Shp2 suppresses fat accumulation in white adipose tissue by activating Wnt/β-catenin signaling following vertical sleeve gastrectomy in obese rats with type-2 diabetes

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Abstract. Adipogenesis and fat accumulation are closely associated with the development of obesity. Sleeve gastrectomy (SG) is an effective treatment for obesity and associated metabolic disorders. Leptin is downregulated after SG and Src homology phosphatase 2 (Shp2) has an important role in leptin signaling. The role of Shp2 in SG and the mechanisms of fat reduction following SG were further investigated in the current study. Sham and SG operations were performed on obese type-2 diabetes model Sprague-Dawley rats. Primary pre-adipocytes were isolated from the inguinal white adipose tissue (ingWAT) of the rats. Shp2 expression in ingWAT pre-adipocytes was silenced using small interfering RNA transfection. Shp2 function was inhibited using the specific inhibitor, SHP099. In addition, Shp2 was overexpressed using lentivirus. Gene and protein expression analysis was performed after adipocyte differentiation. Furthermore, Shp2-overexpressing ingWAT pre-adipocytes treated with the β-catenin inhibitor, PNU-74654, were also used for gene and protein expression analysis. Adipogenic markers, including triglycerides, peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer-binding protein α (Cebpα), adiponectin, fatty acid-binding protein 4 and leptin, were examined. Compared with the sham, triglyceride, leptin, PPARγ and Cebpα levels were significantly reduced in the ingWAT from the SG group. Shp2 expression levels were reduced following leptin treatment. Moreover, genetic analysis demonstrated depot-specific adipogenesis following Shp2 silencing or inhibition in ingWAT pre-adipocytes. Conversely, Shp2 overexpression decreased the expression of adipogenic markers by enhancing β-catenin expression. PNU-74654 treatment abolished the downregulation of adipogenic markers caused by Shp2 overexpression. SG decreased leptin levels in ingWAT, which in turn upregulated Shp2, and Shp2 suppressed fat accumulation and adipogenic differentiation by activating the Wnt/β-catenin signaling pathway. Overall, this may represent a potential mechanism of fat reduction in SG, and Shp2 may serve as a potential therapeutic target for the treatment of obesity and type-2 diabetes.

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Introduction

Obesity is a public health hazard worldwide (1). A previous study revealed that the prevalence of obesity (defined as body mass index ≥30 kg/m^2) increased from 3.2 to 10.8% in adult men and from 6.4 to 14.9% in adult women between 1975 and 2014 (2). Obesity can result in insulin resistance, which substantially increases the risk of type-2 diabetes (T2D) (3,4). For patients with obesity and T2D, sleeve gastrectomy (SG), one of the most popular procedures of bariatric surgery (5), is effective in reducing body fat, improving hepatic insulin resistance and eventually controlling hyperglycemia (6-9). Furthermore, reduced fat accumulation following SG is specifically associated with the amelioration of hepatic insulin resistance (10). However, the concrete mechanism is not fully understood. Previous studies have mainly focused on the effects of food intake and satiety induced by post-operative gut hormone changes on metabolism (11-13). It should be noted that adipose metabolism plays an important role in reducing body weight and improving glycemic control. Thus, further research on the effect of SG on lipid metabolism in adipose tissue is required.

White adipose tissue (WAT) has been recognized as an immunoendocrine organ that regulates energy balance and

metabolism (14). Expansion of WAT leads to obesity with a subsequent imbalance in the production and signaling of adipokines (15). The levels of leptin, an adipose tissue-derived hormone, are increased in both circulation and WAT in obesity, but reduced following SG (16-19). Since leptin is an adipocyte effector protein, its expression is a marker of pre-adipocyte differentiation (20). Recently, Palhinha *et al* (21) suggested that leptin was an inducer of adipogenesis and associated inflammation. Src homology 2 domain-containing phosphatase 2 (Shp2) is encoded by the Ptpn11 gene and involved in leptin signaling downstream of the leptin receptor long form (LepRb) in the hypothalamus (22). This protein plays a notable role in balancing food intake and energy (23), which is a potential target for the treatment of obesity (24). Several studies have demonstrated that Shp2 deficiency in the forebrain causes obesity and diabetes due to disrupted leptin signaling (22,23,25). Moreover, Tao *et al* (26) reported that Shp2 suppresses the early stages of adipogenic differentiation in 3T3-L1 cells. However, to the best of our knowledge, there have been no previous studies examining the role of Shp2 in the lipid metabolism of WAT following SG and adipogenic differentiation of white pre-adipocytes.

In the present study, obese rats with T2D were used to perform vertical SG to investigate the fat metabolism in inguinal WAT (ingWAT) and its underlying mechanism in the regulation of obesity.

Materials and methods

Animals. All animal experiments were approved by the Animal Care and Use Committee of Nanjing Drum Tower Hospital, Nanjing Medical University (approval no. 2019AE02013; Nanjing, China). The present study complies with all relevant ethical regulations of Nanjing Drum Tower Hospital Ethics Committee.

A total of 16 male Sprague-Dawley rats (weight, 150±10 g; age, 4 weeks) were purchased from the animal core facility of Nanjing Medical University (Jiangsu, China). The rats were housed in specific pathogen-free units of the Animal Center at Nanjing Drum Tower Hospital maintained at 60-70% relative humidity and 23±1˚C, with a 12-h light/dark cycle and *ad libitum* access to a 60% high-fat diet (HFD; cat. no. D12492; Research Diets, Inc.) and water. After 8 weeks on HFD, T2D was induced by intraperitoneal injection of 30 mg/kg streptozotocin (STZ; MilliporeSigma). Random blood glucose levels were measured 72 h following STZ injection with a hand-held glucose meter (OneTouch UltraVue; Johnson & Johnson). Rats with random blood glucose levels >16.0 mmol/l on three consecutive days were considered diabetic. Diabetic rats were randomly assigned to the sham or the SG group (n=8/group). Body weight, food intake and fasting glucose (FG) levels were measured pre-operatively and post-operatively at week 2, 4, 6 and 8. An oral glucose tolerance test (OGTT) was performed pre-operatively and at week 8 post-surgery. The body fat content and distribution were also measured at week 8 using dual-energy X-ray absorptiometry (DEXA). The ingWAT was then harvested for subsequent experiments.

SG surgical procedure. Rats were fasted overnight and then anesthetized using isoflurane (3% for induction; 2% for maintenance). Under sterile conditions, a single 3-cm epigastric laparotomy was performed. As presented in Fig. 1A, the greater curvature of the stomach from the antrum to the fundus and glandular stomach was dissected, ~70% of the gastric volume was excised and the gastroesophageal junction and the pylorus were preserved. For the sham group, a similar length incision was made in the anterior gastric wall, then sutured using a 6-0 absorbable thread. Abdominal closure was performed using 3-0 silk suture with a continuous suture technique. The rats received ibuprofen (15 mg/kg of body weight daily) to minimize post-operative pain for 2 days. After allowing 24 h for the anastomoses to heal, the animals were kept on a liquid diet for 3 days before being returned to normal chow for an additional 8 weeks.

Primary pre-adipocytes isolation and culture. Primary pre-adipocytes were isolated from the ingWAT of 4-week-old SD rats. In brief, adipose tissue samples were collected from the inguinal region under sterile conditions and washed in PBS three times. The tissue mass was cut with scissors into ~1-mm sections and digested with 0.1% type-I collagenase at 37˚C for 1 h in a shaking water bath. High-glucose (4.5 g/l) DMEM (Gibco; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) was added to stop digestion. To remove undigested tissue fragments and large cell aggregates, the digested tissue suspension was filtered through 100-μm nylon mesh (Falcon). The cells were collected by centrifugation at 233 x g for 10 min at room temperature (RT). The cells were then resuspended in high-glucose DMEM supplemented with 10% FBS and 1% penicillin and streptomycin (Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2.

For white adipocyte *in vitro* differentiation, fully confluent pre-adipocytes were kept in DMEM + 10% FBS growth medium for 2 days, then treated with induction medium (DMEM + 10% FBS) supplemented with 10 μg/ml insulin, 1 μM dexamethasone and 0.5 mM isobutylmethylxanthine (MilliporeSigma). After 2 days, the cells were kept in medium supplemented with 10 μg/ml insulin for a further 2 days. The culture medium was then replaced with fresh DMEM containing 10% FBS every 2-3 days. The cells were fully differentiated into mature adipocytes on day 9.

Oil Red O staining. Differentiated adipocytes were washed three times with phosphate-buffered saline and were subsequently fixed in 4% paraformaldehyde at RT for 30 min. Afterwards, adipocytes were exposed to Oil Red O for 1 h at RT. To remove the staining solution, cells were washed several times with distilled water. Representative images were taken using a light microscope (Zeiss AG).

