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mTOR Signaling in X/A-Like Cells Contributes to Lipid Homeostasis in Mice

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Gastric mechanistic target of rapamycin (mTOR) signaling is inversely associated with the expression and secretion of ghrelin, a 28-aa peptide hormone produced by gastric X/A-like cells. Ghrelin contributes to obesity and hepatic steatosis. We sought to control global lipid metabolism via the manipulation of gastric mTOR signaling in X/A-like cells. We established a *ghrl-cre* transgene in which the Cre enzyme is expressed in X/A-like cells under the control of the ghrelin-promoter. *mTOR*$^{flox/flox}$ and tuberous sclerosis 1 (*TSC1*)$^{flox/flox}$ mice were separately bred with *ghrl-cre* mice to generate *mTOR-ghrl-cre* or *TSC1-ghrl-cre* mice, within which mTOR signaling was suppressed or activated, respectively. Lipid metabolism in liver and adipose depots was analyzed. Under the control of the ghrelin-promoter, the Cre enzyme was exclusively expressed in stomach X/A-like cells in adult animals. Knockout of mTOR in X/A-like cells increased circulating acyl-ghrelin and promoted hepatic lipogenesis with effects on adipose depots. Activation of mTOR signaling by deletion of its upstream inhibitor, TSC1, decreased ghrelin expression and secretion, altering lipid metabolism as evidenced by resistance to high-fat diet–induced obesity and hepatic steatosis. Both ghrelin administration and injection of rapamycin, an inhibitor of mTOR, altered the phenotypes of *TSC1-ghrl-cre* mice. *Conclusion:* Gastric mTOR signaling in X/A-like cells contributes to organism lipid homeostasis by regulating hepatic and adipose lipid metabolism. Gastric mTOR signaling may provide an alternative strategy for intervention in lipid disorders. (Hepatology 2019;69:860-875).

Mechanistic target of rapamycin (mTOR), a highly conserved serine-threonine kinase, is an intracellular energy sensor.[1,2] mTOR activity is dynamically regulated by nutrients, energy supply, and various hormones. Complex relationships between mTOR and food intake, obesity, and glucose metabolism have been demonstrated in hypothalamus, liver, and skeletal muscle using various approaches including genetic manipulation, diet, and pharmacological methods.[3-6] Less attention has been focused on the possibility of fuel sensing by the gastrointestinal tract, despite its critical role in the regulation of food intake, and glucose and lipid homeostasis.

Ghrelin, the only known peripherally circulating orexigenic hormone, is produced by gastric X/A-like cells, a distinct population that comprises 20%-30% of all endocrine cells in the oxyntic gland.[7] This peptide consists of 28 amino acids. Encoded by 5 exons, preproghrelin undergoes endoproteolytic processing and posttranslational modification to yield des-acyl ghrelin and acyl-ghrelin. For acyl-ghrelin, acylation of the third serine is required for stimulation of growth hormone release and feeding effects.[8] In addition to its orexigenic effects, ghrelin also demonstrates a lipogenic effect through a mechanism independent of energy intake. Although

Abbreviations: BAT, brown adipose tissue; dgat, diacylglycerol O-acyltransferase; DMSO, dimethyl sulfoxide; eWAT, epididymal white adipose tissue; fasn, fatty acid synthase; GLP1, glucagon-like peptide 1; gpam, glycerol-3-phosphate acyltransferase, mitochondrial; HFD, high-fat diet; HSL, hormone-sensitive lipase; mG, mTOR-ghrl-cre; mTOR, mechanistic target of rapamycin; NCD, normal chow diet; pparγ, peroxisome proliferator-activated receptor gamma; pS6k1, phospho-p70 S6k1; RG, ROSA-ghrl-cre; S6K1, S6 kinase beta-1; srebf, sterol regulatory element-binding protein; sWAT, subcutaneous white adipose tissue; TG, TSC1-ghrl-cre; TSC1, tuberous sclerosis 1; ucp, uncoupling protein; WAT, white adipose tissue; and WT, wild type.

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low-dose ghrelin (12 nmol/kg/day) injection has no effect on food intake, it still increases lipogenesis in white-adipose and brown-adipose tissues.[9] Our previous studies also demonstrated that acyl-ghrelin contributes to rapamycin-induced glucose metabolic disorders[10] and promotes high-fat diet (HFD)-induced hepatic steatosis.[11] Conversely, ghrelin receptor (*ghsr*) ablation protects mice from HFD-induced obesity and steatosis, and increases thermogenic capacity.[11-13] Therefore, targeting ghrelin production might provide a strategy for improving global lipid metabolism.

Production of ghrelin from gastric X/A-like cells is tightly linked with organism energy supply.[14,15] Studies using the pharmacological approach suggest that gastric mTOR may serve as a critical molecule mediating the effect of energy supply on the production of ghrelin.[10,14,16] Xu et al. demonstrated that (1) HFD feeding activates gastric mTOR signaling activity while inhibiting ghrelin production; (2) fasting inhibits gastric mTOR activity while stimulating ghrelin production; and (3) rapamycin, an inhibitor of mTOR, increases gastric and circulating ghrelin levels and subsequently stimulates food intake.[10,14,16] These observations suggest that mTOR signaling functions as a fuel-sensing mechanism in the gastric mucosa to link the production of ghrelin with organism energy supply. Because of the limits of pharmacological approaches, the mechanisms by which gastric mTOR signaling contributes to energy metabolism are not well defined.

To better define the specific roles of gastric mTOR signaling on ghrelin production and its control on organism lipid metabolism, we established transgenic animal models in which mTOR signaling is specifically altered in gastric X/A-like cells. Our studies demonstrate that gastric mTOR signaling in X/A-like cells contributes to the regulation of hepatic and adipose lipid metabolism partially through regulation of acyl-ghrelin.

Materials and Methods

ANIMALS

Animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23, revised 1985). All experimental protocols were approved by the Animal Care and Use Committee of Peking University (Permit Number: LA2012-60) and the University of Michigan. *mTOR-Ghrl-/-* (mG) mice or tuberous sclerosis 1 *(TSC1)-Ghrl-/-* (TG) mice were generated by crossing *ghrl-cre* mice with $mTOR^{loxp/loxp}$ mice or $TSC1^{loxp/loxp}$ mice, both of which were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed in standard plastic rodent cages and maintained in a regulated environment (24°C, 12-hour light and 12-hour dark cycles with lights on at 7:00 a.m.). Normal chow and water were available *ad libitum* unless specified otherwise. Interventions were done during the light cycle.

DIETS

Four-week-old male mice were assigned to receive normal chow diet (NCD) (control diet, D12450H; Research Diets, New Brunswick, NJ) or a HFD (60% fat, D12492; Research Diets) for 12 weeks.

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SURGERY AND IMPLANTATION OF OSMOTIC MINI-PUMPS

Mice were anesthetized with ketamine (100 mg/kg body weight) and xylazine (6 mg/kg body weight) by intraperitoneal injection. A 1-cm incision was made in the back skin and mice were implanted subcutaneously with an ALZET osmotic mini-pump (model 1002; DURECT Corp., Cupertino, CA) filled with vehicle (saline) or acyl-ghrelin (11 nmol·kg^{-1}·d^{-1}), or insulin (0.5 U/d), for 14 days. Before implantation, pumps were filled with the test agent and placed in a petri dish with sterile 0.9% saline at 37°C for at least 4 hours before implantation to prime the pumps.

COLD EXPOSURE

Mice were placed in a 4°C cold room for 6 hours and rectal temperature measured every hour during the cold challenge with a rectal probe (Braintree Scientific, Braintree, MA).

TISSUE SAMPLE PREPARATION AND IMMUNOFLUORESCENT STAINING

C57BL/6J mice were deeply anesthetized using pentobarbital (0.07 g·kg^{-1}). Tissues were quickly removed and rinsed thoroughly with phosphate-buffered saline, then fixed in 4% paraformaldehyde (weight per volume), dehydrated, embedded in tissue optimum cutting temperature compound or paraffin, and sectioned at 6 μm. Immunofluorescent staining on paraffin-embedded sections was performed as previously described.[14]

WESTERN BLOTTING AND QUANTITATIVE RT-PCR

Tissues were homogenized in radio immunoprecipitation assay buffer. Proteins were extracted, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membrane, and immunoblotted using the specific antibodies provided in supplemental materials. Total RNA was isolated using Trizol from Invitrogen. Reverse-transcription and real-time quantitative PCR were performed as previously described.[11]

STATISTICAL ANALYSIS

All values were expressed as mean ± SEM. Statistical differences were evaluated by two-way analysis of variance and Newman–Student–Keuls test. Comparisons between the two groups involved use of the Student *t* test. $P < 0.05$ denotes statistical significance.

