

Ginsenoside Ro ameliorates high fat diet-induced obesity and insulin resistance in mice via activation of the TGR5 pathway

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Running Title: Ginsenoside Ro ameliorates obesity via the TGR5 pathway

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Abbreviations: TGR5, G protein-coupled bile acid receptor 5; DIO, diet-induced obese; OGTT, oral glucose tolerance tests; IPITT, intraperitoneal insulin tolerance tests; BAT, brown adipose tissue; UCP1, mitochondrial uncoupling protein 1; GLP-1, glucagon-like peptide-1; HFD, high fat diet; WAT, white adipose tissue; EE, energy expenditure; HE, hematoxylin-eosin; HOMA-IR, homeostasis model assessment of insulin resistance; AUC, area under the curve; TG, triglycerides; TC, total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; FXR, farnesoidX receptor.

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ABSTRACT

Obesity, a well-known risk factor in multiple metabolic diseases, is dramatically increasing worldwide. Ginsenosides extracted from ginseng have been reported against obesity and the associated metabolic disorders. As a subtype of ginsenoside, ginsenoside Ro is a critical constituent of ginseng. However, its specific effects on obesity remains unknown. G protein-coupled bile acid receptor 5 (TGR5, also known as GPBAR1) is a bile acid membrane receptor, widely expressed in human tissues contributing to various metabolic processes to confer the regulations of glucose and lipid homeostasis. TGR5 has displayed potentials as a therapeutic target for the treatment of metabolic disorders. Here, we explore the anti-obesity effect of ginsenoside Ro with TGR5 activation screened by a library of natural products. Our results showed that the ginsenoside Ro (90mg/kg) treatment ameliorated body weight and lipid accumulation in multiple metabolic organs of high fat diet-induced obese (DIO) mice without affecting food intake, and improved oral glucose tolerance tests (OGTT), intraperitoneal insulin tolerance tests (IPITT), and fasting serum glucose. We also found that triglyceride (TG) and total cholesterol (TC) in serum and liver were significantly decreased after ginsenoside Ro treatment. Then we used *Tgr5* knockout mice to explore the role of *Tgr5* in the anti-obesity effect of ginsenoside Ro. Our results further demonstrated that ginsenoside Ro promoted glucagon-like peptide-1 (GLP-1) secretion and energy expenditure in wild type DIO mice. However, the stimulation of ginsenoside Ro on GLP-1 secretion and energy expenditure were restrained in the *Tgr5* knockout mice. In conclusion, our findings demonstrated that ginsenoside Ro ameliorates obesity and insulin resistance in DIO mice via activating TGR5, indicating a potential therapeutic role of ginsenoside Ro to treat obesity and its associated metabolic diseases.

SIGNIFICANCE STATEMENT

Obesity is dramatically increasing worldwide and it contributes to multiple metabolic diseases. TGR5 is a potential therapeutic target for the treatment of metabolic disorders. Ginsenoside Ro, as a oleanane-type ginsenoside, ameliorates obesity and insulin resistance, promotes GLP-1 secretion,

increases energy expenditure via activating TGR5. Ginsenoside Ro could be a potential leading compound for treating obesity and its associated metabolic diseases.

INTRODUCTION

With the accelerated pace of life, high calorie diet has been becoming more popular and results in high prevalence of overweight and obesity (Netto Candido et al., 2018). The incidence of obesity has been greatly increased worldwide during recent years (Bluhner, 2019). For example, around 19.5% of adults were obese in 2015 in OECD countries, and the prevalence of obesity could be increased up to 47% by 2030 suggested by a forecast (2017). Obesity is one of major risk factors in multiple metabolic diseases including diabetes, fatty liver, cardiovascular diseases, stroke, and cancer. A recent study reported that people with obesity had increased risk of severe COVID-19 (Stefan et al., 2020). Behavior and genetics have been recognized as the main factors influencing obesity (Heianza and Qi, 2017). Currently, bariatric surgery is the most effective treatment for morbid obesity and other complications (Arterburn et al., 2020). However, numerous studies were also conducted to identify therapeutic targets for treating obesity. G protein-coupled bile acid receptor 1 (TGR5, also known as GPBAR1) is a bile acid membrane receptor and is expressed in many tissues including intestines, brown adipose tissue, spleen and macrophages (Maruyama et al., 2002). Accumulating evidence suggests that the activation of TGR5 can affect glucose and lipid metabolism in a direct or indirect way. TGR5 was showed to play a crucial role in the vertical sleeve gastrectomy (VSG)-mediated metabolic improvement [6]. In brown adipose tissue (BAT), TGR5 promotes intracellular thyroid hormone activation and induces energy expenditure via regulating the expression of mitochondrial uncoupling protein 1 (UCP1) and the activation of cyclic AMP (cAMP)-dependent iodothyronine deiodinase 2 (Watanabe et al., 2006). Besides, a TGR5 selective agonist, INT-777 was found to promote the release of glucagon-like peptide-1 (GLP-1) secreted by enteroendocrine L cells of the intestinal epithelium. INT-777 also improves insulin sensitivity and

lipid-loading in obese mice (Kumar et al., 2016). In a mouse model, TGR5 was found to be activated by VSG to sustain weight loss, improve fatty liver and insulin resistance (Ding et al., 2016). Meanwhile, bariatric surgery reveals cholic acid-7-sulfate, a gut-restricted TGR5 agonist, to alleviate obesity and type 2 diabetes (Chaudhari et al., 2020). Therefore, it's well-recognized that TGR5 can be therapeutically targeted for obesity and its associated morbidities by developing its ligands.

In our previous study, we demonstrated that the activation of TGR5 could be increased by ginsenoside Ro in vitro. As the major active ingredient in ginseng, ginsenoside is a triterpenoid saponin mainly extracted from *Panax ginseng* C. A. Mey. Ginsenoside has diverse pharmacological activities such as antitumor, anti-inflammatory, and antioxidant (Kiefer and Pantuso, 2003; Wang et al., 2016; Gao et al., 2017b). Previous studies have revealed that ginseng extract and ginsenoside can improve obesity, metabolic syndrome, and type 2 diabetes (Chen et al., 2019). Dammarane-type saponins of ginsenosides such as Rb1, Rg1, and Rg3 show the effects of regulating glucose and lipid metabolism and promoting energy expenditure (Bai et al., 2018; Zhou et al., 2019; Lee et al., 2020). A recent study showed that Changbai Mountain Ginseng extract, whose major compound is ginsenoside Ro, improved exercise performance and energy utilization in mice (Ma et al., 2017). Moreover, the extract affects glucose and triacylglycerols level and increases the mass of BAT in mice (Ma et al., 2017). As a typical oleanane-type saponin, the anti-tumor and anti-inflammation effects (Kim et al., 2015b; Zheng et al., 2019), as well as the protection against platelet aggregation of ginsenoside Ro, have been reported (Kwon, 2019). However, its specific impact on obesity and metabolic diseases remains unknown. In this study, we explored the effects and underlying mechanisms of ginsenoside Ro on the glucose and lipid metabolism in high-fat diet induced obese (DIO) mice.