Small interfering RNA (siRNA) transient transfection. The negative control (NC) and specific siRNA against Shp2 were purchased from HIPPOBIO, Inc. Pre-adipocytes were seeded in 6-well plates at a density of 60-70% confluence and transfected with 50 nM siRNA using Lipofectamine® 3000 transfection reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. After 6 h of transfection, the medium was replaced with normal medium and the cells were cultured for another 18 h at 37˚C with 5% CO_2, and then

Table I. Sequences of siRNA.

Genes	Direction	Sequences (5'-3')
NC siRNA	Sense	UUCUCCGAACGUGUCACGU
	Antisense	ACGUGACACGUUCGGAGAA
Shp2 siRNA	Sense	GUUCCUAAAACCAUUCAGATG
	Antisense	UCUGAAUGGUUUUAGGAACGT

siRNA, small interfering RNA; NC, negative control.

harvested for transfection efficiency evaluation using western blot analysis. The transfected cells were collected to perform subsequent experiments at least 48 h after transfection and transfection was performed every 72 h during differentiation. The NC siRNA consisted of a scrambled sequence that would not cause the specific degradation of any known cellular mRNA. The siRNA sequences are presented in Table I.

Lentiviral transduction. Lentivirus-containing expression cassettes were purchased from Syngentech Co. Ltd., and lentivirus preparation and packaging were performed by the company. Lentiviral transduction was performed following the manufacturer's instructions. Briefly, $5x10^5$ ingWAT pre-adipocytes were seeded in T25 flasks. The next day, these cells were infected with Shp2 lentiviral expression vector (pLV-hef1a-mNeongreen-P2A-Puro-WPRE-CMV-Ptpn11) or control lentiviral expression vector (pLV-hef1a-mNeongreen-P2A-Puro-WPRE-CMV-MCS) at MOI values of 50 at 37˚C with 5% CO_2. The culture media were replaced at 12 h post-transfection, and the cells were incubated for a further 48 h. At 72 h post-transfection, the transfected cells showing green fluorescence were observed under a fluorescence microscope and transduction efficiency was evaluated using western blot analysis. The transfected cells were collected to perform subsequent experiments 4 days after transfection. Stably transduced cells were selected using 2 μg/ml puromycin (Beyotime Institute of Biotechnology) for 3 days.

Histological analysis. At week 8 post-surgery or if a humane endpoint, including listlessness, infection or eating cessation or drinking cessation could not be relieved, rats were euthanized by CO_2 inhalation. The flow rate of CO_2 was 20-30% of the chamber volume per minute. Death was confirmed based on the absence of a heartbeat, respiration and corneal reflex. After the rats were sacrificed, their ingWAT was fixed in 4% paraformaldehyde at RT for 24 h. The samples were then embedded in paraffin and cut into 5-μm coronal slides using a microtome (Thermo Fisher Scientific, Inc.). After at 60˚C for 30 min, the tissue slides from the sham and SG groups were deparaffinized in xylene, rehydrated in gradient ethanol (each for 5 min) and then washed under running water for 3 min. Tissue slides were stained using hematoxylin and eosin (Beyotime Institute of Biotechnology). The slides were immersed in hematoxylin at RT for 3 min and rinsed with running water, sequentially followed by differentiation for 30 sec in 1% acid-alcohol (hydrochloric acid and ethanol) at RT and then rinsed again with running water. The slides were then stained with eosin at RT for 30 sec and rinsed again with water. The slides were air-dried at RT. Subsequently, the slides were sequentially immersed in 75, 85, 95 and 100% ethanol, a solution of 50% ethanol and 50% xylene, and 100% xylene, each for 1 min. The slides were then observed under a light microscope (Zeiss AG). The diameter of the adipocytes was measured using ImageJ (version 1.8.0; National Institutes of Health).

Immunofluorescence staining. After heating at 60˚C for 30 min, histological slides of ingWAT were deparaffinized in xylene, then rehydrated using decreasing concentrations of ethanol ranging from 100 to 50%. The slides were blocked with 5% bovine serum albumin (MilliporeSigma) for 1 h at RT, then incubated with a primary antibody against Shp2 (1:200 dilution; cat. no. sc-7384; Santa Cruz Biotechnology, Inc.) overnight at 4˚C. The slides were washed with PBS and incubated with tetramethylrhodamine-conjugated secondary antibody (1:200 dilution; cat. no. ab6786; Abcam) for 1 h at RT in the dark. The slides were counterstained with DAPI at RT in the dark, and fluorescence images of randomly selected fields (n=3) were obtained using a fluorescence microscope (Zeiss AG).

ELISA. The triglyceride (cat. no. 1025; Applygen Technologies, Inc.) and leptin (cat. no. ml002969; Mlbio) levels in ingWAT samples were measured using ELISA kits according to the manufacturer's protocols. The absorbance was read at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Western blotting. WAT and adipocytes were lysed with RIPA lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing 1 mM phenylmethanesulfonyl fluoride (Beijing Solarbio Science & Technology Co., Ltd.) and 1 mM phosphatase inhibitor cocktail (Bimake). Lysate protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific, Inc.). Equal quantities of protein (10 μg/well) were separated using 10% (w/v) SDS-PAGE (Epizyme, Inc.) and were subsequently transferred to PVDF membranes (MilliporeSigma) according to standard procedures. After the membranes were blocked with 5% (w/v) milk (Biofroxx) for 1 h at RT, the membranes were incubated with primary antibodies (1:1,000 dilution) against Shp2 (cat. no. 3397), β-catenin (cat. no. 84441) (both Cell Signaling Technology, Inc.), peroxisome proliferator-activated receptor γ (PPARγ; cat. no. AP0686; Bioworld Technology, Inc.), CCAAT/enhancer-binding protein alpha (Cebpα; cat. no. ab40764; Abcam), adiponectin

Table II. Primer sequences for reverse transcription-PCR.

Genes	Direction	Sequences (5'-3')
Shp2	Forward	CCGAGAGAAAGGTGTGGACT
	Reverse	TGAACCGGTACTGTGCTTCT
PPARγ	Forward	CTCCAGCATTTCTGCTCCAC
	Reverse	CGCAGGCTCTACTTTGATCG
Cebpα	Forward	GCAAGAGCCGAGATAAAGCC
	Reverse	CCTTGACCAAGGAGCTCTCA
Adiponectin	Forward	GACAAGGCCGTTCTCTTCAC
	Reverse	CCCATACACTTGGAGCCAGA
Fabp4	Forward	ATGTGCAGAAGTGGGATGGA
	Reverse	GTCACGCCTTTCATGACACA
Leptin	Forward	TGTCCAAGATGGACCAGACC
	Reverse	GAGCTATCTGCAGCACGTTT
β-actin	Forward	CCTCTATGCCAACACAGTGC
	Reverse	CACAGAGTACTTGCGCTCAG

PPARγ, peroxisome proliferator-activated receptor γ; Cebpα, CCAAT/enhancer-binding protein alpha; Fabp4, fatty acid-binding protein 4; Shp2, Src homology 2 domain-containing phosphatase 2.

(cat. no. 2789; Cell Signaling Technology, Inc.), leptin (cat. no. DF8583; Affinity Biosciences) and β-actin (cat. no. 4970; Cell Signaling Technology, Inc.) overnight at 4˚C. A horseradish peroxidase-conjugated goat anti-rabbit/mouse IgG (cat. no. BL003A/BL001A; 1:5,000; Biosharp Life Sciences) was used as a secondary antibody for 1 h at RT. All signals were detected using the ChemiDocXRS^{+} Imaging System (Tanon Science and Technology Co., Ltd.). Quantitative analysis of protein densitometry was performed using Image J (version 1.8.0; National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from WAT and adipocytes using the RNA-quick Purification Kit (ES Science) according to the manufacturer's instructions. cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kit (Vazyme Biotech Co., Ltd.) in accordance with the manufacturer's instructions. qPCR was performed using a SYBR Green QPCR kit (Vazyme Biotech Co., Ltd.) on a LightCycler 480 PCR System (Roche Diagnostics). The following thermocycling conditions were used for the qPCR: Initial denaturation at 95˚C for 2 min, followed by 40 cycles of 95˚C for 20 sec, 60˚C for 20 sec and 72˚C for 20 sec, and then a final step at 78˚C for 5 min. The fold changes in the expression levels of each gene were calculated using the $2^{-\Delta\Delta Cq}$ method (27). The mRNA expression levels for each target gene were normalized to those of β-actin. The primer sequences are presented in Table II.

