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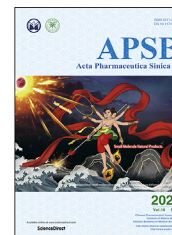

ORIGINAL ARTICLE

Interleukin-27 decreases ghrelin production through signal transducer and activator of transcription 3—mechanistic target of rapamycin signaling

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Received 26 July 2019; received in revised form 6 November 2019; accepted 19 November 2019

KEY WORDS

IL-27;
Ghrelin;
STAT3;
mTOR;
Food intake

Abstract Interleukin-27 (IL-27), a heterodimeric cytokine, plays a protective role in diabetes. Ghrelin, a gastric hormone, provides a hunger signal to the central nervous system to stimulate food intake. The relationship between IL-27 and ghrelin is still unexplored. Here we investigated that signal transducer and activator of transcription 3 (STAT3)—mechanistic target of rapamycin (mTOR) signaling mediates the suppression of ghrelin induced by IL-27. Co-localization of interleukin 27 receptor subunit alpha (WSX-1) and ghrelin was observed in mouse and human gastric mucosa. Intracerebroventricular injection of IL-27 markedly suppressed ghrelin synthesis and secretion while stimulating STAT3−mTOR signaling in both C57BL/6J mice and high-fat diet-induced-obese mice. IL-27 inhibited the production of ghrelin in mHypoE-N42 cells. Inhibition of mTOR activity induced by *mTOR* siRNA or rapamycin blocked the

Abbreviations: DIO, diet-induced-obese; IFN-γ, interferon gamma; IL-27, interleukin-27; mTOR, mechanistic target of rapamycin; S6, ribosomal protein subunit 6; S6K, ribosomal protein subunit 6 kinase; STAT3, signal transducer and activator of transcription 3; WSX-1, interleukin 27 receptor subunit alpha.

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Peer review under responsibility of Institute of Materia Medica, Chinese Academy of Medical Sciences and Chinese Pharmaceutical Association.

https://doi.org/10.1016/j.apsb.2019.12.018

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suppression of ghrelin production induced by IL-27 in mHypoE-N42 cells. *Stat 3* siRNA also abolished the inhibitory effect of IL-27 on ghrelin. IL-27 increased the interaction between STAT3 and mTOR in mHypoE-N42 cells. In conclusion, IL-27 suppresses ghrelin production through the STAT3-mTOR dependent mechanism.

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1. Introduction

Obesity, a disease in which excess body fat accumulates to an extent that health is negatively affected, occurs when caloric intake persistently outpaces energy expenditure[1,2]. Food intake is regulated by a group of anorexigenic hormones including leptin, cholecystokinin, glucagon-like peptide-1 (GLP-1), and the orexigenic gastric hormone ghrelin[1,2]. Ghrelin, the "hunger hormone", is a 28-amino-acid peptide mainly produced by X/A-like cells in the gastric fundus, which functions as a neuropeptide in the central nervous system[3,4]. Besides regulating appetite, ghrelin also plays a significant role in regulating energy metabolism[5]. Ghrelin is initially produced by ghrelinergic cells in the gastrointestinal tract as a 117-amino-acid preproghrelin. Preproghrelin undergoes endoproteolytic processing and posttranslational modification to produce acyl-ghrelin and des-acyl ghrelin[6−9]. Des-acyl ghrelin has the same amino acid sequence as acyl-ghrelin, but its third amino acid (serine 3) is not acylated[10,11]. Ghrelin is unique among peptides due to its acylation with medium-chain fatty acids at the serine-3 residue[3]. Ghrelin is recognized as a potent signal for meal initiation[12]. Systemic administration of exogenous ghrelin has been reported to increase blood glucose levels in rodents and human subjects[13,14]. It increases food intake and blood glucose *via* interacting with growth hormone secretagogue type 1A receptor (GHS-R)[4,13]. Genetic deletion of either ghrelin or ghrelin receptor genes renders mice resistant to obesity and glucose intolerance induced by high-fat diet[15,16]. Precise modulation of ghrelin production is really significant to maintain energy balance[4,6,17]. Investigation of the molecular mechanisms by which ghrelin producing cells modulate the transcription and translation of ghrelin will yield new insights relevant to treatment strategies for human obesity and diabetes.

IL-27, a member of the IL-6/IL-12 cytokine family, consists of two subunits, including Epstein−Barr virus induced gene 3 (EBI3) and P28[18,19]. IL-27 shares structural similarities with IL-12, IL-23 and IL-35[19,20]. WSX-1 (interleukin 27 receptor subunit alpha) and the cytokine receptor GP130 are necessary to mediate signal transduction in response to IL-27[21]. In addition to the immune functions, IL cytokine family also plays a vital role in energy metabolism. Intracerebroventricular injection of IL-6 increases energy expenditure and decreases body fat in rodents[22]. Reduced IL-6 production has been associated with increased body fat mass and decreased energy expenditure in human[23,24]. IL-22 not only improves glucose metabolism but also significantly decreases body weight and food intake in DIO mice[25]. As a member of IL cytokine family, IL-27 also exerts critical function in energy metabolism. It is reported that IL-27 plays a protective role in diabetes through improving STZ-induced hyperglycemia and pancreatic islet inflammation[26]. However, whether IL-27 exerts its function in the regulation of food intake through ghrelin is still unknown. In the current study, we demonstrate that IL-27 inhibits ghrelin production through STAT3−mTOR dependent mechanism. We propose that IL-27 is a potential therapeutic candidate to improve obesity and other metabolic diseases.

2. Materials and methods

2.1. Materials

Interleukin-27 was from Biolegend, Inc. (San Diego, CA, USA). IFN-γ was from Sino Biological Inc. (Beijing, China). Rapamycin was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Rabbit anti-Phospho-STAT3 (Tyr705), anti-phospho-mTOR (Ser 2448), anti-phospho-P70 S6 kinase (Thr389), anti-phospho-S6 (Ser 235/236), anti-mTOR, anti-P70 S6 kinase, anti-S6 antibodies, mouse anti-STAT3, anti-β-actin antibodies, *Stat 3* siRNA, *mTOR* siRNA and control siRNA were purchased from Cell Signaling Technology (Beverly, MA, USA). Mouse anti-ghrelin and rabbit anti-WSX-1 antibodies were from Abcam Inc. (Cambridge, MA, USA). Trizol reagent and reverse transcription (RT) system were purchased from Promega Inc. (Madison, WI, USA). Horseradish peroxidase-conjugated, donkey anti-rabbit IgG and donkey anti-mouse IgG were purchased from Jackson ImmunoResearch (West Grove, PA, USA). Immobilon Western chemiluminescent HRP substrate was purchased from Millipore (Temecula, CA, USA). Lipofectamine was from Invitrogen Inc. (Carlsbad, CA, USA). Goat anti-mouse fluorescein isothiocyanate-conjugated IgG and dylight 594 affinipure donkey anti-rabbit IgG (1:100) were from EarthOx LLC (San Francisco, CA, USA). Anti-rabbit IgG (Cat. A7016) and protein A + G agarose (Cat. P2012) were purchased from Beyotime (Shanghai, China). Total ghrelin enzyme immunoassay kit was purchased from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). Normal chow diet (control diet, D12450B) and high-fat diet (60% fat, D12492) were from Research Diets Inc. (New Brunswick, NJ, USA).

2.2. Animal experiments

4-week-old male C57BL/6J mice were housed in standard plastic rodent cages and maintained at a regulated environment (24 °C, 12 h light, 12 h dark cycle with lights on at 7:00 and off at 19:00) with *ad libitum* access to a normal chow diet (control diet, D12450; Research Diets) or high-fat diet (60% fat, D12492; Research Diets) for 12 weeks. Animals used in this study were handled in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH publication no. 85−23, revised 1996).