MATERIALS AND METHODS

Drug and reagents

Ginsenoside Ro (purity: $\geq 98\%$) was purchased from Chengdu Biopurify Phytochemicals Ltd, Sichuan, China. High fat diet (HFD, D12492i, contains 60% fat, 20% protein, and 20% carbohydrate) and control diet (also named low fat diet, D12450J, contains 10% fat, 20% protein, and 70% carbohydrate) were purchased from Research Diets, Inc. (New Brunswick, USA).

Animal procedures

6-8 weeks male C57BL/6 wild-type mice were purchased from the SLAC Laboratory (Shanghai, China). *Tgr5*^{-/-} mice, *Tgr5*-floxed mice (*Tgr5*^{fl/fl}) and Villin-Cre mice in C57BL/6 background were constructed by the facility of Shanghai Biomodel Organism Science & Technology Development. To generate intestine-specific *Tgr5*-null mice (*Tgr5*^{ΔIN}), homozygous *Tgr5*^{fl/fl} were crossed with mice harboring the cre-recombinase under the control of the villin promoter [Villin-Cremice, from Shanghai Biomodel Organism Science & Technology Development Co., Ltd. [(stock number Jax-021504-B6.Cg-Tg (Vil1-cre) 1000Gum/J), Shanghai, China]. All mice were housed under a 12hr light/12hr dark cycle at 18-22°C in the facility of Shanghai Biomodel Organism Science & Technology Development. All wild-type mice, *Tgr5*^{-/-} and *Tgr5*^{ΔIN} mice were fed with a high-fat diet for six weeks (bodyweight of high fat diet (HFD) induced obese mice was 35.675±3.275 g), and were randomly divided into HFD group and HFD supplemented with ginsenoside Ro powder group at a dose of 90mg/kg (HFD+Ro-H group) or 45mg/kg (HFD+Ro-L group) based on food intake (n=8 per group). The doses were determined based on previous in vivo studies (Matsuda et al., 1990; Matsuda et al., 1991) of ginsenoside Ro and our pre-experiments, and the safety was examined (Supplementary Figure 1). Simultaneously, control group was fed on a low fat diet (lean group, n=8).

After oral administration with ginsenoside Ro for eight weeks, each group mice were performed with oral glucose tolerance tests (OGTT), intraperitoneal insulin tolerance tests (IPITT) and GLP-1 secretion assay. Age- and bodyweight- matched animals were used. After mice were euthanatized by CO₂, serum, liver tissues, white adipose tissue (WAT), and brown adipose tissue (BAT) were collected and snap frozen in liquid nitrogen for RNA extracts or biochemistry studies. All studies were performed according to protocols approved by Shanghai University of Traditional Chinese Medicine's Animal Care and Use Committee.

OGTT, IPITT, and HOMA-IR

OGTT: mice were fasted for 14h prior to OGTT and blood was sampled from the tail vein before and 15, 30, 60, and 120 min after an oral administration of D-glucose at 2.0 g/kg bodyweight. Blood glucose level (mmol/L) was measured by a blood glucose meter. IPITT: mice were fasted for 4h prior to IPITT, and blood was collected from the tail vein before and 15, 30, 60, and 120 min after an intraperitoneal injection of insulin at 0.75 U/kg bodyweight. Blood glucose levels (mmol/L) were measured using a blood glucose meter. The index of the homeostasis model assessment of insulin resistance (HOMA-IR) was calculated according to the following formula: [fasted insulin (μIU/mL)×fasted glucose (mM)]/22.5.

Serum biochemistry measurements

Serum was gathered from blood collected through cardiac puncture. Total serum cholesterol and triglyceride levels were examined by automatic blood biochemical instrument (2700; Olympus, Tokyo, Japan).

GLP-1 release assay

Following 14h fasting, mice were orally administrated with Sitagliptin at 0.3 mg/g body weight 60

min prior to orally treatment with D-glucose at 2.0 g/kg bodyweight. Plasma was collected from the medial canthus vein of eye before and 15, 30 min after D-glucose administration. Plasma active GLP-1 was measured by using Millipore GLP-1 active ELISA kit (Millipore, USA).

Indirect calorimetry

Energy expenditure (EE) was evaluated by an indirect calorimetry system of 16 identical, independent metabolic cages equipped for the continuous monitoring of ambulatory activity and ad libitum access to HFD and water (Columbus Instruments Comprehensive Lab Animal Monitoring System (CLAMS)). Temperature and humidity levels were regulated tightly, Oxygen (O₂) and carbon dioxide (CO₂) levels were monitored constantly. CO₂ consumption and O₂ production of each mouse were measured at 10-min intervals for 1min. EE is calculated utilizing the Weir equation: $\text{Kcal/h} = 60 * (0.003941 * \text{VO}_2 + 0.001106 * \text{VCO}_2)$.

Serum bile acid profile analysis

Chromatographic conditions: chromatographic column used Cortec UPLC[®] C18 1.6 μm , 2.1 \times 100 mm column. And mobile phase A was a-0.01% formal acid, b-actinitrile; mobile phase A was gradient: 0.1 min-5% B, 1.0 min-5% B, 6.0 min-25% B, 13.0 min-30% B, 20 min-40% B, 24.0 min-75% B, 26.0 min-75% B, 26.5 min-95% B, 27.0 min-95% B. Flow rate was 0.3 ml / min. Injection volume was 5 μL . Ion source temperature was 600 °C. CAD parameter was 10 Hz of acquisition frequency.

Mass spectrometry conditions: electrospray ionization source (ESI), ion source temperature was 120°C, capillary voltage 2.8 kV, taper hole voltage 55 V; desolvent gas (N₂) flow rate was 600 L /h, taper hole gas (N₂) flow rate was 50 L /h; desolvent temperature 350°C; negative ion monitoring mode; scanning mode for full scan, scanning range (m/z) 100~1500.

Samples and standards solution preparation: For analyzing bile acid in liver, took appropriate amount of liver samples homogenating with ultrapure water. Then added methanol and centrifuge. Keep the supernatant and blow dry with N₂. Added methanol for redissolution. For serum solution preparation, took appropriate amount of serum and mixed with interior label solution and methanol, following centrifugation, and keeping the supernatant. For standards solution preparation, took appropriate amount of standards, and added methanol to prepare appropriate concentration.

Dual-luciferase reporter assay

The reporter assay was performed by using the Promega Dual-Luciferase Reporter Assay System (Promega, USA) according to the manufacturer's instructions. Briefly, HEK293T (ATCC) cells were seeded on 96-well plates (2×10^4 cells/well) and grown to 80%-90% confluence with high-glucose DMEM containing 10% FBS in an incubator at 37 °C with 5% CO₂. To determine the activation level of mTGR5, pIRESneo3-mTGR5 plasmid and pCRE-luc reporter were co-transfected into HEK293T cells 24h after the seeding. Then cells were incubated with the DMSO as control or ginsenoside Ro (20 μM) for another 24h before the reporter assay. To normalize transfection efficiencies, the renilla luciferase activity was measured. Transfection efficiencies were conducted with Promega® dual luciferase reporter assay kit, and the procedure was carried out in accordance with the kit instruction. The experiments were carried out in triplicate and repeated independently at least three times. All the plasmids were kindly provided by professor Wendong Huang from City of hope medical center (Duarte, CA).

