolol (J). (K) The effect of VP16-CEBPG overexpression on BS3-SV40 promoter activity induced by ATF4. Data are presented as mean ± SEM (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$. Statistical significance was determined by Tukey multiple comparison test.

Among the CEBP family members, CEBPG stands out as the smallest due to its lack of an N-terminal activation domain. Recent research has demonstrated CEBPG's role in regulating cell proliferation and integrated stress response. Several studies have identified CEBPG as an essential ATF4 partner. For instance, CEBPG and ATF4 collaborate in regulating the expression of numerous stress-induced and antioxidant genes by interacting with the common genomic C/EBP-ATF response element. During amino acid deprivation, ATF4 forms heterodimers with CEBPG to facilitate the transcription of genes associated with tRNA charging, folate metabolism, and amino acid synthesis [26]. *ATF4*$^{-/-}$ cells exhibit reduced CEBPG transcription. While previous research has established ATF4's role in promoting GDF-15 expression, CEBPG's regulatory functions remain less understood. An RNA-seq analysis using C2C12 myoblasts to identify CEBPB target genes revealed GDF-15 upregulation upon CEBPB overexpression [27]. Our study further demonstrates that magnolol induces CEBPG expression through ATF4. Subsequently, CEBPG and ATF4 bind to the same DNA sequence in the promoter and intron regions of the *GDF-15* gene, driving magnolol-induced *GDF-15* gene tran-

scription. However, due to its lack of a transcriptional activation domain, CEBPG may function as a dominant negative repressor of gene expression and be considered a general inhibitor of other transcription factors [28]. In this study, we observed that overexpression of wild-type CEBPG decreases ATF4-induced GDF-15 transcriptional activity, while constitutively active CEBPG (VP16-CEBPG) increases it. These findings suggest that CEBPG is essential for GDF-15 expression, and excess CEBPG may act as a dominant negative repressor of ATF4. GDF-15 expression may be regulated by a negative feedback loop. Additionally, CEBPs can recognize noncanonical C/EBP binding sites depending on their heterodimeric partner. C/EBPs can bind to these noncanonical sites and regulate target gene expression [29]. Therefore, besides the classical CEBPG-mediated regulation of GDF-15 expression, other mechanisms may also be involved.

Promoters and enhancers collaborate, and are bound and regulated by transcription factors in a highly cell-type specific manner. Several ATF4 and CEBP binding sites within enhancers

