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Hepatic mTOR-AKT2-Insig2 signaling pathway contributes to the improvement of hepatic steatosis after Roux-en-Y Gastric Bypass in mice

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ABSTRACT

Roux-en-Y Gastric Bypass (RYGB) remains one of the most effective options in treatment of non-alcoholic fatty liver disease (NAFLD). However, the underlying mechanisms are not clear yet. Here, we evaluated the relationship among hepatic mechanistic target of rapamycin (mTOR)-AKT2-insulin-induced gene 2 (Insig2) signaling, lipogenic transcription factors and lipid synthesis enzymes in obese mice with or without RYGB operation. Hepatic mTOR activity and Insig2a were stimulated, while AKT2, sterol response element-binding protein 1c (SREBP1c), peroxisome proliferator-activated receptor γ (PPARγ), lipogenic genes such as acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) were decreased by Roux-en-Y Gastric Bypass in both DMSO and rapamycin treated diet-induced obese (DIO) mice. Increment of hepatic lipogenesis and decline of mTOR signaling induced by rapamycin were significantly reversed by RYGB in DIO mice. RYGB significantly improved high-fat diet- and rapamycin- induced hepatic steatosis by suppression of de novo lipogenesis. Administration of adenovirus-mediated p70 ribosomal protein subunit 6 kinase 1 (Ad-S6K1) from tail vein improved hepatic steatosis. Infusion of Ad-S6K1 suppressed AKT2, SREBP1c, PPARγ, and lipogenesis-related genes while stimulating Insig2a in DIO mice. Ad-S6K1 decreased oleic acid-induced lipid deposition in primary mouse hepatocytes. Our results suggest that mTOR-AKT2-Insig2 signaling pathway contributes to the improvement effect of RYGB on hepatic steatosis induced by high-fat diet.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide. NAFLD is strongly associated with obesity and metabolic syndrome [1]. Current treatment of NAFLD is based on weight reduction [2]. Bariatric surgery is the most effective treatment for morbid obesity and its associated metabolic comorbidities [3,4]. Bariatric surgery has beneficial effects not only in weight loss, but also in the metabolic alterations encompassed by the metabolic syndrome [5,6]. In addition, insulin resistance, lipid profile, inflammation, and adipokines which involved in the development of NAFLD have been changed favorably after bariatric surgery [7]. Roux-en-Y Gastric Bypass (RYGB) is a popular and efficacious form of bariatric operation, which remains one of the most effective options in treatment of NAFLD [8–10]. Although great efforts have been dedicated to elucidate the underlying mechanisms involved in amelioration of fatty liver, the mechanisms are still needed to be further explored. Up-regulated hepatic AMP-activated protein kinase (AMPK) plays a critical role in the resolution of steatosis after RYGB [11]. AMPK regulates lipid and glucose metabolism through direct phosphorylation of its substrates and indirect control over gene transcription [12]. Mechanistic target-of-rapamycin (mTOR) is one of the key downstream targets of AMPK [13].

Abbreviations: ACC, acetyl-CoA carboxylase; Ad-GFP, adenovirus-mediated green fluorescent protein; Ad-S6K1, adenovirus-mediated p70 ribosomal protein subunit 6 kinase 1; AMPK, AMP-activated protein kinase; DGAT, acyl CoA:diacylglycerol transferase; DIO, diet-induced obese; DMSO, dimethyl sulfoxide; FAS, fatty acid synthase; GPAT, glycerol-3-phosphate acyltransferase; GSK3β, glycogen synthase kinase 3β; HE, hematoxylin-eosin; HFD, high-fat diet; Insig2, insulin-induced gene 2; mTOR, mechanistic target of rapamycin; NAFLD, non-alcoholic fatty liver disease; OA, oleic acid; PPARγ, peroxisome proliferator-activated receptor γ; RYGB, Roux-en-Y Gastric Bypass; S6, ribosomal protein S6; S6K1, p70 ribosomal protein subunit 6 kinase 1; SREBP1c, sterol response element-binding protein 1c

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mTOR protein is a serine-threonine kinase belonging to the phosphoinositide 3-kinase (PI3K)-related kinase family that plays key roles in lipid biosynthesis [14–16]. mTOR has been shown to activate the transcription factor, SREBP, which in turn activates ACC, FAS, and stearoyl-coenzyme A desaturase (SCD) enzymes involved in lipogenesis [17–19]. Although the exact mechanism by which SREBP1 and SREBP2 are regulated by mTOR is unclear, it is believed to be mediated by S6K1 [17,18]. The transcriptional regulation of SREBP1c by insulin is not dependent on S6K, whereas post-transcriptional processing of SREBP1c is S6K dependent [20].

mTOR signaling pathway has been described to play an important role in lipid biosynthesis [21–24]. This notion has emerged from observations of clinical trials involving rapamycin or rapalog administration. Side effects of these drugs have been reported such as hyperlipidemia and hypercholesterolemia and activation of gluconeogenesis in liver, a major organ for lipid biosynthesis [25–27]. Furthermore, rodents treated with rapamycin and rapalogs develop NAFLD associated with elevated free fatty acid levels [28].

Although numerous studies have illustrated the positive effects of RYGB on NAFLD [10,29,30], to the best of our knowledge, the underlying mechanism is unknown. Over the past years, several studies revealed that mTOR plays a crucial role in promoting lipid biosynthesis and that such connection could be linked to diseases including obesity and NAFLD [31–33]. Furthermore, hepatic AKT2 is essential for lipid biosynthesis [34]. We thus hypothesized that mTOR-AKT2 pathway is associated with the improvement effect of RYGB on NAFLD. Here, we present evidence that RYGB ameliorates hepatic steatosis induced by high-fat diet through mTOR-AKT2-Insig2 signaling pathway in mice. In the present study, underlying mechanism of RYGB ameliorating hepatic steatosis caused by high-fat diet was explored to provide a novel insight of medical intervention strategies for NAFLD therapy.

2. Materials and methods

2.1. Materials

Rapamycin and DMSO were purchased from Sigma Chemical Co. (St. Louis, MO). Rabbit anti-Phospho-mTOR (Ser2448), anti-Phospho-p70 S6 Kinase (Thr389), anti-Phospho-S6 (Ser235/236), anti-AKT2, anti-PPARγ, anti-acetyl-CoA carboxylase, anti-mTOR, anti-p70 S6 Kinase, anti-S6 antibodies and mouse monoclonal anti-β-actin were from Cell Signaling Technology (Beverly, MA). Rabbit anti-Insig2, anti-fatty acid synthase and mouse monoclonal anti-SREBP1c were from Abcam Inc. (Cambridge, MA). Immobilon western chemiluminescent HRP substrate was from Millipore (Temecula, CA). Horseradish peroxidase-conjugated, donkey anti-Rabbit IgG and donkey anti-Mouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA). Trizol reagent and the reverse transcription (RT) system were from Promega Inc. (Madison, WI). Triglyceride assay kits were from Cayman Chemical Company (Ann Arbor, MI). High-fat diet (60% of kcal as fat, D12492) was from Research Diets, Inc. (New Brunswick, NJ).

