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Mesenchymal stem cells modified by FGF21 and GLP1 ameliorate lipid metabolism while reducing blood glucose in type 2 diabetic mice

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Abstract

Objective: The purpose of the study was to investigate the therapeutic effects of genetically modified mesenchymal stem cells (MSCs) in the treatment of type 2 diabetes mellitues (T2DM), in order to find a new method of diabetes treatment different from traditional medicine, and provide a new way to fundamentally improve or treat diabetes.

Methods: MSCs derived from adipose tissue were modified with over-expression of FGF21 and GLP1, which was achieved through lentiviral particles transduction. The cells were transplanted to the BKS.Cg-Dock7m+/+Leprdb/Nju mice (T2DM mice model). The injections of physiological saline (0.1 mL), Liraglutide (0.5mg/kg) were employed as negative and positive controls respectively. The protein analyses were performed using ELISA or western blot analysis, while quantitative real-time PCR was adopted for gene expression.

Results: The genetic modification had no effects on morphology, differentiation ability, or immunophenotype of MSCs. Moreover, MSC-FGF21+GLP1 cells exhibited significantly increased secretion of FGF21 and GLP1. In T2DM model mice, the transplantation of MSC-FGF21+GLP1 cells ameliorated blood glucose and weight, promoted the secretion of insulin, enhanced the recovery of liver structures, and improved lipid profiles. Moreover, FGF21 and GLP1 exhibited synergistic effects in regulation of glucolipid metabolism through controlling the expression of insulin, srebp1 and srebp2.

Conclusion: The stem cell treatment based on MSCs modified by FGF21 and GLP1 genes is an effective approach for treatment of T2DM.

Introduction

Diabetes mellitus (DM) is a complex metabolic disease characterized by chronic hyperglycemia, insulin resistance, and islet β-cell dysfunction [1-3]. DM is listed as one of the top ten global diseases that cause human death by the World Health Organization (WHO). At present, there are millions of people in the world with diabetes, and the risk of developing diabetes is very high in the future [4]. According to a report published in lancet diabetes and endocrinology, the number of people with T2DM will increase from 406 million in 2018 to 511 million by 2030, which is the result of the continuous growth of global obesity [5]. Diabetes treatment is a long-term process. The long-term use of various chemicals has various limitations and adverse reactions, and even serious complications, such as hypoglycemia and lactic acidosis. So far, there are no drugs to cure diabetes. Chemicals can only control blood glucose. The use of insulin is not only expensive, but also needs to be injected every day. It is not easy for patients to adhere to it, but also need to strictly control the administration time and dosage, otherwise it is easy to cause hypoglycemia. In addition, the chemicals cannot repair damaged tissues. Therefore, there is an urgent need to develop new therapies.

Insufficient secretion of endogenous hormones, cytokines or enzymes, decreased activity or functional defects are closely related to the occurrence of metabolic diseases. For example, insulin resistance and

relative secretion insufficiencies of islet cells are the core causes of diabetes [6]. Therefore, these key molecules to balance glucose and lipid metabolism are also the key targets of drug development for metabolic syndrome. Among them, glucagon-like polypeptide 1 (GLP1) and FGF21 have become important drugs for the treatment of diabetes and obesity [6-10]. GLP1 secreted by L cells of ileum and colon plays an important role in maintaining glucose homeostasis and other physiological processes [10]. GLP1 receptor agonist can promote insulin secretion through glucose dependence to treat T2DM [11]. GLP1 receptor agonists mainly promote insulin release by activating GLP1 receptor. GLP1 receptor enhances calcium influx of calcium ion channel and calcium ion release of endoplasmic reticulum through the cAMP/PKA pathway, activates calmodulin, and finally promotes insulin exocytosis [12]. Studies have shown that GLP1 does not secrete insulin when blood glucose is lower than 4.5mmol/L. Therefore, GLP1 receptor agonist can reduce the risk of hypoglycemia and maintain the balance of blood glucose [13]. In addition to hypoglycemic effects, GLP1R agonists also protect and repair beta cells by inhibiting the secretion of glucagon and stimulating the proliferation and regeneration of beta cells [12, 14]. Fibroblast growth factor 21 (FGF21) is produced in tissues involved in metabolism, such as the liver, adipose tissues, skeletal muscle and pancreas, and has been proved to improve metabolic diseases and induce weight loss in human and mice [15-17]. Recently, a synthetic FGF21 variant, LY2405319, has been shown to reduce low-density lipoprotein (LDL) cholesterol and triglycerides, increase adiponectin levels, improve fasting insulin, and induce weight loss in obese patients with type 2 diabetes [15]. FGF21 administration is associated with decreased levels of sterol regulatory element binding protein (SREBP) , which is necessary for FGF21 induced thermogenesis [18]. Chronic treatment with recombinant FGF21 reduces serum and hepatic triglyceride levels and improves fatty liver in obese mice by inhibiting adipogenesis gene SREBP-1 [7]. In addition to Srebp-1, Srebp-2 has also been identified as a target of FGF21, which is the main regulator of cholesterol biosynthesis by preferentially activating the transcription of key cholesterol producing genes in the liver [19]. All evidence suggests that FGF21 is a promising cytokine for the treatment of metabolic disorders. Interestingly, GLP1 therapy can also activate the iNKT-FGF21 axis in vivo, which contributes to weight loss [20]. That is to say, GLP1 can regulate the expression of FGF21 or play a synergistic role with FGF21 in regulating glucose and lipid metabolism.

An attractive way to treat diabetes is stem cell therapy. Stem cells have the ability of self-renewal and can differentiate into different types of cell. In T2DM, the injection of pluripotent stem cells can differentiate into β-islet cells, thus improving the symptoms of diabetes. At present, the focus of stem cell therapy for T2DM is to induce stem cells to differentiate into islet like cells without considering tissue repair and insulin resistance. Mesenchymal stem cells (MSCs) are derived from different adult tissues and have the ability of long-term self-renewal. Under specific conditions, it can differentiate into a variety of cell types [21]. Under different physiological and pathological conditions, MSCs can maintain homeostasis through multi-directional differentiation. They secrete a large number of cytokines and transmit chemical signals between cells, which are widely used in regenerative medicine research [22-24]. The new treatment method based on MSCs has satisfactory therapeutic effects in clinical applications. In the clinical treatment of diabetes, preliminary animal experiments and clinical evidence have proved that MSCs infusion can effectively decrease blood glucose level, insulin sensitivity of muscle, fat and liver tissue,

and reduce complications such as diabetic nephropathy, diabetic foot and lower extremity vascular disease. More importantly, in the process of MSCs application in clinical research, no serious adverse reactions were reported, which indicates that MSCs has good safety in clinical treatment [25-29]. However, there is a problem in the clinical application of MSCs in diabetes. Due to the different sources of MSCs, there may be significant differences in cell characteristics and therapeutic effects of MSCs. Therefore, new adjuvant therapy is needed to solve the above problems.

To solve these problems of MSCs, we used lentiviral particles with high expression of GLP1 and FGF21 genes to infect adipose derived mesenchymal stem cells. The therapeutic effect of gene modified MSCs on diabetes was verified by infusion of diabetic mice. The results showed that FGF21 and GLP1 gene modified MSCs could significantly improve insulin resistance, and promote the recovery of β - cell function. In addition, the gene modified MSCs have significant hypoglycemic and hypolipidemic activities, which may be due to the decreased expression of SREBP1 and SREBP2 and the increase of insulin expression in lipid metabolism. Based on these results, we have developed a new MSCs for the treatment of metabolic disorders, which will have the potential to fundamentally improve diabetes.

Materials And Methods

1. **Construction of fgf21-glp1-IgG4fc lentiviral expression plasmid**

pGSI-fgf21-glp1-IgG4fc (synthesized by Taihe Biotechnology) was used for subcloning of fgf21 and glp1-IgG4fc gene to the lentivector pCDH-EF1 (Addgene) with EF1α promotor. The amino acid sequence and the nucleotide sequence of the fgf21+ glp1-IgG4fc gene were listed in the supplementary materials. Primers for amplifying the cDNA of the fgf21+ glp1-IgG4fc gene (forward 5'-CGCGGATCCGCCACCATGGACTCGGACGAGACC -3', reverse 5'- ACGCGTCGACTCATTTACCCGGAGACAG -3') were synthesized from TsingKe (Beijing). The glp1-igg4fc gene is abbreviated as glp1 gene.