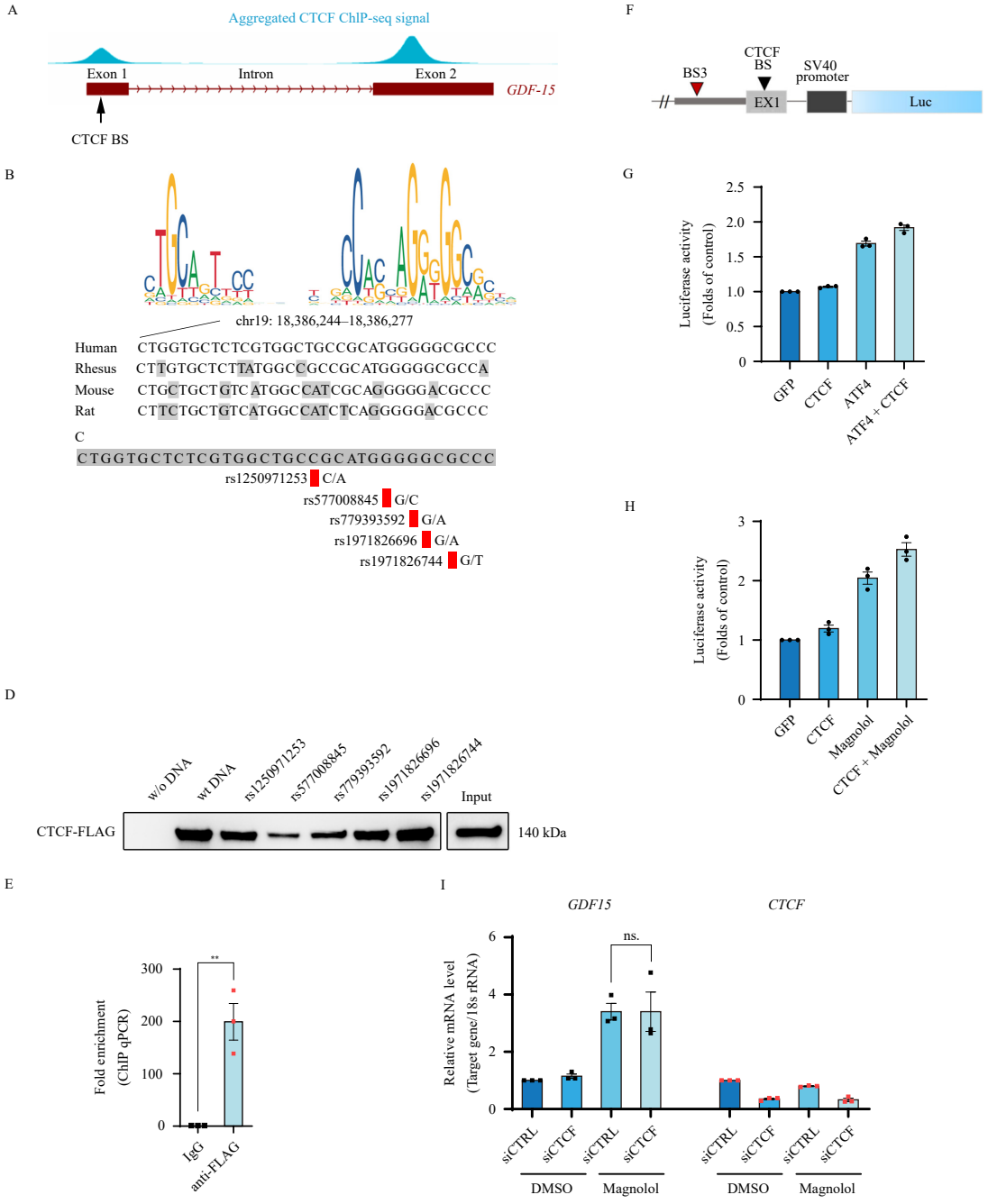
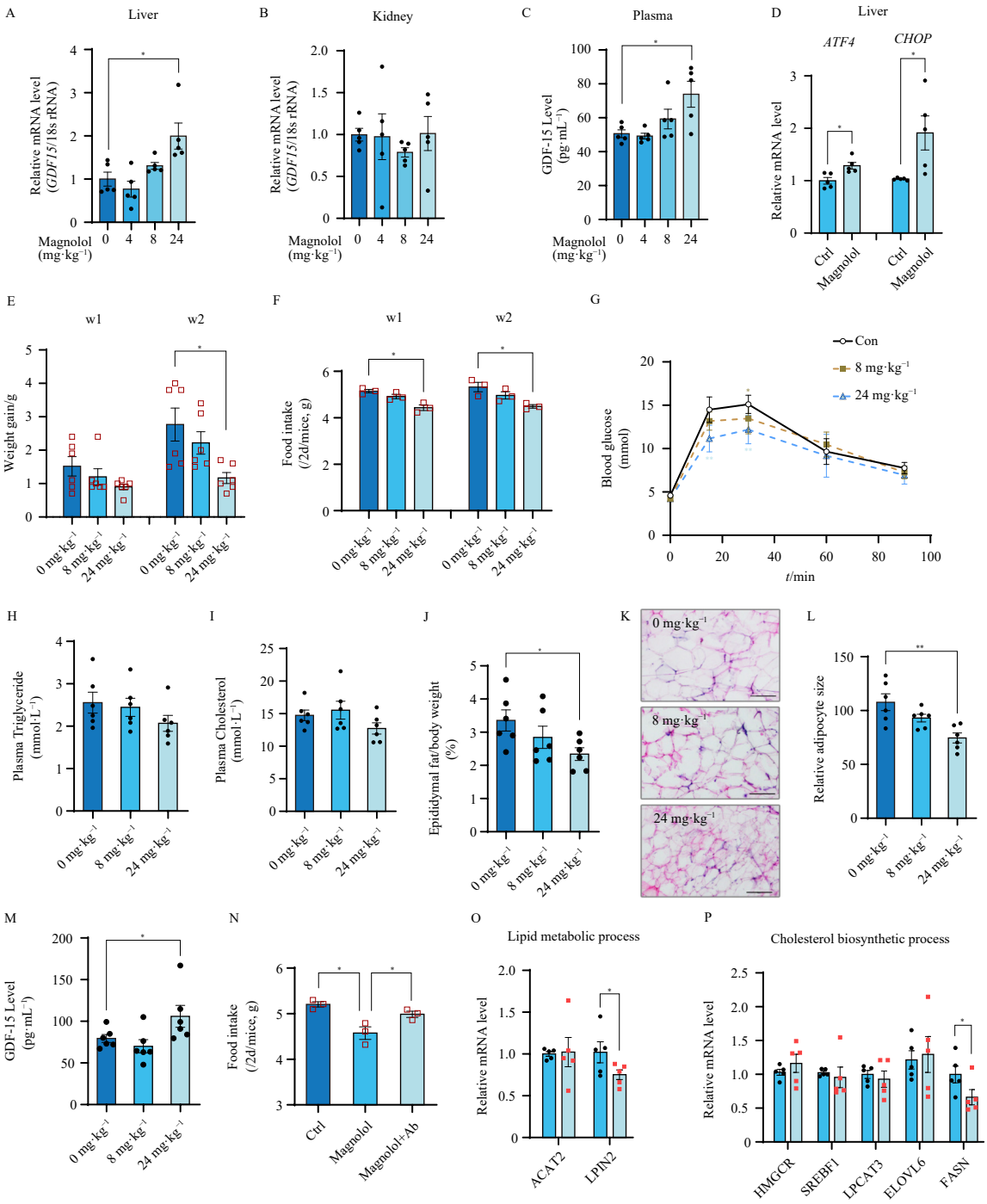