Results

ACTIVATION OF GASTRIC mTOR SIGNALING BY TSC1 KNOCKDOWN DECREASES GHRELIN, FOOD INTAKE, AND HEPATIC LIPID CONTENT

Because ghrelin is known for its orexigenic and lipogenic effects, and is regulated by mTOR[14,15] and its downstream molecular, ribosomal protein S6 kinase beta-1 (S6K1) (Supporting Fig. S1), we hypothesized that mTOR signaling in X/A-like cell affects organism-level metabolism by manipulating ghrelin production. To test this hypothesis, we first used local injection through the left gastric artery to deliver Cre adenovirus into *TSC1*$^{flox/flox}$ mice to knockdown gastric TSC1, an upstream inhibitor of mTOR activity. The feasibility of local injection was validated by typan-blue injection (Fig. 1A), which showed that only the body of the stomach was affected without leak to other organs around the stomach. Knockdown of gastric TSC1 validated by western blot was associated with activation of mTOR signaling, as evidenced by the phosphorylation of its downstream modeluce-S6 (Fig. 1B). Furthermore, activation of gastric mTOR signaling by knocking down TSC1 inhibited ghrelin production (Fig. 1C) and food intake, and showed a trend of decrease in body weight (Fig. 1D). Hepatic triglyceride content was decreased by activation of mTOR signaling in the stomach (Fig. 1E). These observations indicate that it is feasible to deliver modified genes that are relevant to mTOR signaling to the stomach with local expression. Crucially, activation of gastric mTOR signaling inhibits ghrelin mRNA production, food intake, and hepatic lipid content. A limitation of this approach is that mTOR signaling exists in various cell populations in the stomach, and knockdown of the TSC1 gene by left gastric artery injection is not cell-specific. To overcome this difficulty, and to assess the specific effects of mTOR signaling in X/A-like cells, we established a transgenic mouse in which genes related to mTOR signaling are manipulated specifically in X/A-like cells.

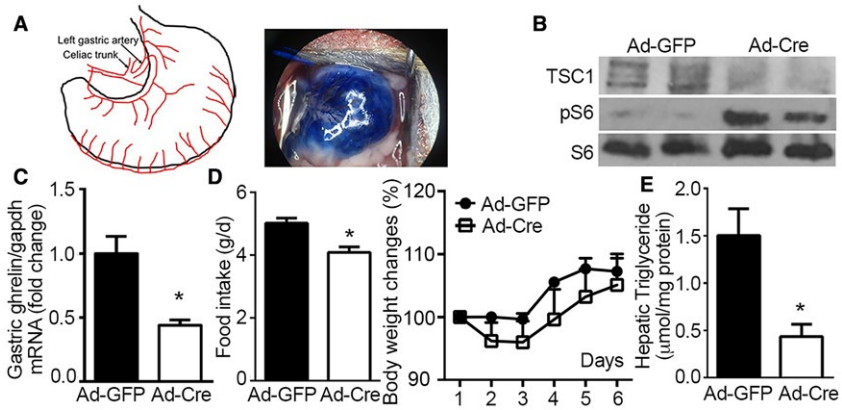

FIG. 1. Effects of local manipulation of gastric mTOR signaling by Cre adenovirus. Laparotomy was performed on 12-week-old *TSC1*$^{flox/flox}$ mice to expose the left gastric artery, through which Cre adenovirus (Ad-Cre) (1×10^9 plaque-forming units) was delivered locally to the stomach. Green fluorescent protein (GFP) adenovirus (Ad-GFP) was used as control. All values are expressed as mean ± SEM. * indicates $P < 0.05$ versus Ad-GFP group, n = 12 mice for each condition. (A) Schematic diagram of blood supply for stomach and location of left gastric artery for injection. Tracing of typan-blue injected through the left gastric artery at a dose of 50 μL showed the feasibility of injection. (B) Knockdown of *TSC1* and activation of mTOR signaling in gastric mucosa were validated by western blotting. (C) Levels of ghrelin mRNA analyzed by real-time quantitative PCR. Glyceraldehyde phosphate dehydrogenase (*gapdh*) was used as reference to normalize ghrelin expression. (D) Food intake and percentage of body weight change were recorded daily for 1 week. (E) One week after injection, hepatic lipid was extracted and assayed for triglyceride. Abbreviations: Ad-Cre, Cre adenovirus; GFP, Green fluorescent protein; Ad-GFP, GFP adenovirus.

GENERATION OF X/A-LIKE CELL-SPECIFIC CRE TRANSGENIC MICE

To specially modulate mTOR signaling in X/A-like cells, we used the ghrelin promotor to drive Cre enzyme expression (Fig. 2A). First-generation pups of different founders were sacrificed to screen for Cre high-expressing lines. Representative lines are shown in Fig. 2B. Only transgenic lines with high expression of Cre mRNA in the stomach were selected to breed with Gt(Rosa)26Sor^{tm1sor}/J mice, which carry the *loxP*-flanked DNA STOP sequence, preventing expression of the downstream *LacZ* gene. In these *ROSA-ghrl-cre* (RG) mice, ghrelin-cre-positive cells express β-galactosidase, because the STOP sequence is removed by the Cre enzyme and *lacZ* is expressed. By detecting β-galactosidase, we evaluated the efficiency of the Cre enzyme and the specificity of different lines. No positive signal was shown in the hypothalamus (Fig. 2C). Because ghrelin is expressed in both the pancreas and stomach during development,[17,18] we monitored gastric and pancreatic β-galactosidase-positive signals at different ages of RG mice (Supporting Fig. S2). Consistent with ghrelin expression levels, β-galactosidase signal was negligible in the neonatal stomach (Supporting Fig. S2A), then significantly increased after weaning (Supporting Fig. S2A) until adulthood (Fig. 2D). Modest levels of Cre activity was detected in neonatal islets (Supporting Fig. S2B). Further, costaining β-galactosidase with chromogranin A (marker for endocrine cells) and insulin (marker for β cells) demonstrated that all β-galactosidase-positive cells are also positive for chromogranin A and most are also insulin-positive (Supporting Fig. 2C,D). This observation indicates that all *ghrl-cre* cells are endocrine cells. After weaning, both ghrelin and β-galactosidase decreased significantly (Supporting Fig. S2B) in pancreatic islets. At 12 weeks of age, most β-galactosidase-positive cells disappeared from the pancreas (Fig. 2E). Of note, no differences in body weight, food intake, serum glucose, plasma ghrelin levels, liver, and epididymal white adipose tissue (eWAT) weights (Supporting Fig. S2E) were detected between *ghrl-cre*-positive animals and their wild-type (WT) littermates.

X/A-LIKE CELL-SPECIFIC KNOCKOUT OF mTOR INCREASES TRIGLYCERIDE ACCUMULATION IN LIVER

After validating the cell specificity of *ghrl-cre*, we bred these mice with *mTOR*$^{flox/flox}$ mice to generate a X/A-like cell-specific mTOR knockout animal