Cell Counting Kit-8 (CCK-8) assay. The viability of pre-adipocytes was assessed using CCK-8 (Dojindo Laboratories, Inc.) assays according to the manufacturer's instructions following treatment with recombinant leptin rat (0, 5, 25, 50, 100 and 200 ng/ml) (Bioworld Technology, Inc.), SHP099 (0, 1, 5, 10, 50, 100 and 200 μM) or PNU-74654 (0, 0.5, 1, 10, 25, 50 and 100 μM) (MedChemExpress) at 37˚C with 5% CO_2 for 24 h. The absorbance at 450-nm wavelength was detected using a microplate reader (Thermo Fisher Scientific, Inc.).

Statistical analysis. SPSS (version 26.0; IBM, Corp.) and GraphPad Prism software (version 8.4; GraphPad Software, Inc.) were used for statistical analysis. Quantitative data are representative of at least three independent experiments. Unpaired two-tailed Student's t-tests were used to compare the means of two groups. Differences between multiple groups were detected using one-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test as appropriate. The data are presented as the mean ± SD. $P<0.05$ was considered to indicate a statistically significant difference.

Results

SG alleviates obesity and associated metabolic disorders. The body shapes of rats 8 weeks after surgery are presented in Fig. 1B. There were no significant differences in body weight or food intake between the sham and the SG group pre-operatively. Throughout the post-operative period, the SG group had a significantly lower body weight and food intake compared with those of the sham group ($P<0.01$; Fig. 1C and D). During the post-operative period, the FG levels declined significantly in the SG group compared with the sham group ($P<0.05$; Fig. 1E). Compared with week 0, the FG levels in the SG group were significantly reduced (70.8±10.1% reduction; $P<0.001$) at week 8 post-surgery. Compared with the pre-operative results (Fig. 1F), the SG group demonstrated marked improvements in glucose control following surgery (Fig. 1G). Moreover, the SG group revealed significantly improved glucose control compared with the sham group post-surgery ($P<0.001$; Fig. 1H). The pre- and post-operative results of the sham group did not differ significantly.

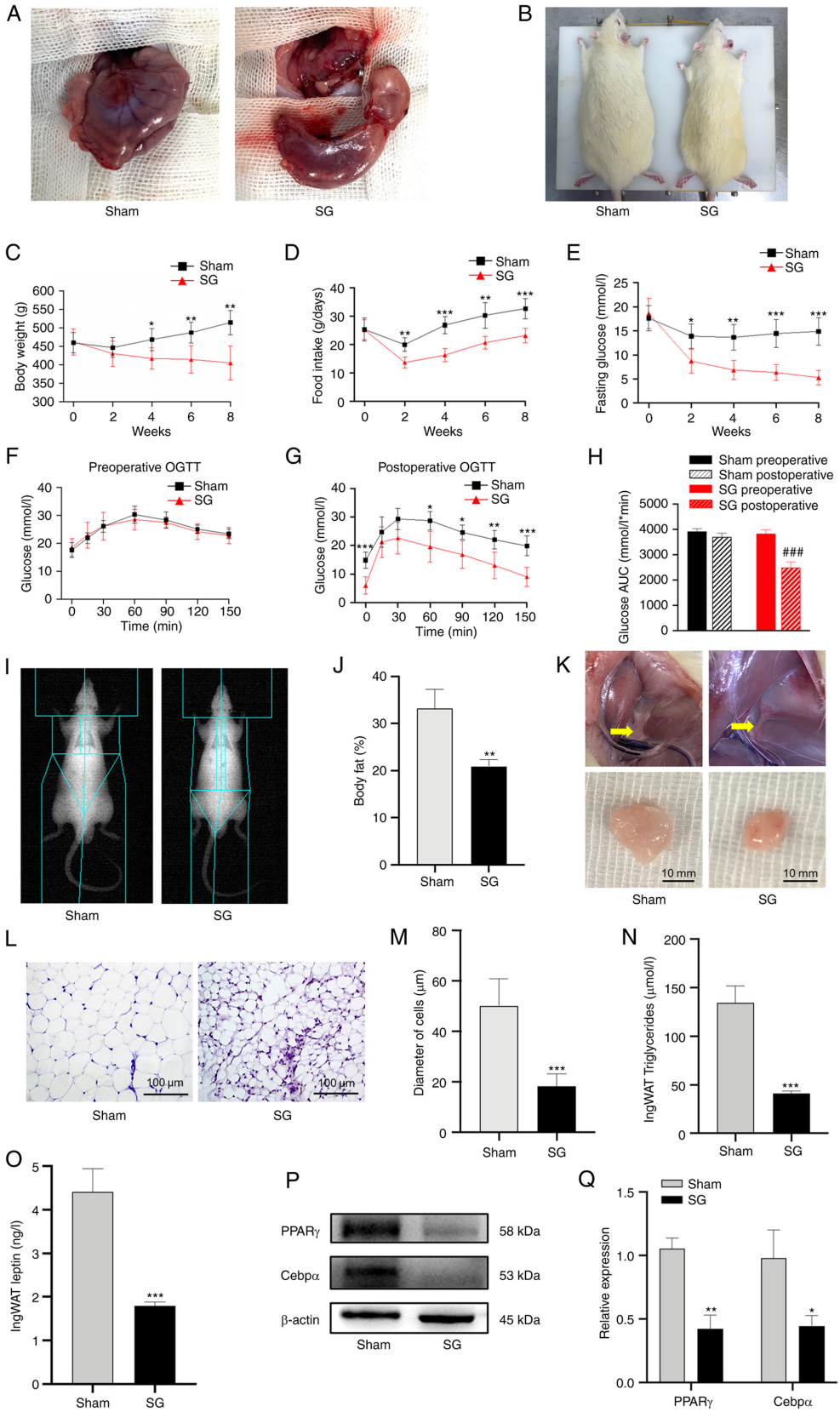

Figure 1. SG alleviates obesity and glucose metabolic disorders in a rat T2D model. (A) Illustration of the sham and SG operations. (B) Appearance of rats in the sham and SG groups were recorded 8 weeks after surgery. Changes in (C) body weight, (D) food intake and (E) fasting glucose levels following surgery. (F) Pre-operative and (G) post-operative OGTT results. (H) AUC for glucose levels. (I and J) Body fat content after SG was measured using DEXA. (K) Adipose tissue in the inguinal region 8 weeks post-operation. Scale bar, 10 mm. (L) H&E staining images of ingWAT were obtained using a light microscope. Scale bar, 100 μm. (M) Diameter of adipocytes of ingWAT in the sham and SG groups. (N) Triglyceride concentration in the ingWAT from the the sham and SG groups. (O) Leptin concentration in the ingWAT from the sham and SG groups. (P) Western blot images and (Q) semi-quantitative analysis of PPARγ and Cebpα protein levels in ingWAT after surgery. The data are presented as the mean ± SD. *P<0.05, **P<0.01 and ***P<0.001 vs. the sham group; $^{\#\#\#}$P<0.001 vs. the SG preoperative level. SG, sleeve gastrectomy; T2D, type-2 diabetes; OGTT, oral glucose tolerance test; AUC, area under the curve; DEXA, dual-energy X-ray absorptiometry; H&E, hematoxylin and eosin; ingWAT, inguinal white adipose tissue; Cebpα, CCAAT/enhancer-binding protein alpha; PPARγ, peroxisome proliferator-activated receptor γ.

