

# Insulin Inhibits Autophagy by Inhibiting the Binding of FoxO1 to the Promoter Region of GABARAPL1



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## ABSTRACT

Hyperinsulinemia and insulin resistance in T2D have a potent suppressive effect on hepatic autophagy, however, the underlying mechanisms remain unclear. To explore the effect of insulin on hepatic autophagy and its possible signaling pathways, HL-7702 cells were treated with insulin with or without insulin signaling inhibitors. The interaction between insulin and the promoter region of GABARAPL1 was assessed through luciferase assay and EMSA. There were significant dose-dependent decreases in the number of intracellular autophagosomes and the protein levels of GABARAPL1 and beclin1 in insulin-treated HL-7702 cells. Insulin signaling inhibitors reversed the inhibitory effect of insulin on rapamycin-induced autophagy and autophagy-related gene upregulation. Insulin blocks the binding of FoxO1 to putative insulin response elements in GABARAPL1 gene promoter, leading to the repressed transcription of GABARAPL1 gene and the suppression of hepatic autophagy. Our study identified GABARAPL1 as a novel target of insulin in suppressing hepatic autophagy.

## Introduction

Diabetes mellitus is posing an increasingly serious threat to human health with an estimated global prevalence of 592 million by 2035 [1]. About 90% of all reported diabetes cases are diagnosed with type 2 diabetes (T2D), which is characterized by hyperinsulinemia and insulin resistance [2]. The epidemiologic studies have revealed a positive correlation between T2D and hepatocellular carcinoma (HCC) [3, 4], a major cause of cancer death worldwide [3], and in-

ulin signaling has been shown to be strongly associated with hepatocarcinogenesis [5, 6]. However, the molecular mechanisms for the association between T2D and HCC are poorly understood.

Autophagy is a self-degradative process in which cytoplasmic components, such as amino-folded or aggregated proteins, damaged organelles, intracellular pathogens, are transported to the lysosomes for degradation. Autophagy is mediated by a unique organelle called autophagosome [7] and plays dual roles in carcinogenesis. In normal cells, autophagy suppresses spontaneous tumorigenesis by maintaining genomic stability, while contributing to

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tumor cell survival once a tumor becomes established and in turn promotes tumor growth and development [8]. The evidence has shown that autophagy-deficient mice may develop benign hepatomas or HCC [9, 10], suggesting that hepatic autophagy is important for suppressing tumor initiation and progression. Since hepatic autophagy is normally inhibited by insulin signaling, it seems that increased insulin secretion compensates for hyperinsulinemia and insulin resistance in T2D has a potent suppressive effect on hepatic autophagy [11], thus promoting tumorigenesis. However, the underlying mechanisms remain unclear. Identifying novel mediators of insulin-induced autophagy suppression may help resolve the etiological puzzle of increased risk of HCC in T2D patients.

Gamma-aminobutyric acid A receptor-associated protein-like 1 (GABARAPL1) belongs to the GABARAP family composed of 5 members: GABARAP, GABARAPL1, GABARAPL2, microtubule-associated protein 1 chain 3 (LC3), and autophagy-related protein 8 (Atg8) [12]. It is well established that GABARAP family members are all involved in the formation of autophagosomes, serving as reliable biomarkers for autophagy [13]. A recent study demonstrated that mRNA and protein expression of *GABARAPL1* were significantly downregulated in HCC tissues compared with para cancer tissues, and low *GABARAPL1* expression was correlated with poor prognosis in HCC patients [14]. Based on these findings, we hypothesized that autophagy-related genes, such as *GABARAPL1*, might mediate crosstalk between insulin signaling and autophagy suppression to facilitate hepatocarcinogenesis.

In the present study, we treated human normal hepatic cell line HL-7702 with different concentrations of insulin to explore its effect on autophagy. Selective insulin signaling inhibitors were used to investigate the possible signaling pathways involved in insulin-modulated autophagy and related gene expression. In addition, we also examined the interaction between insulin and the promoter region of *GABARAPL1* to address the molecular mechanism. Our results demonstrated that insulin suppresses autophagy in a phosphatidylinositol 3-kinase (PI3K)-dependent manner through interfering the binding of transcription factor forkhead box protein O1 (FoxO1) to insulin response element (IRE) in the promoter of *GABARAPL1* gene, leading to downregulation of *GABARAPL1* expression. Our data provide a valuable clue to a better understanding of autophagy dysregulation in T2D and HCC.