Cyclic AMP (cAMP) release assay

HEK293T cell line was transiently transfected with mTGR5 and pCRE-luc plasmids and treated with LCA and ginsenoside Ro for 30min in Krebs ringer buffer without serum added with 100 mM RO

20-1274 and 500 mM IBMX (Sigma, St. Louis, MO). According to the instructions of cAMP- Glo kit (Promega), the cell lysates were used to determine the content of cAMP.

Real-time PCR

At 8 weeks after treatment, the mouse liver was obtained and subjected to total RNA extraction using Trizol reagent. Relative levels of amplifiants were identified utilizing SYBR Green qPCR mix (applied biosystems) on Applied Biosystems Real-Time PCR System (Table 1.).

Histological analysis of liver and adipose tissue

Hematoxylin-eosin (HE) staining: liver and adipose tissues were fixed in 4% paraformaldehyde and embedded in paraffin, and sectioned at 5mm onto poly-L-lysine-coated slides. Tissue sections were stained in Mayer's hematoxylin. Oil red O staining: liver tissue was embedded in Tissue-Tek OCT cryostat molds and frozen at -80°C. Liver tissue was used to generate 10-mm-thick sections in a cryostat. And tissue sections were stained in 0.5% oil red O and then counterstained with Mayer's hematoxylin. Images were quantified by image J 1.51k.

Statistics

All data were expressed as mean \pm SEM. All statistical analyses were carried out by one-way analysis of variance with Dunn's post-test Graphpad Prism 7.0 (7.0 version, GraphPad, La Jolla, CA).

P values < 0.05 was considered statistically significant.

RESULTS

Ginsenoside Ro improves high fat diet (HFD) induced obesity.

It has been demonstrated that the activation of TGR5 inhibits HFD induced obesity. Given our previous finding that ginsenoside Ro activates TGR5 in vitro, we hypothesized that ginsenoside Ro

might have an anti-obesity effect. The C57BL/6 wild-type mice were randomly divided into Lean group (Low fat diet), HFD group (60 Kcal% high fat diet), HFD+Ro-L group (60 Kcal% high fat diet with 45mg/kg ginsenoside Ro) and HFD+Ro-H (60 Kcal% high fat diet with 90mg/kg ginsenoside Ro) group. The HFD group was compared with Lean group, and both HFD+Ro-L and HFD+Ro-H group were compared with HFD group.

First, we tested the activation effect of ginsenoside Ro on TGR5 in vitro by dual-luciferase reporter assay and cyclic AMP (cAMP) release assay. The results showed that treatment with ginsenoside Ro (20 μ M) which is a typical oleanane-type saponin (Fig. 1A) increased luminescence signal and the cAMP release (Fig. 1B, C).

We then recorded the bodyweight and food intake of DIO mice and examined the histopathology of BAT and white adipose tissue (WAT) by Hematoxylin-eosin (HE) staining. The results showed that high-dose ginsenoside Ro significantly reduced the body weight and fat mass (Fig. 1D-G) of the mice without affecting the food intake (Fig. 1H) compared to the HFD group. However, low-dose ginsenoside Ro had no effect on either body weight or fat mass. In addition, histological examination showed a substantial decrease of adipocytes volume in BAT and inguinal white adipose tissue (iWAT) (Fig. 1 I, J) in both high- and low-dose ginsenoside Ro treated mice, compared to those in the HFD group.

Ginsenoside Ro improves the glucose metabolism of DIO mice.

To examine the effect of ginsenoside Ro on glucose metabolism, glucose tolerance, insulin secretion, insulin tolerance, and fasting serum glucose level were tested. High-dose ginsenoside Ro significantly improved the glucose tolerance and insulin tolerance (Fig. 2 A-C) in DIO mice. Both

high- and low-doses ginsenoside Ro had no effect on fasting insulin (Fig. 2 E). Besides, high dose, but not the low-dose ginsenoside Ro markedly descended the fasting serum glucose and HOMA-IR index (Fig. 2D, F).

Ginsenoside Ro improves lipid metabolism of DIO mice.

To explore the effect of ginsenoside Ro on lipid metabolism, the lipid accumulation in the liver was visualized by oil red O staining and HE staining, and serum triglycerides (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-c), and low density lipoprotein cholesterol (LDL-c) were tested. Meanwhile, the contents of TC and TG in the liver were also measured. Lipid accumulation was observed to be reduced in the liver of both high- and low- dose treated DIO mice (Fig. 3A, B, I). The contents of TC and TG in serum and liver were significantly reduced by the treatment of high-dose ginsenoside Ro (Fig. 3C-F). The serum HDL-c level was also markedly raised in response to the treatment of high-dose ginsenoside Ro (Fig. 3G). However, no significant difference in the effects on serum TC, TG and LDL-c was seen between the low-dose ginsenoside Ro treated group and the HFD group. Unexpectedly, low-dose ginsenoside Ro treatment significantly decreased liver TC, but increased serum HDL-c level (Fig. 3 E, G) compared to the HFD group. High-dose ginsenoside Ro significantly improved lipid metabolism, and low-dose ginsenoside Ro also had partial improvement effect.

Ginsenoside Ro alters bile acids profile of DIO mice.

Bile acids primarily serving as emulsifier to facilitate fat absorption have been considered as crucial signaling molecules involved in glucose and lipid metabolism. To explore the underlying

mechanisms of ginsenoside Ro on regulating glucose and lipid metabolism, we analyzed the bile acids profile with ginsenoside Ro treatment. Total bile acids in serum and liver were both upregulated in high-dose ginsenoside Ro treated mice (Fig. 4A-B). It has been demonstrated that farnesoidX receptor (FXR) is an important Bile acid nuclear receptor involving in the regulation of bile acid circulation and synthesis, and the inhibition of liver FXR promotes the synthesis and secretion of bile acids. We thus measured the hepatic mRNA levels of FXR, Shp, Cyp7a1 and Cyp7b1 to test whether ginsenoside Ro treatment disturbs synthesis pathway of bile acids. As showed in Figure 4C, ginsenoside Ro treatment suppressed mRNA expression of FXR and Shp in DIO mice, but the levels of Cyp7a1 and Cyp7b1 were promoted, which indicates that ginsenoside Ro regulates bile acid synthesis in the liver by modulating the transcription of genes. Besides, we also found that compared to the HFD group, the concentration of serum glycine conjugated bile acids GUDCA, GCA, and GCDCA increased in the ginsenoside Ro treated group (Fig. 4D). However, ginsenoside Ro treatment did not affect the concentration of hepatic glycine conjugated bile acids (Fig. 4E). Furthermore, taurine conjugated bile acids level in serum and liver were significantly raised following ginsenoside Ro treatment (Fig. 4F-G). The concentration of free bile acids including serum β -MCA, serum UDCA (Fig. 4H), and hepatic β -MCA were also increased significantly after ginsenoside Ro treatment (Fig. 4I).