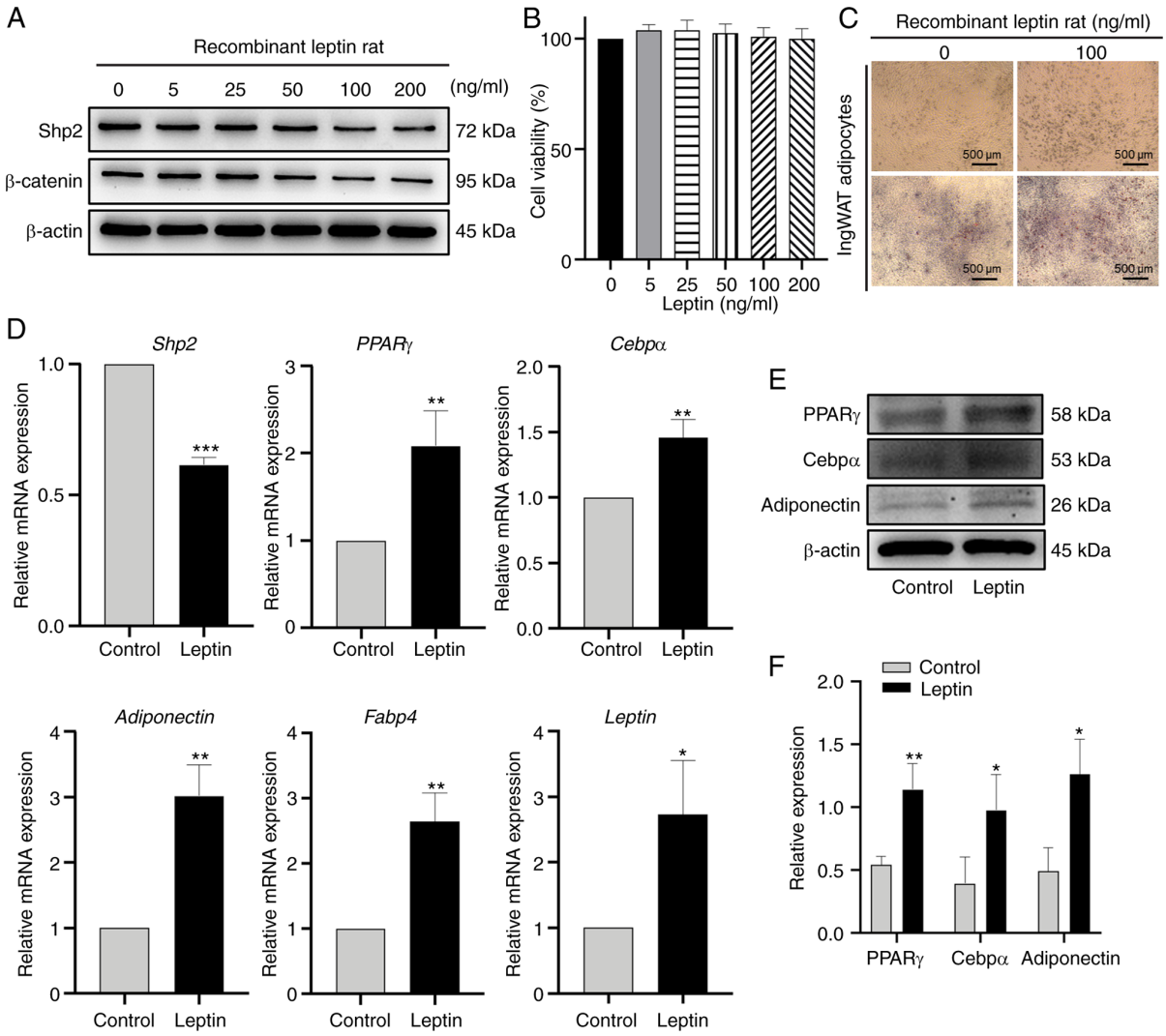

Figure 2. Leptin downregulates Shp2/β-catenin protein levels and promotes adipogenic differentiation. (A) Western blot analysis of Shp2 and β-catenin protein levels in ingWAT adipocytes treated with 0, 5, 25, 50, 100 or 200 ng/ml leptin. (B) IngWAT pre-adipocytes were treated with different concentrations of leptin, and cell viability was measured using Cell Counting Kit-8 assays. (C) Oil Red O staining following 100 ng/ml leptin supplementation at differentiation day 9. Scale bar, 500 μm. (D) Reverse transcription-quantitative PCR analysis of mRNA levels of adipogenic markers in adipocytes treated with leptin. (E) Western blotting and (F) quantitative analysis of PPARγ, Cebpα and adiponectin protein levels in adipocytes treated with leptin. The data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. control group. Shp2, Src homology 2 domain-containing phosphatase 2; ingWAT, inguinal white adipose tissue; PPARγ, peroxisome proliferator-activated receptor γ; Cebpα, CCAAT/enhancer-binding protein α; Fabp4, fatty acid-binding protein 4.

SG suppresses fat accumulation in vivo and downregulates leptin levels in adipose tissue. The SG group had significantly lower body fat compared with the sham group (P<0.01; Fig. 1I and J). As presented in Fig. 1K, the volume of adipose tissue of the SG group was smaller than those of the sham group. Histological analysis of adipose tissue sections revealed smaller adipocyte sizes in rats from the SG group compared with the sham (P<0.001; Fig. 1L and M). At week 8 post-surgery, the SG group exhibited significantly lower triglyceride (P<0.001) and leptin (P<0.001) levels in adipose tissue than the sham group (Fig. 1N and O). Furthermore, the expression of the adipogenic markers, PPARγ and Cebpα, were significantly downregulated in ingWAT from the SG group compared with the sham group (P<0.05; Fig. 1P and Q).

Leptin downregulates the expression of Shp2 and β-catenin and upregulates the expression of adipogenic markers. To investigate the effect of leptin on the expression of Shp2, ingWAT pre-adipocytes were treated with different concentrations of leptin (0, 5, 25, 50, 100 and 200 ng/ml) during differentiation. The results indicated that Shp2 and β-catenin protein levels were markedly reduced by leptin in a dose-dependent manner (Fig. 2A). Marked changes in Shp2 and β-catenin protein expression were observed after 100 and 200 ng/ml leptin treatment. CCK-8 assay was performed to evaluate the cell viability, the results of which suggested that there was no effect on cell viability at 100 ng/ml leptin (Fig. 2B). Subsequently, 100 ng/ml leptin was used to stimulate ingWAT pre-adipocytes during differentiation. As presented in Fig. 2C, leptin treatment markedly increased the lipid accumulation of adipocytes. In addition, the mRNA expression level of Shp2 and fatty acid-binding protein 4 (Fabp4), and the mRNA or protein expression levels of the other adipogenic markers, including PPARγ, Cebpα, adiponectin and leptin significantly increased (all P<0.05; Fig. 2D-F).

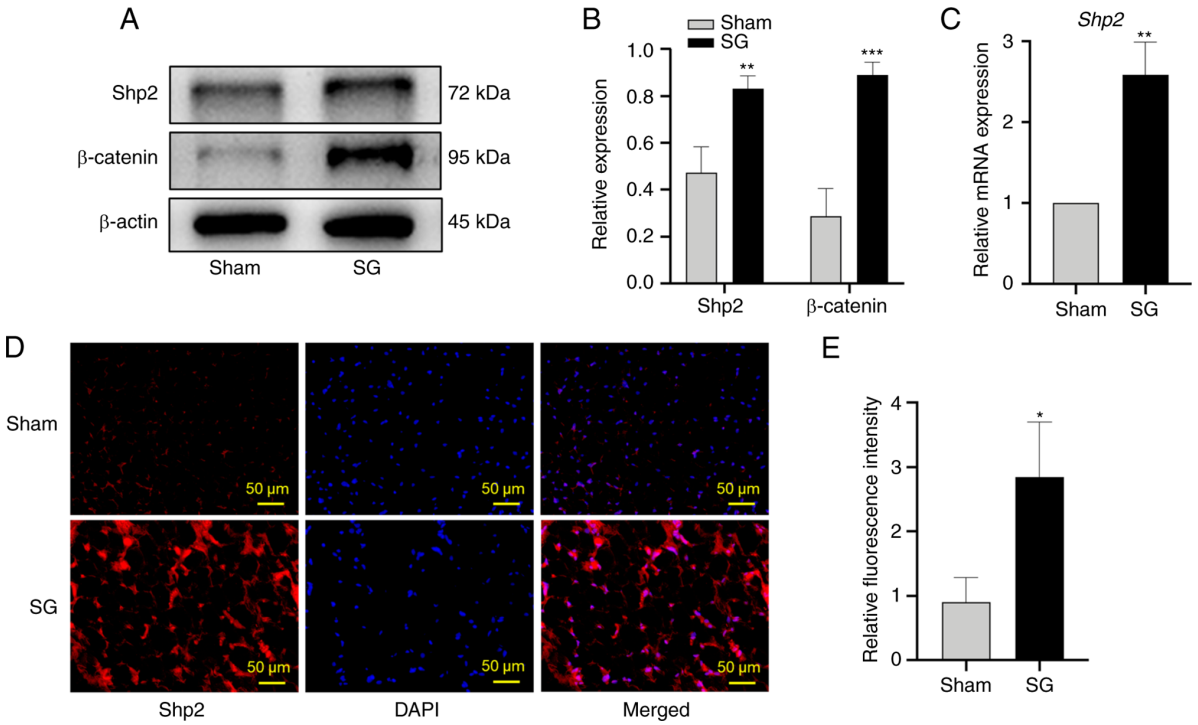

Figure 3. SG upregulates Shp2 and β-catenin expression levels in ingWAT. (A) Western blotting and (B) quantitative analysis of Shp2 and β-catenin proteins in ingWAT after operation. (C) Reverse transcription-quantitative PCR analysis of the mRNA level of Shp2 in ingWAT. (D) Fluorescence images and (E) quantitative analysis of Shp2 fluorescence intensity in ingWAT. Scale bar, 50 μm. The data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. sham group. SG, sleeve gastrectomy; Shp2, Src homology 2 domain-containing phosphatase 2; ingWAT, inguinal white adipose tissue.