## Experimental

### Chemical reagents

Recombinant human insulin solution was purchased from Shanghai No.1 Shenghua Pharmaceutical Industry Co., Ltd. (Shanghai, China). Rapamycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Wortmannin and LY29400 were obtained from Medchem Express (Monmouth Junction, NJ, USA).

### Cell culture

A human normal hepatic cell line HL-7702 was purchased from Jenio-bio (Guangzhou, Guangdong, China) and maintained in DMEM (HyClone, South Logan, Utah, USA) supplemented with 10% fetal calf serum (HyClone), penicillin (50 IU/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO<sub>2</sub> at 37 °C. NI-

H/3T3 mouse embryo fibroblast cells were obtained from and maintained in the First Affiliated Hospital of University of South China (Hengyang, Hunan, China).

### Monodansylcadaverine (MDC) staining for autophagic vacuoles

MDC staining was performed as previously described by Eom et al. (2010) [15]. Briefly, HL-7702 cells were plated in 6-well plates at a density of  $5 \times 10^5$  cells/well and allowed to adhere overnight. Cells were then starved in Earle's Balanced Salt Solution (Hyclone, GE Healthcare, Chicago, IL, USA) for 8 hours followed by incubation with different dose of insulin (1, 10, or 100 nM) overnight at 37 °C. Untreated cells were used as a blank control. After treatment, cells were incubated with 0.05 mmol/L MDC at 37 °C for 45 minutes before fixing with 4% paraformaldehyde for 5 minutes at 4 °C. Images were acquired using Olympus X71 fluorescence microscopy (Olympus, Tokyo, Japan) at magnification 400×. The fluorescence intensity was quantified in 5 randomly selected fields using Image J2X (NIH, Bethesda, MD, USA).

### Transmission electron microscopy (TEM) analysis

After treatment, HL-7702 cells were prefixed with 2.5% glutaraldehyde for 2 hours and postfixed with 1% osmic acid for additional 2 hours at 4 °C, followed by gradient dehydration in 30%, 50%, and 70% ethanol (10 min each), 80%, 90%, and 95% acetone (10 min each), and 100% acetone (10 min twice). Cells were then embedded in the resin and stained with lead citrate. The stained cells were observed and imaged under a Jeol1230 TEM (NRI-MCDB Microscopy Facility, Santa Barbara, CA, USA).

### Cloning and mutagenesis

The 1829-bp mouse *GABARAPL1* promoter region spanning -1715 bp to +114 bp was PCR-amplified from mouse genomic DNA extracted from NIH/3T3 mouse embryo fibroblast cells using the primer sets 5'-TG TAGAGCTCCACCGACATGCAGTAACGTAGT-3' (forward) and 5'-GCCACTCGAGGCACTACTGCGTAAACGTCCAG-3' (reverse). PCR products were then ligated and subcloned into luciferase expression vector pGL4.10 to prepare recombinant plasmid *GABARAPL1*-promoter/pGL4.10 or GEC1 expressing the entire promoter region of mouse *GABARAPL1*. The sequencing results were compared with mouse *GABARAPL1* cDNA sequence reported by GenBank database. Putative insulin response elements (IRE) in *GABARAPL1* promoter were predicted using the Motif finder software. Four deletion mutants, GEC1-D1 (-1204/+114), GEC1-D2 (-818/+114), GEC1-D3 (-437/+114), and GEC1-D4 (-91/+114), expressing 1318-, 932-, 551-, and 205-bp fragments of the mouse *GABARAPL1* promoter region, respectively, were generated using GEC1 (-1715/+114) as a template and primer sets as shown in ▶ **Table 1**. They were subcloned into pGL4.10 vector to generate plasmids GEC1-D1-pGL4.10, GEC1-D2-pGL4.10, GEC1-D3-pGL4.10, and GEC1-D4-pGL4.10, respectively.