Ginsenoside Ropromotes GLP-1 secretion via activating intestinal TGR5.

We further tested the mRNA level of intestinal *Tgr5* in DIO mice and found the mRNA expression is enhanced by ginsenoside Ro (Fig. 5A). Intestinal TGR5 activation was reported to promote GLP-1 secretion, which involved in the regulation of glucose and lipid metabolism. We subsequently

explored whether GLP-1 is regulated by ginsenoside Ro using intestinal TGR5 conditional knockout mice (*Tgr5^{ΔIN}*). Our results revealed that ginsenoside Ro treatment significantly increased GLP-1 level in WT mice but not in *Tgr5^{ΔIN}* mice (Fig. 5B, D). In addition, fasting serum glucose level, glucose tolerance and insulin tolerance were not increased in *Tgr5^{ΔIN}* mice either (Fig. 5C, E &F).

The metabolic benefits of ginsenoside Ro treatment are absent in *Tgr5^{-/-}* mice.

We then investigated whether ginsenoside Ro could affect the phenotype of *Tgr5^{-/-}* mice. First of all, we measured and found that the bodyweight of *Tgr5^{-/-}* mice were not affected by ginsenoside Ro treatment (Fig. 6A). But the serum TC level still decreased in *Tgr5^{-/-}* mice after ginsenoside Ro treatment (Fig. 6B). Furthermore, the effect of ginsenoside Ro on serum TG was restrained by *Tgr5^{-/-}* knockout (Fig. 6C). And the enhancement of mRNA expressions of *Dio2*, *Ucp1*, *Ucp3*, and *Cpt1β* observed in BAT of WT mice was not shown in *Tgr5^{-/-}* mice (Fig. 6D). Consistently, the improvements of ginsenoside Ro mediated lipid accumulation in BAT and iWAT in WT mice were abolished in *Tgr5^{-/-}* mice (Fig. 6E).

Ginsenoside Ro promotes energy expenditure via TGR5.

Finally, we evaluated the energy expenditure by an indirect calorimetry system of 16 identical, independent metabolic cages. As expected, ginsenoside Ro promoted oxygen consumption, carbon dioxide output and energy consumption during the day and night in WT mice (Fig. 6F, H & J). However, the energy metabolism was not affected by ginsenoside Ro in *Tgr5^{-/-}* mice (Fig. 6G, I&K).

DISCUSSION

As a traditional Chinese medicine, *Panax ginseng* has long been used against metabolic diseases such as type 2 diabetes and obesity (Bai et al., 2018). Ginsenosides, a main active ingredient in ginseng, have been reported to modulate glycolipid metabolism including gluconeogenesis reduction, glucose transport and lipid regulation (Bai et al., 2018). The regulation of ginsenosides on energy metabolism has also been illustrated (Li and Ji, 2018). Based on the structure differentiation of the sapogenins, ginsenosides are classified into two main types including oleanane-type saponin and dammarane-type saponin (Supplementary Figure 2). Compared with dammarane-type saponins, the pharmacological study of oleanane-type ginsenosides is limited. Ginsenoside Rb1 has protective effects on obesity, hyperglycemic, and diabetes through regulating mitochondrial energy metabolism, improving insulin resistance (Zhou et al., 2019). Ginsenoside Rg1 reduced gluconeogenesis through increased AMPK expression and decreased FOXO1 activity (Liu et al., 2017). It also improved lipid metabolism, anti-apoptotic, and anti-inflammatory by inhibiting JNK pathway (Tian et al., 2017). Ginsenoside Rg1 and Rg3 can reduce intestinal glucose uptake by inhibit SGLT1 (Wang et al., 2015; Gao et al., 2017a). Rg3 affected lipid-regulating dependent on the STAT5-PPAR γ pathway (Lee et al., 2017). However, ginsenoside Ro only has been reported to increase the secretion of lipase activity in 3T3-L1 cells (Masuno et al., 1996). In the present work, we showed that ginsenoside Ro reduced bodyweight and lipid accumulation of peripheral adipose tissue in obese mice without affecting the food intake. Meanwhile, ginsenoside Ro improved insulin resistance and fatty liver. Our data suggest that ginsenoside Ro plays a similar role as many other dammarane-type saponins in contributing to the anti-obesity effects of ginseng by ameliorating glucose and lipid metabolism. Furthermore, ginsenoside Ro promoted GLP-1 secretion and energy expenditure in HFD-induced obese mice,

indicating that the anti-obesity effects of ginsenoside Ro were likely due to promoting GLP-1 release and energy consumption. However, some ginsenoside Ro treatment induced metabolic improvements were absent in *Tgr5*^{-/-} mice or *Tgr5*^{ΔIN} mice, indicating that ginsenoside Ro improves metabolism through TGR5 activation.

TGR5 is an important bile acid receptor involved in the regulation of glucose, lipid, and energy metabolism. There is growing evidence that activated TGR5 can be a promising target for treating obesity, type 2 diabetes, and non-alcoholic steatohepatitis (Duboc et al., 2014). TGR5 transmits its signal by rising intracellular concentrations of cAMP, causing rapid phosphorylation of downstream kinases (van Nierop et al., 2017). Bile acids are internal activator of TGR5, but complex enterohepatic bile acid cycling limits the exposure of some of these target tissues to the receptor ligand. To investigate how to utilize TGR5 as a therapeutic target, a number of TGR5 abiotic agonists including selective TGR5 agonists INT-777, MN6, intestinal TGR5 agonist (Chen et al., 2018), and a newly reported TGR5 agonist RDX8940 that function in metabolic diseases have been identified and studied recently (Finn et al., 2019). On the other hand, several natural products especially triterpenoids such as oleanolic acid and betulinic acid were also proved to affect the activity of TGR5 (Lo et al., 2016; Liu et al., 2019). In the present study, we demonstrated that ginsenoside Ro activated TGR5 in vitro and increased the mRNA expression of *Tgr5* in vivo, suggesting the effects of ginsenoside Ro on obesity and glycolipid metabolism through TGR5. We next clarified whether the endogenous TGR5 ligands-bile acids were altered due to the lower bioavailability of ginsenoside Ro (Qi et al., 2013). We examined the content and composition of bile acids in ginsenoside Ro treated mice, and the results showed that both of them were affected. Ginsenoside Ro raised the concentration of various bile acids, such as CDCA, UDCA, and its

conjugated bile acids. DCA, CDCA, UDCA, and its conjugated bile acids are reported to be TGR5 agonists (Fiorucci and Distrutti, 2019). Together, these results suggested that the regulation through bile acids is likely one of the potential mechanisms of how ginsenoside Ro improves obesity.