A 26-gauge stainless steel guide cannula (Plastics One, Roanoke, VA, USA) projecting into the third cerebral ventricle was implanted into each mouse using flat-skull coordinates from bregma (antero-posterior, −0.825 mm; medio-lateral, 0 mm;

dorso-ventral, −4.8 mm)[27]. At the end of experiments, the cannula implantation was validated by trypan blue.

2.3. Intracerebroventricular (icv) microinjections

For the animal treatment, IL-27 or interferon gamma (IFN-γ) was dissolved in saline on the day of treatment, and micro injected into third ventricle 1 h before onset of the dark phase. After 12 h fasting, lean C57BL/6J mice received either IL-27 (100 ng) or saline in a total volume of 2 µL by slow infusion in mice. High-fat diet-induced-obese mice received saline, IL-27 (100 ng), IFN-γ (500 pg) or IL-27 plus IFN-γ in a total volume of 2 µL. The mice were returned to their home cages with free access to a pre-measured amount of chow and water, and the effect of icv microinjection of IL-27 or IFN-γ on feeding was determined. Changes in food intake were measured at selected time points after treatments. Twenty-four hours after injection, blood samples and tissues were harvested.

2.4. Cell culture and transfection

Embryonic mouse hypothalamic cell line N42 (mHypoE-N42) (Cellutions Biosystems Inc., Burlington, NC, USA), a hypothalamic cell line in which ghrelin is abundantly expressed, was used for *in vitro* analysis of ghrelin modulation. mHypoE-N42 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and maintained in high glucose DMEM medium supplemented with 10% FBS. Cells were plated at optimal densities and grown for 24 h, then transfected with control siRNA, *mTOR* siRNA or *Stat 3* siRNA using lipofectamine reagent according to the manufacturer's instruction. After 24 h transfection, the cells were then treated with saline, IL-27 (50 nmol/L) or IFN-γ (5 pg/mL) for another 24 h.

2.5. Co-immunoprecipitation (Co-IP)

For co-immunoprecipitation, mHypoE-N42 cells treated with IL-27 (50 nmol/L) or saline for 24 h were lysed with RIPA lysis buffer for 30 min and centrifuged at 12,000×*g* for 15 min. Total proteins (500 µg) were incubated with indicating primary antibodies such as STAT3 or mTOR. The mixture was gently rotated at 4 °C overnight. Anti-rabbit IgG antibody was served as negative control. The immunocomplex was collected with protein A + G agarose, and the precipitates were washed five times with ice-cold PBS. Finally, proteins were released by boiling in sample buffer and utilized for Western blot analysis.

2.6. Recruitment of human subjects and collection of gastric biopsies

Participation in this study was voluntary, and written informed consent was obtained from each participant. The guidelines of the Declaration of Helsinki of the World Medical Association were followed. All protocols were approved by the Research Ethics Committee of the First Affiliated Hospital of Jinan University (Guangzhou, China).

The biopsy specimens were obtained under protocols approved by the Research Ethics Committee of the First Affiliated Hospital of Jinan University (Guangzhou, China). Informed consent was obtained from all patients. All animal experiments were undertaken with approval from the Laboratory Animal Ethics Committee of Jinan University (Guangzhou, China).

2.7. Reporter assays

Construction of human ghrelin promoter-luciferase expression vectors was performed as described previously[28]. For transient transfection, mHypoE-N42 cells were plated onto 24-well tissue culture plates at optimal densities and grown for 24 h. Cells were then transfected with the ghrelin promoter-luciferase reporter gene constructs (500 ng), which were mixed with an internal control pSV-β-galactosidase (25 ng) per well using lipofectamine reagent according to the manufacturer's instruction. Cells were grown overnight, and then treated with chemicals indicated for 24 h. Cell lysates were analyzed for luciferase activity with the luciferase reporter assay system using a luminometer (Monolight 2010; Analytical Luminescence Laboratory, San Diego, CA, USA) according to the manufacturer's instruction. β-Galactosidase activity was measured according to the manufacturer's protocol.

2.8. Western blot analysis

The tissues and cultured cells were quickly harvested, rinsed thoroughly with PBS, then homogenized on ice in the lysis buffer. Protein concentration was measured by Bradford's method. Proteins were loaded onto SDS-PAGE gels, and then transferred to nitrocellulose membranes. The membranes were incubated for 1 h at room temperature with 5% fat-free milk in Tris-buffered saline containing Tween 20, 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, West Grove, PA, USA). A Western blotting luminol reagent was used to visualize bands corresponding to each antibody.

2.9. RNA extraction, quantitative real-time PCR, and reverse transcription-PCR analysis

For gene expression analysis, RNA was isolated from tissues or mHypoE-N42 cells 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, USA). Triplicate samples were collected for each experimental condition to determine relative expression levels. Sequences for the primer pairs used in this study follow:

Mouse *Wsx-1* (accession No. NM_016671.3):

sense 5′-GCTCTGCCCTGGTTTCTGTC-3′,
antisense 5′-CTCCTTGATGTAAGGTTGCCC-3′.

Mouse ghrelin (accession No. NM_021488):

sense 5′-CCATCTGCAGTTTGCTGCTA-3′,
antisense 5′-GCAGTTTAGCTGGTGGCTTC-3′.

Mouse β-actin (accession No. NM_007393.5):

sense 5′-ATCTGGCACCACACCTTC-3′,
antisense 5′-AGCCAGGTCCAGACGCA-3′.

For reverse transcription-PCR, *Wsx*-1 mRNA was amplified in 25 mL and the amplification parameters consisted of initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 30 s and extension at 72 °C for 1 min. The resulting products were visualized by electrophoresis on a 2% agarose gel.

2.10. Immunohistochemistry

Gastric mucosal biopsies were postfixed in 4% paraformaldehyde, dehydrated, embedded in wax, and sectioned at 6 μm. Paraffin-embedded sections were dewaxed, rehydrated, and rinsed in PBS. After boiling for 10 min in 0.01 mol/L sodium citrate buffer (pH 6.0), sections were blocked in 5% goat preimmune serum in PBS for 1 h at room temperature and then incubated overnight with rabbit anti-Phospho-STAT3 (Tyr705, 1:100), or rabbit anti-WSX-1 (1:50) combined with mouse monoclonal antibody to ghrelin (1:100). Tissue sections were then incubated at 22 °C for 2 h with a mixture of the following secondary antibodies: goat anti-mouse fluorescein isothiocyanate-conjugated IgG (1:50) and dylight 594 affinipure donkey anti-rabbit IgG (1:100). Controls included substituting primary antibodies with mouse IgG or rabbit IgG. Photomicrographs were taken under a confocal laser-scanning microscope (Leica, Berlin, Germany).

2.11. Measurements of ghrelin

Blood samples were collected after anesthesia in the presence of aprotinin (2 μg/mL) and EDTA (1 mg/mL). HCl was added into plasma and cell culture medium for a final concentration of 0.1 mol/L to prevent ghrelin from degradation. Plasma and cell culture medium were harvested and stored at −80 °C before use. Total ghrelin was measured using an enzyme immunoassay kit according to the manufacturer's instruction.

2.12. Statistical analysis

All values are expressed as means ± SEM (standard error of mean). Statistical differences were evaluated by two-way ANOVA and Newman−Student−Keuls test. Comparisons between two groups involved use of the Student's *t* test. P value < 0.05 denotes statistical significance.