Mutagenesis of putative forkhead box protein O1 (FoxO1) binding sites located at *GABARAPL1* promoter was performed using GEC1-D3-pGL4.10 (-437/+114) as a template and a QuikChange site-directed mutagenesis kit (Agilent, Santa Clara, CA, USA) following the manufacturer's instruction. The primer sets are shown

► **Table 1** Primer sets for deletion mutants of mouse *GABARAPL1*.

Name	Primers	Size (bp)
GEC1-D1-F	5'- TTCTGAGCTCCTAATTGTATTTGGGTCAAGGGT-3'	1318
GEC1-D2-F	5'- ACTAGAGCTCGTCTCCACCAGCATGCCAGTCAG-3'	932
GEC1-D3-F	5'-TCCAGAGCTCGACTCCTGATTCCATTGCTCTGGA -3'	551
GEC1-D4-F	5'-ACAAGAGCTCTGTCCACCCGGATCCAGCAGGTCC -3'	205
GEC1-D-R	5'- GCCACTCGAGGCACTACGTGGCTAAACGTCCAG-3'	

► **Table 2** Primer sets for site-directed mutagenesis of mouse *GABARAPL1*.

Name	Sequence (5'–3')
IRE1-F	GTCACAGCACCCCTGGATACCTTGCTTTCCAGCTCTTGG
IRE1-R	CCTTGAGCTGGAACACAAGGTATCCAGGGCGGTGCTGTGAC
IRE2-F	GGGTGAAAAGAAGATACATACCTAAACAAGCTTCTGTCCACC
IRE2-R	GGTGGACAGAAGCTTTGTTTAGGTATGTATCTTCTTTACCC
IRE3-F	CCTTAATATCAATCCTCATACCTAAAAACCCGACTACATG
IRE3-R	CATGTAGTCGGGTTTTTAGGTATGAGGATTGATATTAAGG
IRE4-F	GGATTTCTCTCTGTGTTAGACCTGGGCATAGACCAAGAACTTG
IRE4-R	CAAGTTCTTGGTCTATGCCAGGTCTAACACAGAGGAAGAAAATCC

in ► **Table 2**. All mutations/substitutions in the DNA were confirmed by DNA sequencing (data not shown).

### Luciferase assay

Luciferase assay was performed using a luciferase reporter assay system (Promega, Madison, WI, USA), luciferin was used as the substrate to detect firefly luciferase, and then coelenterazine was used as the substrate to detect renilla reniformis luciferase, following the manufacturer's instruction. Briefly, HL-7702 cells were seeded in 24-well plates at a density of  $1 \times 10^5$  cells per well and transfected with recombinant plasmids using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The renilla luciferase reporter plasmid pRL-TK (Promega) was used as an internal control. Cells were lysed after 24 hours of transfection using lysis buffer (Promega) and the luciferase activity was immediately determined using a luminometer (Thermo). The results were normalized to corresponding pRL-TK activity. Independent experiments were performed at least three times.

### Electrophoretic mobility shift assay (EMSA)

Nuclear proteins from HL-7702 cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce biotechnology, USA). The double-stranded probe of transcription factor FoxO1 was designed based on the sequence of IRS1 in mouse *GABARAPL1* promoter (upper strand: 5'-TTCTCTGTGTTGTTTGGGC-3', lower strand: 5'-GCCAAAACAACACAGAGGA-3') and was end-labeled with biotin, followed by incubation with nuclear extracts at 15 °C for 30 minutes. In addition, parallel binding was run in the presence

of excessive unlabeled cold probes, anti-FoxO1 IgGs or normal IgGs. The reaction mixtures were electrophoresed in 6% polyacrylamide nondenaturing gel at 4 °C and visualized by autoradiography.