2. **Lentivirus production**

Lentiviral vector plasmids and the packaging plasmids (psPAX and pMD.2G) were purchased from Addgene. Lentiviral particles with pCDH-EF1-FGF21, pCDH-EF1-FGF21+GLP1 and pCDH-EF1-GLP1 produced through transfection of HEK293T packaging cells with 3rd generation plasmids system. 293T cells were transfected with 24 μg of plasmids, 48 μl of Lipofectamine LTX and 24 μl of PLUS reagents, and the proportions of pMD. 2G, psPAX, and pCDH-EF1 plasmid were 1:2:3. The supernatants were collected at 24 and 48h after transfection, filtered by 0.45 μm filters, and harvested by ultrafiltration with 100 kDa spin column (Millipore) at 4°C and 4,000g for 30 min. Lentiviral particles were dispensed in aliquots, and stored at - 80 °C until use. Transfection efficiency was examined by EGFP using flow cytometry (Beckman), and viral titer was determined according to the equation: virus titer (pfu/ mL) = cell number in each well × virus dilution factor × 10/ volume of added virus fluid (mL).

3. **Mesenchymal stem cell culture, flow cytometry analysis, and characterization**

Adipose tissue-derived mesenchymal stem cells were donated by Xijing Hospital and cultured in the same way as traditional ones. Briefly described as follows, to obtain the upper adipose tissue the healthy adult adipose tissue extracted by liposuction was transferred to a 50mL centrifuge tube, fully washed with PBS, and centrifuged at 1500rpm for 5 minutes. 0.2% mixed collagenase (Type I, II and IV collagenases=1:1:1) was prepared, and adipose tissue: collagenase =1:1 was added to the mixed collagenase digestion solution. The adipose tissue was digested in a 37 °C shaker for 30 minutes. Digested adipose tissue was immediately added to α-mem cell culture medium containing 10% FBS(Gibco), namely complete medium. To precipitate cells and tissue clumps, the mixture was centrifuged at 1500rpm for 10 min. Cells were resuspended using complete medium and the undigested tissue was removed by nylon mesh. The cells were inoculated in a culture flask and incubated at 37 ℃ in a 5% CO2 incubator. Two days later, the unadhered cells were discarded and the adhered cells were washed gently with PBS. The cells continued to be cultured in complete medium.

MSCs were harvested from passage 5, washed three times with PBS, and then incubated with 0.1–10 μg/mL of conjugated antibody (BD Biosciences) for 30-min without light at room temperature. Then, the cells were examined by flow cytometer analysis. In total, above 95% of cells expressed CD73, CD90, and CD105, while 2% or less of cells expressed CD45, CD34, and HLA-DR. Released cells were negative for pathogenic microorganisms, HBV, HCV, HIV, cytomegalovirus, syphilis, and ALT, and endotoxin levels were found within 40 IU/L and 0.5 EU/mL, respectively. The total cells were counted, and cell viability (≧ 85%) was determined by Trypan blue staining.

4. **Transduction of MSCs with lentiviral particles and detection of target gene expression**

The MSCs (<3 passages) were transduced with concentrative lentivirus with a multiplicity of infection (MOIs) of 40 for 6 h in the α-MEM medium containing 8 μg/ml polybrene. To detect the expression patterns of the FGF21 and GLP1 in MSCs, western blot analyses of cellular supernatant using anti-FGF21 and human IgG4-Fc monoclonal antibody were performed. To further measure the secreted the FGF21 and GLP1, culture medium (CM) of MSCs and MSCs transduced with pCDH-EF1-FGF21, pCDH-EF1-FGF21+GLP1, pCDH-EF1-GLP1, or pCDH-EF1-vector lentiviral particles were collected after incubation for 48 h. The secreted FGF21 and GLP1 in the medium of MSC culture were measured by ELISA (Abcam) according to the manufacturer's protocol. When collecting the culture supernatant for testing, in order to ensure the same sample quantity, we inoculated different kinds of cells with uniform density, and then added the same amount of medium. After 48 hours of culture, the same amount of centrifuged culture supernatant was detected by ELISA and WB. For testing the proliferation of secreted MSCs, each MSCs were seeded in 96-well plates at 5 × 104 cells/well and preconditioned in culture medium. After 48 h incubation, 20 μl of CCK8 was added to each well and incubated for 4 h at 37 °C and absorbance was

measured at 570 nm with a Quant microplate reader. All samples were analyzed as duplicates and samples with coefficient of variation (CV) values > 15% were excluded.

5. **Adipogenic and osteogenic differentiation**
   MSCs were cultured in a 24-well plate in complete a-MEM medium supplemented with adipogenic and osteogenic-inducing agents (Sigma Aldrich) at an initial cell density of 1×10^4 cells/well. After 2-3 weeks, cells were washed twice with PBS and fixed by 4% paraformaldehyde at room temperature for 30 min. Oil-red-O or alkaline phosphatase staining was applied to detect adipogenic and osteogenic differentiation.

6. **Western blotting**
   The cells were washed with PBS buffer and subsequently lysed using cell lysis buffer (Tiangen) with complete protease inhibitor mix (Biotool). Liver tissue were grinded and subsequently lysed using lysis buffer (Tiangen) with complete protease inhibitor mix (Biotool). The lysate and protein marker were run in SDS-PAGE gels (12% or 15%) respectively and transferred onto nitrocellulose membranes (MIllipore). Membranes were blocked with 5% milk in Tris-buffered saline plus Tween 20 (TBST) and exposed to primary antibodies against rabbit or mouse (1:3000, Abcam or Cell Signaling). Blots were probed with horseradish peroxidase (HRP)-conjugated goat anti-rabbit (or mouse) IgG (H+L) secondary antibodies and visualized using a Pierce ECL Western Blotting Substrate kit (Thermo Scientific) for signal detection.

7. **Relative quantitative real time polymerase chain reaction (RT-PCR)**

Total RNA was extracted from cells using RNA extraction reagent (TRizol; Sigma). The total RNA isolated was reversed into cDNA using BioScript All-in-One cDNA Synthesis SuperMix (Biotool). Quantitative real-time PCR reactions were performed using the SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara) in 7500 Real-Time PCR System (Applied Biosystems). For normalization, threshold cycles (Ct-values) were normalized to β-actin/GAPDH within each sample to obtain sample-specific ΔCt values (ΔCt ¼ Ct gene of interest Ct β-actin/GAPDH). The values of $2^{-\Delta\Delta Ct}$ were calculated to obtain fold expression levels. Primers for quantitative analyses of FGF21 gene (forward 5'- ATCGCTCCACTTTGACCCTG -3', reverse 5'- GGGCTTCGGACTGGTAAACA -3'), GLP1-IgG4Fc gene (forward 5'- CCCCAAAACCCAAGGACACT -3', reverse 5'- GCCATCCACGTACCAGTTGA -3'), srebp1c gene (forward 5'- CACTGTGACCTCGCAGATCC -3', reverse 5'- ATAGGCAGCTTCTCCGCATC -3'), insulin gene (forward 5'- TCTCTACCTAGTGTGCGGGG -3', reverse 5'- GCTGGTAGAGGGAGCAGATG -3'), β-actin gene (forward 5'- CCTGGCACCCAGCACAAT -3', reverse 5'- GGGCCGGACTCGTCATAC -3') and GAPDH gene (forward 5'- GGAGCGAGATCCCTCCAAAAT -3', reverse 5'- GGCTGTTGTCATACTTCTCATGG -3') were synthesized by TsingKe Company (Beijing).

8. **Animal experiments**

In our study, BKS.Cg-Dock7m+/+Leprdb/Nju mice (T2DM model mice) model was used, and they were purchased from the Model Animal Research Center of Nanjing University. Thirty-six male BKS mice aged 6-8 weeks (>20 g body weight) were randomly divided into six groups. Each group contained six mice kept in two cages. The experiment was divided into six groups. Control group was injected with 100 μ l saline intraperitoneally. Liraglutide group was injected with 100 μ l liraglutide drug (0.5mg/kg, twice a week until the end of the experiment). MSC group (containing pCDH-EF1-vector lentiviral particles), MSC-FGF21 group, MSC-FGF21+GLP1 group and MSC-GLP1 group was injected with 100 μ l cell suspension (1×106 MSC cells suspended in 0.1 mL of physiological saline, once a week for three weeks). The drugs were administered by intravenous injection. Tail blood glucose was measured every week during the experiments. On day 28, peripheral blood was collected from the retro-orbital sinus of each mouse.