2.2. Animals and treatments

Four weeks-old male C57BL/6J mice were purchased and fed with a high-fat diet for 16 weeks. Mice were housed on a 12:12-h light/dark cycle. Animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publications No. 8023, revised 1978). All animal protocols were approved by the Animal Care and Use Committee of Jinan University.

At the time of surgery, obese mice were divided into sham and RYGB groups. After 4-week recovery from surgery, mice were ip injection with DMSO or rapamycin (1 mg/kg), or injected with Ad-GFP (10^9 pfu) or Ad-S6K1 (10^9 pfu) from tail veins for 9 consecutive days before sacrifice.

2.3. Gastrointestinal surgery and postoperative care

Male diet-induced obese mice underwent either RYGB or sham operations, as described previously [35–37].

In the RYGB group, a small gastric pouch at approximately 5% of the normal gastric volume was generated and anastomosed with the jejunum by 16–18 interrupted stiches with 11–0 nylon suture. The Roux limb and the biliopancreatic limb were 5 to 6 cm in length. Before closing of the abdominal cavity, the intestine was arranged in an “S” position to avoid intestinal obstruction.

In the sham group, the perigastric ligaments were cut, and then a 3 mm incision was made in the stomach wall and closed with a titanium clip. In addition, the jejunum was transected 2 cm distal to the ligament of Treitz.

For postoperative care, mice were maintained on a standardized post-operative protocol [37,38] during which a liquid diet was provided on post-surgery days 2 through 5. On post-surgery days 6 and 7, 0.25 g high-fat diet was provided, and on post-surgery day 8, high-fat diet was provided ad libitum.

2.4. Histological analysis

Liver samples were harvested, fixed in 4% paraformaldehyde, paraffin-embedded, cut into 6 μm sections and stained with hematoxylin-eosin according to standard procedures. For Oil Red O staining, frozen sections of liver were stained in 0.5% Oil Red O solution for 2 h in a 50 °C oven and then in 85% propylene glycol solution for 5 min. Sections were rinsed in distilled water, stained in Gill's hematoxylin for 2 s, washed, and mounted with aqueous mounting medium.

2.5. Determine of hepatic and plasma triglyceride

Liver tissues were homogenized in 1 mL of 2:1 chloroform/methanol mix on ice and placed at 4 °C for 18 h. Two hundred microliters of distilled water was added to the homogenates. The mixture was vortexed, then centrifuged for 10 min at 3000 rpm, 4 °C. The lower phase was collected, lyophilized and resolved in 5% Triton X-100 in PBS for measurements of lipids. 100 μl blood sample was collected in a sterile bottle and allowed to clot for about an hour at 37 °C. Hepatic and plasma triglyceride were measured according to the manufacturer's instructions. Values were normalized to protein concentration.

2.6. Western blot analysis

The tissues were homogenized on ice in the lysis buffer. After centrifugation and protein quantification, proteins were loaded onto SDS-PAGE gels, and then transferred to nitrocellulose membranes. The membranes were incubated in 5% nonfat dry milk in TBST for 1 h at room temperature, followed by incubation overnight at 4 °C with the primary antibodies. The antibodies were detected using 1:10,000 horseradish peroxidase-conjugated, donkey anti-Rabbit IgG and donkey anti-Mouse IgG (Jackson ImmunoResearch, PA). A western blotting luminol reagent was used to visualize bands corresponding to each antibody. The band intensities were quantitated by Image J software.

2.7. RNA extraction, quantitative real-time PCR

For gene expression analysis, RNA was isolated from mouse tissues using Trizol and reverse-transcribed into cDNAs using the First-Strand Synthesis System for RT-PCR kit. SYBR Green-based real-time PCR was performed using the Mx3000 multiplex quantitative PCR system (Stratagene, La Jolla, CA). Sequences for the primer pairs used in this study were shown in Table 1.

Table 1
List and sequences of primers used in RT-PCR experiments.

	Upstream primer(5′-3′)	Downstream primer(5′-3′)	Accession number(s)
Mouse AKT2	GAGCATAGATTCTTCCTCAGCATC	GTGGTGGCAGAGGGCTGCTCACTC	XM_006539481.3
Mouse Insig2a	CCCTCAATGAATGTACTGAAGGATT	TGTGAAGTGAAGCAGACCAATGT	NM_178082.3
Mouse PPARγ2	CTCTGGGAGATTCTCCTGTTGA	GGTGGGCCAGAATGGCATCT	XR_001785108.1
Mouse SREBP1c	GGAGCCATGGATTGCACATT	GGAAGTCACTGTCTTGGTTGTTGA	XM_006532716.2
Mouse FAS	TGGGTTCTAGCCAGCAGAGT	ACCACCAGAGACCGTTATGC	NM_007988.3
Mouse ACC	TGGTCGTGACTGCTCTGTGC	GTAGCCGAGGGTTCAGTTCC	XM_006531957.3
Mouse GPAT	CACACGAGCAGGAAAGATGA	GGACTGCATAGATGCTGCAA	XM_006526693.3
Mouse DGAT1	TAGTGAGCGTTCCCCTGC	CAAATGCCATCCCCAAGAG	XM_017316426.1
Mouse DGAT2	CGTGACGTGCATTGGCTTC	TGGAGGGCTGAGAGGATGC	NM_026384.3
Mouse β-actin	CCACAGCTGAGAGGGAAATC	AAGGAAGGCTGGAAAAGAGC	NM_007393.5

2.8. Statistical analysis

All data are expressed as mean values ± SEM. Statistical differences were evaluated by factorial design analysis of variance (ANOVA) or one-way ANOVA followed by Newman-Student-Keuls test. Differences were significant with p values < 0.05.

3. Results

3.1. RYGB reverses hepatic steatosis induced by high-fat diet or rapamycin

To examine the effects of RYGB on hepatic steatosis induced by high-fat diet or rapamycin, male C57BL/6J mice fed with high-fat diet for 16 weeks were divided into four groups: sham-operated mice receiving dimethyl sulfoxide (DMSO), sham group receiving rapamycin (1 mg/kg, ip injection for 9 days), RYGB-operated mice receiving either vehicle or rapamycin (Fig. 1A). Associated with significant weight loss, Oil red O staining and HE staining showed decreased lipid accumulation in RYGB-operated mice compared to sham animals. In contrast with RYGB, rapamycin induced hepatic steatosis. Moreover, RYGB suppressed high-fat diet- and rapamycin-induced hepatic lipid deposition (Fig. 1B–D).