Fig. 5 CTCF is bound to the first exon of the *GDF-15* gene. (A) SCREEN dataset illustrating the CTCF binding region of the *GDF-15* gene. (B) The predicted CTCF binding site and its conservation across species. (C) SNPs located within the CTCF binding site. (D) *In vitro* DNA binding assay of CTCF-FLAG bound to biotinylated oligonucleotides containing various polymorphisms. Results are representative of three independent experiments. Representative images are shown. (E) HEK-293 cells were transfected with plasmid expressing CTCF-FLAG. ChIP assay was performed using antibodies as indicated. (F) Construction of pGL3-BS3 wt-CTCF BS-SV40 promoter reporter. (G) HEK-293 cells were co-transfected with pGL3-BS3 wt-CTCF BS-SV40 promoter reporter and pGFP, pCTCF-FLAG, pATF4 or pCTCF-FLAG plus pATF4. Luciferase activity was measured 24 h post-transfection. (H) HEK-293 cells were co-transfected with pGL3-BS3 wt-CTCF BS-SV40 promoter reporter and pGFP or pCTCF-FLAG, then exposed to magnolol (30 μmol·L^{-1}), and the luciferase activity assay was conducted. (I) The ECs were transfected with scrambled or CTCF siRNA for 32 h and subsequently treated with magnolol (30 μmol·L^{-1}) for an additional 16 h. The mRNA expression of GDF-15 was quantified using the qRT-PCR method. Results are derived from three independent experiments. Data are presented as mean ± SEM (n = 3). ns. no significance; $^{**}P < 0.01$. For G–I, statistical significance was determined by Tukey multiple comparison test. For E, statistical significance was calculated based on biological replicates using an unpaired Student's t-test.