3. Results

3.1. Expression and co-localization of WSX-1 or pSTAT3 and ghrelin in mouse and human gastric mucosa

IL-27 binds to a receptor complex called IL-27R, which is composed of WSX-1 and gp130, to exercise its physiological functions. WSX-1 is unique to IL-27R while GP130 is a ubiquitously expressed signal-transducing receptor[21]. To examine the expression of WSX-1, we analyzed the expression of WSX-1 in different tissues and mHypoE-N42 cells, a hypothalamic cell line in which ghrelin is abundantly expressed. As shown in (Fig. 1A), *Wsx*-1 mRNA was detected in hypothalamus, stomach, ileum, liver, pancreas and muscle. *Wsx-1* is also expressed in mHypoE-N42 cells. After binding with the WSX-1/gp130 complex, IL-27 activates the STAT3 signaling pathway[19−21,29]. In the present study, double immunofluorescent staining was used to localize WSX-1, or phosphorylated-STAT3 (pSTAT3, Tyr705) and ghrelin in the mouse gastric mucosa. Antibodies recognizing WSX-1, pSTAT3 and ghrelin demonstrated strong positive reactivity in stomach. Virtually 100% of ghrelin-positive cells stained positively for WSX-1, and 90 ± 3% WSX-1-positive cells expressed ghrelin (Fig. 1B). Nearly all gastric X/A-like cells stained positively for pSTAT3, and 70 ± 6% pSTAT3-positive cells expressed ghrelin (Fig. 1C).

In human stomach, about 90% of ghrelin-positive cells stained positively for WSX-1, while 85 ± 5% WSX-1-positive cells expressed ghrelin (Fig. 1E).

3.2. Effects of exogenous IL-27 on ghrelin synthesis in hypothalamus and stomach

Short-term effects of exogenous IL-27 on ghrelin synthesis and secretion were examined in C57BL/6J mice fed with standard chow. We found that 3rd icv injection of IL-27 (100 ng/2 μL, 24 h) significantly decreased protein (Fig. 2A and B) and mRNA (Fig. 2C and D) levels of ghrelin in both hypothalamus and stomach, as well as circulating ghrelin (Fig. 2E). Our previous studies suggest that mTOR functions as a critical fuel sensor to integrate nutritional and hormonal signals with the synthesis and secretion of ghrelin[28,30]. Thus, gastric and hypothalamic mTOR signaling was examined. IL-27 significantly enhanced the phosphorylation of mTOR, ribosomal protein subunit 6 kinase (S6K) and ribosomal protein subunit 6 (S6), indicating the activation of mTOR signaling. Consistent with the decrease in ghrelin production induced by IL-27, IL-27 inhibited 1, 4 and 12 h food intake in the dark cycle (Fig. 2F).

3.3. IL-27 reverses the effects of IFN-γ on ghrelin synthesis and STAT3−mTOR signaling in DIO mice

Similar effects of IL-27 on ghrelin were also observed in high fat diet induced obese mice. Central administration of IL-27 significantly down-regulated protein (Fig. 3A) and mRNA (Fig. 3B) levels of ghrelin as well as circulating ghrelin (Fig. 3C) in diet-induced obese mice. Interferon gamma (IFN-γ) is a cytokine with important roles in tissue homeostasis, immune and inflammatory responses and tumor immunosurveillance[31]. It is reported that IFN-γ stimulates food intake in mice[32]. Consistent with this finding, we found that central administration of IFN-γ significantly up-regulated protein and mRNA levels of ghrelin in stomach, as well as circulating ghrelin (Fig. 3A−C), contributing to the increased food intake in dark cycle (Fig. 3D). Furthermore, 3rd icv injection of IFN-γ significantly inhibited the phosphorylation of mTOR, S6K, S6 and STAT3 (Fig. 3A).

Sharma et al.'s research[33] proved that IL-27 inhibits IFN-γ induced autophagy *via* concomitant induction of JAK/PI3K/AKT/mTOR cascade. Here we show that IL-27 abolished the orexigenic effect of IFN-γ. IL-27 reversed the stimulation of ghrelin synthesis and secretion as well as the suppression of STAT3−mTOR signaling induced by IFN-γ in DIO mice (Fig. 3A−D).

3.4. Effects of IL-27 on STAT3−mTOR signaling and ghrelin expression in mHypoE-N42 cells

As shown in Fig. 1A, *Wsx-1* is detected in mHypoE-N42 cells. We next examined the direct effects of IL-27 on ghrelin synthesis and secretion in mHypoE-N42 cells, a hypothalamic ghrelin producing cell line. We found that IL-27 stimulated the phosphorylation of mTOR, S6K, S6, and STAT3 in mHypoE-N42 cells, indicating an activation of STAT3−mTOR signaling in a concentration-dependent manner (Fig. 4A). Associated with the change of STAT3−mTOR signaling, IL-27 reduced ghrelin mRNA (Fig. 4C) and protein levels (Fig. 4A), as well as medium content of ghrelin (Fig. 4E). Moreover, IL-27 at 50 nmol/L time-dependently stimulated the STAT3−mTOR signaling and decreased ghrelin mRNA and protein levels as well as ghrelin secretion in mHypoE-