Consistently, RYGB significantly decreased plasma total triglyceride and hepatic triglyceride in DIO mice, while rapamycin increased plasma and hepatic triglyceride contents. RYGB improved the worse lipid profile induced by rapamycin (Fig. 2).

3.2. Effects of RYGB on hepatic mTOR-AKT2-Insig2 signaling and de novo lipogenesis in DIO mice

Our previous studies showed that RYGB increased ileal mTOR signaling activity [37]. Interestingly, RYGB also significantly stimulated the hepatic mTOR signaling as evidenced by the increase in the phosphorylation levels of mTOR and its downstream targets: S6K and S6 (Fig. 4). Some studies have reported that decreases in hepatic lipid accumulation and steatosis are phenotypes described for the liver-specific knockout of AKT2 [34,39]. Associated with the activation of mTOR signaling, decrease in the mRNA (Fig. 3A) and protein levels (Fig. 4) of hepatic AKT2 was observed in RYGB-operated mice versus sham animals. Although attenuation of AKT2 expression, phosphorylation of AKT at Ser473 and glycogen synthase kinase 3 β (GSK3β) were significantly up-regulated especially in RYGB-operated mice under administration of insulin (Supplemental Fig. 1). It is reported that Insig proteins inhibits hepatic SREBP1c and lipogenesis [40]. Here we found RYGB enhanced Insig2a expression, while inhibited SREBP1c. RYGB inhibited mRNA and protein levels of SREBP1c, the master regulators of lipogenesis and its targets gene required for endogenous fatty acid synthesis such as ACC and FAS (Figs. 3–4). Similar results were observed in another lipogenic transcription factor, PPARγ2 but not in glycerol-3-phosphate acyltransferase (GPAT), acyl CoA: diacylglycerol transferase (DGAT1) and DGAT2 (Figs. 3–4).

Rapamycin, a mTOR-specific inhibitor significantly stimulated AKT2 and inhibited Insig2a in sham-operated mice but not in RYGB-operated animals (Figs. 3A and 4). Consistent with this observation, rapamycin inhibited the mTOR signaling and up-regulated the expression of AKT2 in primary mouse hepatocytes (Supplemental Fig. 2). Consistent with the increased triglyceride content (Fig. 2), the decline of mTOR signaling activity and upregulation of lipogenic enzymes genes expression induced by rapamycin were reversed by RYGB (Figs. 3–4).

3.3. Both Ad-S6K1 and RYGB ameliorated NAFLD induced by high-fat diet

We next examined whether activation of hepatic mTOR signaling would contribute to the improvement of NAFLD. S6K1 is a well-known downstream target of mTOR. Diet-induced obese mice were randomly divided into sham- or RYGB-operated group receiving tail vein injection with either Ad-GFP (10^9 pfu) or Ad-S6K1 (10^9 pfu). Similar with RYGB, infusion of Ad-S6K1 markedly improved hepatic steatosis (Fig. 5A–C). Both RYGB and administration of Ad-S6K1 improved the deteriorated lipid profiles induced by high-fat diet (Fig. 6). Moreover, our in vitro data shows Ad-S6K1 significantly reduced OA-induced lipid deposition in primary hepatocytes (Supplemental Fig. 3).

3.4. Additional effects of S6K1 over-expression and RYGB on mTOR-AKT2-Insig2 signaling and hepatic lipogenesis in DIO mice

Since over-expression of S6K1 ameliorated fatty liver induced by high-fat diet, Ad-S6K1 was expected to change the level of hepatic lipogenesis. Infusion of Ad-S6K1 (10^9 pfu) significantly increased both phospho-S6K and phospho-S6 levels in livers (Fig. 8), suggesting the activation of mTOR signaling. Similar to RYGB, Ad-S6K1 decreased hepatic AKT2 expression (Figs. 7A and 8). Both Ad-S6K1 and RYGB significantly enhanced Insig2a, thus inhibited SREBP1c (Fig. 7B, D and 8). Tail-vein injection of Ad-S6K1 resulted in a significant reduction in lipogenesis-related enzyme genes including ACC and FAS mRNA levels, but it had no effect on GPAT, DGAT1 and DGAT2 (Fig. 7). RYGB combined with administration of Ad-S6K1 also significantly stimulated mTOR signaling activity and inhibited SREBP1c, PPARγ, ACC and FAS expression compared to sham-operated mice receiving Ad-GFP (Figs. 7–8).

4. Discussion

The major finding of the present study is that the hepatic mTOR-AKT2-Insig2 pathway contributes to the improvement of lipid metabolism after Roux-en-Y Gastric Bypass in high-fat diet- induced obese mice. This conclusion is supported by the following distinct observations: 1) RYGB reversed both high-fat diet-induced and rapamycin-induced steatosis in macroscopic and microscopic examination of mouse livers. 2) RYGB stimulated hepatic mTOR signaling and Insig2a, while decreasing AKT2 expression. 3) Rapamycin stimulated AKT2 and