SG upregulates Shp2 and β-catenin levels in ingWAT. The mRNA and protein expression levels of Shp2 and β-catenin were significantly upregulated following SG (P<0.01 and P<0.001; Fig. 3A-C). Moreover, the fluorescence intensity of Shp2 in ingWAT was significantly increased in the SG group compared with the sham group (P<0.05; Fig. 3D and E).

Expression pattern of Shp2 in ingWAT pre-adipocyte differentiation. To confirm that the observed changes in Shp2 were associated with inhibition of pre-adipocyte differentiation at day-2 post-confluence, growth-arrested ingWAT pre-adipocytes were induced into mature adipocytes. The differentiation status of ingWAT pre-adipocytes was monitored by Oil Red O staining, which is indicative of terminal differentiation (Fig. 4A). The results suggested that the mRNA and protein levels of Shp2 were significantly reduced following treatment with inducers (P<0.001), and the mRNA expression levels of adipogenic markers significantly increased (P<0.01 and P<0.001; Fig. 4B and C).

Knockdown or inhibition of Shp2 accelerates ingWAT pre-adipocyte differentiation. To examine the role of Shp2 during ingWAT pre-adipocyte differentiation, siRNA was used to knock-down the expression of Shp2. The results demonstrated that Shp2 knockdown resulted in increased lipid accumulation throughout differentiation (Fig. 5A). Additionally, Shp2 siRNA significantly inhibited both the mRNA and protein expression of Shp2 at day 9 post-induction (P<0.05 and P<0.001; Fig. 5B-D). Triglyceride levels were also significantly increased (P<0.001; Fig. 5E) and there were significant increases in the mRNA levels of adipogenic markers (P<0.01; Fig. 5F). Analysis of protein expression confirmed the adipogenic effects of Shp2 knockdown. PPARγ, Cebpα and adiponectin protein levels were significantly increased at differentiation day 9 in the Shp2 knockdown group compared with the NC group (P<0.05; Fig. 5G and H). Although the protein expression levels of leptin increased following Shp2 knockdown, this was not statistically significant. Furthermore, the expression of β-catenin, a protein involved in the Wnt signaling pathway, was significantly reduced in the Shp2 knockdown group compared with the NC group (P<0.05; Fig. 5G and H).

Furthermore, similar experiments were carried out in ingWAT pre-adipocytes in the presence of the Shp2 inhibitor, SHP099, in the induction medium at different time points. IngWAT pre-adipocytes were treated with different concentrations of SHP099, and cell viability was measured using CCK-8 assays, with the results showing that cell viability decreased with increasing SHP099 concentration (Fig. 6A). The optimal protective effect was conferred by 10 μM, so this concentration was selected for subsequent experiments. As presented in Fig. 6B-F, SHP099 had a significantly positive effect on cellular lipid accumulation (P<0.001; Fig. 6B and C) and significantly increased the mRNA and protein levels of adipogenic markers compared with the NC group (P<0.05; Fig. 6D-F). Moreover, the protein expression of β-catenin was decreased significantly compared with that in the NC group (P<0.05; Fig. 6E and F). These results suggested that knockdown or inhibition of Shp2 promoted ingWAT adipocyte differentiation, and these adipogenic effects may be mediated by the Wnt/β-catenin signaling.

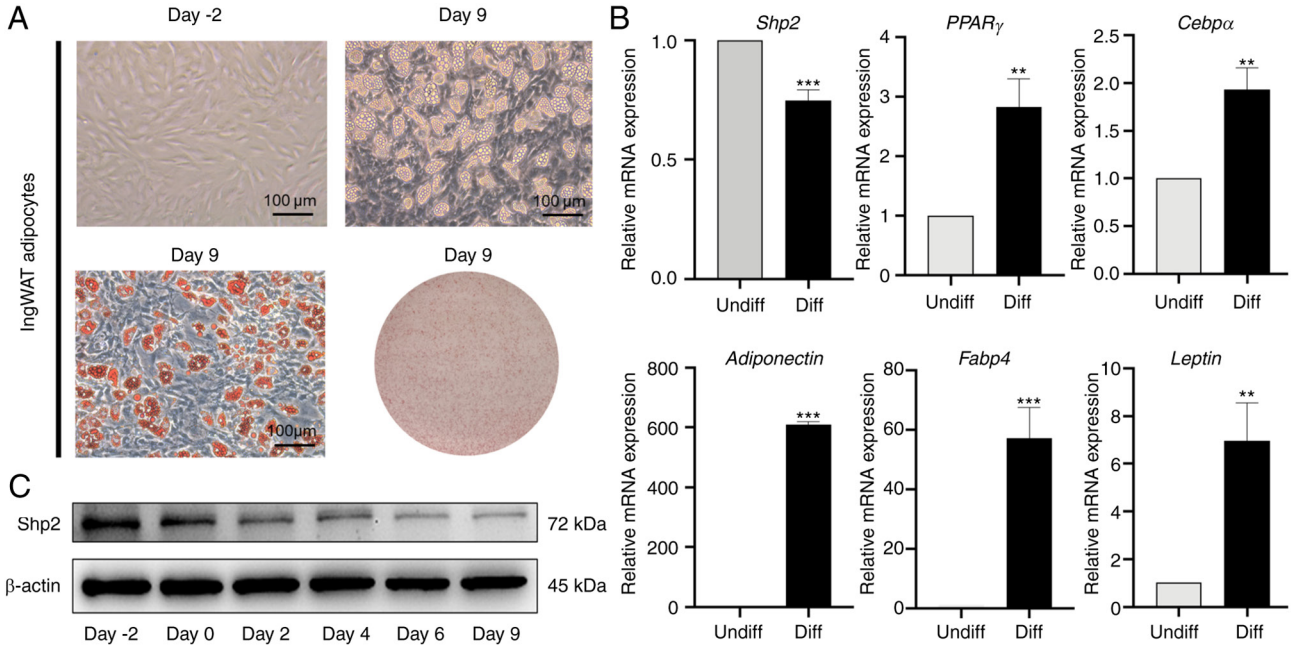

Figure 4. Shp2 expression is reduced during ingWAT pre-adipocyte differentiation. (A) Oil Red O staining of ingWAT pre-adipocytes on day-2 and day 9 of differentiation. Scale bar, 100 μm. (B) Reverse transcription-quantitative PCR analysis of mRNA levels of Shp2 and adipogenic markers in undifferentiated and differentiated adipocytes. (C) Shp2 protein expression was assessed using western blot analysis during adipocyte differentiation. The data are presented as the mean ± SD (n=3). **P<0.01 and ***P<0.001 vs. undifferentiated groups. Shp2, Src homology 2 domain-containing phosphatase 2; ingWAT, inguinal white adipose tissue; undiff; undifferentiated; diff, differentiated; PPARγ, peroxisome proliferator-activated receptor γ; Cebpα, CCAAT/enhancer-binding protein α; Fabp4, fatty acid-binding protein 4.

Shp2 inhibits ingWAT pre-adipocyte differentiation by activating the Wnt/β-catenin signaling pathway. To determine whether the Wnt/β-catenin signaling pathway was involved in the effects of Shp2 on pre-adipocyte differentiation, a small-molecule inhibitor of the Wnt/β-catenin pathway, PNU-74654 (28,29), was used to test this hypothesis. The pre-adipocytes were treated with different concentrations for 24 h, cell viability was measured using CCK-8 assays. When the concentration reached 10 μM, cell viability was not significantly affected, and an optimal concentration of 10 μM was selected for subsequent experiments (Fig. 7A). Shp2 was overexpressed in ingWAT pre-adipocytes using lentiviral transduction. At day 9 of differentiation, lipid accumulation markedly decreased in the Shp2-overexpressing cells (Fig. 7B). Moreover, the triglyceride levels of Shp2-overexpressing adipocytes were significantly reduced compared with the vector group (P<0.01; Fig. 7C). Furthermore, the overexpression of Shp2 decreased the mRNA and protein levels of adipogenic markers (P<0.05; Fig. 7D-F), while the protein expression level of β-catenin was significantly upregulated (P<0.05; Fig. 7E and F). These results suggested that Shp2 may suppress adipocyte differentiation through the Wnt/β-catenin pathway. Next, PNU-74654 was added to the inducing medium at different time points, and the protein expression of β-catenin was significantly decreased compared with the Shp2 overexpression group (P<0.05; Fig. 7E and F). As presented in Fig. 7B, Shp2-overexpressing adipocytes treated with PNU-74654 displayed markedly increased lipid accumulation. In addition, triglyceride levels were also significantly increased compared with the Shp2 overexpression group (P<0.01; Fig. 7C). Furthermore, the mRNA and protein expression of adipogenic markers were significantly increased compared with the Shp2 overexpression group following treatment with PNU-74654 (P<0.05; Fig. 7D-F). These results suggested that Shp2 suppressed ingWAT adipocyte differentiation by activating the Wnt/β-catenin signaling pathway.