TGR5 contributing metabolic improvements are mainly attributable to GLP-1 secretion in ileum and energy expenditure in adipose tissue. GLP-1 plays a critical role in maintaining blood glucose homeostasis, and both secretion and sensitivity of insulin can be promoted by GLP-1. Simultaneously, GLP-1 inhibits the release of glucagon (Nadkarni et al., 2014). In a T2DM mice model, ginsenoside Rg3 increased GLP-1 secretion and decreased hyperglycemia through a sweet taste receptor-mediated signal transduction pathway (Kim et al., 2015a). Ginseng total saponins and ginsenoside Rb1 exhibit antidiabetic effects by promoting GLP-1 secretion in cultured NCI-H716 cells (Liu et al., 2014). Compound K (CK), a major metabolite of ginsenosides, induced GLP-1 secretion in NCI-H716 cells via TGR5 activation (Kim et al., 2014). The promoting effect on GLP-1 secretion is one of the major mechanisms of TGR5 treatment against obesity. The selective TGR5 agonist improves obesity, glucose tolerance, insulin, GLP-1 secretion, and insulin sensitivity in both liver and muscle of TGR5-Tg mice with a high-fat diet, but not in TGR5 knockout (*Tgr5*^{-/-}) mice. TGR5 overexpression or selective TGR5 agonist treatment increases energy expenditure and reduces hepatic steatosis and obesity. In the present study, ginsenoside Ro increased GLP-1 secretion in WT mice but not the intestinal *Tgr5* knockout mice, suggesting that ginsenoside Ro enhanced GLP-1 secretion via TGR5 activation.

Besides, activation of TGR5 increases energy expenditure by regulating the activity of type 2 iodothyronine deiodinase (D2) and the subsequent activation of thyroid hormone in BAT and muscles. In the present study, ginsenoside Ro increased energy expenditure in the WT but not the

Tgr5 knockout mice. Moreover, ginsenoside Ro had no effects on the body weight, serum TG level, and lipid in the *Tgr5* knockout mice, suggesting that ginsenoside Ro ameliorated obesity and improved energy expenditure by activating TGR5.

CONCLUSION

Ginsenoside Ro treatment ameliorates obesity and insulin resistance in diet-induced obese mice. The anti-obesity activity of ginsenoside Ro is mediated through TGR5. The findings of this study provide new insights into the mechanisms on improving metabolic disease by ginsenoside Ro or herbs containing ginsenoside Ro and indicate ginsenoside Ro a potential leading compound for treating obesity and its associated metabolic disease.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Wang, Ding and Yang.

Conducted experiments: Jiang, Li, Zhuang, Yu, Sun and Ju.

Performed data analysis: Jiang and Li.

Wrote or contributed to the writing of the manuscript: Jiang, Li and Ding.

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FOOTNOTES

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Conflict of Interest

The authors declare that they have no conflict of interest.

FIGURE LEGENDS

Fig. 1. Ginsenoside Ro activates TGR5 in vitro and improved HFD induced obesity. Wild-type male mice were feed with high-fat diet supplemented with ginsenoside Ro for 8 weeks. (A) Schematic of ginsenoside Ro chemical structural. (B) Fold changes of luminescence signal normalized to the control group. (C) The relative percentage of cAMP release. (D) Bodyweight curves of the mice treated with ginsenoside Ro for eight weeks. Bodyweight of the mice in each group at the 8th week following ginsenoside Ro treatment. (F) The relative bodyweight gain of the mice in each group. (G) Fat mass. (H) Food intake of the mice in each group at the 8th week following ginsenoside Ro treatment. (I) HE staining of BAT. (J) HE staining of iWAT. Magnification bar: 50 μ M. Data are represented as mean \pm SEM. [#]*P*<0.05, versus Lean, ^{***}*P*<0.001, ^{*}*P*<0.05 versus DMSO or HFD controls by one-way analysis of variance with Graphpad Prism 7.0.

Fig. 2. Ginsenoside Ro improves glucose metabolism in DIO mice. (A) Experimental groups underwent OGTT at 6 weeks following ginsenoside Ro treatment, and the area under the curve (AUC) measurements of OGTT between 0 and 120min was calculated. Experimental groups underwent (B) IPGTT and (C) IPITT at 7 weeks following ginsenoside Ro treatment, and the area under the curve (AUC) measurements of IPGTT and IPITT between 0 and 120 min were calculated. (D) Fasting serum glucose was measured after a 16h period of food-restriction. (E) Fasting insulin was measured after a 16h period of food-restriction and oraladministration of glucose. (F) HOMA-IR was calculated according to the following formula: [fasted insulin (μ IU/mL) \times fasted glucose (mM)]/22.5. Data are represented as mean \pm SEM. [#]*P*<0.05, versus Lean, ^{*}*P*<0.05 versus HFD controls by one-way analysis of variance with Graphpad Prism 7.0.

Fig. 3. Ginsenoside Ro improves the lipid metabolism of DIO mice. (A) HE staining of the liver. Magnification bar: 50 μ M. (B) Red oil staining of the liver. Magnification bar: 100 μ M. (C-D) Serum cholesterol and triglycerides are determined at eight weeks following ginsenoside Ro treatment. (E-F) Liver cholesterol and triglycerides were measured by liver homogenate supernatant. (G-H) Serum HDL-C and LDL-C. (I) Quantitative analysis of oil red staining images. Data are represented as mean \pm SEM. [#] P <0.05, ^{###} P <0.001 versus lean, ^{*} P <0.05, ^{***} P <0.001 versus HFD controls by one-way analysis of variance with Graphpad Prism 7.0.

Fig. 4. Effects of ginsenoside Ro on bile acids profile in DIO mice. The concentration of bile acids was analyzed by UPLC-MS / MS. (A-B) Total bile acids in serum and liver. (C) Relative mRNA expression of *Fxr*, *Shp*, *Cyp7a1*, *Cyp7b1*, *Cyp8b1* and *Cyp27a1*. (D-E) Glycine conjugated bile acids in serum and liver. (F-G) Taurine conjugated bile acids in serum and liver. (H-I) Free bile acids in serum and liver. Data are represented as mean \pm SEM. [#] P <0.05 versus lean, ^{*} P <0.05 versus HFD controls by one-way analysis of variance with Graphpad Prism 7.0.

Fig. 5. Ginsenoside Ro promotes GLP-1 secretion via TGR5. (A) Relative mRNA expression of *Tgr5* in the intestine of WT mice. (B) Active GLP-1 release between 0 and 30min was measured after administration with 2g/kg D-glucose in WT mice. (C) Fasting serum glucose in intestinal *Tgr5* knockout mice after eight weeks of ginsenoside Ro treatment. (D) Active GLP-1 release in *Tgr5*^{ΔIN} mice. (E-F) The OGTT and IPITT of *Tgr5*^{ΔIN} mice were measured at six weeks and seven weeks following ginsenoside Ro treatment, respectively. The area under the curve (AUC) measurements of

OGTT or IPITT between 0 and 120min were calculated. Data are represented as mean \pm SEM.

[#] $P < 0.05$ versus lean, ^{*} $P < 0.05$ versus HFD controls by one-way analysis of variance with Graphpad Prism 7.0.