9. **Glucose stimulated insulin secretion (GSIS)**

Rat INS-1 pancreatic β cell line was purchased from CCTCC (China Center for Type Culture Collection). The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2. The culture medium was RPMI medium 1640 containing 11 mM glucose and supplemented with 10% FBS, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 μM mercaptoethanol. The culture medium was replaced every second day, and cells were passaged once a week following trypsinization.

To determine the effect of gene modified MSCs on GSIS, INS-1 cells were seeded onto 12-well plates and cultured for 24 hours. Then, the cells were washed two times with Krebs-Ringer bicarbonate buffer (KRBB, 129 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 5 mM $NaHCO_3$, 0.1% BSA, 10 mM HEPES, (pH 7.4) and 2.8 mM glucose) and starved for 2 hours in KRBB. The cells were incubated in fresh KRBB containing different MSCs conditioned medium for 1 hour in the presence of glucose. The supernatants were collected to measure insulin concentration.

10. **Fasting glucose and glucose tolerance tests**

For the weekly fasting glucose test, the mice were starved overnight to assess glycemia. At the end of the experiment, after overnight fasting, the mice were administered glucose (1 g/kg) by oral gavage, and blood samples were collected from the tail vein for glucose determination. Glycemia was assessed using an Accu-Chek glucometer (Roche, Basel, Switzerland, http://www.roche.com), and the area under the curve was calculated.

11. **Statistical analysis**

All statistical analyses were conducted using the software of SPSS. Data were analyzed using one-way ANOVA followed with the Tukey's post-doc test or two-way ANOVA followed with the Bonferroni's post-hoc test for the differences among the treatment means. $P < 0.05$ was considered significant.

Results

1. **Morphology and immunophenotypic characterization of adipose derived MSCs**

MSCs isolated from human adipose tissue showed spindle like morphology similar to fibroblasts under phase contrast microscopy (Figure 1A). In vitro differentiation analysis confirmed that MSCs could differentiate into osteoblasts and adipocytes (Figure 1B). In order to further identify adipose derived MSCs, a panel of surface markers were analyzed by flow cytometry. Adipose derived MSCs were negative for CD34, CD45 and HLA-DR, but positive for CD73, CD90 and CD105 (Figure 1C).

2. **FGF21 and GLP1 expression in transduced MSCs**

In order to explore the most suitable infection conditions, we infected MSCs with lentivirus particles expressing EGFP (pCDH-EF1-EGFP), and then detected and evaluated these MSCs. EGFP modified MSCs were examined by fluorescence microscope (**Figure 2A**). Flow cytometry analysis of EGFP was performed with the cells at 48 h after transduction, and EGFP-positive cells ranged from 79 to 98% (**Figure 2B**). Flow cytometry analysis showed that when the infection multiplicity (MOI) was 40, the number of EGFP positive cells was more than 95%, and the transfection efficiency was not significantly improved between MOI 40 and 55 (**Figure 2B**). Therefore, the optimal MOI of the transduction scheme was 40. Then, the differentiation ability and surface markers of MSCs transfected with FGF21 + GLP1 were detected. The results showed that lentiviral particle transduction did not affect the biological characteristics of MSCs (**Figure 2C, D**).

In order to further verify the expression of FGF21 and GLP1, we performed quantitative RT-PCR analysis. The results showed that the expression of FGF21 and GLP1 mRNA in MSCs transfected with FGF21 or/and GLP1 was significantly higher than that of control MSCs ($P < 0.05$ for all) (**Figure 3A**). The content of FGF21 and GLP1 in the supernatant was detected by ELISA. The results showed that MSCs in FGF21 or/and GLP1 gene modified group could secrete a large number of FGF21 and GLP1 cytokines (**Figure 3B**). In addition, Western blot analysis also showed that the expression of FGF21 and GLP1 protein in the supernatant of FGF21 or/and GLP1 gene modified MSCs was significantly increased (**Figure 3C**).

3. **FGF21+GLP1 modified MSCs transplantation ameliorated blood glucose and weight in T2DM mice**

The effects of MSC-FGF21+GLP1 cells in system metabolic disturbance were investigated in our study. BKS.Cg-Dock7m+/+Leprdb/Nju mice (BKS mice) with leptin receptor deficient is characterized by obesity, insulin resistance, hyperglycemia, and dyslipidemia. MSCs and GLP1 analogues (liraglutide) were used as the controls. After 3 weeks of cell therapy, the weight gain trend of MSC-FGF21+GLP1 group was significantly inhibited (**Figure 4A**). And compared with the untreated group, the weight of adipose tissue of BKS mice treated with MSC-FGF21+GLP1 also decreased. Interestingly, MSC-FGF21+GLP1 had similar effects to liraglutide (**Figure 4B**). Meanwhile, fasting blood glucose level of BKS mice was measured once a week for 4 weeks. As shown in **Figure 4C**, MSC-FGF21+GLP1 cells significantly reduced fasting blood

glucose levels in BKS mice. The results showed that the hypoglycemic effect of MSC-FGF21+GLP1 was slightly higher than that of other cell groups. The same phenomenon was observed in the results of the oral glucose tolerance test (**Figure 4D**). In addition, after MSC-FGF21+GLP1 treatment, the plasma insulin level of BKS mice increased slightly (**Figure 4E**). These observations suggested that MSC-FGF21+GLP1 could enhance insulin secretion.

4. **FGF21+GLP1 modified MSCs transplantation could improve lipid disorders in T2DM mice**

The histopathological analysis was performed to determine the potential target tissues or organs of MSC-FGF21+GLP1 in T2DM mice. We found that MSC-FGF21+GLP1 cells could improve the histological structures of liver and adipose in BKS mice. The diagnosis index of fatty liver is fatty change or balloon like change of hepatocytes. In pathological section, there are a lot of blank areas in hepatocytes. In the mice treated with msc-fgf21 + GLP1, pathological sections showed that the blank area decreased, indicating that the degree of hepatic steatosis was reduced, indicating that the treatment was effective (**Figure 5A**). Histological observation of adipose tissue showed that the size of abdominal adipocytes in mice treated with MSC-FGF21+GLP1 decreased substantially, but no significant change was observed in the other groups (**Figure 5A, B**). This is interesting because adipose tissue response to MSC-FGF21+GLP1 treatment may partly explain the effects of weight loss and lipid-lowering. In accordance with the above results, MSC-FGF21+GLP1 significantly improved the lipid profile of mice, which was manifested by the significant decrease of triglyceride (TG), cholesterol (TC), low density lipoprotein cholesterol (LDL) and highdensity lipoprotein cholesterol (HDL) (**Figure 5C**).

5. **FGF21+GLP1 modified MSCs significantly suppressed srebp1c transcription and promoted insulin expression**

To preliminarily verify the reasons why MSC-FGF21+GLP1 can correct glucose and lipid metabolism, we primarily detected the expression of SREBP and insulin, the key genes affecting glucose and lipid metabolism. In order to detect the effects of MSC cells on srebp1 gene and insulin gene expression, we used conditional media of different gene-modified MSC cells (FGF21 or/and GLP1 modified) to treat human HepG2 cells and rat INS-1 cells, respectively. As shown in **Figure 6A** and **6B**, the supernatant isolated from MSC-FGF21+GLP1 treatment group significantly inhibited srebp1c mRNA level and increased insulin mRNA level, and the activity of MSC-FGF21+GLP1 was significantly higher than that of MSC-FGF21, MSC-GLP1, and even liraglutide, the positive control drug. GSIS experiment also confirmed that MSC-FGF21+GLP1 can promote insulin secretion. The results suggested that MSC-FGF21+GLP1 could significantly stimulate insulin secretion in INS-1 cells (**Figure 6C**). Therefore, FGF21 and GLP1 double gene modified MSCs have significant synergistic effect on regulating glucose and lipid metabolism, especially in regulating srebp1c and insulin genes expression.