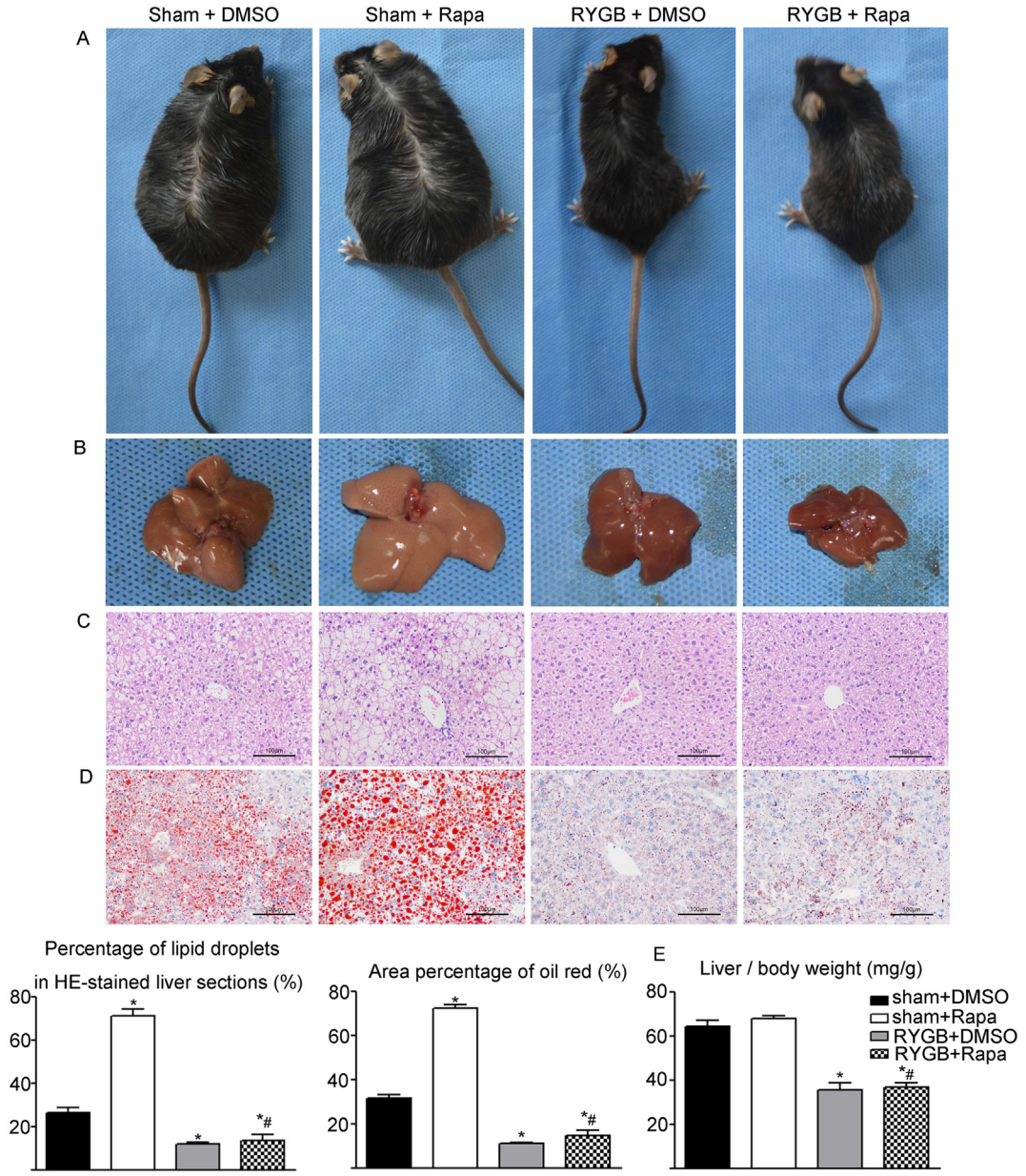

Fig. 1. Effects of RYGB on high-fat diet- and rapamycin-induced hepatic steatosis in DIO mice.
After 4 weeks of post-surgical recovery, mice were assigned to receive ip injection of rapamycin or DMSO. (A–B) Post-mortem representative macroscopic photomicrographs of sham- or RYGB-operated mice receiving DMSO or rapamycin. Gross morphology of mice (A), livers (B), HE staining of liver sections (C), Oil red O staining of liver (D), percentage of lipid droplets in HE-stained liver sections and area percentage of oil red. Liver to total body weight ratio (E). n = 6, *p < 0.05 vs. sham-operated mice receiving DMSO. #p < 0.05 vs. sham-operated mice receiving rapamycin.

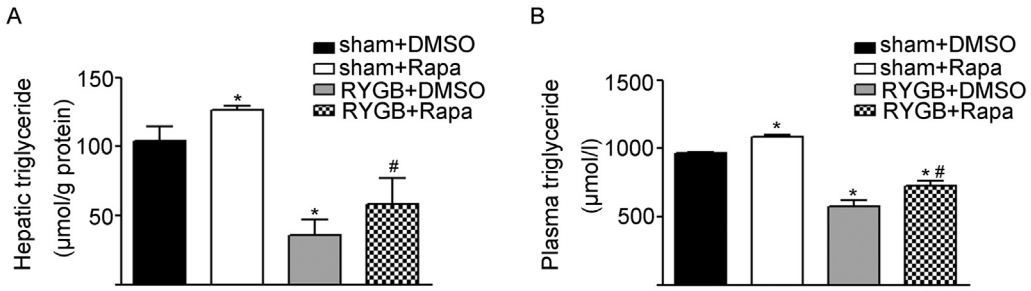

Fig. 2. Effects of RYGB and rapamycin on hepatic and plasma triglyceride content.
Hepatic triglyceride content, (B) plasma triglyceride levels. Six samples were examined for each condition. *p < 0.05 vs. sham-operated mice receiving DMSO. #p < 0.05 vs. sham-operated mice receiving rapamycin.