Fig. 6. Effects of Ginsenoside Ro on *Tgr5*^{-/-} mice and energy expenditure. *Tgr5*^{-/-} mice were feed with a regular high-fat diet or the high-fat diet containing ginsenoside Ro for 8 weeks. (A) Final bodyweight of *Tgr5*^{-/-} mice. (B-C) Serum cholesterol and triglycerides of *Tgr5*^{-/-} mice. (D) Relative mRNA expression of *Dio2*, *Ucp1*, *Ucp3* and *Cpt1 β* . (E) HE staining of BAT and WAT. Magnification bar: 100 μ M. (F-G) Oxygen production volume of the period over 24h. (H-I) Carbon dioxide consumption volume of the period over 24h. (J-K) Energy expenditure of the period over 24h. Data are represented as mean \pm SEM. ^{###} $P < 0.001$ versus lean, ^{*} $P < 0.05$, ^{**} $P < 0.01$ versus HFD controls by one-way analysis of variance with Graphpad Prism 7.0.

Table 1. Sequences of the mouse primers used in real-time PCR.

Gene	Sense primer	Anti-sense primer
<i>Gapdh</i>	GGCCGAGAATGGGAAGCTTGT	ACATACTCAGCACCGGCCTCA
<i>Tgr5</i>	AGGTGTCTACGAGTGCTT	CATTGGCTACTGGTGTGG
<i>Dio2</i>	CGTCTCCAATCCTGAATCA	AAGTCAAGAAGGTGGCATT
<i>Ucp1</i>	GAAACACCTGCCTCTCTC	ACCTTCACGACCTCTGTA
<i>Ucp3</i>	ACCCGATACATGAACGCTCC	CACAAATCCTTTGTAGAAGGCCG
<i>Cpt1β</i>	AAGAGACAGACTTGCTACAG	TAGAGCCAGACCTTGAAGA