6. **The mechanisms of FGF21 and GLP1 synergistically improving lipid metabolism.**

To further clarify the mechanism of MSC-FGF21+GLP1 synergistically regulating lipid metabolism, we detected the key lipid metabolism regulation genes upstream and downstream of srebp genes. First, we added conditioned medium containing different gene modified MSCs (FGF21 or/and GLP1) to human HepG2 cells for 48 hours. The cytoplasmic protein and nuclear protein were extracted and the protein expression level was detected. Secondly, because MSCs mainly stay in the liver after being reinfused into mice through vein, we took the liver of mice for grinding, and then extracted the cytoplasmic protein and nuclear protein to detect the expression of the target protein. As shown in **Figure 7**, both in vitro and in vivo experiments, MSC-FGF21+GLP1 could significantly increase the phosphorylation level of AMPK, which was much higher than that of single gene modified MSCs. Next, we detected the expression of SREBP1 and SREBP2 genes in the nucleus. In vitro and in vivo, it was found that the expression of splicing active protein (base band) and integrity protein (top band) of SREBP1 decreased in the nucleus, while SREBP2 was not affected in MSC-FGF21+GLP1 group, and the splicing active protein (base band) was significantly decreased in MSC-FGF21+GLP1 group. In accordance with the above results, the phosphorylation level of ACC protein and Fas protein in SREBP downstream were decreased, and the phosphorylation level of HSL an enzyme associated with promoting fat decomposition, was significantly increased.

The summary mechanism of FGF21 and GLP1 synergistically improving lipid metabolism was shown in **Figure 8**, the diagram illustrates the three existing methods for regulating lipid metabolism. The red box represents known metabolic regulation of PCSK9 and HMC-CoA reductase. At present, antibodies and statins are mainly used to block the expression of PCSK9 and HMC-CoA reductase, thereby reducing cholesterol and lipid synthesis. In this study, we focused on cytokines that regulate lipid metabolism. FGF21 and GLP1 bind to their receptors, and then they synergistically enhanced AMPK phosphorylation. Activated AMPK further inhibited the expression of SREBP1/2 gene and mature SREBP1/2 protein into nucleus, and finally regulated the expression of enzymes involved in lipid metabolism.

Discussion

Diabetes is a chronic disease with high morbidity and mortality in the world. Traditionally, diabetes is divided into type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), among which the incidence of T2DM is more than 90% [30]. Current studies have shown that insulin resistance (IR) and islet β - cell secretion deficiency are the two major pathogenesis of T2DM [30-33]. Of course, diabetes can also be considered a combination of the other three diseases. Firstly, it is an endocrine disease, involving a variety of hormone level disorders, including insulin, glucocorticoids and adrenal hormones [34]. Secondly, it is a metabolic disease with abnormal glucose, lipid metabolism, mitochondrial function, nucleic acid regulation and so on [35-37]. Thirdly, it is a systemic disease characterized by decreased insulin sensitivity in metabolic tissues and organs, which can damage the structure and function of

various tissues and organs in the body [38, 39]. Based on the above theory, in order to efficiently change the symptoms of diabetes and achieve tissue repair, stem cells, regeneration factors, nutrients and other comprehensive functions are needed, as shown in the following figure. However, the current therapeutic strategies strategy of diabetes mainly focuses on the control of glucose and lipid metabolism. which is difficult to fundamentally improve insulin resistance and tissue repair. In order to improve this situation, we designed double gene modified MSCs (FGF21 and GLP1) to achieve multiple repair effects in the treatment of diabetes.

The metabolism of sugar, fat and protein is the most basic metabolism of human body. The mutual regulation of metabolic organs forms a complex regulatory network, which involves neuroendocrine system, growth factors and enzymes. Generally speaking, there are three types of endogenous molecules involved in the regulation of regeneration factor metabolism. The first one is hormones, including insulin, glucagon, GLP1 and glucocorticoid. The second group of factors is the growth factors of cell with hormone like function, including FGF19, FGF21 and FGF23. The third category is involved in metabolic regulation or cell signal transduction enzymes, such as PI3K and HSL. These endogenous hormones, cytokines or enzymes are closely related to the occurrence of metabolic diseases. In this study, we selected GLP1 and FGF21 as the joint repair factors. We chose these two factors mainly because they can interact with each other through the iNKT-FGF21 axis in vivo, so as to achieve the regulation of body weight [20]. The second reason was that the safety of these two factors in human body had been demonstrated [9,15]. There are many drugs approved by FDA for marketing and drugs under phase II clinical trials, such as liraglutide, dulaglutide and LY2405319. This ensures the safety of MSC-FGF21+GLP1 in clinical transformation. In this study, we used GLP1 sequence and FGF21 sequence to refer to dulaglutide and LY2405319, respectively. GLP1 plays a hypoglycemic role by increasing insulin synthesis and secretion, inhibiting the emptying of gastric contents, and inhibiting the excitation of the feeding center [10]. At the same time, GLP1 can correct the expression of GLUTs in liver and muscles of patients with T1DM and T2DM, thus changing the glucose intake of cells [40]. FGF21 regulates glucose and lipid metabolism in adipose tissue through endocrine pathways, improves insulin sensitivity, insulin resistance and stimulates glucose uptake of skeletal muscle through GSIS [7, 8, 41]. FGF21 has been developed as a drug for the treatment of metabolic diseases [6]. FGF21 can improve fatty liver by inhibiting SREBP-1, reducing triglyceride levels in serum and liver of obese mice, and reduce liver cholesterol production by inhibiting SREBP-2 [18]. In vivo, GLP1 therapy can also activate the iNKT-FGF21 axis, which contributes to weight loss [20]. Therefore, we suggest that GLP1 can further regulate SREBP expression through FGF21 signaling, which may have synergistic effect on regulating glucose and lipid metabolism. In our study, the combined application of FGF21 + GLP1 could significantly reduce the expressions of srebp1 and srebp2, and significantly increase the expression of insulin, which was better than that of single administration. The results revealed the synergy between GLP1 and FGF21.

Although GLP1 and FGF21 can effectively alleviate the glucose and lipid metabolism of diabetic patients, there are multiple application barriers in the application. The most important obstacle is the extremely short half-life of the drug, and patients need to be injected with large quantities of both drugs daily or weekly, which will cause high cost and drug resistance. Therefore, FGF21 and GLP1 gene modification of

MSCs is a good way to solve these problems. Although the concentrations of FGF21 and GLP1 secreted by MSC-FGF21+GLP1 were low, the effect of MSC-FGF21+GLP1 was better than that of drug therapy alone. MSCs can survive for a long time in vivo and secrete cytokines continuously, which will help patients to solve the problem of long-term drug injection. Of course, MSCs treatment also has some limitations, its operation is more complex than the general drug treatment, and the price is generally more expensive, so the use of the population also has a tendency. At the same time, due to the cell transfusion, there are many uncontrollable factors, so we need to carry out a detailed physical examination before cell therapy. Therefore, we will demonstrate the biosafety and pharmacokinetics of MSC-FGF21+GLP1 in the following studies.

The molecular mechanism of MSCs in the treatment of diabetes remains unclear. The possible mechanisms include: promoting islet cell regeneration, reducing insulin resistance in peripheral tissues, increasing insulin sensitivity, regulating immune system, protecting islet beta cells, and improving diabetic complications [26, 41-45]. However, if MSCs cells are used alone without gene modification, there will be many problems. Diabetic patients are in hyperglycemia state. In their body, high concentration of blood glucose will promote the expression of PPAR-γ and C/EBP-α genes in reinfusion MSCs or autologous MSCs, which makes MSCs more likely to differentiate into adipocytes and osteoblasts. This was not conducive to the repair of damaged islets by MSCs [46,47]. At the same time, with the increase of age in patients with diabetes, the activity of MSCs in patients will decrease [48-50]. The use of allogeneic MSCs has some problems, such as short survival time of MSCs [51] and foreign gene contamination. Therefore, two kinds of gene modified MSCs (FGF21 and GLP1) were used as the compensation scheme. Of course, lenticular transmission might not be approved for clinical trials. We could consider preconditioning of MSCs to make them express FGF21 or GLP1 endogenously.

To date, the understanding of metabolic kinetics of MSCs in vivo mainly comes from animal experiments. Due to the chemotaxis of MSCs to damaged tissues and organs, there are differences in the metabolic kinetics between healthy body and diseased subjects. After peripheral intravenous injection, most MSCs remained in the lungs and then reached the liver, kidney and spleen along with blood flow. This may be due to the concentration gradient of substance P released from damaged tissue in vivo, which attracts MSCs to migrate to the injured site along the concentration gradient for repair [41]. In this study, MSC-FGF21+GLP1 could significantly reduce liver injury, which may be due to this reason. We will discuss the homing site and survival time of MSC-FGF21+GLP1 in the following experiments.