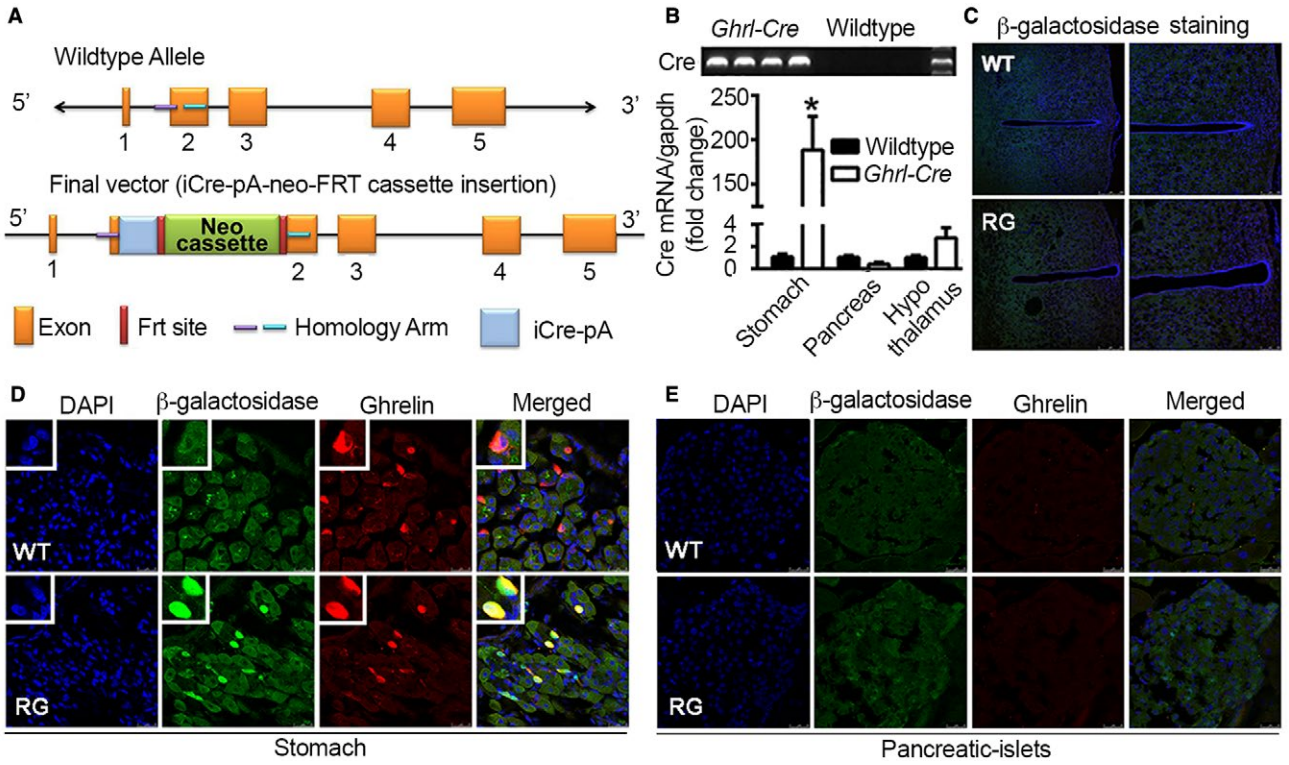

FIG. 2. Establishment of ghrelin-cre (*ghrl-cre*) transgenic mice. (A) Schematic diagram for the generation of ghrelin-cre mice. A ghrelin bacterial artificial chromosome construct containing the 59.36-kb sequence upstream the anti-thymocyte globulin code was used. The first 29 base pair of the ghrelin coding sequence was replaced by the coding sequence of the *iCre* gene followed by an SV40 polyadenylation (pA) signal. (B) Screening for the transgenes with high expression of the *cre* gene. Primers were designed to target the *cre* sequence. mRNAs of stomach, pancreas, and hypothalamus from WT and *ghrl-cre* animals were used for complementary DNA preparation. (C-E) Validation of the Cre enzyme in hypothalamus (C), stomach (D), and pancreatic islets (E). The *ghrl-cre* mice were bred with Gt(Rosa)26Sor^{tm1sor}/J mice (RG), which carry the β-galactosidase reporter gene. RG mice and WT littermates at 12 weeks old were perfused with 4% paraformaldehyde. Brain, stomach, and pancreas were harvested, sectioned, and stained for β-galactosidase (green) and ghrelin (red). Nuclei were stained with DAPI (blue). n =12 and 15 for WT littermates and transgene, respectively.

model. Only *ghrl-cre*-positive and *mTOR*$^{flox/flox}$ homozygous mice were selected as mG (Supporting Fig. 3A). Immunofluorescent staining of stomach showed colocalization of phospho-p70 S6k1 (pS6k1), a downstream molecule of mTOR signaling, and ghrelin, in WT mice. Significant reduction of pS6K1 was observed in ghrelin-positive X/A-like cells of mG mice, indicating a decrease in mTOR signaling in these cells (Fig. 3A). Consistent with our previous finding that inhibition of mTOR signaling by systemic rapamycin injection increases ghrelin secretion,[14] circulating active-ghrelin (acyl-ghrelin) was significantly increased in mG mice relative to WT littermates (Fig. 3B). Phenotype analysis of this animal model showed that overall body weight and most tissue weights were similar between mG mice and WT littermates. Stomach weight and eWAT weights were higher (Supporting Fig. S3B). Interestingly, hepatic triglyceride content was increased in mG mice (Supporting Fig. S3C,D and Fig. 3C), whereas circulating triglyceride and cholesterol remained virtually unaltered. Associated with the alteration in hepatic triglyceride content was a significant increase in mRNA levels of lipogenesis-related genes, such as sterol regulatory element-binding protein 1 (*srebf1*), peroxisome proliferator-activated receptor gamma (*pparγ*), fatty acid synthase (*fasn*), glycerol-3-phosphate acyltransferase, mitochondrial (*gpam*), and diacylglycerol O-acyltransferase 1 (*dgat1*). Genes related to β-oxidation and lipid transport remained largely unchanged (Fig. 3D). Moreover, genes related to lipid absorption in the small intestine remained largely

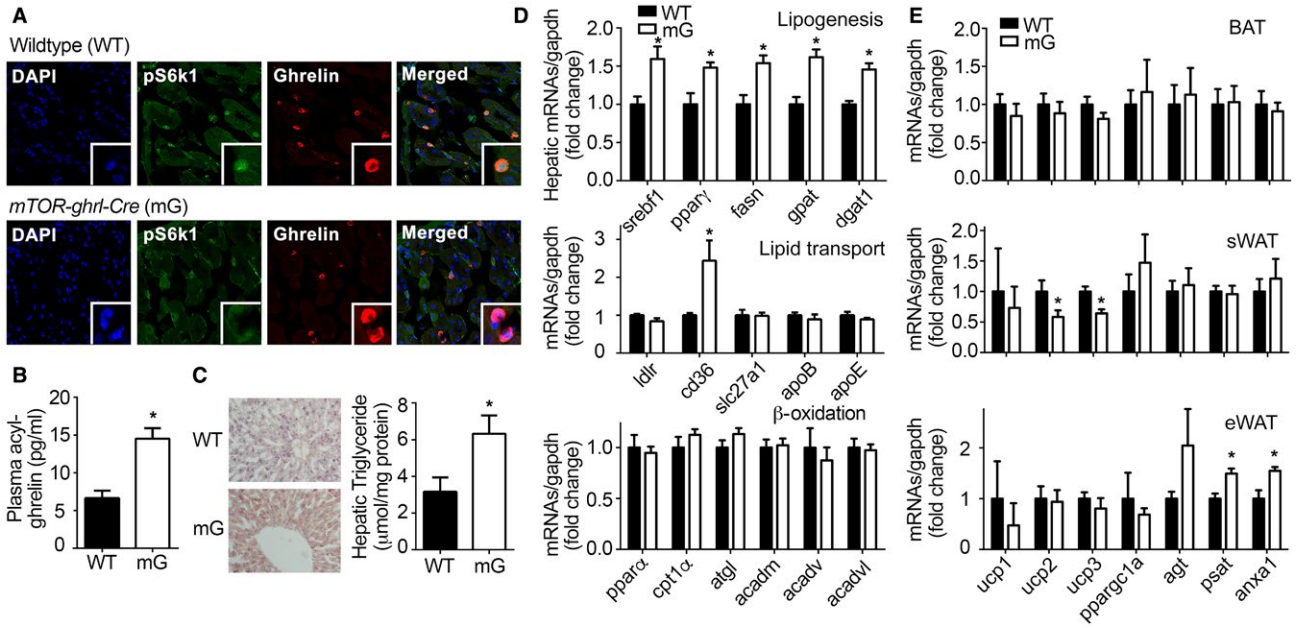

FIG. 3. Effects of mTOR suppression in X/A-like cells. The *ghrl-cre* mice were bred with *mTOR* $^{flox/flox}$ mice to generate X/A-like cell-specific knockout of mTOR mice (mG mice). Eight-week-old animals were used to assess mTOR signaling in X/A-like cells and analysis of lipid metabolism in liver and adipose tissues. Results were expressed as mean ± SEM. n = 8 for each group. **P* < 0.05 versus WT. (A) Colocalization of pS6k1 (green) and ghrelin (red) in stomach of WT and mG mice. Nuclei were stained with DAPI (blue). (B) Plasma levels of acyl-ghrelin. (C) Oil Red O staining and hepatic triglyceride content were measured by colorimetric assay and normalized by protein level. (D) mRNA levels of lipogenesis-related, lipid transport–related, and β-oxidation-related genes were determined by real-time quantitative PCR and normalized by *gapdh*. (E) mRNAs from BAT, sWAT, and eWAT were analyzed for expression levels of brown (*ucp1-3* and *ppargc1γ*) or white adipose (*agt*, *psat,* and *anxa1*) markers; *gapdh* was used to normalize results. Abbreviation: mG mice, *mTOR-ghrl-cre* mice.

unaltered (Supporting Fig. S3E) except the gene of *FABP4*, indicating that the increase of lipid content in transgenic mice may not be due to increased absorption of lipids.