Figure 1

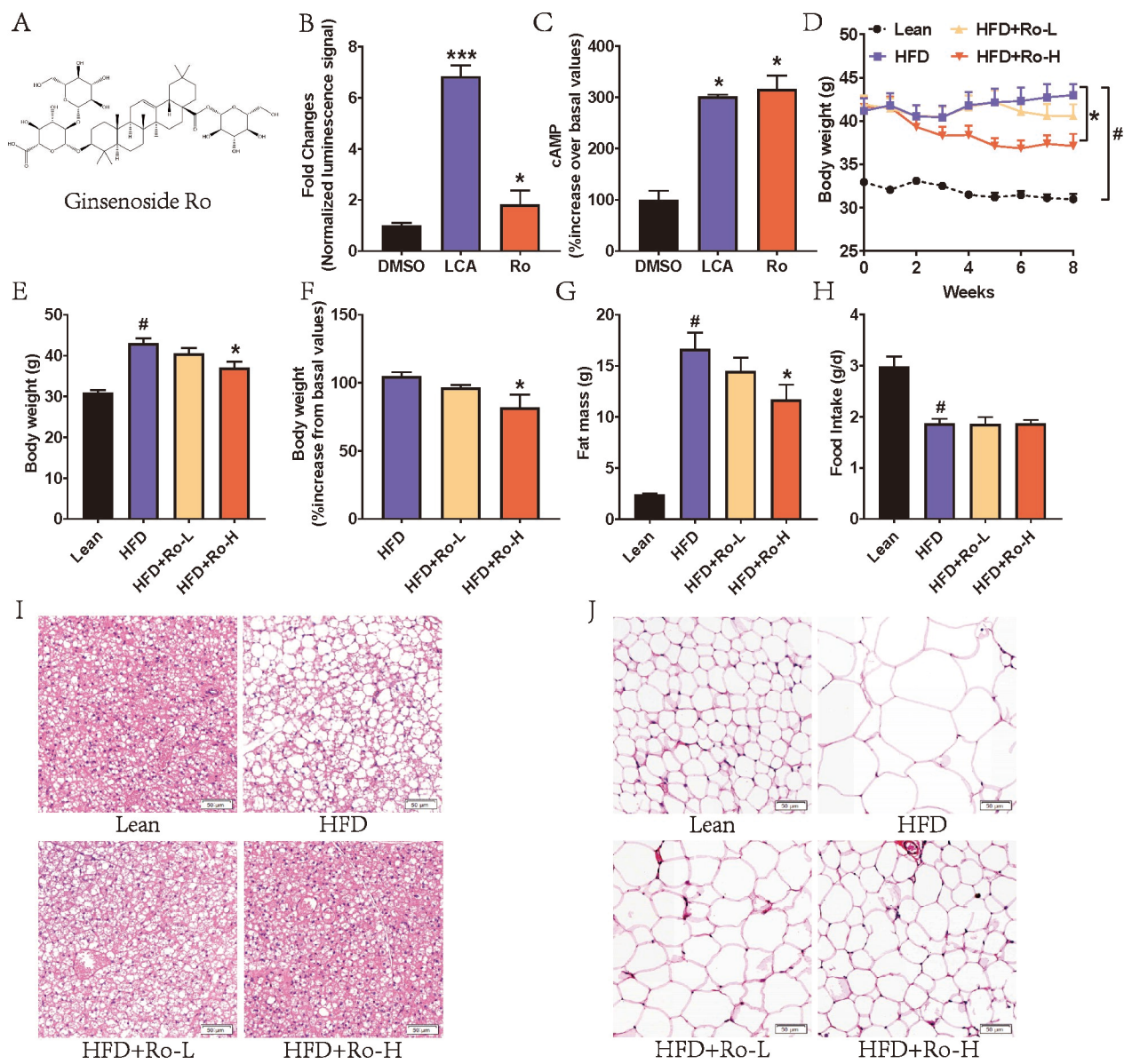


Figure 2

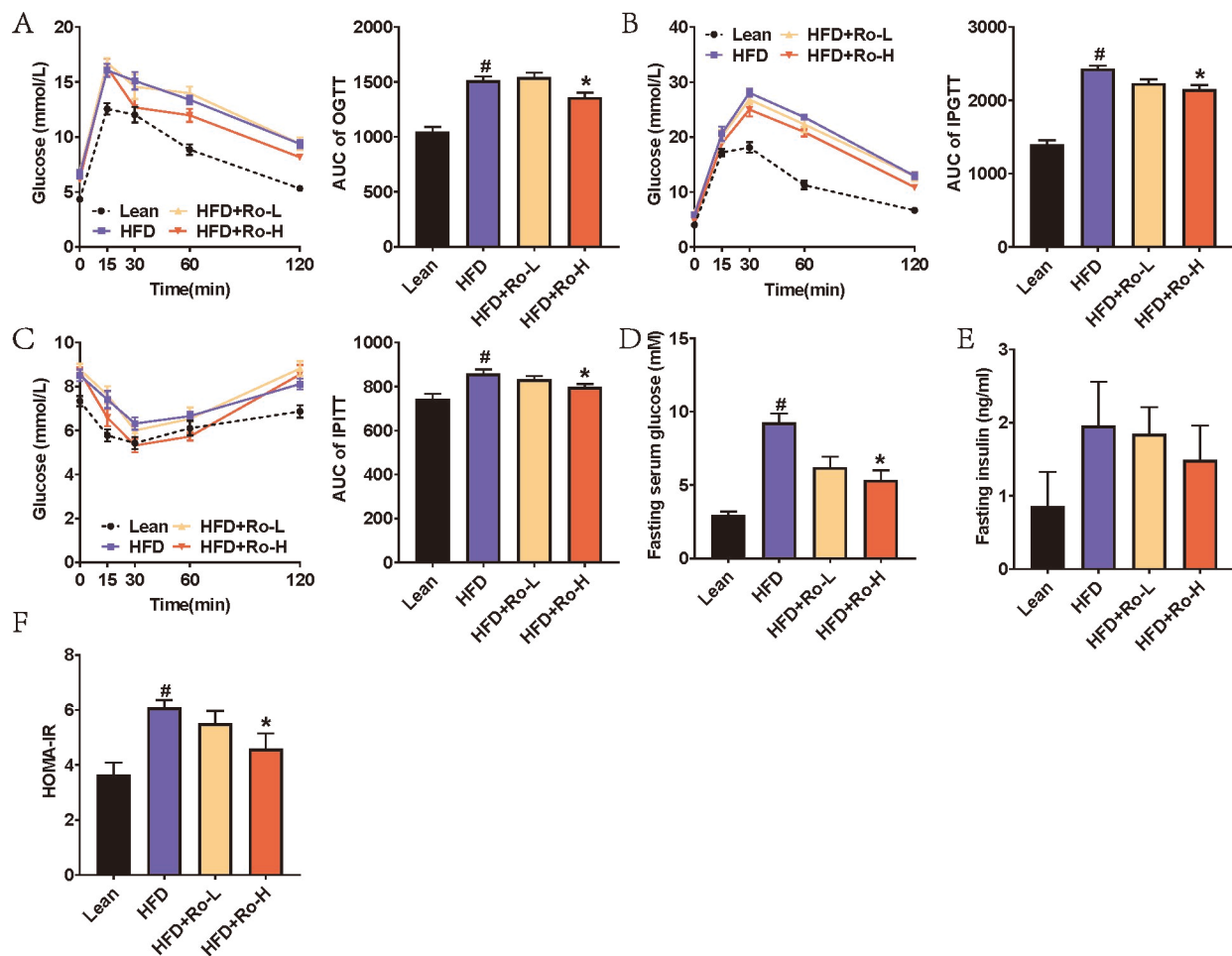


Figure 3

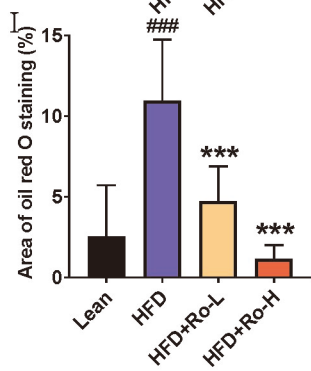
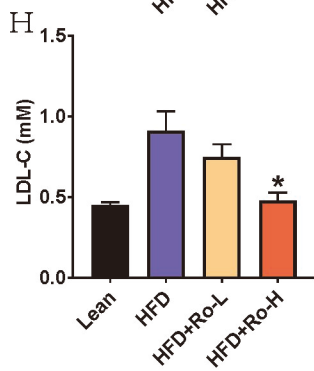
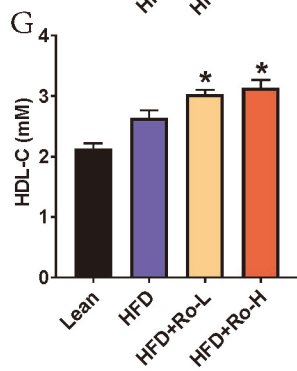
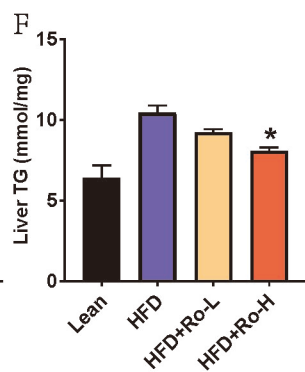
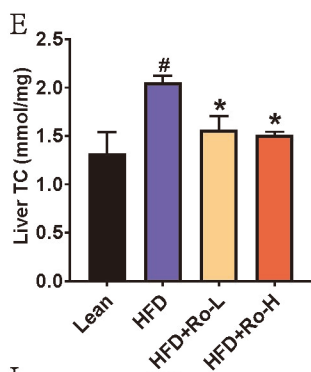
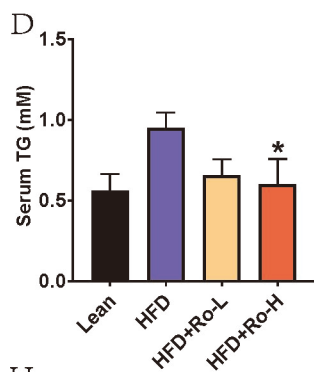
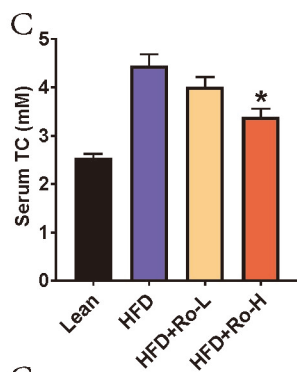
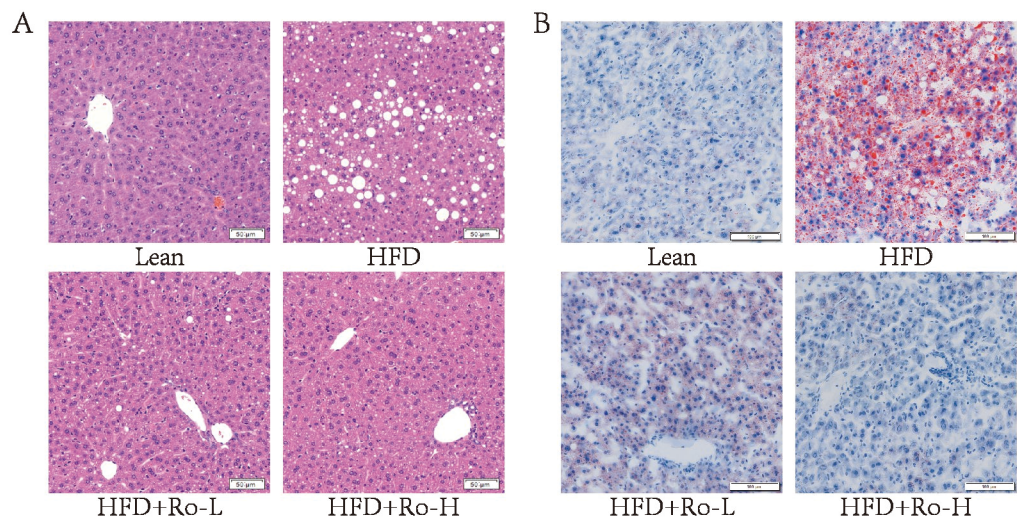