Conclusions

In conclusion, this study has shown a new approach that combines FGF21 and GLP1 gene therapy with MSC cell therapy to treat type 2 diabetic mice. We found that infusion of FGF21 and GLP1 modified MSCs could significantly improve significantly insulin sensitivity and glucose metabolism, promote the recovery of liver structure, increase plasma insulin content, and play a synergistic role in regulating glucose and lipid metabolism.

Abbreviations

MSCs: Mesenchymal stem cell; CM: Culture medium; DM: Diabetes mellitus; T1DM: Type 1 diabetes mellitus; T2DM: Type 2 diabetes mellitus; GLP1: glucagon-like polypeptide 1; FGF21: fibroblast growth factor 21; SREBP: sterol regulatory element binding protein; GSIS: Glucose stimulated insulin secretion; TG: Triglyceride; TC: Cholesterol; LDL: Low-density lipoprotein cholesterol; HDL: High-density lipoprotein cholesterol.

Declarations

Declare to Stem Cell Research & Therapy

The laboratory animals were handled in accordance with Guidelines for the Care and Use of Laboratory Animals and the Animal Welfare Act in China and approved by the Animal Use and Care Committee of the Academy of Military Sciences. The protocol of MSCs preparation was approved by the General Logistics Department of the PLA.

Ethics approval and consent to participate

The care and use of laboratory animals were approved by the Animal Use and Care Committee of the Academy of Military Sciences.

The care and use of adipose derived mesenchymal stem cells were approved by the Xijing Hospital of Airforce Medical University, permit number of ethics approval was 201909044425.

Consent for publication

Not applicable.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

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Authors' contributions

BHX performed the scientific design, analyzed all experiments, and drafted the manuscript. HFD and LJQ performed the scientific design and revised the manuscript. XXX, TTY, JX, and TN performed the experiments and revised the manuscript critically; XHX, QHJ, LW and SFM contributed to the data and statistical analyses. All authors read and approved the final manuscript.

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Competing Interests

The authors declare that they have no competing interests.

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Figures

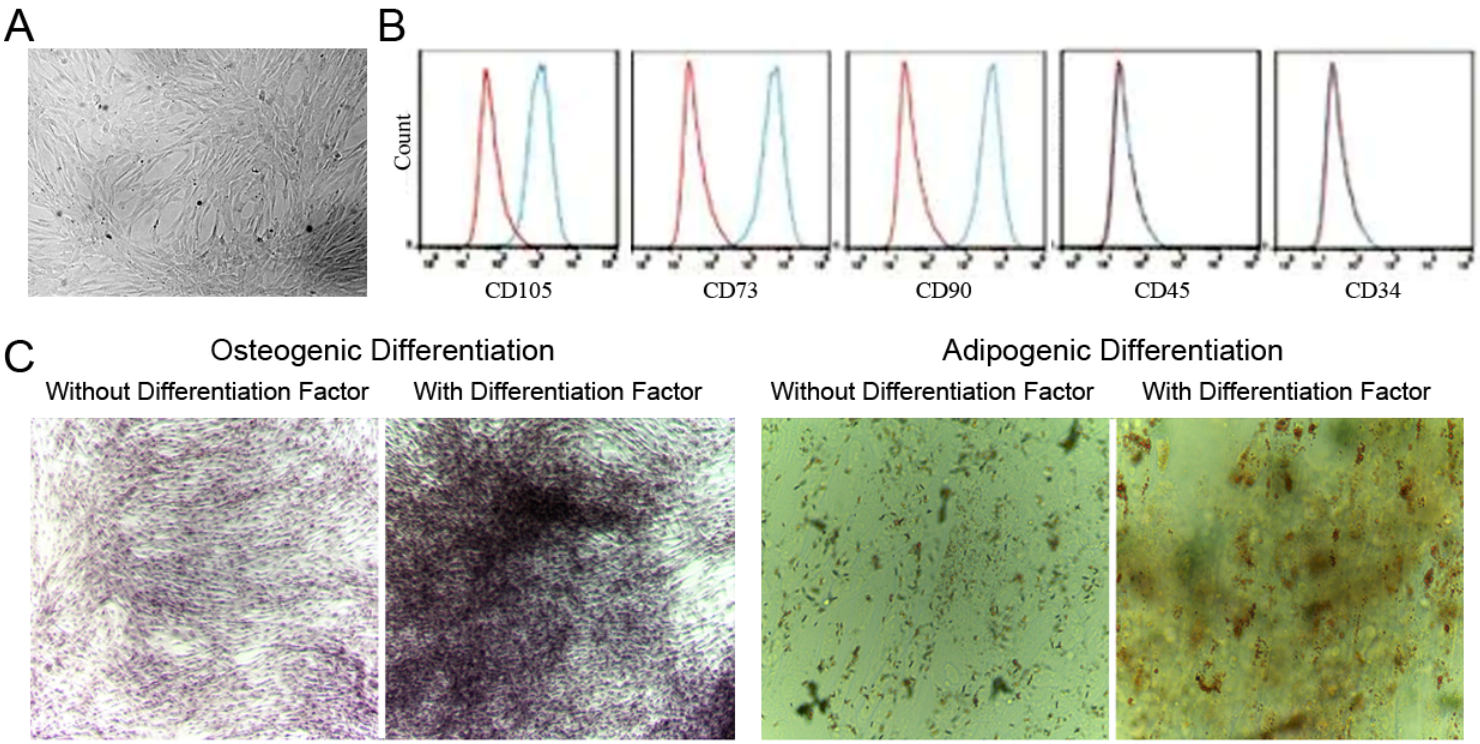

Figure 1

Morphology and multilineage differentiation capacity of MSCs. A: Adipose derived MSCs showed a homogeneous spindle-shaped morphology. B Flow cytometry analysis of phenotype characterization of MSCs. Phenotype of CD73, CD90, CD105, CD34 and CD45 of MSCs was detected by flow cytometry. The green lines indicate the fluorescence intensity of cells stained with the corresponding antibodies, and the red lines represent isotype-matched negative control cells. C: Osteogenesis was examined by von Kossa staining for mineral nodule deposition. Adipogenesis as observed by the presence of lipid vesicles and confirmed by oil red O staining.