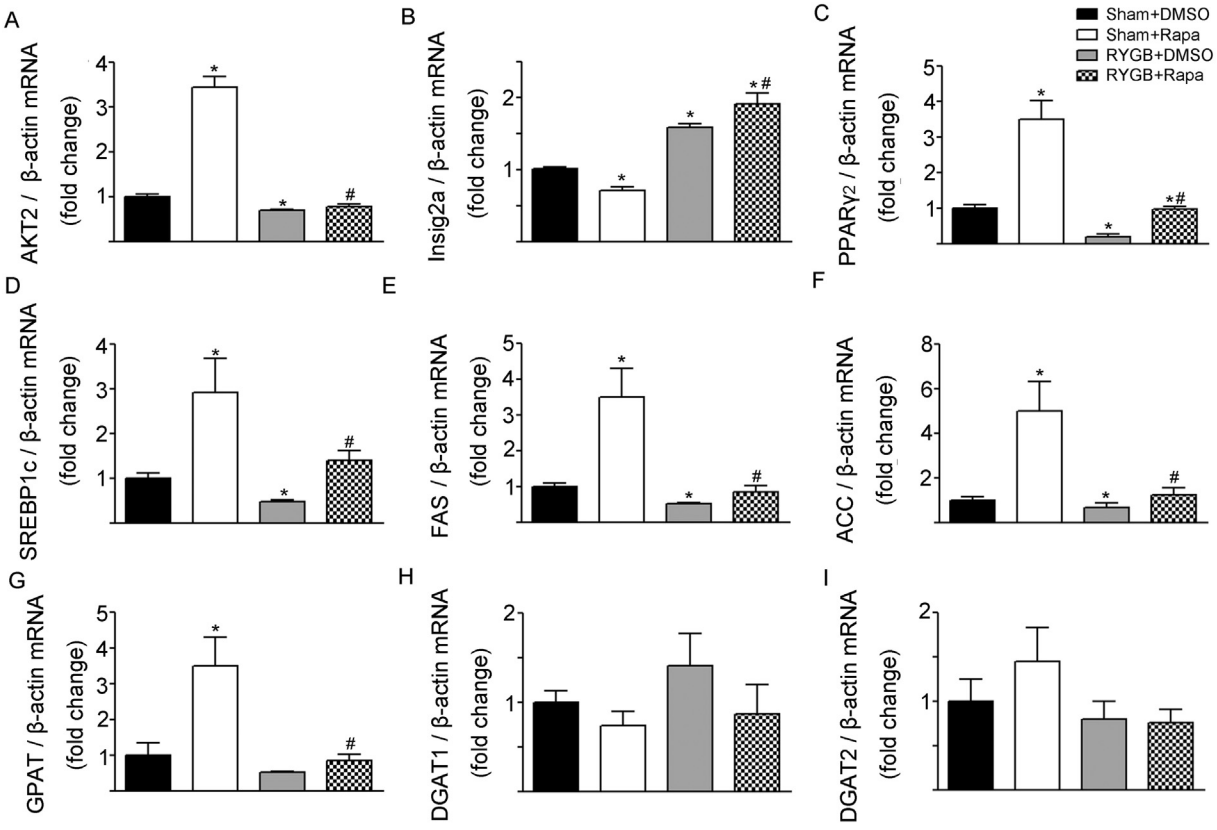

Fig. 3. Effects of RYGB and rapamycin on AKT2, Insig2, lipogenic transcription factors and genes in DIO mice.
Results of quantitative PCR analysis of AKT2 mRNA (A), Insig2a mRNA (B), PPARγ2 mRNA (C), SREBP1c mRNA (D), FAS mRNA (E), ACC mRNA (F), GPAT mRNA (G), DGAT1 mRNA (H), and DGAT2 mRNA (I) in mouse livers are expressed as fold change from control using β-actin as loading control. n = 6, *$p < 0.05$ vs. sham-operated mice receiving DMSO. #$p < 0.05$ vs. sham-operated mice receiving rapamycin.

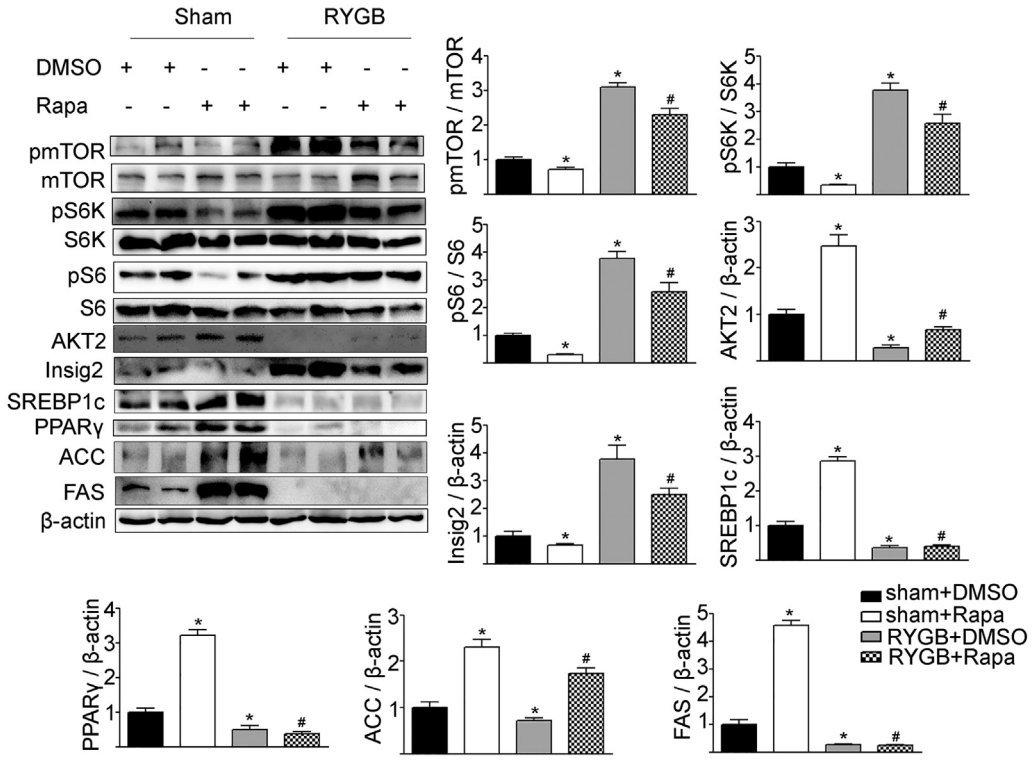

Fig. 4. Effects of RYGB on mTOR-AKT2-Insig2 signaling and hepatic lipogenesis in DIO mice receiving ip injection of DMSO or rapamycin.
Representative western blot from obese sham- or RYGB-operated mice that received ip injection of DMSO or rapamycin (Rapa, 1 mg/kg). pmTOR, pS6K, pS6, AKT2, Insig2, SREBP1c, PPARγ, ACC, and FAS were detected using specific antibodies. mTOR, S6K, S6, and β-actin were used as loading controls. Quantification of image analysis of mTOR, S6K, S6 phosphorylation and AKT2, Insig2, SREBP1c, PPARγ, ACC, FAS expression is expressed as mean values ± SEM. n = 6, *$p < 0.05$ vs. sham-operated mice receiving DMSO. #$p < 0.05$ vs. sham-operated mice receiving rapamycin.