To further examine therapeutic potential, hepatic enzymes and genes relative to proliferation and senescence, apoptosis and fibrosis, were assayed. Hepatic alanine aminotransferase content was markedly increased in mG mice fed with HFD relative to the obese WT littermates (Supporting Fig. S3F). Immunostaining of p16 and proliferating cell nuclear antigen, as well as TUNEL (terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling) staining, showed no obvious change in mG mice and WT littermates (Supporting Fig. S3G). There were no significant changes in genes related to proliferation and senescence. Interestingly, genes related to fibrosis such as α smooth muscle actin and transforming growth factor β1 were increased in mG mice fed HFD (Supporting Fig. S3H). However, ghrelin only demonstrated a mild effect on proliferation-related and fibrosis-related genes in cultured LX2 cells, a hepatic stellate cell line (Supporting Fig. S3I). Together with our previous studies showing the presence of functional ghrelin receptor and its direct stimulation of *de novo* lipogenesis in hepatocytes, this observation focused on subsequent experiments on lipid metabolism.

No distinguishable alteration in adipose tissue markers was observed in brown adipose tissue (BAT). mRNA levels for uncoupling protein (*ucp*) 2 and *ucp3* decreased significantly in subcutaneous white adipose tissue (sWAT) from mG mice. White adipose tissue (WAT) development–related genes such as *psat* and *anxa1* were increased in eWAT from mG mice versus WT littermates (Fig. 3E). Because fat depots are highly innervated, sympathetic tone significantly affects the adipose phenotype. We recorded heart rate and mean blood pressure as a measurement of the

sympathetic activity; neither showed any difference between mG and WT littermates (Supporting Fig. S3J).

X/A-LIKE CELL-SPECIFIC ACTIVATION OF mTOR SIGNALING ALTERS ORGANISM LIPID METABOLISM

To activate mTOR signaling in X/A-like cells, we knocked out TSC1, an upstream inhibitor of mTOR signaling, by breeding the *TSC1*$^{flox/flox}$ mice with *ghrl-cre* mice to generate TG mice (Supporting Fig. S4A). Activation of mTOR signaling in X/A-like cells was validated by the costaining of pS6k1 and ghrelin. Relative to WT littermates, nuclear translocation of pS6k1 was observed (Fig. 4A) in X/A-like cells of TG mice. S6K1 has been reported to exist in both cytoplasmic and nuclear compartments under basal conditions and to translocate into nuclei on activation of mTOR signaling.[19] Strikingly, TG mice showed lower body weights (Supporting Fig. S4B), and less eWAT and sWAT weights relative to WT littermates under both NCD and HFD conditions (Supporting Fig. S4C). There was a trend of decreasing food intake in TG mice compared with the WT littermates, but it was not statistically significant. Hypothalamic genes related to regulation of food intake (*NPY, AgRP,* and *POMC*) were not altered in TG mice (Supporting Fig. S4D).

To explore the mechanisms by which a lean phenotype is induced by X/A-like cell–specific activation of mTOR signaling, production of ghrelin in the stomach was examined. Protein expression and secretion of ghrelin were significantly decreased in TG mice compared with WT littermates (Fig. 4B). Consistent with the pro-lipogenesis function of ghrelin in both liver and adipose tissues, decreased circulating ghrelin was associated with lower hepatic lipid content, as shown by Oil Red O staining in HFD animals (Fig. 4C). We detected lower hepatic and circulating triglyceride levels in TG mice fed a HFD for 12 weeks (Fig. 4D). Levels of lipogenesis-related transcriptional factors (*srebf1*, *srebf2*, *pparγ*, and *pparγ2*), enzymes (*acaca, fasn, dgat1, dgat2, gpam,* and *scd1*), and lipid transporter (*cd36*) were significantly decreased in liver from TG mice fed HFD. In addition, levels of β-oxidation-related genes were markedly increased in TG mice fed either NCD or HFD (Fig. 4E). No change in either hepatic or plasma cholesterol contents was observed (Supporting Fig. 4E). Transcription of genes related to cholesterol metabolism demonstrated no statistical difference (Supporting Fig. 4F). Intestinal lipid absorption–related genes were not changed between TG mice and their WT littermates fed either NCD or HFD (Supporting Fig. 4G), indicating that the lipid-lowering effects are not due to malabsorption.

To further examine therapeutic potential, mRNA levels of genes related to proliferation, senescence, and fibrosis were examined (Supporting Fig. 4H). Both *cyclinD1* and *myc* were significantly increased in TG mice under the condition of NCD. For genes related to senescence, only *p21* showed a significant reduction in TG mice fed NCD. No obvious change of fibrosis-related genes was detected.

In addition to amelioration of HFD-induced liver steatosis, TG mice were resistant to HFD-induced hypertrophy in WAT depots. Histology of BAT, sWAT, and eWAT showed that adipocyte sizes in TG mice were smaller than those in WT littermates under either NCD or HFD conditions (Fig. 5A,C,E). *De novo* lipogenesis related genes were significantly decreased under the HFD condition, whereas lipid uptake–related gene expression remained unchanged in TG mice (Supporting Fig. S5A). To further investigate whether lipolysis also contributes to the lean phenotype of TG mice, phosphorylation of hormone-sensitive lipase (HSL) was detected in eWAT. Although both phosphor-HSL and HSL protein levels increased significantly in TG mice, the ratio of its phosphorylation level remained unaltered (Supporting Fig. S5B). Adipocyte marker genes related to browning, including *ucp 1-3* and *ppargc1a*, were significantly increased, whereas genes related to WAT development, such as *agt, psat, ednra* and *anxa1*, were decreased in TG mice fed NCD or HFD relative to WT littermates (Fig. 5B,D,F). Moreover, the thermoregulatory gene (*Cox8b*) and mitochondrial adenosine triphosphate synthase (*ATP5B*) were also elevated in sWAT of TG mice relative to WT littermates (Supporting Fig. S5C). At room temperature, the respiratory quotient was decreased in TG mice fed with HFD, indicating that TG mice consume more lipids (Supporting Fig. S5D). Moreover, the activity of TG mice was also higher than WT littermates under the HFD condition (Supporting Fig. S5D). Consistently, TG mice were resistant to 4°C cold exposure–induced body temperature drop relative to