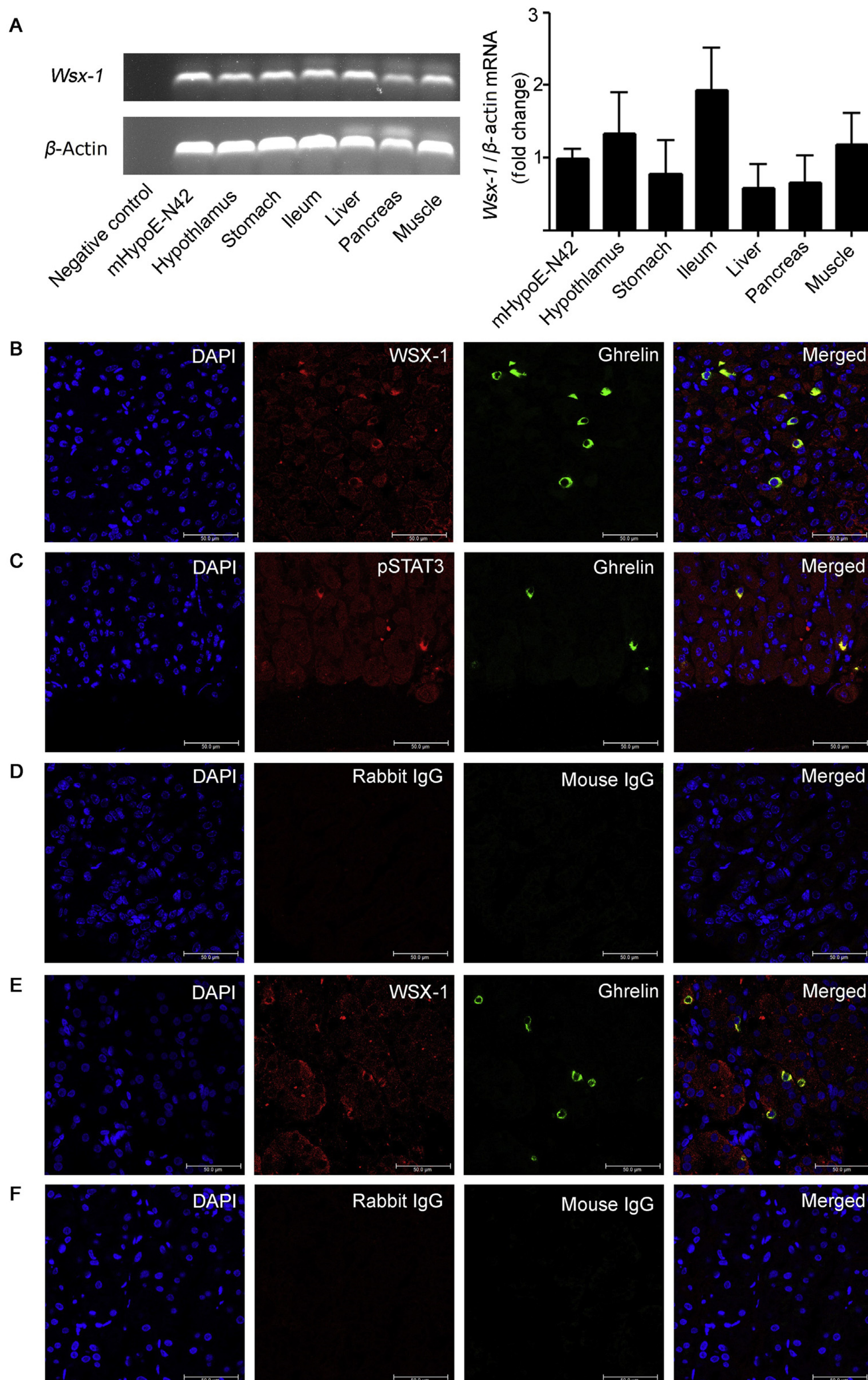

Figure 1 Expression and co-localization of WSX-1, pSTAT3 and ghrelin in mouse and human gastric mucosa. (A) Expression of *Wsx-1* mRNA in tissues and mHypoE-N42 cells. Shown is the representative of three individual reverse transcription-PCR and real-time PCR. β-Actin was used as internal control. (B) Images depicting WSX-1 (red) and ghrelin (green) in mouse gastric mucosal cells. Merged image illustrates co-localization of WSX-1 and ghrelin (orange). Results are expressed as mean±SEM. (C) Expression and co-localization of phospho-STAT3 (red) and ghrelin (green) in mouse gastric mucosal cells. Merged image illustrates co-localization of pSTAT3 and ghrelin (orange). (D) Controls included substituting primary antibodies with rabbit IgG and mouse IgG. (E) Images depicting WSX-1 (red) and ghrelin (green) in human gastric mucosa. Merged image illustrates co-localization of WSX-1 and ghrelin (orange). (F) Rabbit IgG and mouse IgG were used as negative control. Scale bar, 50 μm.

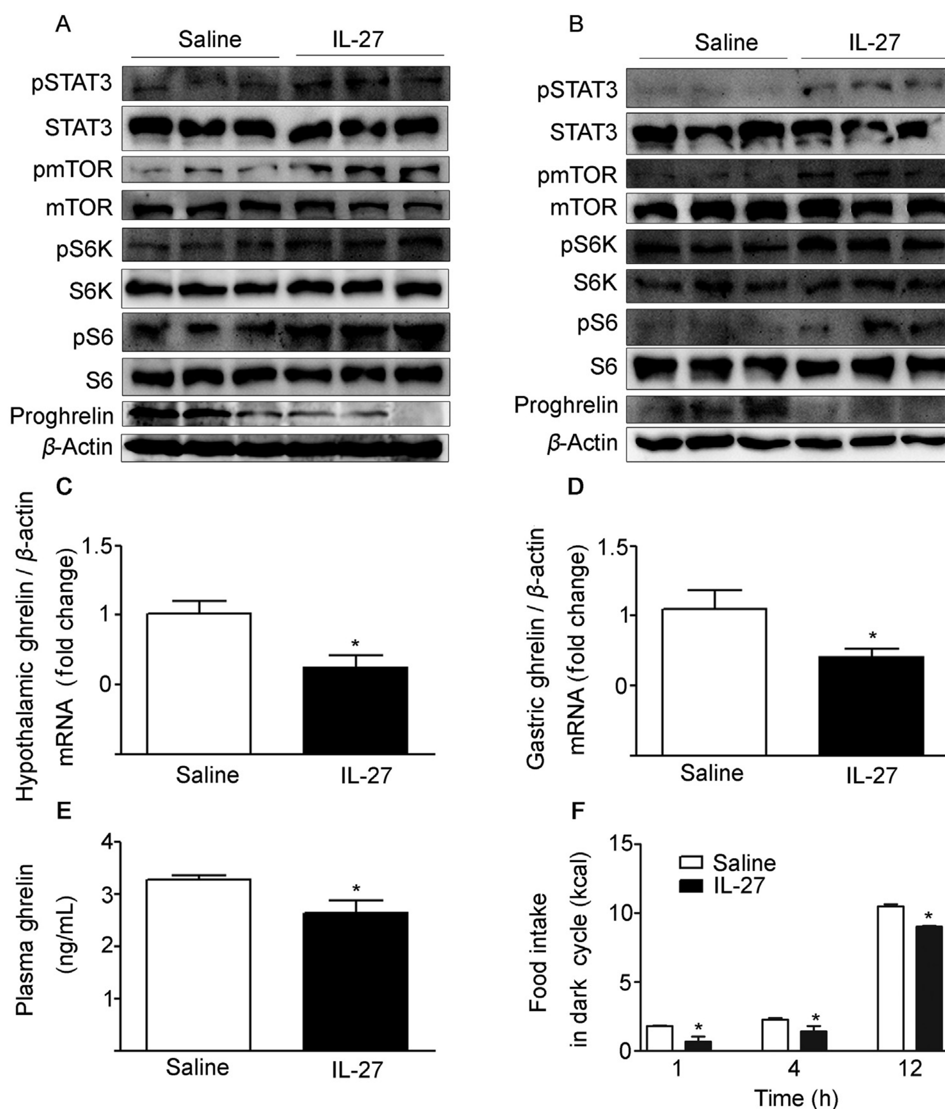

Figure 2 Intracerebroventricular administration of IL-27 inhibits ghrelin synthesis and food intake in lean C57BL/6J mice. (A) and (B) Representative Western blots from 16-week-old male C57BL/6J mice that received 3rd icv of saline (2 μL) or IL-27 (100 ng/2 μL). (A) Hypothalamic and (B) gastric pSTAT3, STAT3, pmTOR, mTOR, pS6K, S6K, pS6, S6, proghrelin and β-actin were detected using specific antibodies. (C) and (D) Hypothalamic and gastric ghrelin mRNA levels were measured using quantitative PCR analysis. (E) Plasma total ghrelin was detected by ELISA. (F) Food intake was measured 1, 4, and 12 h after injection. Results are expressed as mean±SEM, $n=6$; $^{*}P < 0.05$ *vs.* saline treatment.

N42 cells (Fig. 4B, D, and F). The significant inhibition of ghrelin occurred as early as 6 h and lasted up to 24 h (Fig. 4B, D, and F). As shown in Fig. 4G, IL-27 markedly inhibited ghrelin promoter activity in a concentration-dependent manner in mHypoE-N42 cells.

3.5. Effects of IFN-γ on STAT3−mTOR signaling and ghrelin production in mHypoE-N42 cells

Consistent with the *in vivo* study, exposure of mHypoE-N42 cells to IFN-γ at the doses of 0.625−10 pg/mL for 24 h caused a concentration-dependent inhibition of STAT3−mTOR signaling (Fig. 5A) and stimulation of ghrelin mRNA (Fig. 5C) and protein levels (Fig. 5A), as well as medium content of ghrelin (Fig. 5E). Furthermore, IFN-γ at 5 pg/mL significantly decreased STAT3−mTOR signaling (Fig. 5B) but increased ghrelin mRNA and protein levels, as well as ghrelin secretion in a time-dependent manner in mHypoE-N42 cells (Fig. 5B, D, and F).

3.6. IL-27 reverses the stimulation of ghrelin induced by IFN-γ in mHypoE-N42 cells

Further, pretreatment of mHypoE-N42 cells with IL-27 abolished the increase in ghrelin mRNA (Fig. 6B) and protein levels of proghrelin (Fig. 6A), as well as ghrelin secretion (Fig. 6C) induced by IFN-γ. IL-27 also blocked the suppression of STAT3−mTOR signaling caused by IFN-γ (Fig. 6A).