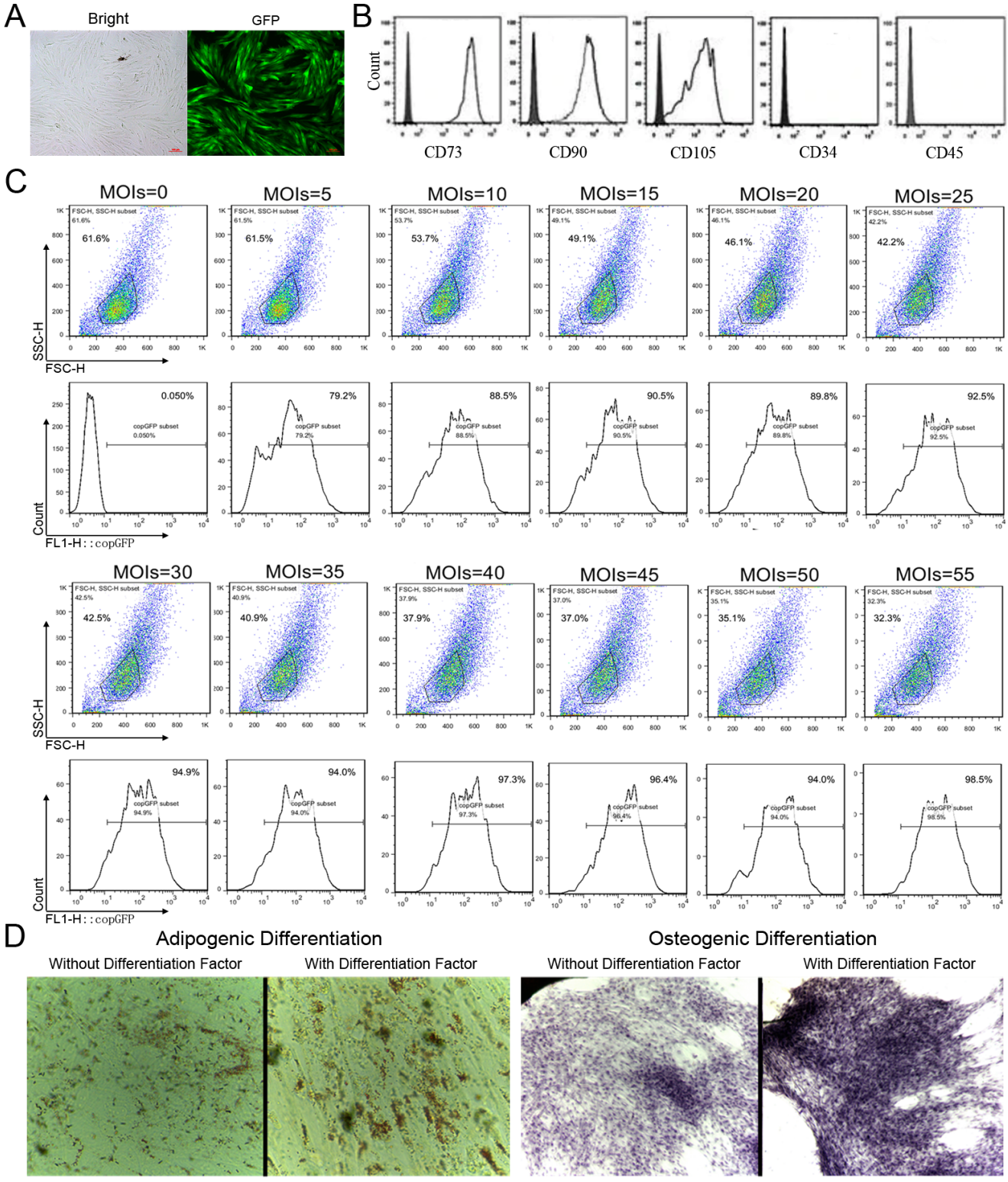

Figure 2

Transduction of MSCs with lentiviral vector particles. A: Expressing of EGFP in transduced MSCs with MOI of 40 under fluorescence microscopy. B: Flow cytometry analysis of phenotype characterization of MSC-FGF21+GLP1. Phenotype of CD73, CD90, CD105, CD34 and CD45 of MSCs was detected by flow cytometry. The white areas indicate the fluorescence intensity of cells stained with the corresponding antibodies, and the gray areas represent isotype-matched negative control cells. C: Analysis of EGFP

fluorescence by flow cytometry at 48 h after transduction with different MOI. The MOI ranges from 0 to 55, with every five as an interval. D: The FGF21+GLP1 lentiviral transduced MSCs could differentiate into osteoblasts and lipids.

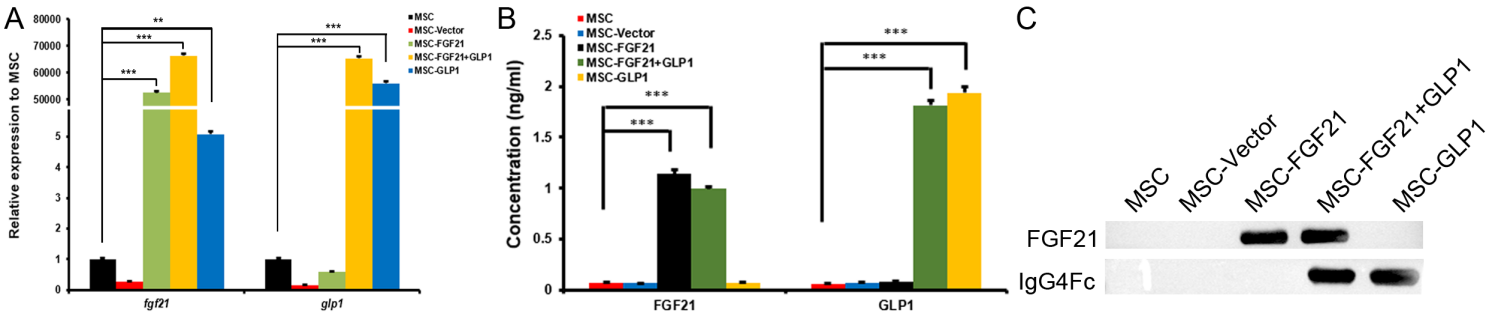

Figure 3

The expressions of FGF21 and GLP1 in the gene modified MSCs. A: Quantitative real-time PCR detected the expressions of FGF21 and GLP1 mRNA in the FGF21 or/and GLP1 modified cells, unmodified MSCs and blank vector modified MSCs were controls. The intracellular β-actin gene was used as a reference gene, **P<0.01, ***P<0.001. B: ELISA analyzed the expression of FGF21 and GLP1 in FGF21 or/and GLP1 modified MSCs cell culture medium, unmodified MSCs and blank vector modified MSCs were controls., ***P<0.001. C: Western blot analysis shown strong FGF21 and GLP1 band in the MSCs transduced with FGF21or/and GLP1.

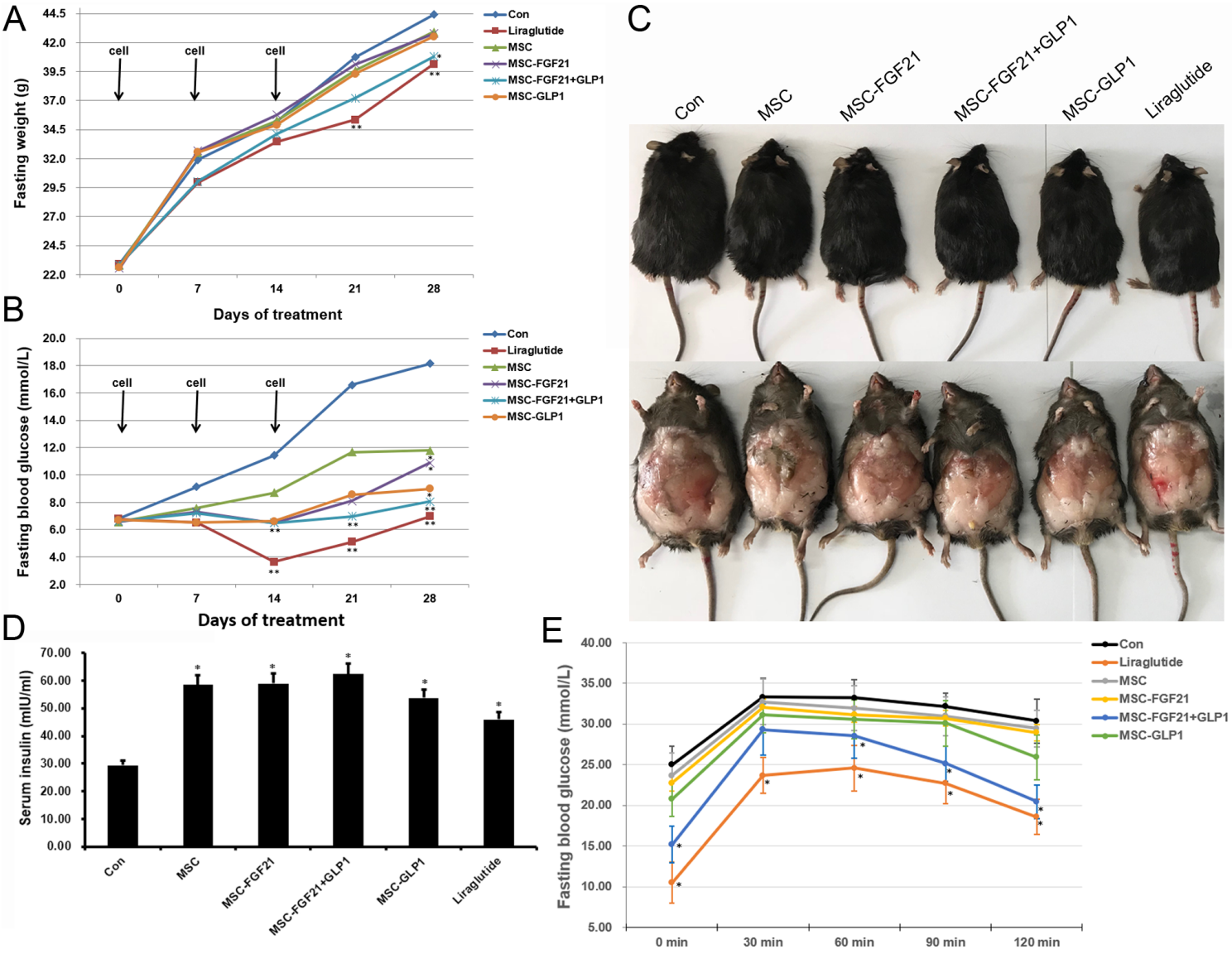

Figure 4

FGF21 and GLP1 modified MSCs could reduce blood glucose and weight in T2DM mice. A: The time course of body weight during 4 weeks in BKS mice injected i.p. with saline (Con), MSCs, liraglutide, FGF21 or/ and GLP1 transduced MSCs. The arrow position represents the time of cell injection, n=6, *P<0.05, **P<0.01, compared to Con. B: Time course of the fasting blood glucose concentrations of BKS mice injected i.p. with saline (Con), MSCs, liraglutide, FGF21 or/ and GLP1 transduced MSCs. C: Gross appearance of BKS mice injected i.p. with saline (Con), MSCs, liraglutide, FGF21 or/ and GLP1 transduced MSCs, *P<0.05, **P<0.01, compared to Con. D: The serum insulin levels in each group, *P<0.05, compared to Con. E: Blood glucose concentration from oral glucose tolerance tests in BKS mice injected i.p. with saline (Con), MSCs, liraglutide, FGF21 or/ and GLP1 transduced MSCs, * represent significant differences between groups, P<0.05, compared to Con.