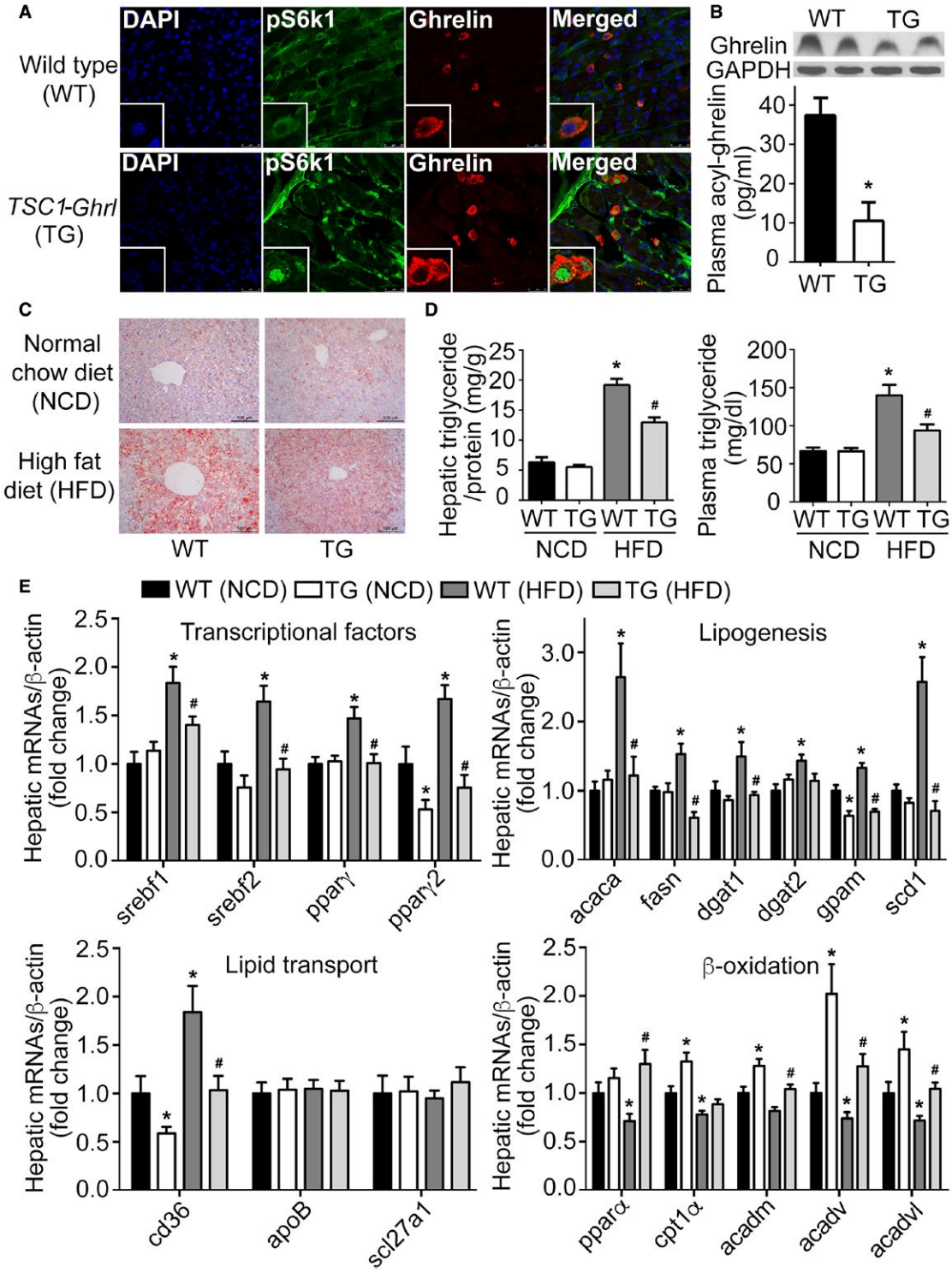

FIG. 4. Activation of mTOR signaling in X/A-like cells alters HFD-induced hepatic steatosis. *TSC1*$^{flox/flox}$ mice were bred with *ghrl-cre* mice to generate TG animals, in which mTOR signaling is specifically activated in X/A-like cells. Results were expressed as mean ± SEM. n = 20 for each group. *P < 0.05 versus WT (NCD); #P < 0.05 versus WT (HFD). (A) Colocalization of pS6k1 (green) and ghrelin (red) in stomach of 12-week-old WT littermates and TG mice. Nuclei were stained with DAPI (blue). (B) Gastric and plasma ghrelin were measured by western blot and enzyme-linked immunosorbent assay. (C) Oil Red O staining. (D) Hepatic and circulating triglyceride contents were measured by colorimetry. (E) Levels of lipogenesis-related, lipid transport–related, and β-oxidation-related genes were determined by real-time quantitative PCR and normalized to *β-actin*.

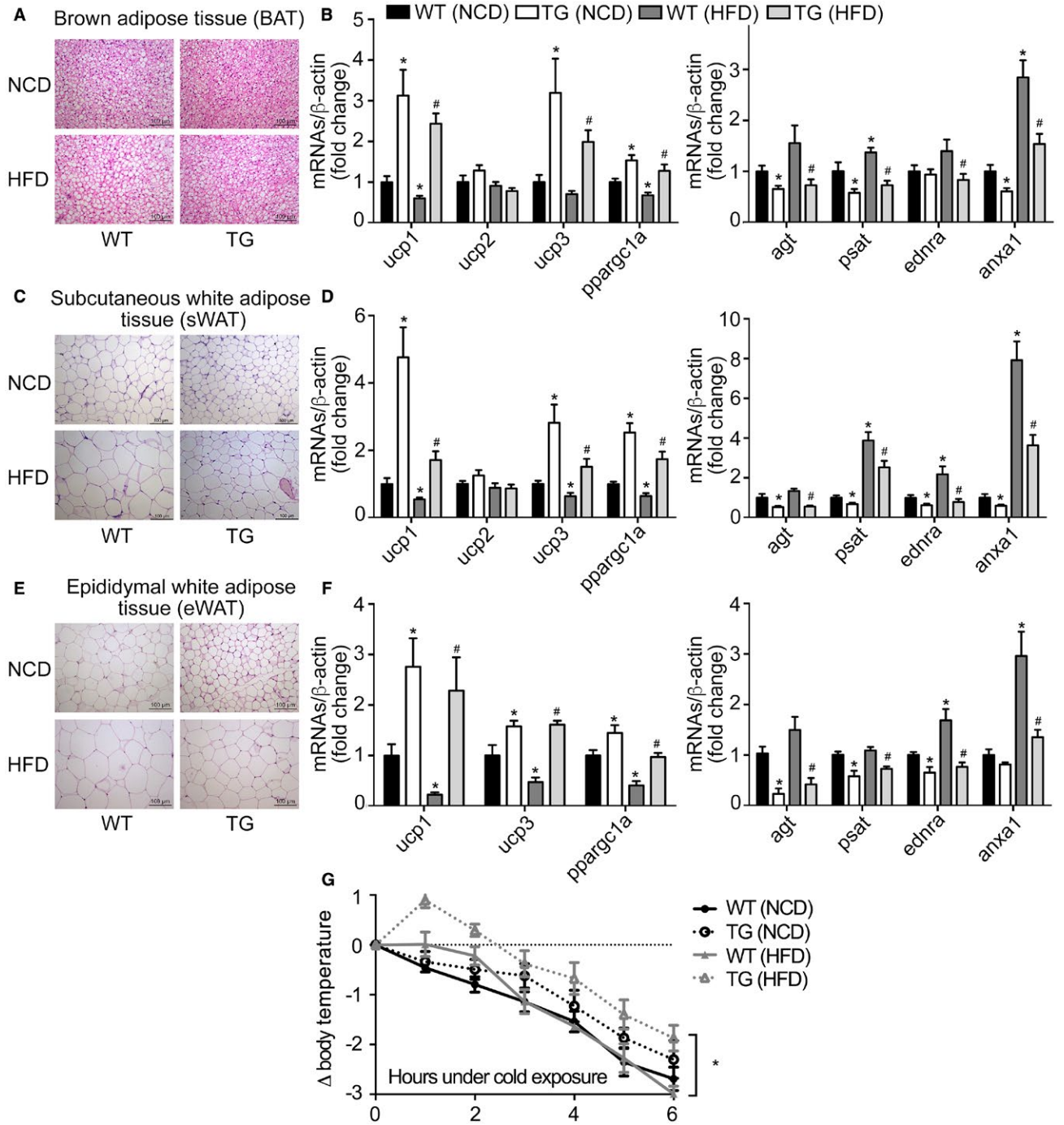

FIG. 5. Activation of mTOR signaling in X/A-like cells increases the brown phenotype of BAT and WAT depots. Histology of BAT (A), sWAT (C), and eWAT (E) shown by hematoxylin and eosin staining. mRNA levels of brown-adipocyte and white-adipocyte marker genes were detected by real-time quantitative PCR in BAT (B), sWAT (D) and eWAT (F), and normalized to *β-actin*. Fold changes were expressed as mean ± SEM. n = 20 for each group. $^{*}P < 0.05$ versus WT mice fed NCD; $^{\#}P < 0.05$ versus WT littermates fed HFD (Student *t* test). (G) Mice were placed in cold room (4°C) for 6 hours and rectal temperature was measured every hour. n = 6 per group. $^{*}P < 0.05$ indicates TG versus WT mice fed HFD.