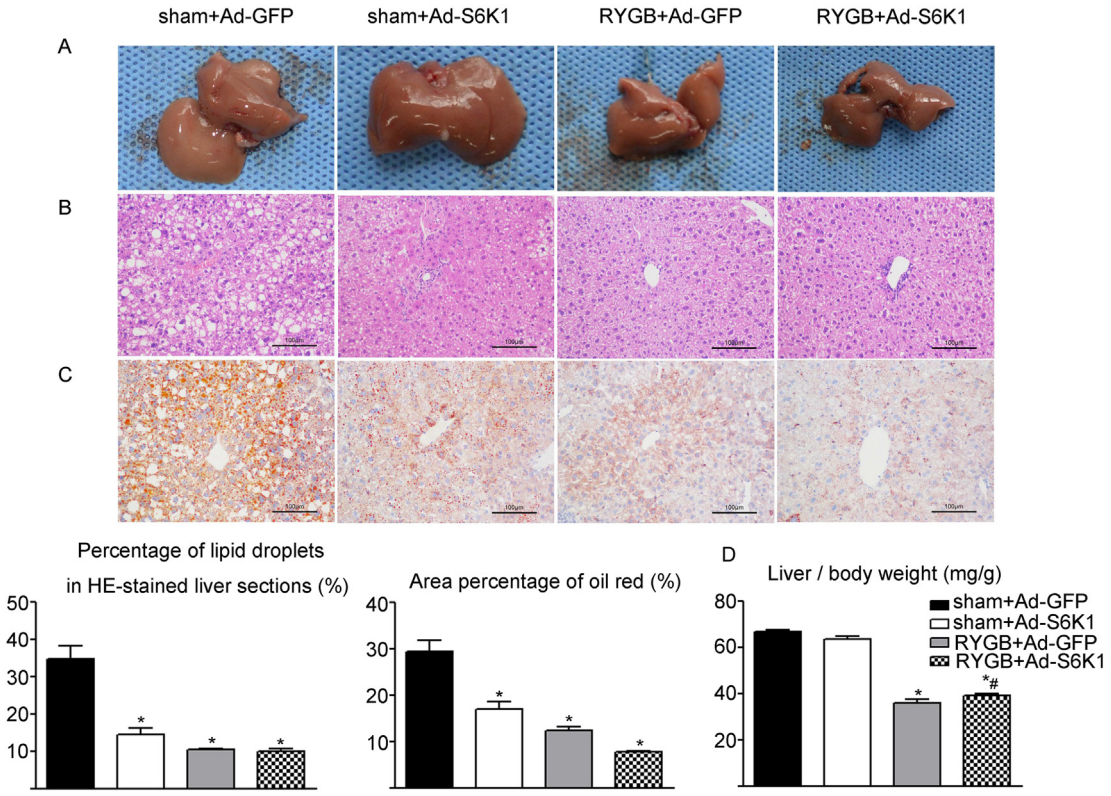

Fig. 5. Effects of RYGB and tail-vein administration of Ad-S6K1 on NAFLD in DIO mice.
Post-mortem representative macroscopic photomicrographs of sham-operated mice or RYGB mice receiving tail vein administration of Ad-GFP (10^9 pfu) or Ad-S6K1 (10^9 pfu). Gross morphology of livers (A), HE staining of liver (B), Oil red O staining of liver (C), percentage of lipid droplets in HE-stained liver sections and area percentage of oil red. Liver to total body weight ratio (D). n = 6, *p < 0.05 vs. obese sham-operated mice receiving Ad-GFP. #p < 0.05 vs. sham-operated mice receiving Ad-S6K1.

inhibited Insig2a in sham animals but not in RYGB-operated mice. 4) RYGB attenuated the increment of SREBP1c and its target lipogenic genes induced by high-fat diet and rapamycin. 5) Tail vein injection of Ad-S6K1 improved high-fat diet-induced hepatic steatosis. 6) RYGB and infusion of Ad-S6K1 reduced while rapamycin increased hepatic and plasma triglyceride levels in high-fat diet-induced mice. 7) Ad-S6K1 decreased OA-induced lipid deposition in primary mouse hepatocytes. 8) Administration of Ad-S6K1 suppressed AKT2 while increased Insig2a, which was associated with significant decreases of SREBP1c and lipogenic genes.

Roux-en-Y Gastric Bypass (RYGB) is proven to be a safe and effective treatment of obesity and related co-morbidities including NAFLD [4,9]. RYGB is likely to have potential benefits in ameliorating the factors such as insulin resistance, lipid profile, inflammation, weight loss, and adipokines that contribute in a marked way to the pathogenesis of NAFLD [7]. RYGB protects the liver against HFD-induced fatty liver disease by attenuating ER stress and excess apoptosis [41]. According to Andriy's study, change of serum bile composition is responsible for the suppression of lipogenesis in mice after VSG surgery [42]. Here we show that RYGB resolves steatosis induced by high-fat diet via alteration of hepatic mTOR-AKT2-Insig2 signaling pathway. The inhibition of hepatic AKT2 substantially elicits an improvement in Insig2a and repression of lipogenic genes after RYGB.

De novo lipogenesis plays a substantial role in the pathogenesis of NAFLD, accounting for 26% hepatic triglycerides in human subjects [43]. The notion that mTOR promotes lipogenesis and may contribute to NAFLD came from a series of observations showing the positive effects of mTOR on SREBP1c expression and activity that lead to de novo lipid synthesis [17,18,21,24]. In vitro study showed that mTOR signaling pathway promotes de novo lipid synthesis. In isolated rat hepatocytes, insulin-mediated stimulation of SREBP1c processing requires mTOR, which is also required for insulin-mediated SREBP1c mRNA

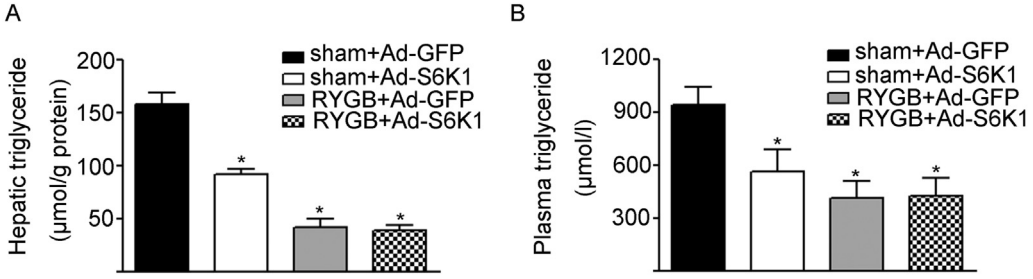

Fig. 6. Effects of Ad-S6K1 and RYGB on hepatic and plasma triglyceride contents.
Hepatic triglyceride content, (B) plasma triglyceride levels. Six samples were examined for each condition. *p < 0.05 vs. obese sham-operated mice receiving Ad-GFP.