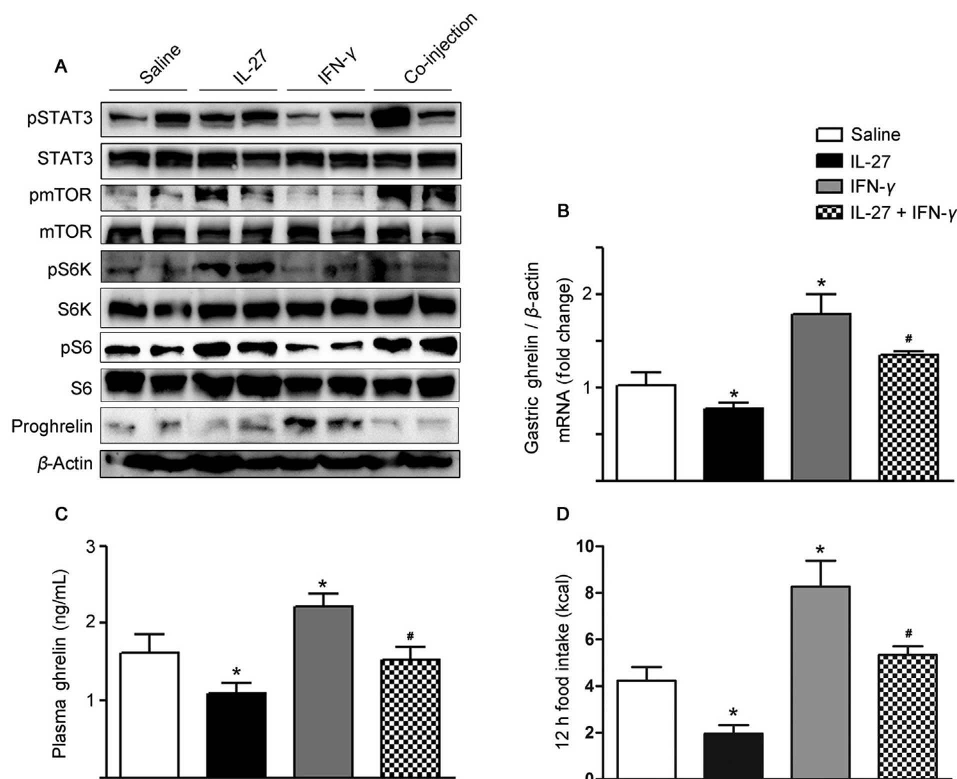

Figure 3 IL-27 reverses the effect of IFN-γ on ghrelin synthesis and STAT3−mTOR signaling in high-fat diet-induced-obese mice. (A) Representative Western blot from 16-week-old DIO mice that received 3rd icv injection of saline, IL-27 (100 ng), IFN-γ (500 pg) or IL-27 plus IFN-γ in a total volume of 2 μL. (A) Gastric pSTAT3, STAT3, pmTOR, mTOR, pS6K, S6K, pS6, S6, proghrelin and β-actin were blotted; mTOR, S6K, S6, STAT3 and β-actin were used as loading controls. (B) Gastric ghrelin mRNA. (C) Plasma ghrelin. (D) Food intake. Results are expressed as mean±SEM, $n=6$; $^{*}P<0.05$ *vs.* saline treatment; $^{\#}P<0.05$ *vs.* IFN-γ treatment.

3.7. Pharmacological inhibition of STAT3−mTOR blocks the suppression of ghrelin elicited by IL-27

Our previous studies demonstrate that activation of mTOR signaling inhibits ghrelin synthesis[28,30]. We thus examined whether mTOR signaling mediates the effect of IL-27 on ghrelin production. As shown in Fig. 7, inhibition of mTOR signaling activity by rapamycin blocked the decrease in ghrelin production induced by IL-27 (Fig. 7A−C). Moreover, rapamycin also inhibited the phosphorylation of STAT3 (Fig. 7A).

3.8. IL-27 decreases ghrelin production through activating STAT3-mTOR signaling

In addition to suppressing mTOR activity, siRNA knockdown of mTOR blocked STAT3 activation (Fig. 8A) and suppression of ghrelin (Fig. 8C and E) induced by IL-27 in mHypoE-N42 cells. *Stat 3* siRNA also abolished the activation of mTOR signaling (Fig. 8B) and suppression of ghrelin (Fig. 8D and F) elicited by IL-27 in mHypoE-N42 cells. Co-IP was further employed to investigate whether mTOR and STAT3 interact with each other. IgGs, against mTOR and STAT3 co-immunoprecipitated with each other, indicating an interaction between mTOR and STAT3. IL-27 enhanced the interaction between mTOR and STAT3 (Fig. 8G and H).

4. Discussion

The major finding of the present study is that IL-27 inhibits ghrelin *via* stimulating STAT3−mTOR signaling pathway. This conclusion is supported by the following distinct observations: (1) *Wsx-1* is expressed in mouse hypothalamus, stomach and mHypoE-N42 cells; (2) Co-localization of WSX-1, or pSTAT3 and ghrelin is observed in both mouse and human gastric fundus; (3) Administration of exogenous IL-27 significantly activates the STAT3-mTOR signaling in hypothalamus as well as stomach of lean and obese mice, which is associated with the inhibition of ghrelin and food intake; (4) IL-27 stimulates STAT3−mTOR activity and inhibits ghrelin synthesis and secretion in mHypoE-N42 cells; (5) IFN-γ decreases STAT3−mTOR signaling activity but increases ghrelin production in mHypoE-N42 cells; (6) The effects of IFN-γ on STAT3−mTOR and ghrelin can be antagonized by IL-27; (7) Pharmacological and genetic interference of mTOR signaling block the activation of STAT3−mTOR signaling and the suppression of ghrelin induced by IL-27; (8) Genetic interference of *Stat 3* inhibits the effects of IL-27 on STAT3-mTOR signaling as well as ghrelin expression; and (9) IL-27 increases the interaction between STAT3 and mTOR.

Interleukin 27 is a member of the IL-6/12 cytokine family. It consists of EBI3 and p28. IL-27 is expressed by antigen presenting cells and interacts with IL-27 receptor, which consists of WSX-1 and GP130[21,29]. WSX-1 is unique to IL-27R, whereas the GP130