WT littermates, with larger extent in HFD-induced obese animals (Fig. 5G). Body temperature was lower in HFD-induced obese mice (39.05°C in NCD versus 38.39°C in HFD) (Supporting Fig. S5E), which is consistent with previous reports.[20,21] Because oleic acid (18:1) has been reported to activate oxidation of fatty acids in the differentiated C2C12 cells,[22] we next examined the free fatty acid profile to determine whether mono-unsaturated fatty acids contribute to cold resistance in TG mice. As shown in Supporting Fig. S5F, oleic acid (18:1) and several saturated fatty acid species (e.g., 18:0, 20:0, and 22:0) were significantly increased in TG mice fed NCD. Of note, free fatty acids 18:1 and 22:0 in TG mice fed HFD demonstrated no significant difference relative to WT littermates fed HFD. Consistent with the elevation of β-oxidation-related gene expression in liver, these genes were also increased in skeletal muscle of TG mice fed HFD, indicating an increase in the global fatty acid use in these transgenic mice (Supporting Fig. S5G). In addition, heart rate and mean blood pressure were unchanged in TG mice (Supporting Fig. S5H), indicating that the lipid-lowering effects may not act through effects of the sympathetic system.

GLOBAL LIPID-LOWERING EFFECTS OF mTOR ACTIVATION IN X/A-LIKE CELLS ARE INDEPENDENT OF INSULIN DEFICIENCY

Unexpectedly, we observed a significant reduction in insulin levels, impaired glucose tolerance, and no change in insulin sensitivity in the TG mice. Neither change was observed in ghrelin-positive cells at neonatal stage nor pS6 staining at adult stage in islets of TG mice (Supporting Fig. S6A,B). To rule out that the lipid-lowering phenotypes in TG mice is caused by the insulin deficiency, we supplemented TG mice with exogenous insulin (0.5 U/d) for 2 weeks by subcutaneous osmotic mini-pump and assayed the glucose and lipid metabolism. Although insulin administration normalized the serum insulin levels and glucose to levels of WT littermates (Supporting Fig. S6C,D), genes related to lipogenesis and lipid transportation in liver, browning markers, and whiting markers in sWAT and BAT were not affected, similar to TG mice receiving saline treatment (Supporting Fig. S6E-G). These data demonstrate that the lipid-lowering effects in TG mice are independent of insulin levels.

EXOGENOUS ACYL-GHRELIN PARTIALLY ELIMINATES THE LEANER PHENOTYPES IN TG MICE

The observation that ghrelin production and secretion in TG mice were significantly reduced prompted us to examine whether resistance to HFD-induced obesity and steatosis in these transgenes relies on the suppression of ghrelin. We rescued plasma acyl-ghrelin in TG mice to levels observed in WT littermates by infusing acyl-ghrelin peptide through osmotic min-pump implantation. Administration of acyl-ghrelin demonstrated no significant effect on the activation of mTOR signaling in X/A-like cells as measured by co-immunostaining of phosphor-mTOR and ghrelin (Supporting Fig. S7A-B) in TG mice. Consistent with our previous study,[11] ghrelin directly stimulated lipogenesis in primary hepatocytes (Supporting Fig. S7C), leading to the increase of hepatic lipid accumulation in WT animals. Further, the decrease of hepatic lipid storage in TG mice was partially reversed by exogenous acyl-ghrelin (Fig. 6A). Administration of acyl-ghrelin partially reversed the reduction of hepatic and circulating triglyceride in TG mice (Fig. 6B). Exogenous ghrelin also reversed the suppression of lipogenesis-related genes including *srebf, pparγ, fasn, dgat,* and *gpam* in TG mice to levels similar to WT littermates (Fig. 6C). Application of exogenous acyl-ghrelin demonstrated little effect on the expression of lipid transport–related and β-oxidation-related genes. Similarly, the decrease of adipocyte size was partially reversed by acyl-ghrelin administration (Fig. 6D and Supporting Fig. S7D,F). Elevation of browning marker genes in sWAT of TG mice were also reversed by acyl-ghrelin. Among the white adipocyte marker genes, *anxa1* was recovered (Fig. 6E). The upregulation of brown marker genes in BAT from TG mice were partially reversed by acyl-ghrelin administration (Supporting Fig. S7E), whereas *agt* was the only white adipocyte gene reversed by acyl-ghrelin (Supporting Fig. S7E). In eWAT of TG mice, *ucp1* was significantly suppressed

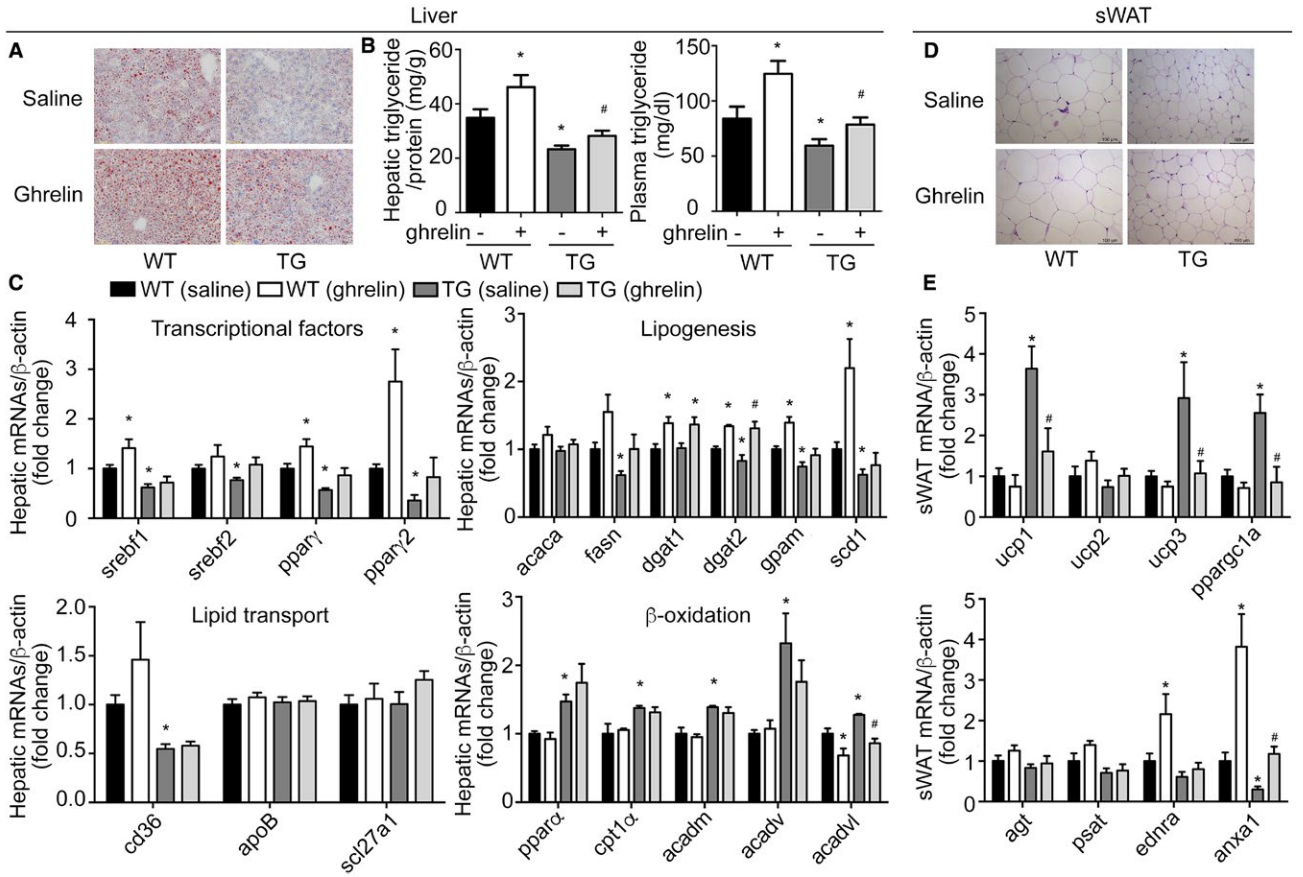

FIG. 6. Effects of ghrelin supplementation in TG mice. WT littermates and TG mice were fed HFD for 12 weeks and administered with acyl-ghrelin (11 nmol/kg/day) for 2 weeks through a subcutaneous osmotic mini-pump. Levels of mRNAs were determined by real-time quantitative PCR and normalized to *β-actin*. Fold changes were expressed as mean ± SEM. n = 10 for each group. $^{*}P < 0.05$ versus WT (saline). $^{\#}P < 0.05$ versus TG (saline). (A) Oil Red O staining. (B) Hepatic and circulating triglyceride content measured by colorimetric assay. (C) Hepatic mRNA levels of lipogenesis-related, lipid transport–related, and β-oxidation-related genes. (D) Representative hematoxylin and eosin staining of sWAT. (E) mRNAs levels of brown-marker or white-marker genes in sWAT.

by acyl-ghrelin, whereas the decrease of white adipocyte marker genes in TG mice was recovered by acyl-ghrelin (Supporting Fig. S7G).