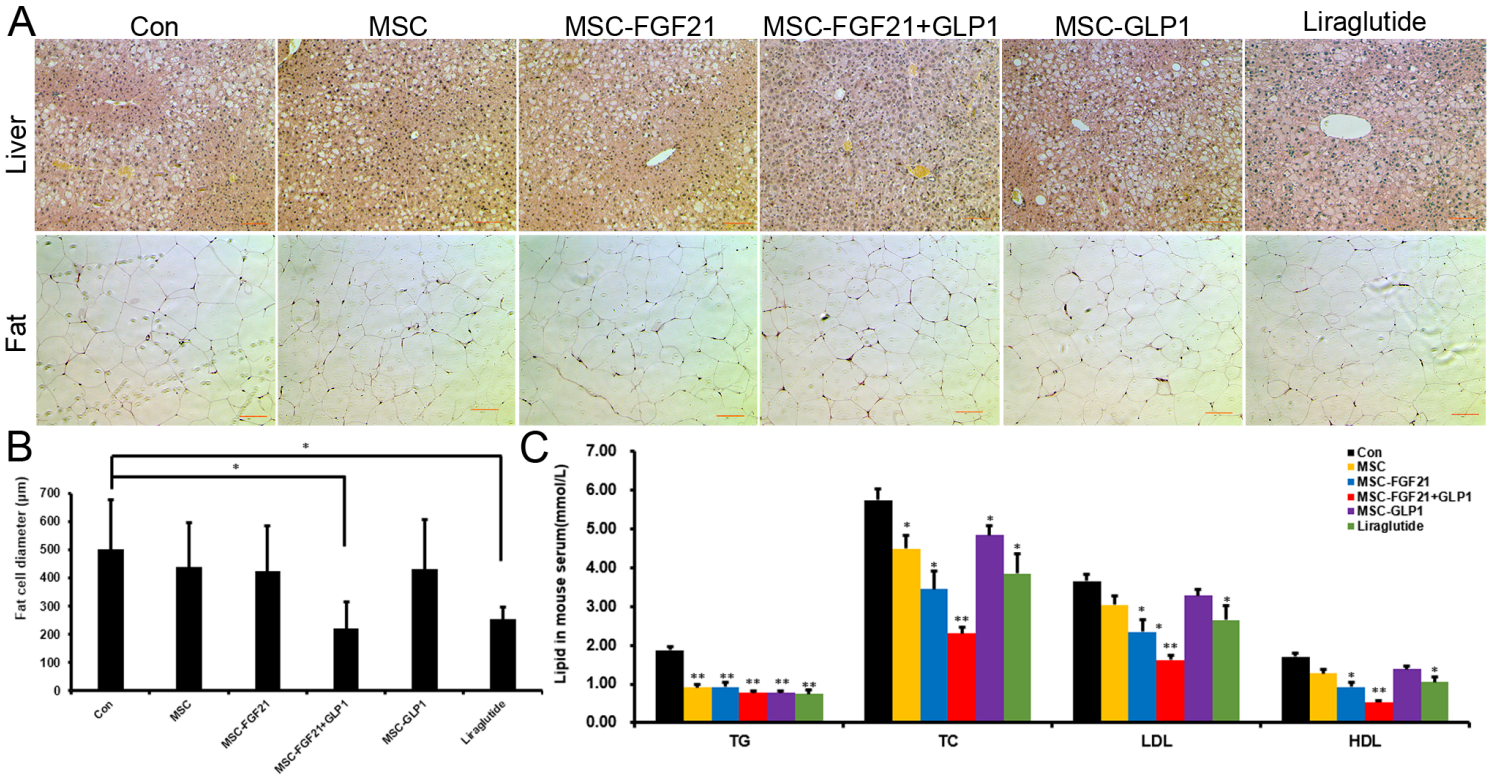

Figure 5

FGF21 and GLP1 modified MSCs could improve lipid metabolism in T2DM mice. A: Hematoxylin and eosin staining of representative liver and adipose sections obtained from mice of the indicated groups (scale bars = 100 mm). B: Statistics on the diameter of fat cells, *P<0.05. C: The serum TG, TC, HDL-C, and LDL-C levels in the indicated groups, *P<0.05, **P<0.01, compared to Con.

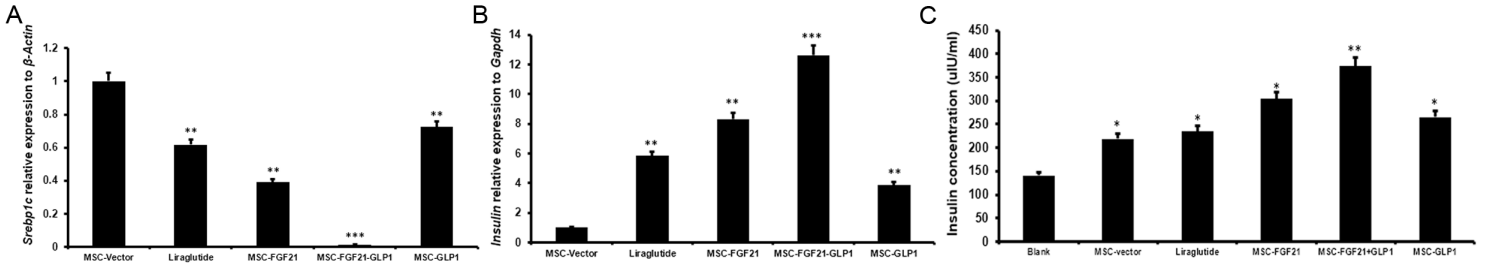

Figure 6

. FGF21 and GLP1 modified MSCs could improve glucolipid metabolism in vitro. A: Quantitative real-time PCR detected the effect of FGF21 or/and GLP1 modified MSCs on the expressions of srebp1c mRNA in the HepG2 cells. blank vector modified MSCs was negative control, and liraglutide was employed as positive control. The intracellular β-actin gene was used as reference genes, **P<0.01, ***P<0.001, compared to MSC vector. B: Quantitative real-time PCR detected the influence of FGF21 or/and GLP1 modified MSCs on the expressions of insulin mRNA in the INS-1 cells. blank vector modified MSCs was negative control and liraglutide was positive control. The intracellular GAPDH gene was used as reference genes, **P<0.01, ***P<0.001, compared to MSC vector. C: Insulin secretion in INS-1 cells incubated at conditioned medium of different modified MSCs. Blank group was KRBH medium without any added

reagent. blank vector modified MSCs and liraglutide acted the negative and positive controls, respectively, *P<0.05, **P<0.01, compared to MSC vector.