Discussion

The present study demonstrated that SG resulted in significant improvements in body weight, glucose control and fat accumulation. The major findings of the present study were as follows: i) Leptin expression was decreased and Shp2 expression increased in ingWAT following SG; ii) leptin inhibited Shp2 expression and promoted adipogenic differentiation in white pre-adipocytes; iii) both Shp2 knockdown and inhibition promoted the lipid accumulation of white adipocytes, while overexpression of Shp2 reduced it; and iv) Shp2 inhibited the adipogenic differentiation of ingWAT pre-adipocytes by activating the Wnt/β-catenin signaling pathway. These findings revealed a novel role of Shp2 in lipid metabolism in WAT, which may provide insight into the mechanism through which SG sustains long-term weight loss.

Gut hormones, bile acid and gut flora are considered major causes of the effects of SG on body weight and metabolic changes (13). Moreover, reduced fat accumulation after SG also results in body weight and metabolic changes (30,31). Obesity is characterized by excessive accumulation of body fat, especially in WAT. Adipocytes play a notable role in lipid storage and metabolism (32). The suppression of adipogenesis can affect the formation of WAT, which is conducive to alleviating the accompanying metabolic disorders (33). The present study identified an important role for SG in this process.

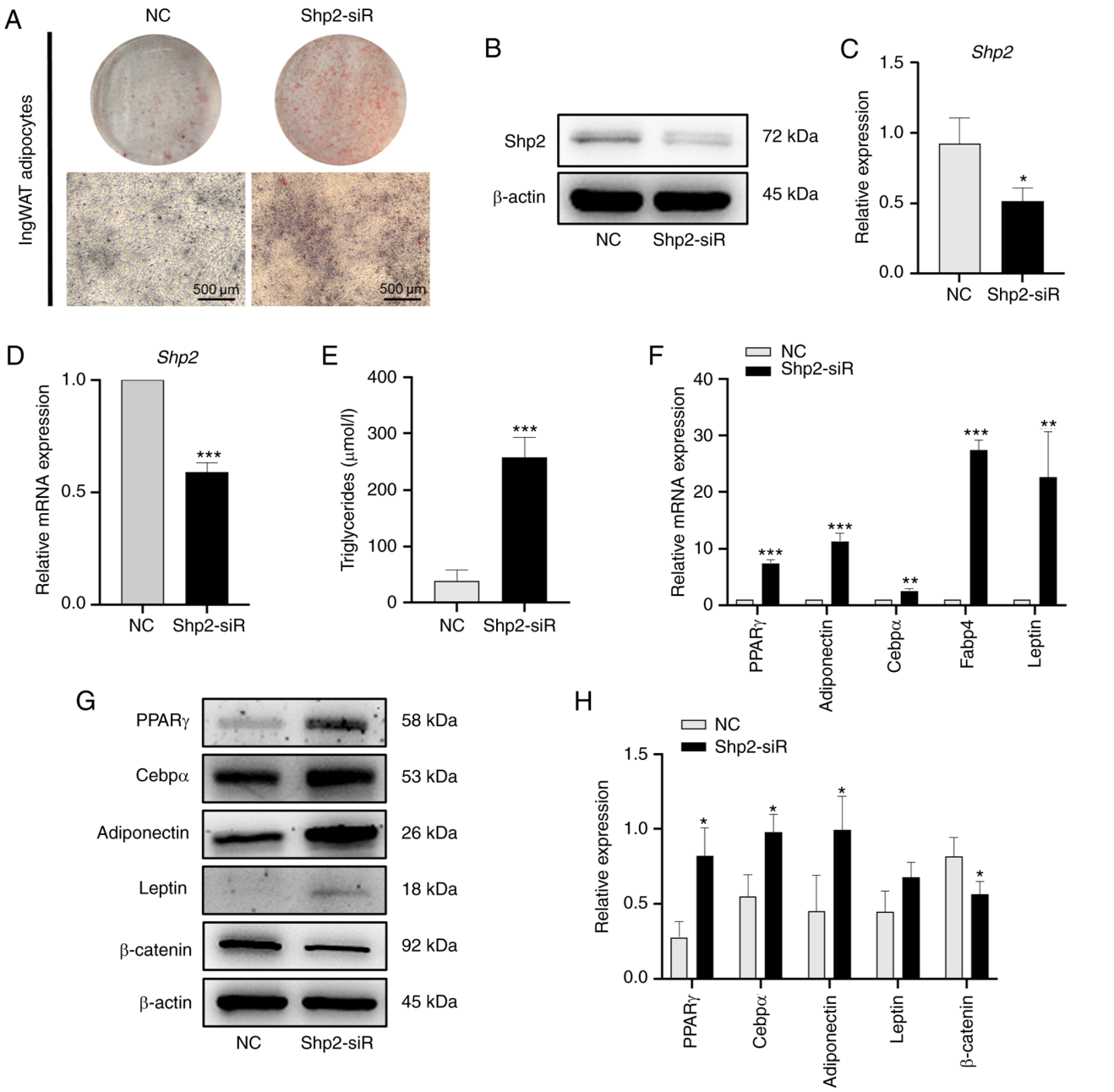

Figure 5. Shp2 knockdown accelerates ingWAT pre-adipocyte differentiation. (A) Oil Red O staining of Shp2 knockdown at differentiation day 9. Scale bar, 500 μm. (B) Western blotting and (C) semi-quantitative analysis of Shp2 knockdown. (D) RT-qPCR analysis of mRNA level of Shp2 knockdown. (E) Triglyceride concentration of adipocytes with Shp2 knockdown. (F) RT-qPCR analysis of mRNA levels of adipogenic markers in Shp2 knockdown adipocytes. (G) Western blotting and (H) semi-quantitative analysis of adipogenic markers and β-catenin in Shp2 knockdown adipocytes. The data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. NC groups. Shp2, Src homology 2 domain-containing phosphatase 2; ingWAT, inguinal white adipose tissue; siRNA/siR, small interfering RNA; RT-qPCR, reverse transcription-quantitative PCR; NC, negative control; PPARγ, peroxisome proliferator-activated receptor γ; Cebpα, CCAAT/enhancer-binding protein α; Fabp4, fatty acid-binding protein 4.

The present findings indicated that a HFD increases fat accumulation and that SG could reverse this phenomenon. This suggests that SG contributes to weight loss by reducing fat accumulation. Moreover, the SG group had improved glucose control and lipid metabolism compared with the sham group, which was consistent with previous reports (34,35). A significant post-operative reduction in leptin levels was also observed in the ingWAT. Leptin is an adipokine that acts centrally to regulate food intake and metabolism by activating LepRb in the hypothalamus (36,37). With WAT expansion in patients with obesity, high leptin levels are detectable in the circulation, which impairs the hypothalamus and leads to leptin resistance (38). A partial reduction in plasma leptin level in obesity restores hypothalamic leptin sensitivity, which contributes to weight loss and improves insulin sensitivity (39). Thus, decreased leptin levels after SG contributes to fat consumption and weight loss.

Reduced fat accumulation allows sustained weight loss. In the present study, continuous leptin supplementation had a positive effect on the differentiation of pre-adipocytes from ingWAT. The expression levels of the adipogenic factors PPARγ, Cebpα, adiponectin, Fabp4 and leptin were upregulated