INHIBITION OF mTOR SIGNALING BY RAPAMYCIN ELIMINATES METABOLIC BENEFITS ON LIPIDS IN TG MICE

To further confirm that mTOR signaling in X/A-like cells contributes to the improvement in lipid metabolism in TG mice, rapamycin, an inhibitor of mTOR activity, was intraperitoneally injected for 2 weeks into TG mice and WT littermates. This approach has been demonstrated to suppress gastric mTOR signaling in previous reports[10,14] and Supporting Fig. S8A in the current study. As shown in Fig. 7A, rapamycin injection in TG mice increased the circulating acyl-ghrelin to levels identical to WT littermates. The decrease in levels of hepatic and circulating triglyceride in TG mice was totally reversed to levels observed in WT littermates (Fig. 7B). mRNA levels of hepatic lipid metabolism-related genes were also restored by rapamycin (Fig. 7C). Similarly, elevation of most brown adipocyte marker genes in sWAT and BAT from TG mice were reversed to levels of WT littermates (Fig. 7D,E and Supporting Fig. S8B,C). The decrease of white adipocyte marker genes in eWAT was reversed by rapamycin injection (Supporting Fig. S8D,E).

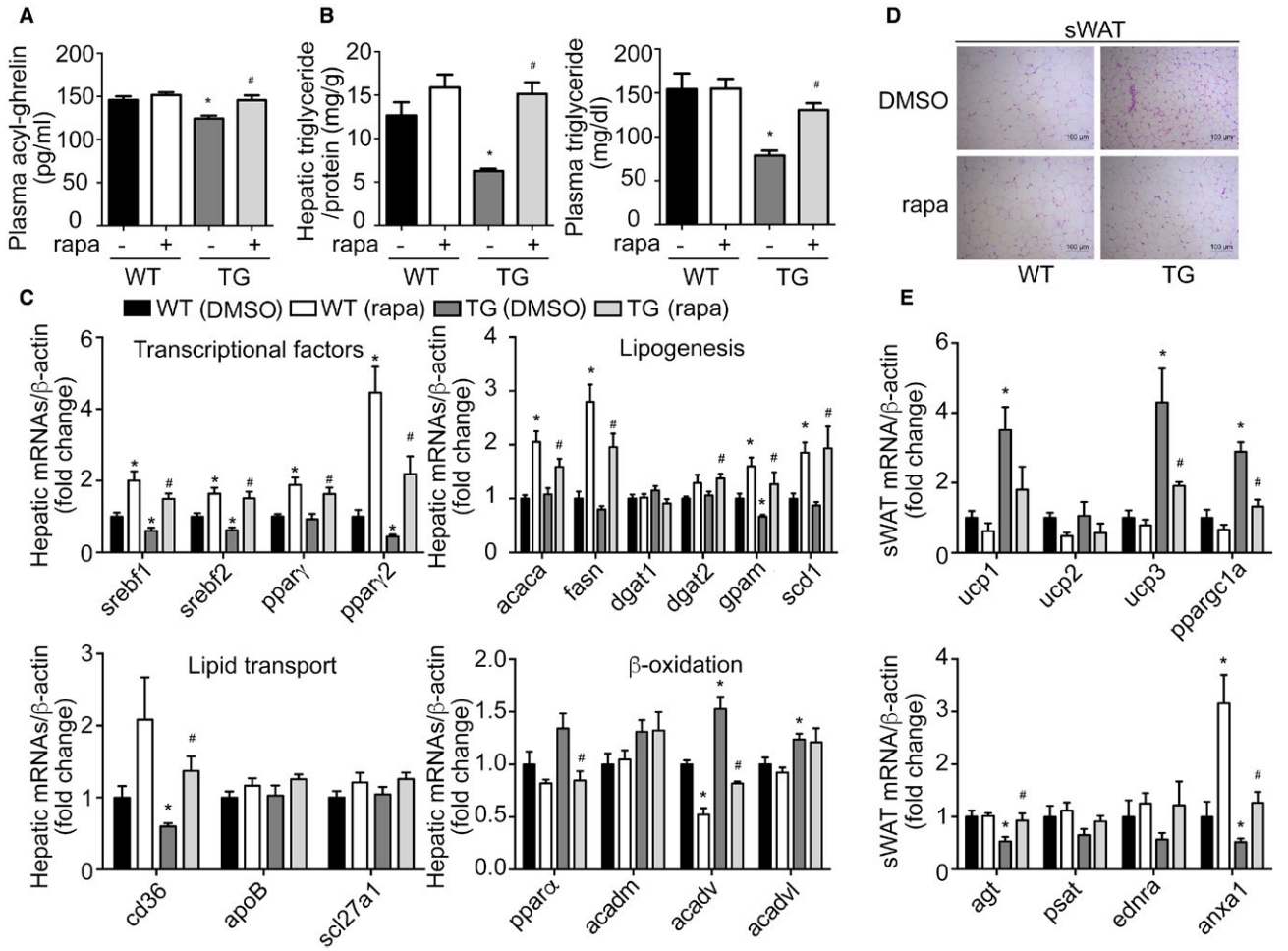

FIG. 7. Rapamycin reverses the lipid metabolic effects in TG mice. Rapamycin (1 mg/kg/day) was intraperitoneally injected into 16-week-old WT littermates or TG mice for 2 weeks. Levels of mRNAs were measured by real-time quantitative PCR and normalized to *β-actin*. Fold changes were expressed as mean ± SEM. n = 9 for each group. $^{*}P < 0.05$ versus WT (dimethyl sulfoxide [DMSO]), $^{\#}P < 0.05$ versus TG (DMSO). (A) Circulating levels of acyl-ghrelin in WT and TG mice treated with rapamycin (rapa) or control DMSO. (B) Hepatic and circulating triglyceride content measured by colorimetric assay. (C) Hepatic mRNA levels of lipogenesis-related, lipid transport–related, and β-oxidation-related genes. (D) Representative hematoxylin and eosin staining of sWAT. (E) mRNAs levels of brown-marker or white-marker genes in sWAT.

Discussion

By this gain-of-function or loss-of-function genetic approach, our studies provide evidence that mTOR signaling pathway in X/A-like cells plays a role in the regulation of organism lipid homeostasis. Using gastric targets to inhibit orexigenic hormonal signal may therefore provide a therapeutic strategy for intervention in obesity and lipid disorders. This concept is supported by the following findings: (1) Establishment of transgenic mice in which mTOR signaling gene in gastric X/A-like cells is specifically manipulated under the control of the ghrelin promoter allows us to explore the role of mTOR signaling pathway in the metabolic function of these cells; (2) deletion of mTOR genes in X/A-like cells increases acyl-ghrelin levels and subsequently induces hepatic steatosis in mice fed NCD; (3) deletion of TSC1 activates mTOR signaling in X/A-like cells, decreases ghrelin production, and protects animals from HFD-induced obesity and hepatic steatosis; (4) the metabolic effects of activation of mTOR signaling in X/A-like cells partially rely on suppression of ghrelin; and (5) inhibition of mTOR signaling by rapamycin reverses the phenotype of TG mice.

IN *GHRL-CRE* TRANSGENIC MICE, THE SPECIFICITY OF CRE EXPRESSION IN GASTRIC X/A-LIKE CELLS AND PANCREATIC ISLETS ARE AGE-DEPENDENT

Although we detected exclusive expression of Cre enzyme in X/A-like cells in adult animals, there were Cre-positive cells in the pancreas during early stages of development (Fig. 2 and Supporting Fig. S2B). This observation is consistent with previous studies demonstrating that ghrelin is transiently expressed in pancreatic islets during embryonic development.[17] The physiological significance of the transient expression of this hormone in pancreatic islets is unknown. Previous studies have shown that deficiency of Nkx2.2 or Pax4 transcriptional factors that are critical for the development of β-cells leads to expansion of ghrelin-producing cells at the expense of β-cells in pancreatic islets.[23,24] Because the Cre enzyme is constitutively activated in our animal model, we cannot exclude the possibility that ghrelin might influence organism lipid metabolism indirectly by altering the development and differentiation of endocrinal cells in pancreas. Our observation that neither ghrelin-positive cells at neonatal stage nor mTOR signaling activity at adult stage was changed in the islets of TG mice (Supporting Fig. S6A,B) does not support this concept. Although TG mice demonstrate insulin deficiency due to loss of β-cells, the lipid-lowering effects in these transgenic mice are independent of insulin. Supplementation of exogenous insulin normalizes the glucose metabolism, but demonstrates no effects on lipid metabolism (Supporting Fig. S6C-G). Further studies could use inducible Cre activation to exclude indirect effects of pancreatic endocrine cells during early developmental stages.