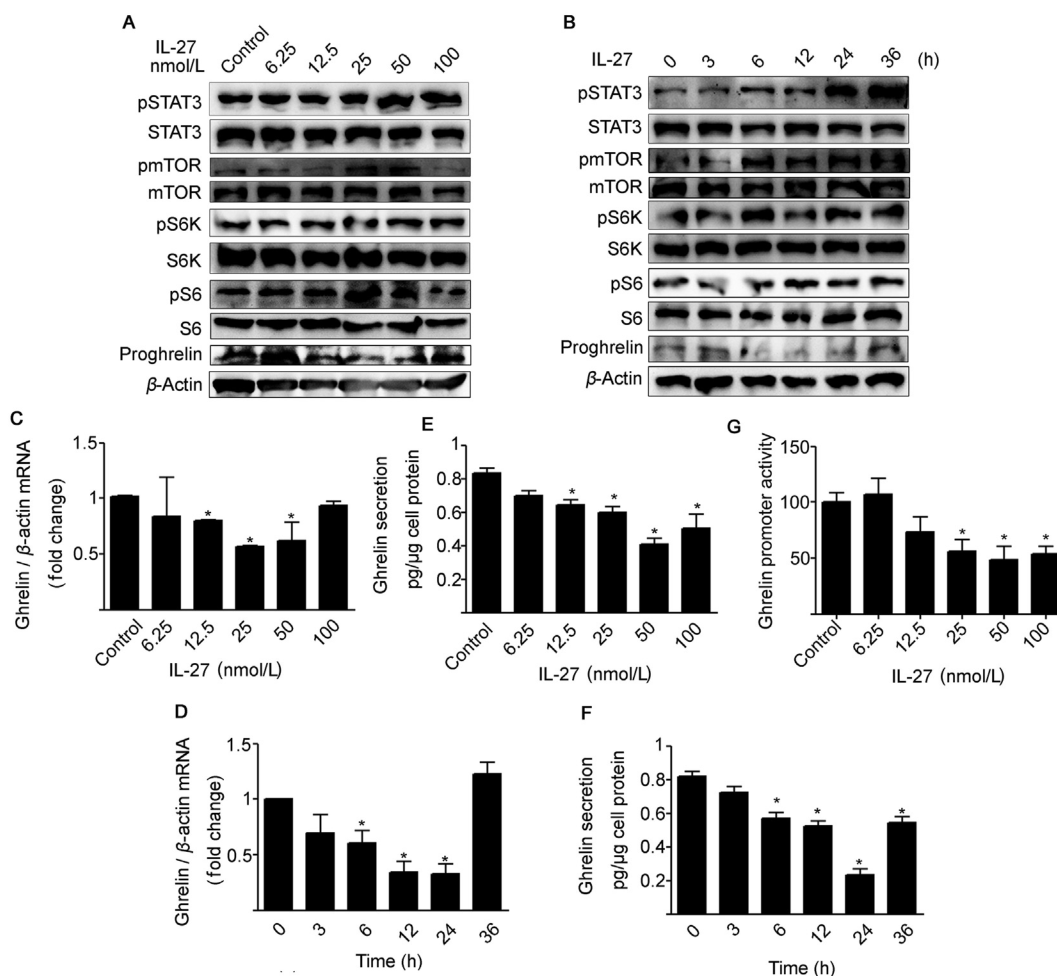

Figure 4 Inhibition of ghrelin synthesis and secretion by IL-27 in mHypoE-N42 cells. Cultured mHypoE-N42 cells were treated with (A), (C), and (E) various concentrations of IL-27 (6.25−100 nmol/L) for 24 h, or (B, D, and F) IL-27 (50 nmol/L) for time indicated. (A) and (B) Representative Western blots of pmTOR, mTOR, pS6K, S6K, pS6, S6, pSTAT3, STAT3, proghrelin and β-actin. Ghrelin (A) and (B) protein and (C) and (D) mRNA were measured by Western blotting and real-time PCR, respectively. (E) and (F) Medium ghrelin was determined by enzyme immunoassay. (G) Relative luciferase activity in mHypoE-N42 cells transfected with ghrelin promoter-luciferase plasmid following exposure to various concentrations of IL-27. Results are expressed as mean±SEM. Experiments were repeated for three times. $^{*}P<0.05$ *vs.* control.

subunit of IL-27R is expressed ubiquitously and shared with receptors for IL-6, IL-35 and other cytokines[21]. In order to explore whether it exists a relationship between IL-27 signaling and ghrelin production, co-localization of WSX-1, or pSTAT3 and ghrelin is first observed in both mouse and human gastric fundus in present study. WSX-1 and GP130 constitute a signal-transducing receptor for IL-27[21]. Moreover, IL-27 activates the STAT signaling pathway through WSX-1/GP130 complex[19−21,29], we thus speculate that both WSX-1 and GP130 are required for the process of IL-27-inhibited ghrelin production. A main effect of IL-27 is participating in differentiation of the diverse populations of T cells in the immune system[19,20,34], while recent studies indicate that IL cytokine family also plays a vital role in energy metabolism. IL-6 increases GLP-1 secretion, contributing to hyperinsulinemia and blood glucose lowering[35,36]. IL-10 induces STAT3 phosphorylation in arcuate nucleus POMC neurons, and thereby ameliorates hyperphagia and obesity caused by leptin deficiency[37]. IL-1β inhibits ghrelin expression in ghrelin-producing cells[38]. IL-27 reduces lipid accumulation of foam cell *via* JAK2/STAT3[39]. Although the functions of IL cytokine family in energy metabolism have been reported, the functional role and underlying mechanism of IL-27 in food intake remain unclear.

Ghrelin is a 28-amino-acid peptide that is mainly produced by the neuroendocrine cells (named "X/A-like" in mice and "P/D1" in humans) in the oxyntic mucosa of the gastric fundus[4]. Ghrelin increases food intake and weight gain in rodents and human[4,40]. Infusion of ghrelin into the cerebral ventricles of rats markedly enhances food intake apparently through actions on the hypothalamus[41]. Investigation of the molecular mechanisms to modulate the transcription and translation of ghrelin may provide a new target for the treatment of obesity. Apart from the gastrointestinal tract, ghrelin expression has been identified in a number of tissues[42]. Ghrelin peptide has been shown to be expressed in the hypothalamus. Cowley et al.[43] discovered expression of ghrelin in a previously uncharacterized group of neurons adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei. Furthermore, the secretion of ghrelin is regulated by the central nervous system[44]. Thus, we chose to infuse the IL-27 in 3rd icv. According to our data, IL-27 receptor is expressed in both hypothalamus and stomach, the action of IL-27

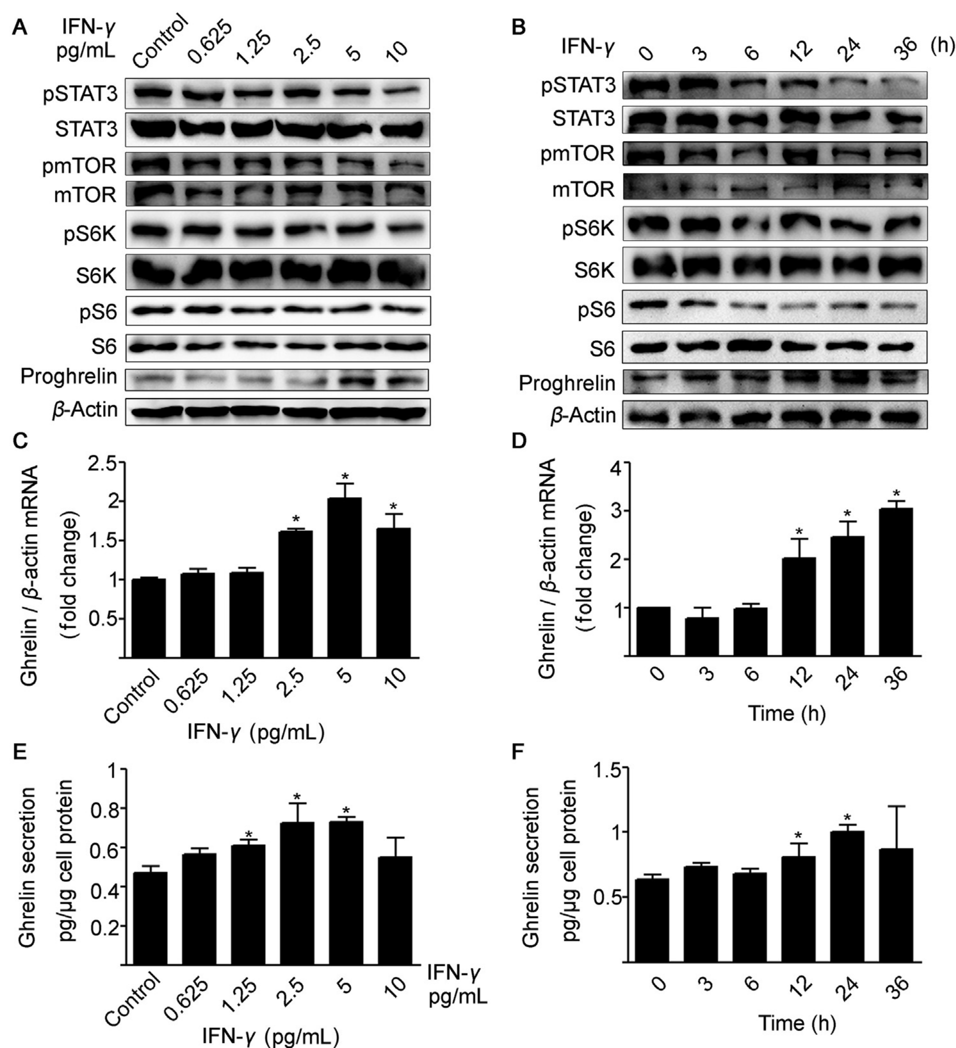

Figure 5 Stimulation of ghrelin by IFN-γ in mHypoE-N42 cells. Cultured mHypoE-N42 cells were treated with various concentrations of IFN-γ (0.625−10 pg/mL) for 24 h, or IFN-γ (5 pg/mL) for the time indicated. Ghrelin (A) and (B) protein and (C) and (D) mRNA were analyzed by Western blotting and real-time PCR, respectively. (E and F) Medium ghrelin was determined by enzyme immunoassay. Results are expressed as mean±SEM. Experiments were repeated for three times. $^{*}P<0.05$ *vs*. control.