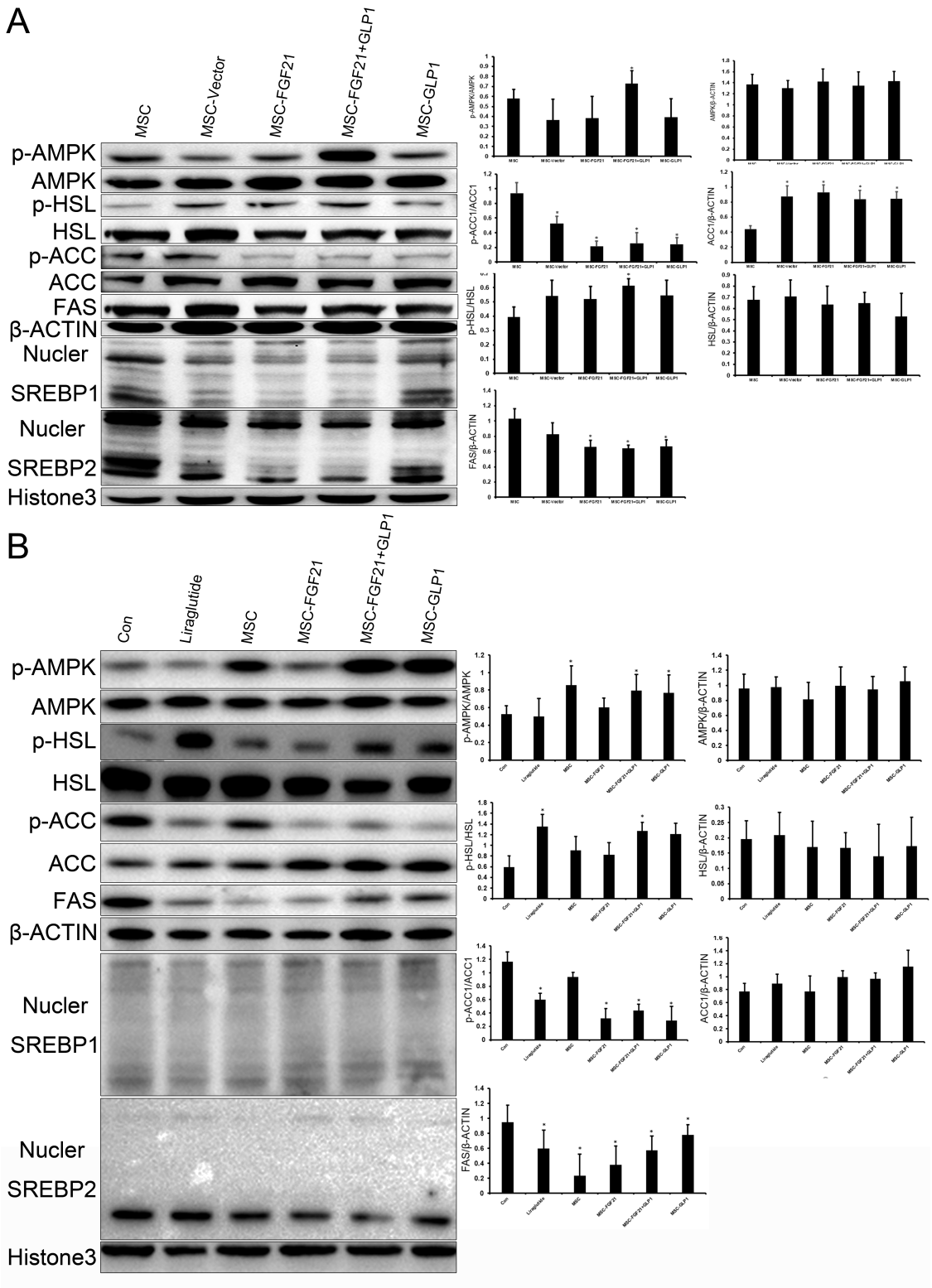

Figure 7

Signaling pathways of MSC-FGF21+GLP1 regulating lipid metabolism. Western blot verified the signaling pathway of MSC-FGF21+GLP1 cells regulating lipid metabolism. β-actin and GAPDH were reference proteins. p-AMPK, p-ACC and p-HSL respectively represented the phosphorylation levels of these proteins.

Cytoplasmic proteins were extracted for detecting AMPK, ACC, FAS, and HSL. Nuclear proteins were extracted for detecting SREBP1 and SREBP2. AMPK: adenosine monophosphate activated protein kinase, SREBP: sterol regulatory element binding proteins, ACC: acetyl-coenzyme A carboxylase, FAS: fatty acid synthase, HSL: hormone sensitive lipase. A: Western blot analyzed the expression of above genes in HepG2 cells, *P<0.05, compared to Con. B: Western blot analyzed the expression of above genes in liver, *P<0.05, compared to Con.

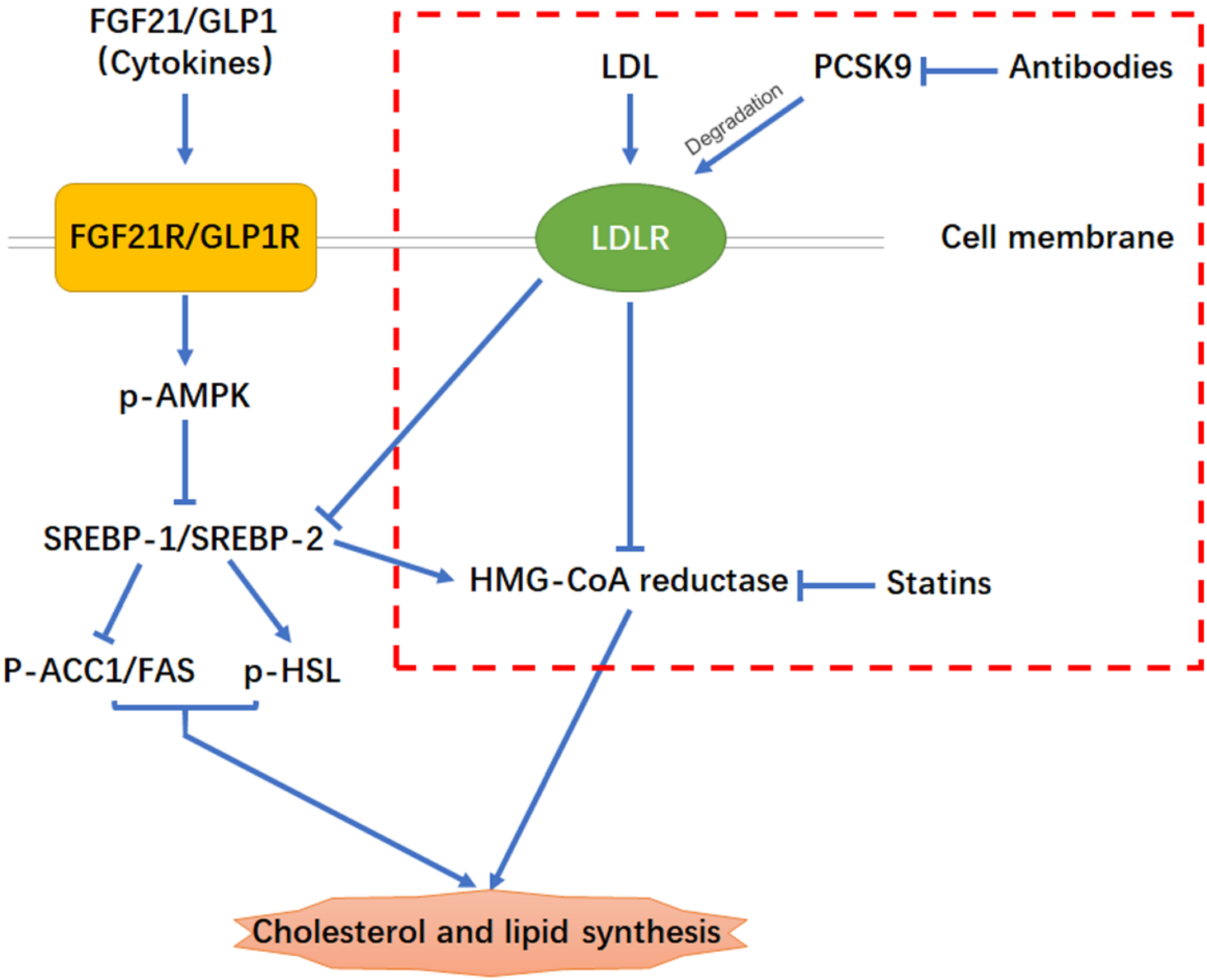

Figure 8

The mechanisms of FGF21 and GLP1 synergistically improving lipid metabolism. The diagram illustrates three existing approaches to regulate lipid metabolism. The red box represents the known metabolism regulation aimed at PCSK9 and HMC-CoA reductase. Currently, antibodies and statins are mainly used to

block the expression of PCSK9 and HMC-CoA reductase so as to reduce cholesterol and lipid synthesis. In this study, we focused on cytokines that regulate lipid metabolism. FGF21 and GLP1 bind to their receptor, then they synergistically enhanced AMPK phosphorylation levels. Activated AMPK further inhibits the expression of SREBP1/2 genes and mature SREBP1/2 protein into the nucleus. At last the expression of enzymes directly involved in lipid metabolism is significantly inhibited.

Supplementary Files

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