Figure 4

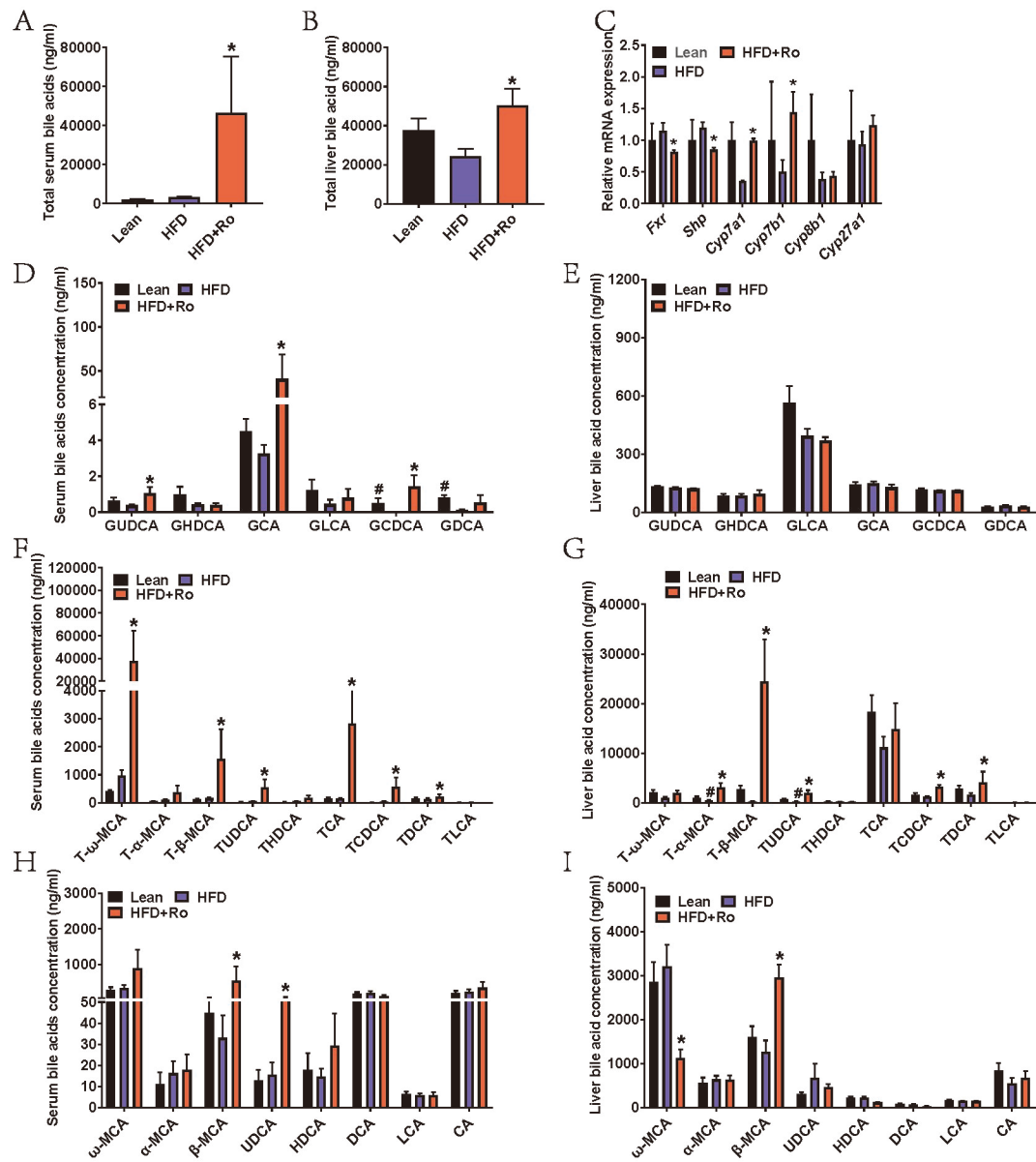


Figure 5

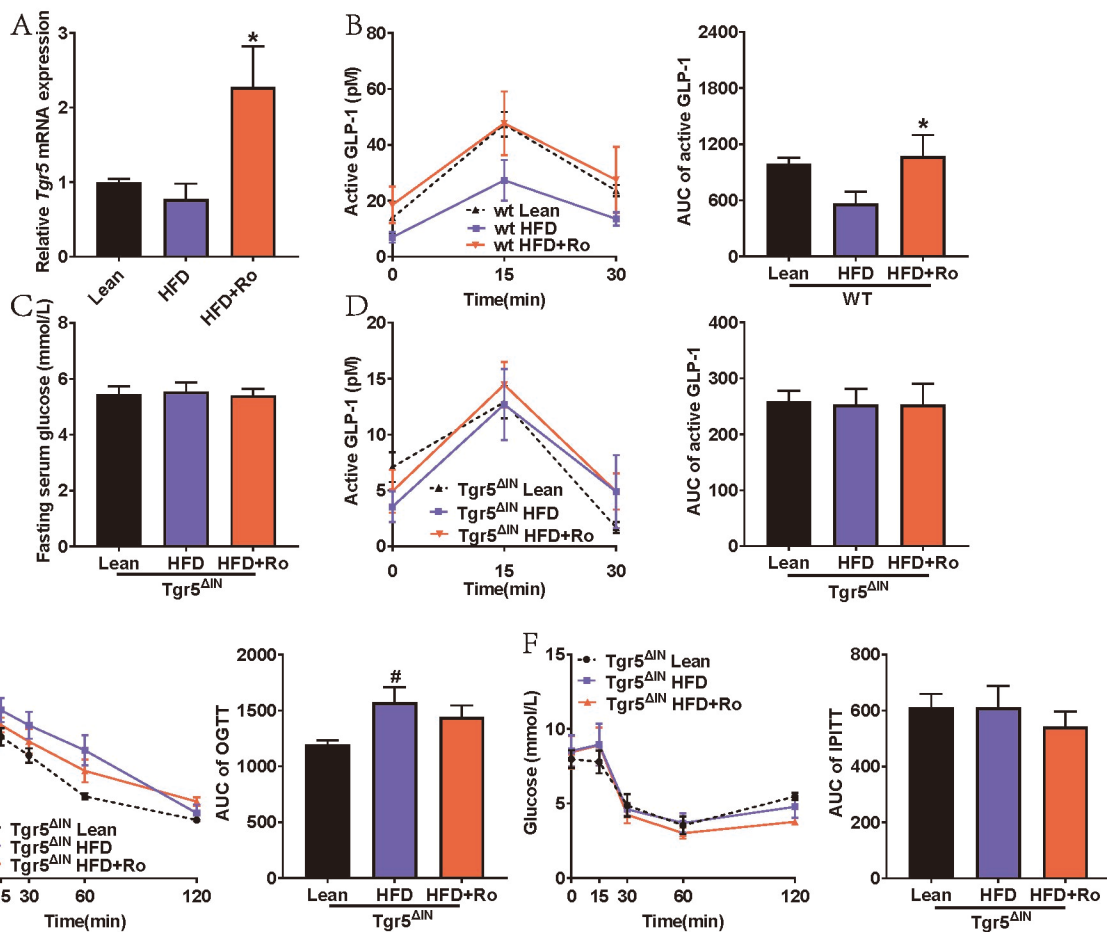


Figure 6