on ghrelin production may be centrally or peripherally orchestrated. In the present study, intracerebroventricular administration of IL-27 significantly decreased the production of ghrelin and food intake in lean or obese mice. Time- and concentration-dependent suppression of ghrelin induced by IL-27 was observed in mHypoE-N42 cells. In sum, these results support that IL-27 suppresses ghrelin synthesis and secretion *in vivo* and *in vitro*. Hyperghrelinaemia has been reported to cause progressive weight gain and hyperphagia in patients with Prader−Willi syndrome (PWS) and bulimia nervosa[45−47]. Thus, IL-27 may be a potential therapeutic candidate to improve PWS and bulimia nervosa by inhibiting ghrelin production.

Our previous study showed that mTOR is a gastric fuel sensor whose activity is linked to the regulation of energy intake through ghrelin[28]. IL-27 binds to the IL-27 receptor to exert its functions through JAK−STAT3 and P38 MAPK pathways[19,20]. Exogenous IL-27 can induce the activation of mTOR through JAK/PI3K pathway[33]. Here we demonstrate that IL-27 inhibits ghrelin by activating the STAT3−mTOR signaling pathway. Intracerebroventricular injection of IL-27 not only stimulates phosphorylation of STAT3 but also enhances phosphorylation of mTOR and its downstream targets such as S6K and S6 in mouse hypothalamus and stomachs, contributing to the decrease in ghrelin expression and food intake. *In vitro* study, both pharmacological and genetic interference of mTOR and STAT3 block the inhibition of ghrelin induced by IL-27 in mHypoE-N42 cells. Gene silence of *Stat 3* by *Stat3* siRNA contributes to the inhibition of mTOR signaling, while both *mTOR* siRNA and rapamycin decrease the phosphorylation of STAT3. After exposure to IL-27, phosphorylation of STAT3 and mTOR are elevated, IL-27 also enhances the interaction between STAT3 and mTOR. The concurrent stimulation of STAT3 and mTOR leads to the subsequent inhibition of ghrelin production.

Extensive clinical data and experimental models demonstrate the involvement of cytokines in the pathogenesis of autoimmune diseases. Obesity appears to be a major environmental factor contributing to the onset and progression of autoimmune

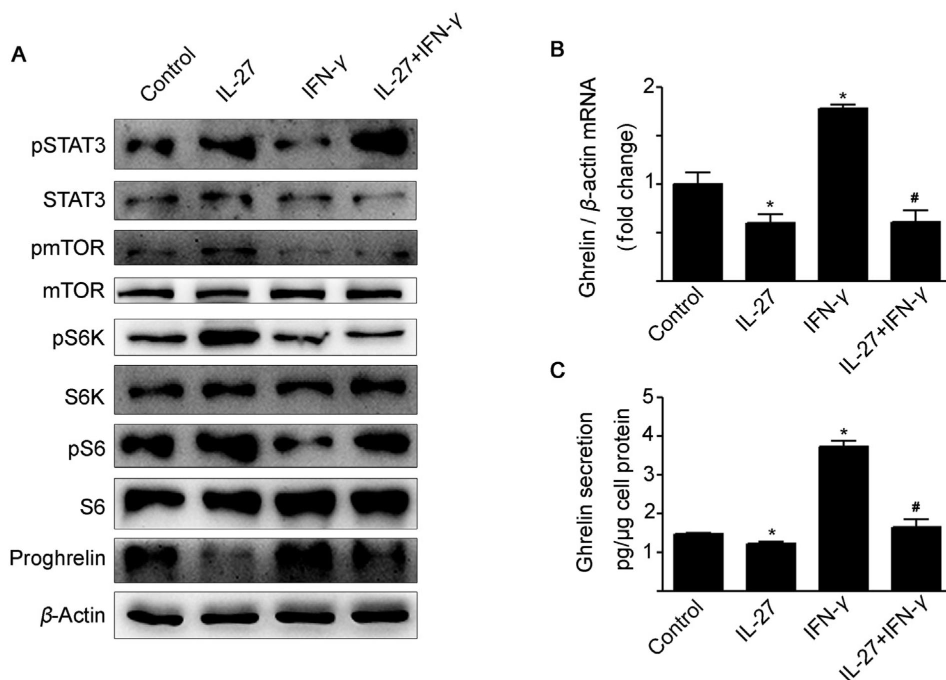

Figure 6 IL-27 reverses the stimulation of ghrelin caused by IFN-γ in mHypoE-N42 cells. mHypoE-N42 cells were treated with saline, IL-27 (50 nmol/L), IFN-γ (5 pg/mL), IL-27 plus IFN-γ for 24 h. (A) Representative Western blots of pmTOR, mTOR, pS6K, S6K, pS6, S6, pSTAT3, STAT3, proghrelin and β-actin. (B) Ghrelin mRNA. (C) Medium ghrelin. Results were expressed as mean±SEM. Experiments were repeated for three times. $^{*}P<0.05$ *vs*. control, $^{\#}P < 0.05$ *vs*. IFN-γ treatment.

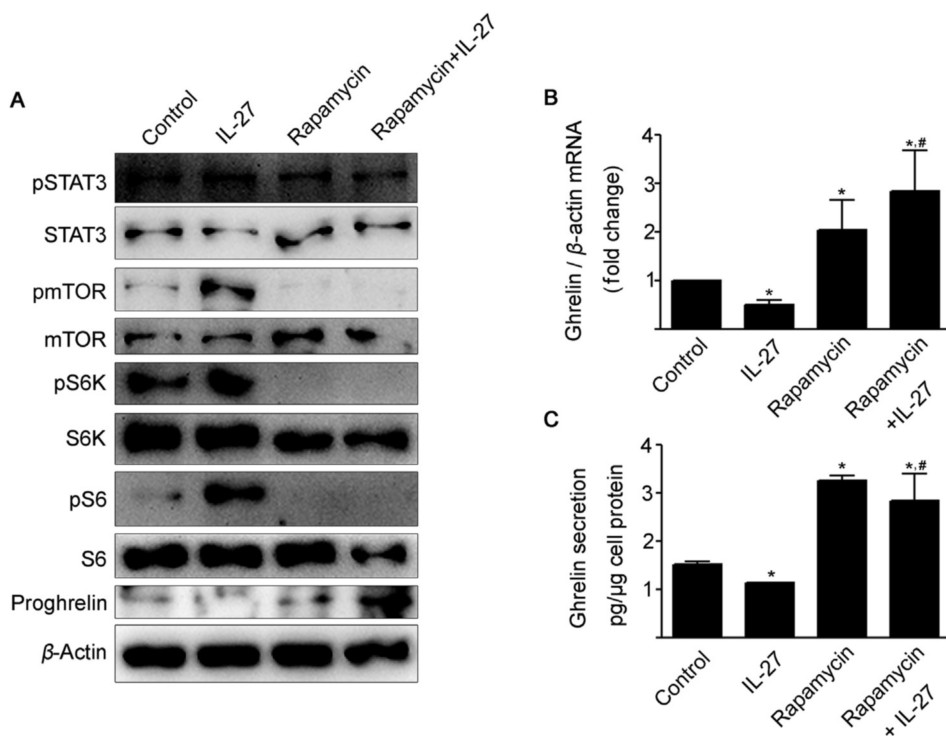

Figure 7 Pharmacological inhibition of STAT3−mTOR blocks the suppression of ghrelin induced by IL-27. mHypoE-N42 cells were treated with DMSO, IL-27, rapamycin (20 nmol/L), and IL-27 plus rapamycin for 24 h. (A) Representative Western blots of pmTOR, mTOR, pS6K, S6K, pS6, S6, pSTAT3, STAT3, proghrelin and β-actin. (B) Ghrelin mRNA. (C) Medium ghrelin. Results were expressed as mean ± SEM. Experiments were repeated for three times. $^{*}P<0.05$ *vs*. control, $^{\#}P < 0.05$ *vs*. IL-27 treatment.