have been identified. For instance, ATF4 can bind to the hybrid motif as a heterodimer with CEBPB to induce ER stress-related genes in differentiating human mesenchymal stem cells [30]. ChIP analysis revealed a novel ATF4 binding site in the erythropoietin 3'-enhancer region [31]. The expression of the daunorubicin drug export pump ATP binding cassette subfamily B member 1 is dynamically regulated in leukemia cells through an ATF4-bound enhancer [32]. ATF4 also stimulates the transcription of BCL11 transcription factor A by binding to its enhancer and promoting enhancer-promoter interactions [33]. This study reports a new candidate enhancer E1944285, with a proximal enhancer-like signature, located approximately 0.7 kb from the transcription start site of the *GDF-15* gene. Both ATF4 and CEBPG can bind to the E1944285 enhancer and regulate *GDF-15* gene transcription. CTCF, a highly conserved zinc finger protein, functions as a transcriptional activator, repressor, or insulator protein. CTCF binding to an insulator sequence inhibits the interaction between enhancer and promoter, blocking gene transcription, as observed in *β*-globin and IGF-2 [34]. However, studies have also suggested that CTCF binding is not always necessary to maintain target gene expression. For example, a ChIP-chip assay detected four CBSs upstream of the *homeobox D cluster* gene and seven sites downstream of the cluster. Nonetheless, CTCF does not affect *homeobox D cluster* gene expression. CTCF binding is also not required to maintain *homeobox A cluster* gene expression in acute myeloid leukemia cells [35]. In this study, we identified CTCF presence at the first exon of the *GDF-15* gene and demonstrated that CTCF binding is not essential for maintaining *GDF-15* gene expression. The present study's data reveal several novel observations illustrating the roles of ATF4, CEBPG, and CTCF in regulating GDF-15 gene expression induced by magnolol and stress conditions. Further research is needed to determine if the CTCF response ele-



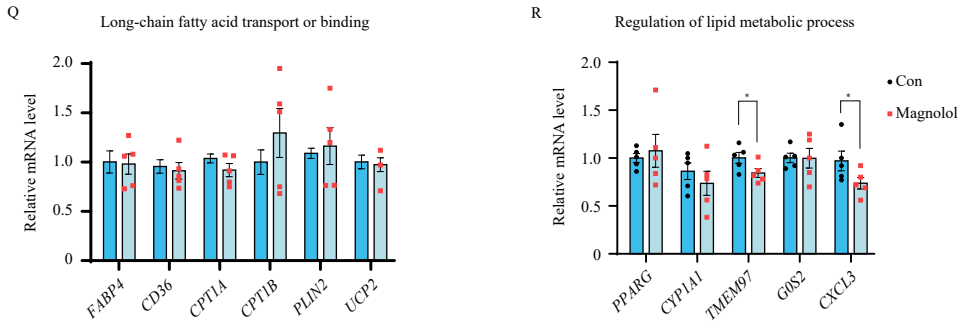


Fig. 6 Magnolol regulates food consumption partially through GDF-15. (A–D) C57BL/6 mice were administered magnolol *via* gavage (4, 8, and 24 mg·kg^{-1}). Sixteen hours post-administration, the mRNA expressions of *GDF-15* in the liver (A) and kidney (B) were quantified using qRT-PCR (n = 5). Plasma GDF-15 levels in mice were determined by ELISA assay (C) (n = 5). The mRNA expressions of *ATF4* and *CHOP* in the livers of mice administered 24 mg·kg^{-1} were analyzed using qPCR (D) (n = 5). Data are presented as mean ± SEM (n = 5). Statistical significance was calculated using Tukey multiple comparison test. (E, F) The effect of magnolol on body weight gain (E) and food intake (F) in high-fat diet-fed *ApoE*$^{-/-}$ mice. Data are presented as means ± SEM from 6 mice per group. (G) Results of OGTT of *ApoE*$^{-/-}$ mice treated with magnolol for 2 weeks. Data are presented as means ± SEM from 6 mice per group. (H, I) Plasma levels of total triglyceride (H) and cholesterol (I) in the HFD-fed *ApoE*$^{-/-}$ mouse. Data are presented as means ± SEM (n = 6). (J) Effects of magnolol on epididymal fat mass in *ApoE*$^{-/-}$ mice fed high-fat diet. Data are presented as means ± SEM from 6 mice per group. (K, L) Effects of magnolol on adipocyte size in *ApoE*$^{-/-}$ mice fed a high-fat diet. Representative images of hematoxylin and eosin-stained sections of eWAT (K). Scale bars, 100 μm. (L) Quantification of adipocyte size of F. Data are presented as means ± SEM from 6 mice per group. (M) Magnolol treatment elevates GDF-15 levels in the plasma of *ApoE*$^{-/-}$ mice. Data are presented as means ± SEM from 6 mice per group. (N) *ApoE*$^{-/-}$ mice were orally administrated magnolol. Six hours later, mice were injected with anti-GDF-15 through tail vein injection. Five mice were included in each group, and the daily food intake of each group of mice was measured to calculate the average daily food intake per group. This procedure was repeated three times to obtain three biological replicates for each sample. (O–R) The expression of genes in eWAT of *ApoE*$^{-/-}$ mice fed a high-fat diet involved in lipid metabolic process (O), cholesterol biosynthetic process (P), long-chain fatty acid transport or binding (Q), and regulation of lipid metabolic process (R). Data are presented as means ± SEM (n = 5). $^{*}P < 0.05$, $^{**}P < 0.01$. Statistical significance was calculated using Tukey multiple comparison test.