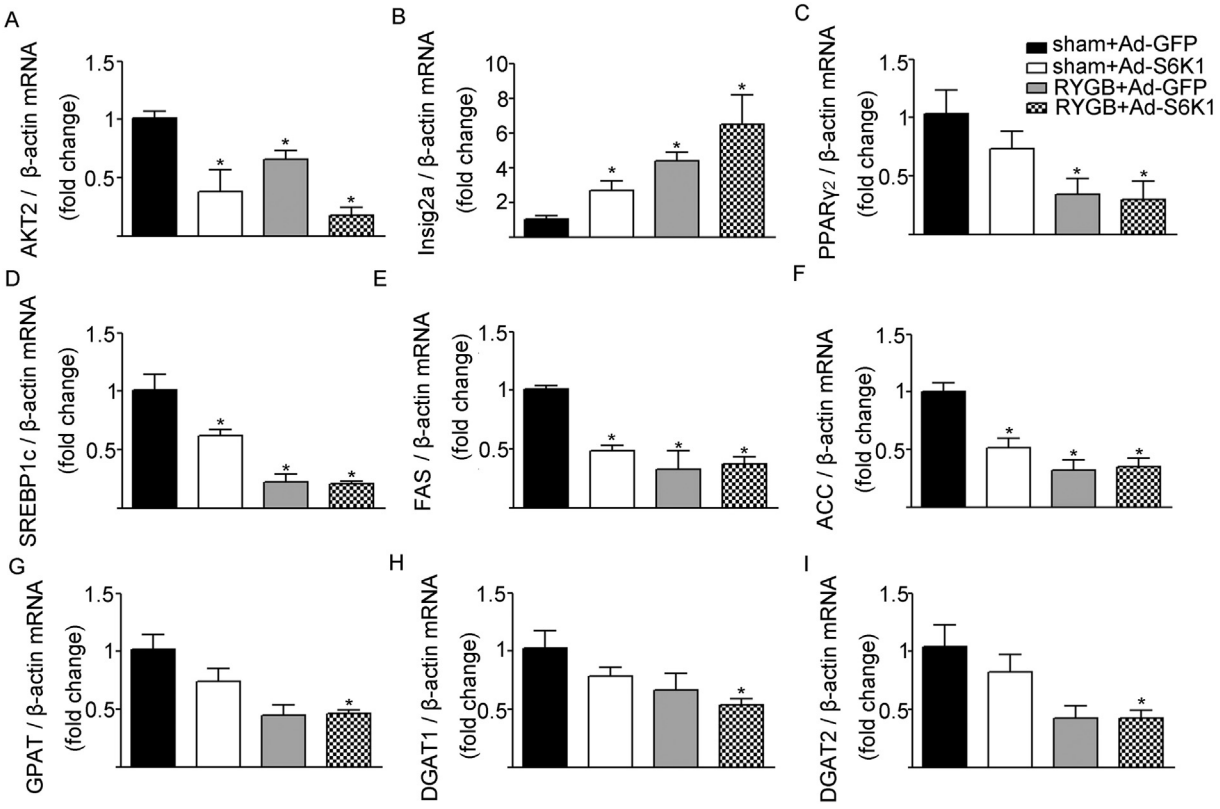

Fig. 7. Effects of Ad-S6K1 and RYGB on AKT2, Insig2 and transcription factors correlating with lipid synthesis and lipogenesis-related enzyme in DIO mice. Representative real-time-PCR from sham- or RYGB-operated mice that received tail vein injection of Ad-GFP or Ad-S6K1. AKT2 (A), Insig2a (B), PPARγ2 (C), SREBP1c (D), FAS (E), ACC (F), GPAT (G), DGAT1 (H) and DGAT2 (I). n = 6, *$p < 0.05$ vs. obese sham-operated mice receiving Ad-GFP.

induction [20]. Rapamycin decreased expression of genes encoding acetyl-coenzyme A carboxylase I and mitochondrial glycerol phosphate acyltransferase in isolated hepatocytes [24]. However, mTOR hyperactivity per se does not induce steatosis, but instead, protects against high-fat diet-induced steatosis in mice. Mice with a liver-specific deletion of tuberous sclerosis complex 1 (TSC1) show increased mTOR activity, but have defective SREBP1c activation and lipogenesis due to an attenuation of AKT signaling [44]. Elevated FGF21 expression is considered to explain the improvement of lipid metabolism in liver-specific TSC1 knockout mice [45]. Moreover, plasma FGF21 is significantly increased in post-RYGB subjects [46,47]. Our studies demonstrate for the first time that mTOR signaling in liver is necessary in the process of RYGB reversing high-fat diet and rapamycin-induced steatosis. Hepatic mTOR signaling activity is elevated significantly after RYGB in rodents. Both RYGB and over-expression of S6K1, representing hyperactivation of mTOR signaling, protect the liver against HFD-induced fatty liver disease. Consistently, Ad-S6K1 decreased OA-induced lipid deposition in vitro study. Transcription factors SREBP1C and PPARγ, and key enzymes encoded by genes such as ACC and FAS were reduced in response to RYGB or Ad-S6K1.

AKT2, the major isoform of AKT expressed in livers mediating lipogenesis by the stimulation of de novo lipogenesis. Recent reports have found that AKT2 is required for hepatic lipid accumulation in models of insulin resistance [34]. Rapamycin selectively activates the AKT2 isoform in vascular smooth muscle cells [48]. In the present study, we show that hepatic AKT2 expression is decreased dramatically in RYGB-operated mice. Inhibition of mTOR signaling by rapamycin markedly increased but infusion of Ad-S6K1 decreased AKT2 in liver. Rapamycin also stimulated AKT2 in primary mouse hepatocytes. Therefore, hepatic mTOR contributes to the modulation of lipogenic genes and de novo lipogenesis. In sum, RYGB ameliorates hepatic steatosis induced by high-fat diet and rapamycin through mTOR-AKT2 signaling pathway. mTOR nucleates at least two distinct multi-protein complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Hagiwara et al. report that mTORC2-AKT signaling promotes hepatic de novo lipogenesis [49]. It is worth of noting that mTORC2-AKT signaling pathway may involve the effects of RYGB surgery on NAFLD. Further studies on mTORC2-AKT signaling pathways will address this potential.

According to Jessica's study, AKT2 is responsible for Insig2a suppression, leading to up-regulating of SREBP1c and lipogenesis in liver [44]. In present study, RYGB promoted Insig2a expression, while inhibited SREBP1c. In contrast with RYGB, rapamycin decreased Insig2a expression. Tail-vein injection of Ad-S6K1 significantly enhanced Insig2a, thus inhibited SREBP1c.

Increased hepatic phosphorylation of AKT is associated with the amelioration of steatosis in diabetic mice [50]. As expected, both RYGB and Ad-S6K1 treated livers exhibited elevated phosphorylation of AKT at Ser473. Hepatic pAKT473 was inhibited by rapamycin, which was blocked by RYGB operation. Akt-mediated phosphorylation and inhibition of GSK3 stimulates glycogen synthesis and promotes SREBP stability thus enhances lipid production [51]. The mRNA levels of GSK3β showed a substantial decrease after RYGB in subjects and rats [52,53]. Our study found RYGB significantly promoted the phosphorylation of AKT at Ser473 and GSK3β, while the expression of SREBP1c is down-regulated in RYGB-operated mice. It suggests that AKT2-Insig2 signaling but not AKT-GSK3β-SREBP contributes to the improvement of hepatic steatosis after Roux-en-Y Gastric Bypass in mice.

Rapamycin is commonly used as an immunosuppressant following renal transplant, and more recently, its analogs have gained FDA approval for use in human tumors such as renal cell carcinoma and subependymal giant cell astrocytoma [54]. Posttransplantation diabetes is very common [55], it is conceivable that in this clinical setting, rapamycin adversely affects a preexisting metabolic syndrome and is toxic