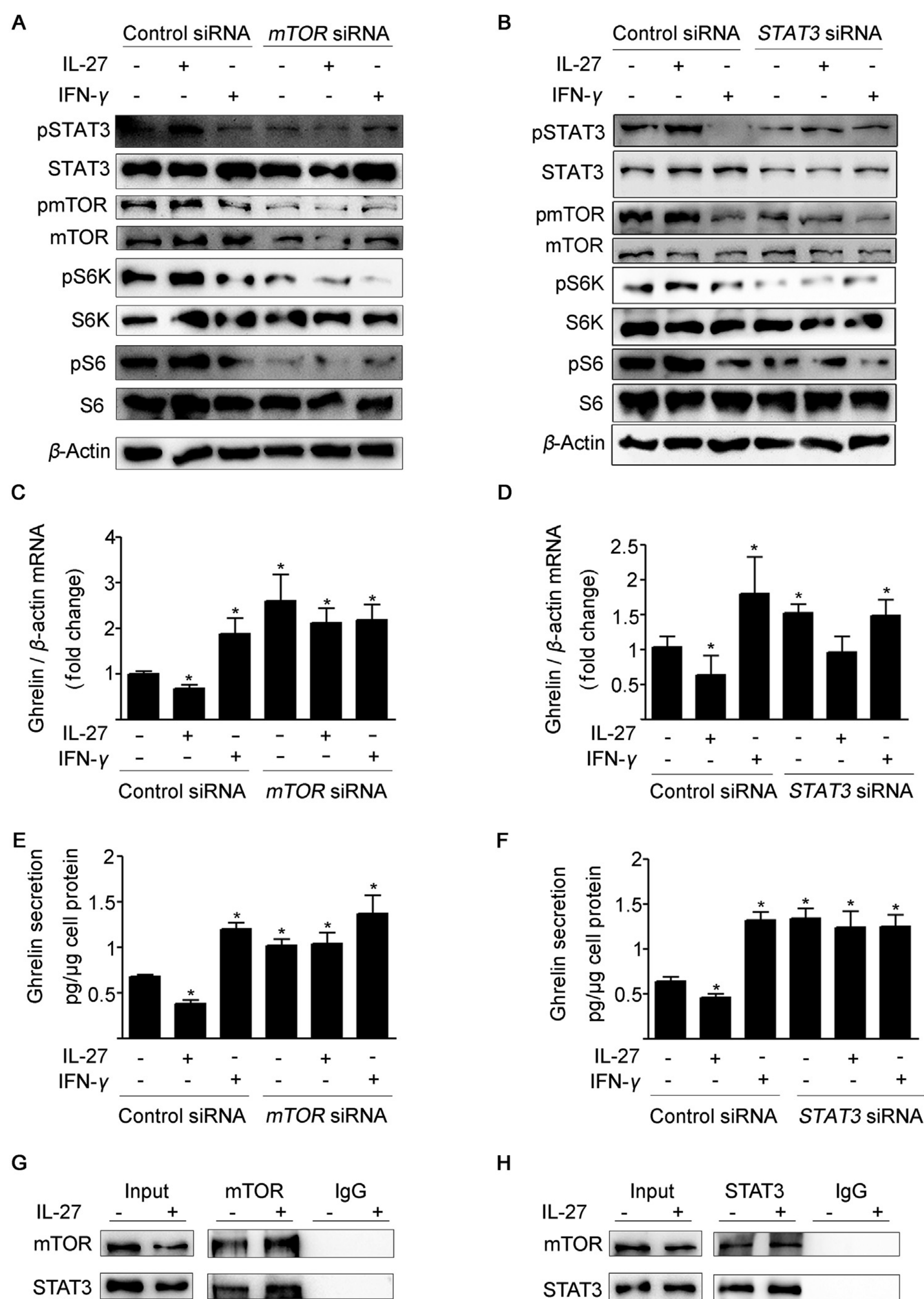

Figure 8 Modulation of ghrelin production by IL-27 through STAT3−mTOR signaling. (A), (C), and (E) Genetic interference of mTOR blocks the suppression of ghrelin induced by IL-27. mHypoE-N42 cells were transfected with control siRNA or *mTOR* siRNA and then treated with saline, IL-27 or IFN-γ. (B), (D), and (F) Genetic silence of STAT3 blocks the suppression of ghrelin induced by IL-27. mHypoE-N42 cells were transfected with control siRNA or *Stat3* siRNA and then treated with saline, IL-27 or IFN-γ. (A) and (B) Representative Western blots of pmTOR, mTOR, pS6K, S6K, pS6, S6, pSTAT3, STAT3 and β-actin. (C) and (D) Ghrelin mRNA was analyzed by real-time PCR. (E) and (F) Medium ghrelin was determined by enzyme immunoassay. (G) and (H) Immunoprecipitations/Immunoblot assaying for interaction between endogenous mTOR and STAT3. mHypoE-N42 cells were treated with saline or IL-27 (50 nmol/L) for 24 h. The interaction between mTOR and STAT3 was detected by co-immunoprecipitation. (G) mTOR was precipitated using anti-mTOR antibodies and co-precipitated STAT3 was immunoblotted under saline or IL-27 treatment. (H) STAT3 was precipitated using anti-STAT3 antibodies and co-precipitated mTOR was immunoblotted under saline or IL-27 treatment. Results are expressed as mean ± SEM. Experiments were repeated for three times. $^{*}P<0.05$ *vs*. control.

diseases[48−50]. IL-27 is a multifaceted heterodimeric cytokine with immunoregulatory functions[34]. We find that IL-27 decreases ghrelin production through the STAT3−mTOR signaling pathway, demonstrating a profound cross-talk between the immune system and the metabolic system.

5. Conclusions

In the present study, we show that IL-27 inhibits ghrelin synthesis and secretion in C57BL/6J mice fed either normal chow or high-fat diet by activating STAT3−mTOR signaling. Thus,

understanding this mechanism of action may open the door to new therapeutic approaches or lead to a more judicious use of existing drugs for the intervention of obesity.

Acknowledgments

We thank Dr. Zhinan Yin from Jinan University for critical comments and advice of the manuscript. This work was supported by grants from the National Natural Science Foundation of China (81770794 and 314010010), the Special Grants from the Guangzhou Pearl River Young Talents of Science and Technology (201610010079, China), the Fundamental Research Funds for the Central Universities (21617457, China).

Author contributions

Geyang Xu designed research; Heng Zhang, Qingjie Li, Yuxin Teng, Yubi Lin, Shaojian Li, Tingfeng Qin, Linxi Chen, Jiana Huang, Hening Zhai and Quan Yu performed research; Heng Zhang, Qingjie Li, Yuxin Teng, Yubi Lin 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.

Conflicts of interest

The authors declare no conflicts of interest.

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