ment is involved in the gene-gene interaction between *GDF-15* and other genes.

SNPs can function as biological markers due to their association with various complex diseases. Pharmacotherapy responses and the risk of adverse reactions are also significantly influenced by SNPs. One mechanism through which this occurs is that differences in the binding sequences of transcription factors can affect their recruitment and function. This study demonstrates that the polymorphic binding sites in the *GDF-15* gene exhibit distinct affinities for ATF4, CEBPG, and CTCF. Some of these SNPs are more likely to act as transcriptional repressors of the *GDF-15* gene. Furthermore, GDF-15 is recognized as a stress response gene and has been extensively studied as a biomarker for disease prognosis. Notably, these SNP genotypes also influence the regulatory effects of cisplatin and hypoxia (Data not shown), which are known to induce GDF-15 production, on GDF-15 expression. Consequently, further investigation into the genetic effect of these SNPs on the application of GDF-15 as a biomarker for disease monitoring, as well as their impact on magnolol-induced GDF-15 production in humans *in vivo*, would be of significant interest.

Research has indicated that the GDF-15 gene suppresses appetite for high-fat diets and mitigates obesity [16] while also reducing body weight and adiposity in obese mice through the leptin pathway [12]. A study conducted in 2013 revealed that, in C57BL/6 mice fed a high-fat diet (HFD), magnolol and honokiol (approximately 17 mg/kg body weight per day) can decrease white adipose tissue weight by enhancing adipose fatty acid oxidation and reducing fatty acid synthesis, as well as adipocyte differentiation. This occurred without altering daily food intake, body weight, or hepatic fat accumulation [3]. Additionally, another study demonstrated that oral administration of magnolol (100 mg/kg body weight per day) for 13 weeks reduced fasting blood glucose and plasma insulin levels in non-obese type 2 diabetic Goto-Kakizaki GK rats, without affecting body weight [36]. In this study, we demonstrated that magnolol administration slightly inhibits food intake and epididymal fat deposit in high fat-dieted *ApoE*$^{-/-}$ mice. These findings suggest a beneficial effect of magnolol on glycemia-related parameters.

The utilization of natural products as primary active agents for treating various diseases, including metabolic disorders, has garnered increasing attention [37,38]. However, it is essential to consider that magnolol can induce ER stress, and prolonged ER stress contributes to the pathogenesis of metabolic disorders. Additionally, magnolol's low water solubility and rapid metabolism result in poor bioavailability *in vivo*. Consequently, the clinical effects of magnolol on metabolic diseases and GDF-15 production require further investigation. Furthermore, we explored potential targets of magnolol using SuperPred, a web server for compound target prediction [39]. Among these targets, we noted that IRE1A functions as a sensor of unfolded proteins in the ER, leading us to hypothesize that magnolol directly binds to IRE1A. We initially tested this hypothesis using a DARTS approach. However, magnolol did not reduce the sensitivity of IRE1A protein to protease degradation (Supplementary data 4A, B), suggesting that IRE1A is not a binding target of magnolol. Elucidating the cellular targets of magnolol remains an intriguing subject for future research.

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Supporting information

Supporting information for this study is available upon request *via* email to the corresponding authors.

Declaration of competing interest

These authors have no conflict of interest to declare.

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