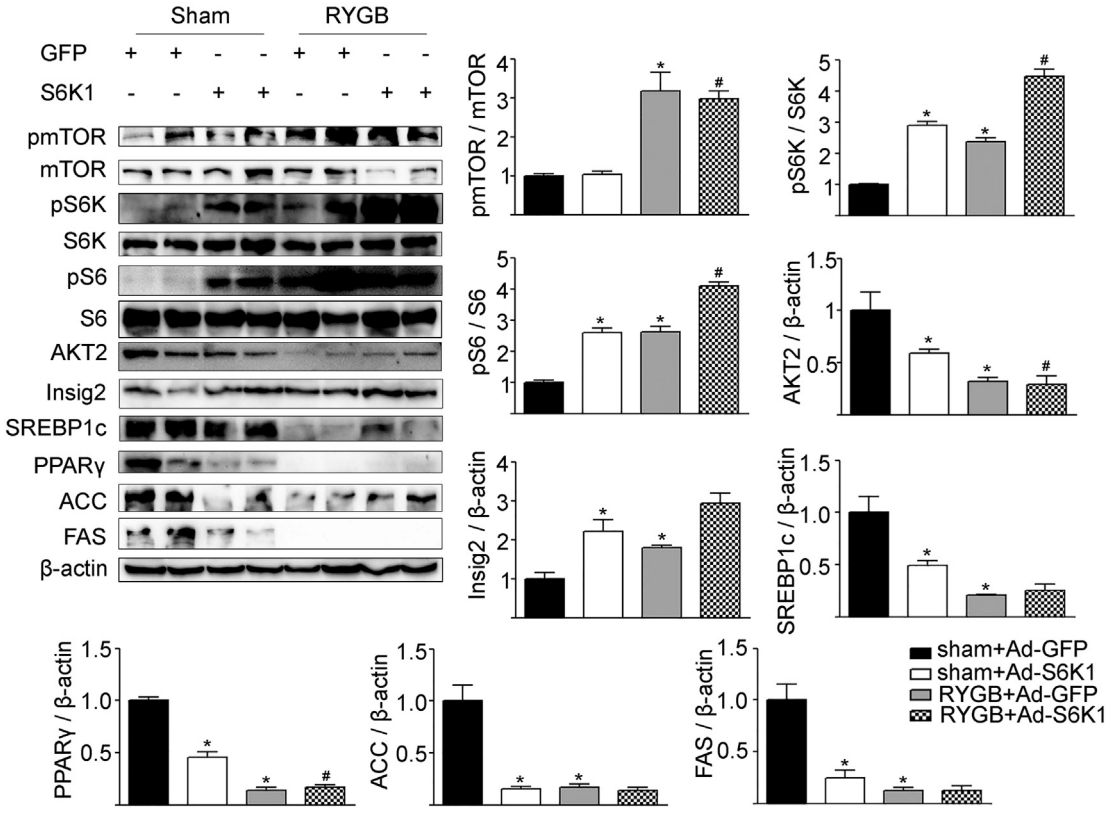

Fig. 8. Effects of RYGB on mTOR-AKT2-Insig2 signaling and hepatic lipogenesis under tail vein infusion of Ad-S6K1.
Representative western blot from obese sham- or RYGB-operated mice that received administration of Ad-GFP or Ad-S6K1. pmTOR, pS6K, pS6, AKT2, Insig2, SREBP1c, PPARγ, ACC and FAS were detected using specific antibodies. mTOR, S6K, S6, and β-actin were used as loading controls. Quantification of image analysis of mTOR, S6K, S6 phosphorylation and AKT2, Insig2, SREBP1c, PPARγ, ACC, FAS expression is expressed as mean values ± SEM. n = 6, *p < 0.05 vs. sham-operated mice receiving Ad-GFP. #p < 0.05 vs. sham-operated mice receiving Ad-S6K1.

to endogenous or transplanted pancreatic islets [56]. Reports of rapamycin-induced numerous features of the metabolic syndrome including hyperlipidemia, hypercholesterolemia, and insulin resistance in humans and in mice [57–59] are consistent with our observations. Chronic rapamycin treatment increased hepatic lipid deposition and plasma triglyceride level measured by oil-red staining and enzymatic assay kits in mice fed HFD. Considerable studies have showed that RYGB is associated with marked improvement in NAFLD induced by high-fat diet, our current study showed that RYGB also contributes to the improvement of rapamycin-induced steatosis. Stimulation of hepatic mTOR activity may therefore provide a potential therapeutic strategy for deteriorated lipid metabolism associated with rapamycin treatment after transplantation. The mechanism by which stimulation of mTOR generate an decrease in lipogenesis involves a multitude of factors that includes AKT2, Insig2a, SREBP1c and PPARγ that interplay with each other to regulate the lipogenic gene program.

In summary, our studies demonstrate that RYGB alters mTOR-AKT2-Insig2 signaling activity in liver and mTOR signaling is necessary to the amelioration of NAFLD by down-regulating de novo lipid synthesis (Fig. 9). Furthermore, high-fat diet-induced and rapamycin-induced steatosis could be reversed by RYGB in mice. Hepatic mTOR signaling may represent a novel mechanism responsible for the metabolic benefit of RYGB on NAFLD, thus providing a potential target for the therapy of NAFLD by “pharmaceutical gastric bypass”.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Statement of ethics

Animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publications No. 8023, revised 1978). All animal protocols were approved by the Animal Care and Use Committee of Jinan University.

Disclosure statement

None.

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Author contributions

Geyang Xu designed research; Qinling Pan, Tingfeng Qin, Yuan Gao, Shaojian Li, Danjie Li, Miao Peng and Hening Zhai performed research;

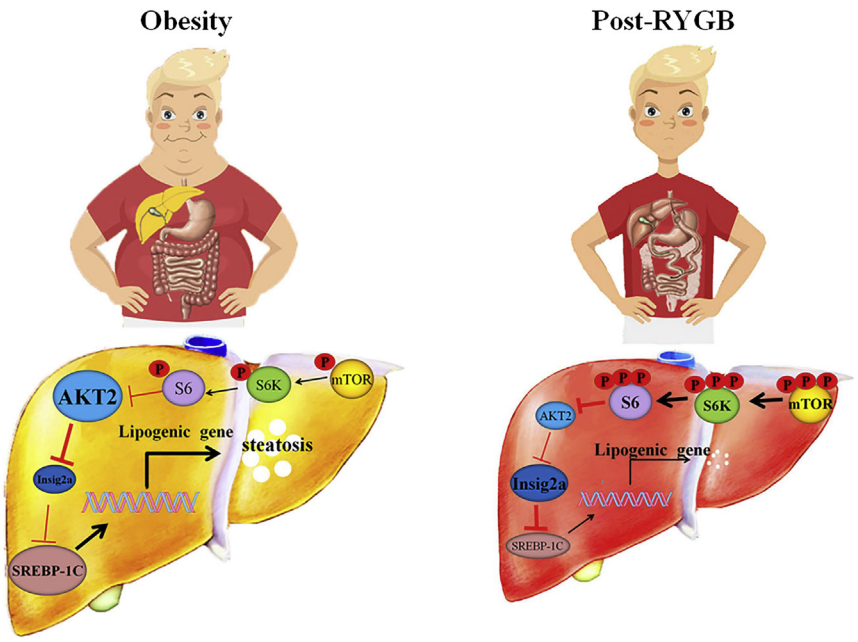

Fig. 9. Summary of the Roux-en-Y Gastric Bypass–mediated development of NAFLD through mTOR-AKT2-Insig2 signaling.
After RYGB, hepatic mTOR activity and Insig2a are activated, whereas AKT2 is decreased. The suppression of hepatic AKT2 substantially elicits an enhancement in Insig2a and repression of lipogenic genes after RYGB.

Qinling Pan, Tingfeng Qin and Geyang Xu analyzed data; Geyang Xu wrote and edited the paper. All authors contributed to the discussion and revised the article and all approved the final versions of the manuscript. Geyang Xu is responsible for the integrity of the work as a whole.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.12.014.

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