GASTRIC mTOR SIGNALING IN X/A-LIKE CELLS INFLUENCES HEPATIC LIPID METABOLISM

The existence of gut–liver communication has been well-studied in terms of regulating hepatic glucose metabolism either through the vagal–vagal reflex or endocrine hormones.[25-27] Intestinal mucosa is able to sense luminal nutrients such as lipids and glucose, and alter hepatic gluconeogenesis and glycogenolysis, leading to change in net hepatic glucose output through neuronal network.[25-27] Intestinal hormones, like cholecystokinin from I cells and glucagon-like peptide 1 (GLP1) from L cells, are also well-known for their regulatory effects on glucose metabolism as well.[28-30] Our studies extend the gut–liver axis to gastric X/A-like cells. Alteration of mTOR signaling in X/A-like cells contributes to the regulation of hepatic lipid metabolism. Together with our previous studies demonstrating that mTOR activity in X/A-like cells is closely related to organism energy levels, we propose mTOR signaling as a fuel-sensing mechanism in the stomach, whose activity coordinates organism energy levels with hepatic lipid metabolism.

In the present study, we also demonstrate that mTOR activity in X/A-like cells regulate hepatic lipid metabolism partially through ghrelin. Ghrelin, a 28 amino acid peptide hormone, is the only circulating orexigenic hormone.[31] The primary action site for ghrelin has been proposed to be within the hypothalamus.[32] Interestingly, no significant alteration in hypothalamic genes relative to appetite control is detected in TG mice. This observation indicates that alternative pathway may contribute to the regulation of global lipid metabolism by mTOR signaling in gastric X/A-like cells. Recent studies have also suggested a direct peripheral action of ghrelin in the regulation of hepatic lipogenesis and adipogenesis.[11,33] Consistently, GLP1 has been found to regulate hepatic lipid metabolism by its direct activation of the GLP1 receptor on hepatocytes.[34] These observations indicate an alternative pathway for ghrelin, and a direct gut–liver interaction instead of relay through the central nervous system.

Our previous study demonstrated that acyl-ghrelin infusion promotes lipogenesis, but has little effect on lipid transport and β-oxidation.[11] In line with this observation, reduction of acyl-ghrelin in TG mice increases lipogenesis, whereas increase of acyl-ghrelin in mG mice is associated with an upregulation in expression of lipogenesis-related genes. Interestingly, activation of mTOR signaling in X/A-like cells not only suppresses hepatic lipogenesis but also stimulates β-oxidation. Furthermore, supplementation of acyl-ghrelin partially reverses the improvement of lipid metabolism in TG mice. Taken together, these findings suggest that in addition to acyl-ghrelin, other signals from X/A-like cells may contribute to the metabolic effects observed in these transgenic mice. Nesfatin-1, an

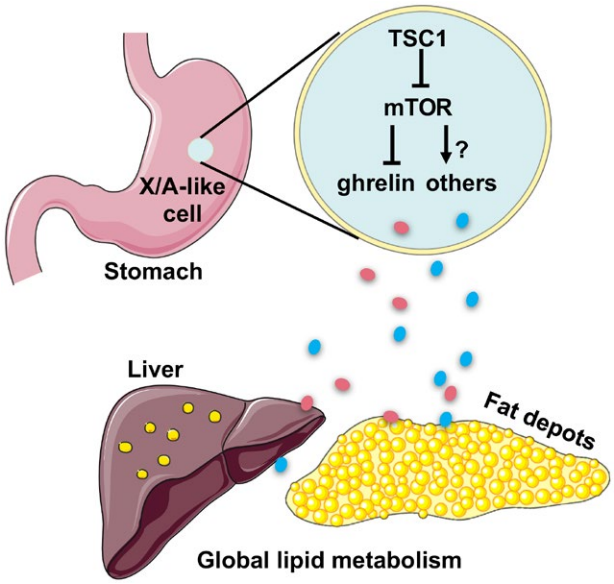

FIG. 8. Graphic highlight of findings.

82-aa peptide derived from a 396-aa precursor protein nucleobindin 2, is identified in X/A-like cells, but in different vesicles than ghrelin. Although secreted from the same cell, nesfatin-1 often exerts opposing metabolic functions than ghrelin. Previous studies have demonstrated that activation of mTOR signaling increases the production of nesfatin-1 in a manner opposite to the ghrelin. Consistently, increase of nesfatin-1 has been detected in TG mice in which mTOR signaling is activated specifically in X/A-like cells (data not shown). In addition, we have demonstrated that nesfatin-1 promotes the differentiation of brown adipocytes.[35] However, until the receptor for nesfatin-1 is characterized, it is not possible to efficiently block the action of nesfatin-1, and therefore to confirm that increase of nesfatin-1 in TG mice contributes to metabolic effects in TG mice. In addition to ghrelin and nesfatin-1, there might exist other unknown secretory factors from gastric X/A-like cells that contribute to the regulation of global lipid metabolism. Future investigation will focus on seretome profiling of TG and mG mice to characterize these molecules.

GASTRIC mTOR SIGNALS ADIPOSE TISSUE BROWNING

Emerging evidence supports the concept that "brown conversion" of white fat is an inherent property of most or all white fat cells.[36,37] Because of great potential in the therapy of obesity and metabolic diseases, attention has been focused on the identification of molecules critical for browning. Although a range of transcriptional factors such as FoxC2, IκB kinase ε, PGC-1a, PRDM16, and RIP140, and secreted molecules including cardiac natriuretic peptides, fibroblast growth factor 21, irisin and bone morphogenetic proteins, have been identified,[37] little attention has been focused on the possibility of gastrointestinal control of browning, despite its critical role in the regulation of food intake and energy balance. Our studies suggest that mTOR activity in gastric X/A-like cells contributes to the browning of WAT. Suppression of mTOR signaling in X/A-like cells reduces, whereas activation of its activity increases, the expression of browning genes. More importantly, our functional test demonstrated that activation of mTOR signaling in X/A-like cells attenuates the decline of body temperature induced by cold exposure.

Our studies also indicate that ghrelin may mediate the effects of gastric mTOR signaling on browning of WAT. There exists a negative relation between circulating levels of ghrelin and gastric mTOR activity and levels of browning genes. Suppression of gastric mTOR signaling in mG mice increases circulating ghrelin, which is associated with a decrease of browning genes in sWAT. Activation of gastric mTOR signaling in TG mice reduces circulating ghrelin, leading to the subsequent increase in browning. The effects of mTOR activation is blocked by rapamycin, a mTOR inhibitor. Importantly, the effect of mTOR activation in X/A-like cells on browning are partially reversed by exogenous ghrelin. Ghrelin might act in a concentration-specific manner. In high acyl-ghrelin concentration models such as mG mice (Fig. 3E) and exogenous acyl-ghrelin infusion (Fig. 6D,E and Supporting Fig. S7B-G), ghrelin demonstrates only modest effects on either brown-marker or white-marker genes. However, in a low acyl-ghrelin animal model (TG mice), the brown-marker genes are significantly increased and white-marker genes decreased. Intriguingly, almost all brown-marker genes in sWAT and BAT, as well as white marker genes in eWAT from TG mice, are affected by acyl-ghrelin infusion. These observations suggest that ghrelin is important for maintenance of phenotypes of adipose tissue, and that the effects of ghrelin may be tissue-specific.

In conclusion, this study demonstrates that the mTOR signaling in X/A-like cells is important for organism lipid homeostasis by regulating hepatic and adipose tissue lipid metabolism. These effects occur partially through ghrelin (Fig. 8). These findings support the endocrine functions of the stomach and highlight potential strategies for dyslipidemia treatment by targeting this organ.

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Supporting Information

Additional Supporting Information may be found at onlinelibrary.wiley.com/doi/10.1002/hep.30229/suppinfo.