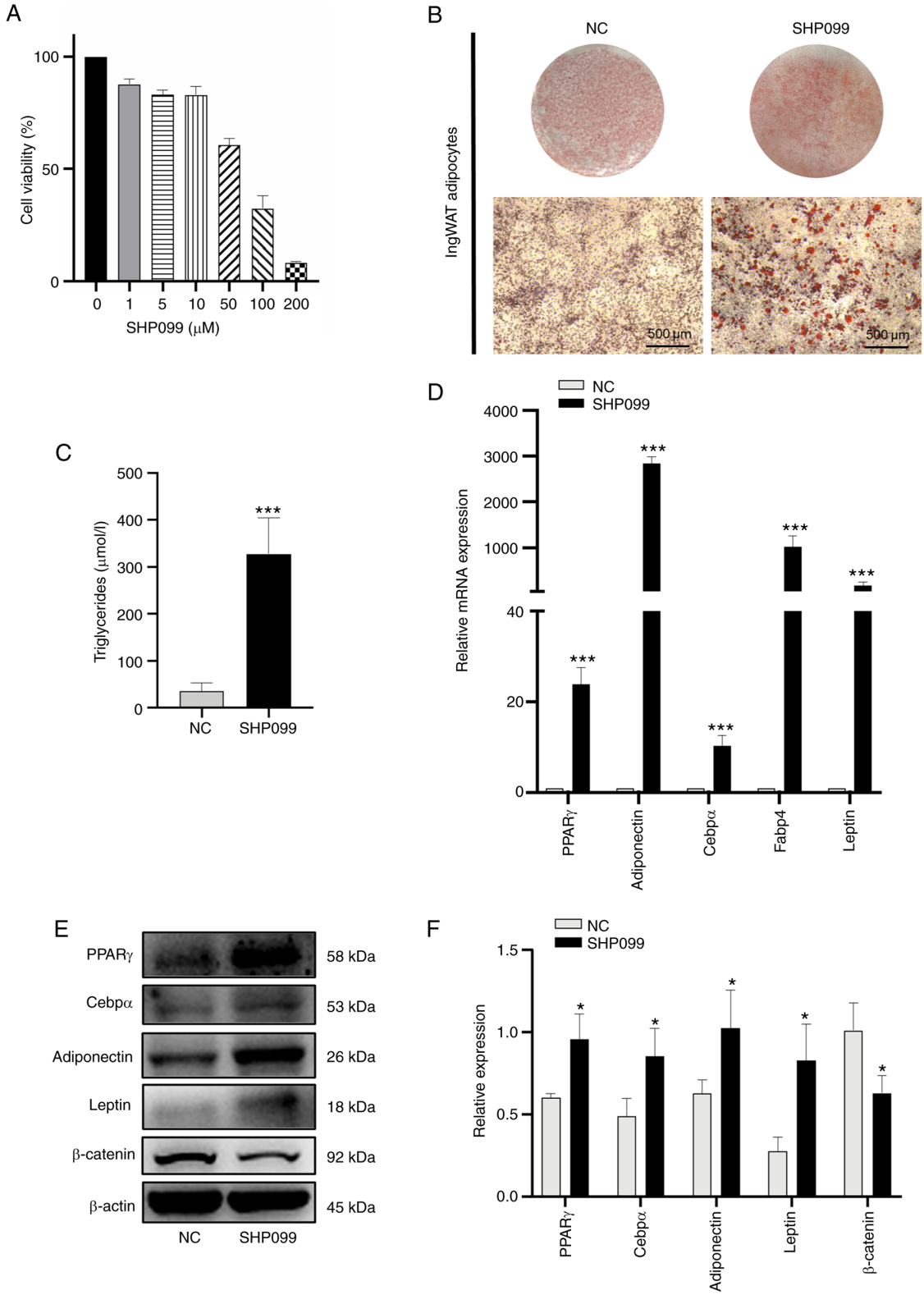

Figure 6. Inhibition of Shp2 accelerates ingWAT pre-adipocyte differentiation. (A) IngWAT pre-adipocytes were treated with different concentrations of the Shp2 inhibitor, SHP099, and cell viability was measured using Cell Counting Kit-8 assays. (B) Oil Red O staining of adipocytes treated with SHP099 at differentiation day 9. Scale bar, 500 μm. (C) Triglyceride concentration in adipocytes following SHP099 treatment. (D) Reverse transcription-quantitative PCR analysis of the mRNA levels of adipogenic markers in adipocytes treated with SHP099. (E) Western blotting images and (F) semi-quantitative analysis of adipogenic markers and β-catenin in adipocytes treated with SHP099. The data are presented as the mean ± SD (n=3). *P<0.05 and ***P<0.001 vs. NC group. Shp2, Src homology 2 domain-containing phosphatase 2; ingWAT, inguinal white adipose tissue; NC, negative control; PPARγ, peroxisome proliferator-activated receptor γ; Cebpα, CCAAT/enhancer-binding protein α; Fabp4, fatty acid-binding protein 4.

in mature adipocytes. It was also observed that leptin down-regulated the expression of Shp2 in ingWAT pre-adipocytes. Previous studies have indicated that Shp2 participates in leptin signaling immediately downstream of LepRb in the

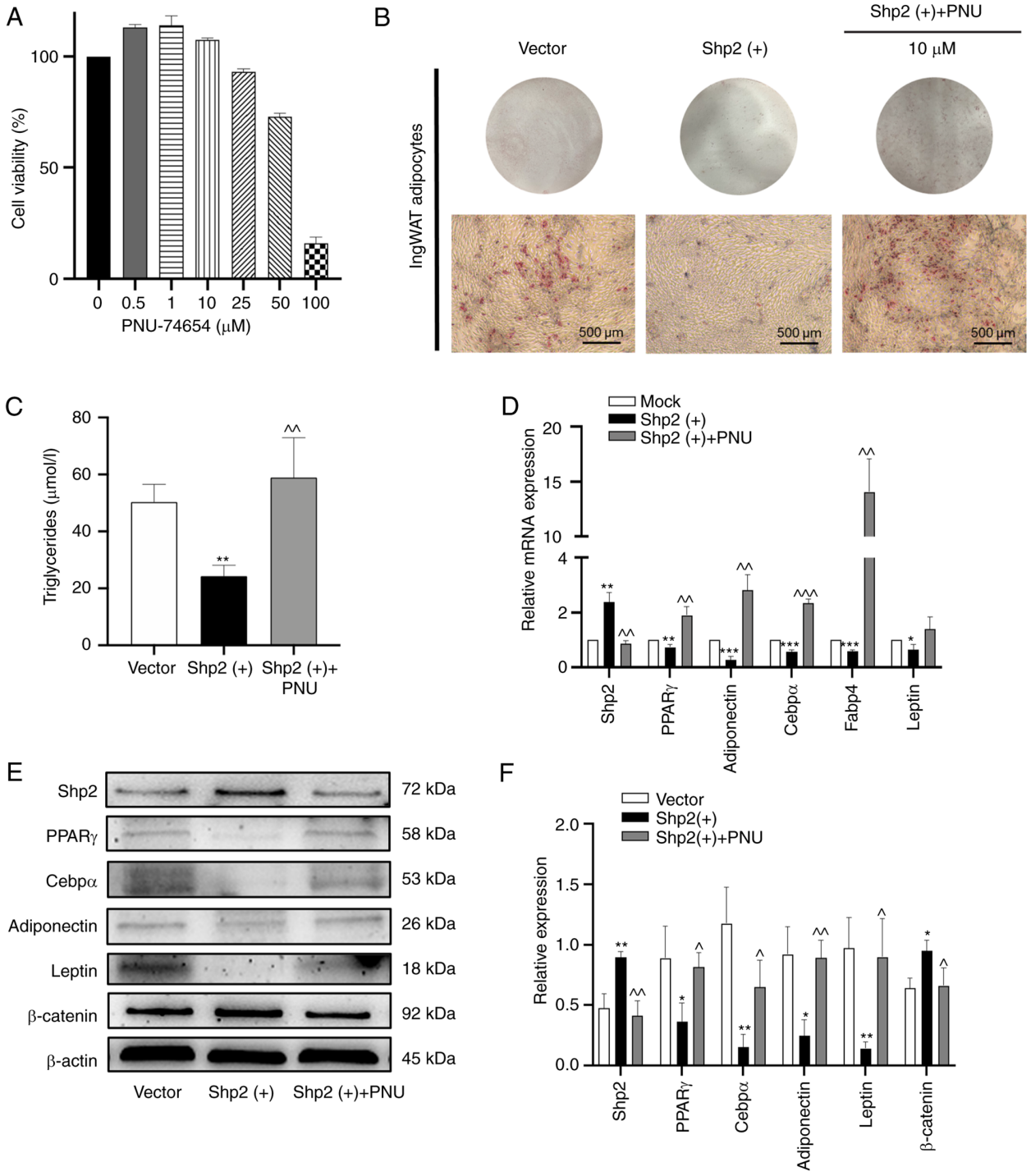

Figure 7. Shp2 inhibits ingWAT pre-adipocyte differentiation by activating the Wnt/β-catenin signaling pathway. (A) IngWAT pre-adipocytes were treated with different concentrations of the β-catenin inhibitor PNU-74654, and cell viability was measured using Cell Counting Kit-8 assays. (B) Oil Red O staining of adipocytes following Shp2 overexpression with or without PNU-74654 treatment at differentiation day 9. Scale bar, 500 μm. (C) Triglyceride concentration in adipocytes following Shp2 overexpression with or without PNU-74654 treatment. (D) Reverse transcription-quantitative PCR analysis of mRNA levels of adipogenic markers in adipocytes following Shp2 overexpression with or without PNU-74654 treatment. (E) Western blotting images and (F) semi-quantitative analysis of adipogenic markers and β-catenin in adipocytes following Shp2 overexpression with or without PNU-74654 treatment. The data are presented as the mean ± SD (n=3). *P<0.05, **P<0.01 and ***P<0.001 vs. the vector group; ^P<0.05, ^^P<0.01 and ^^^P<0.001 vs. the Shp2 overexpression group. Shp2, Src homology 2 domain-containing phosphatase 2; ingWAT, inguinal white adipose tissue; PNU, PNU-74654; PPARγ, peroxisome proliferator-activated receptor γ; Cebpα, CCAAT/enhancer-binding protein α; Fabp4, fatty acid-binding protein 4.

hypothalamus, which could alleviate leptin resistance in obesity (22,40). However, only a few have documented the role of Shp2 in leptin signaling in adipocytes, and its involvement in the regulation of adipogenic differentiation remains controversial.

A previous study suggested that Shp2 suppressed adipogenic differentiation in 3T3-L1 cells (26). By contrast, He *et al* (41) revealed that Shp2 knockout in embryonic stem cells could inhibit adipocyte differentiation and that adipose tissue-specific deletion of Shp2 resulted in lipodystrophy.

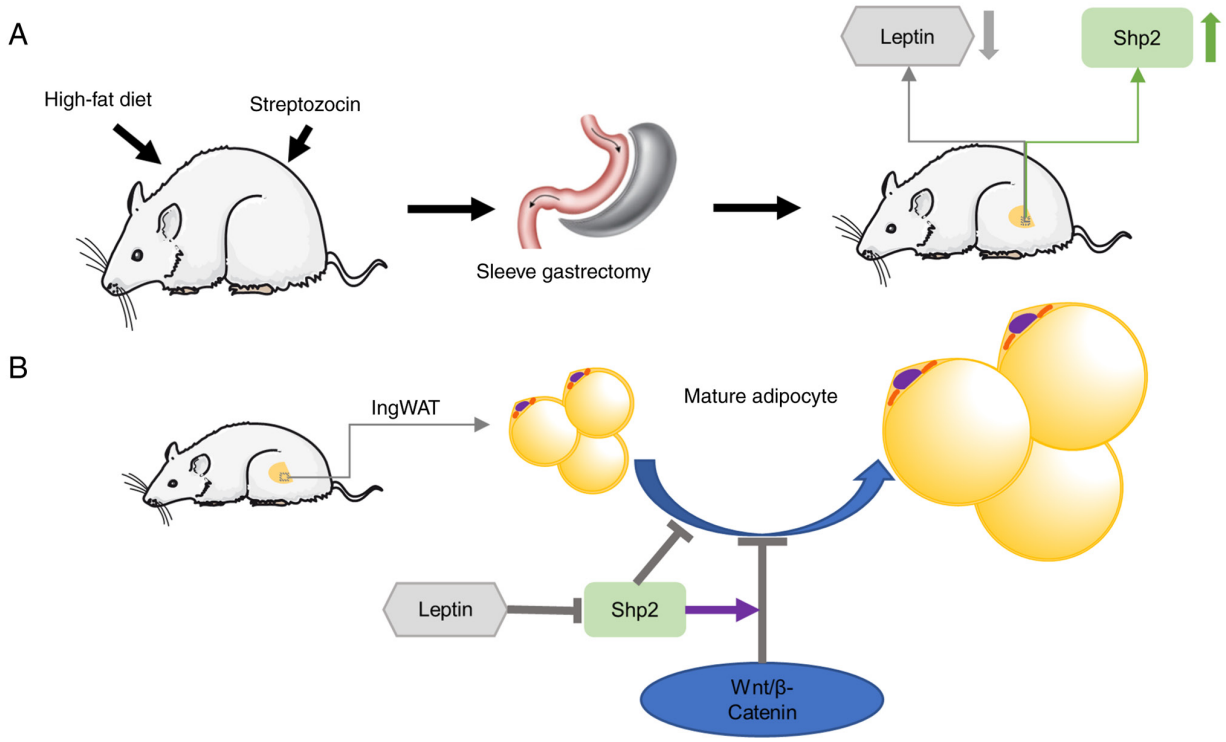

Figure 8. Schematic diagram of the cellular and molecular mechanisms of reduced fat accumulation in SG. (A) SG decreased leptin levels in ingWAT, which in turn upregulated Shp2. (B) Shp2 suppressed the adipogenic differentiation of ingWAT pre-adipocytes by activating the Wnt/β-catenin signaling pathway. Shp2, Src homology 2 domain-containing phosphatase 2; ingWAT, inguinal white adipose tissue.

In addition, Bettaieb *et al* (42) demonstrated that adipose tissue-specific Shp2 deletion did not affect the development of adipose tissue. These results indicate that Shp2 may play different roles in adipocyte differentiation and adipose tissue formation. The present study demonstrated that the knockdown of Shp2 promoted ingWAT pre-adipocyte differentiation. Consistent with this finding, SHP099, a Shp2 inhibitor, also promoted adipogenic differentiation. Conversely, overexpression of Shp2 markedly inhibited ingWAT pre-adipocyte differentiation. These findings led to the hypothesis that Shp2 may be an inhibitor of adipogenesis and that the observed increase in Shp2 expression in ingWAT following SG may represent an integral part of the reduced fat accumulation. Notably, the elaborate mechanism between leptin and Shp2 remains to be clarified in future studies.

The Wnt/β-catenin signaling pathway plays a significant role in various biological processes, especially in the regulation of cell proliferation and differentiation, which has been demonstrated to inhibit adipocyte differentiation *in vitro* (43,44). Shp2 has been reported to signal via various kinases, including Erk, MAPK and Wnt/β-catenin (41,45,46). However, to the best of our knowledge, the association between Shp2 and the Wnt/β-catenin pathway in adipogenic differentiation has not been reported previously. In the present study, SG upregulated β-catenin expression levels in ingWAT. β-catenin expression after adipogenic differentiation of ingWAT pre-adipocytes was reduced following the knockdown or inhibition of Shp2, but increased following Shp2 overexpression. Moreover, when using the β-catenin inhibitor PNU-74654, the adipogenic differentiation of ingWAT pre-adipocytes was enhanced compared with Shp2 overexpression alone. These results suggested that Shp2 may serve as a positive regulator of the Wnt/β-catenin pathway. And more key proteins of Wnt/β-catenin signaling will be investigated in future studies.

There are some limitations to address for the present study. First, a restricted diet group, in which the food intake was equivalent to that of SG group to rule out the effect of diet on fat reduction was not used. However, previous studies have confirmed that the reduced fat accumulation caused by SG is not due to reduced food intake alone (10,17). Secondly, the post-operative outcomes were only observed at week 8. Furthermore, only ingWAT was used as a representative WAT for ease of morphological comparison and for consistency *in vivo* and *in vitro*, and more types of WAT are needed for further investigation. Finally, we did not measure the protein expression level of Fabp4 due to the lack of antibody. Fabp4 regulates leptin secretion and increases with leptin synergistically during adipose inflammation process (47). The expression level of Fabp4 may be reflected by leptin level partially. The protein expression level of Fabp4 could be investigated in future studies via western blotting.

Nevertheless, the results of the present study demonstrated that the Shp2/Wnt/β-catenin pathway was important for adipogenesis and sustained weight loss following SG. This study provides further insight into the mechanisms through which SG modulates body weight through reduced fat accumulation. Meanwhile, decreased triglyceride levels through fat consumption may contribute to glucose and lipid metabolism.

In the present study, SG was considered to alleviate obesity and related metabolic disorders. Moreover, it inhibited fat

accumulation and upregulated Shp2 level in ingWAT (Fig. 8A). SG reduced leptin levels in ingWAT, which in turn upregulated Shp2/β-catenin levels. The findings further revealed that Shp2 suppressed ingWAT adipocyte differentiation by activating the Wnt/β-catenin signaling pathway, while the inhibition of β-catenin reversed the effects of Shp2 on adipogenic differentiation (Fig. 8B). These observations raise the possibility that Shp2 and Shp2-associated signaling may serve as potential therapeutic targets for the treatment of obesity and T2D.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

XYQ and ZS conceived the study and drafted the manuscript. XL, YJ and JQ performed the *in vivo* experiments and analyzed the data. SC and PS performed the *in vitro* experiments. JQ and ZQ carried out the statistical analysis. XSQ and LT supervised the experiments, analyzed the data and revised the manuscript. XYQ and ZS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were approved by and performed in accordance with the Animal Care and Use Committee of Nanjing Drum Tower Hospital, Nanjing Medical University (approval no. 2019AE02013). All animal care and use protocols were approved by the Committee on the Ethics of Animal Experiments.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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