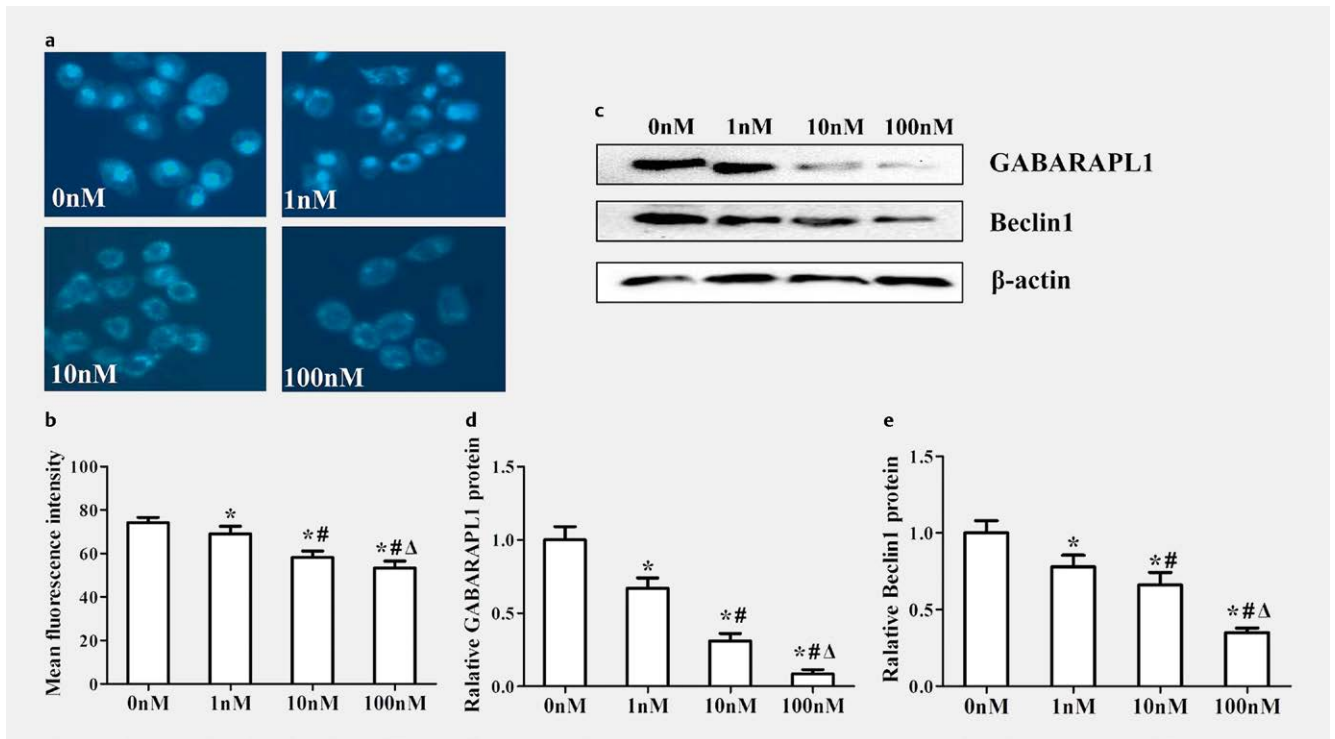
### Statistical analysis

Data are expressed as mean  $\pm$  SD. Statistical analysis was performed by the SPSS 17.0 software. Comparisons among different groups were analyzed by one-way ANOVA, then post hoc Dunn and multiple comparison tests.  $p < 0.05$  was considered significant.

## Results

### Insulin suppresses autophagy and autophagy-related gene expression

To explore the effect of insulin on autophagy, human normal hepatic cell line HL-7702 cells were treated with insulin at various concentrations (0, 1, 10, or 100 nM) followed by MDC staining of autophagic vacuoles. As shown in ► **Fig. 1a, b**, MDC fluorescent intensity was significantly reduced by insulin treatment in a dose-dependent manner. Consistently, the protein expression of two autophagy markers *GABARAPL1* [12] and *Beclin1* [16] was also dose-dependently downregulated in insulin-treated cells compared with untreated ones (► **Figs. 1c–e**). These results suggest that insulin may exert a suppressive effect on hepatic autophagy.



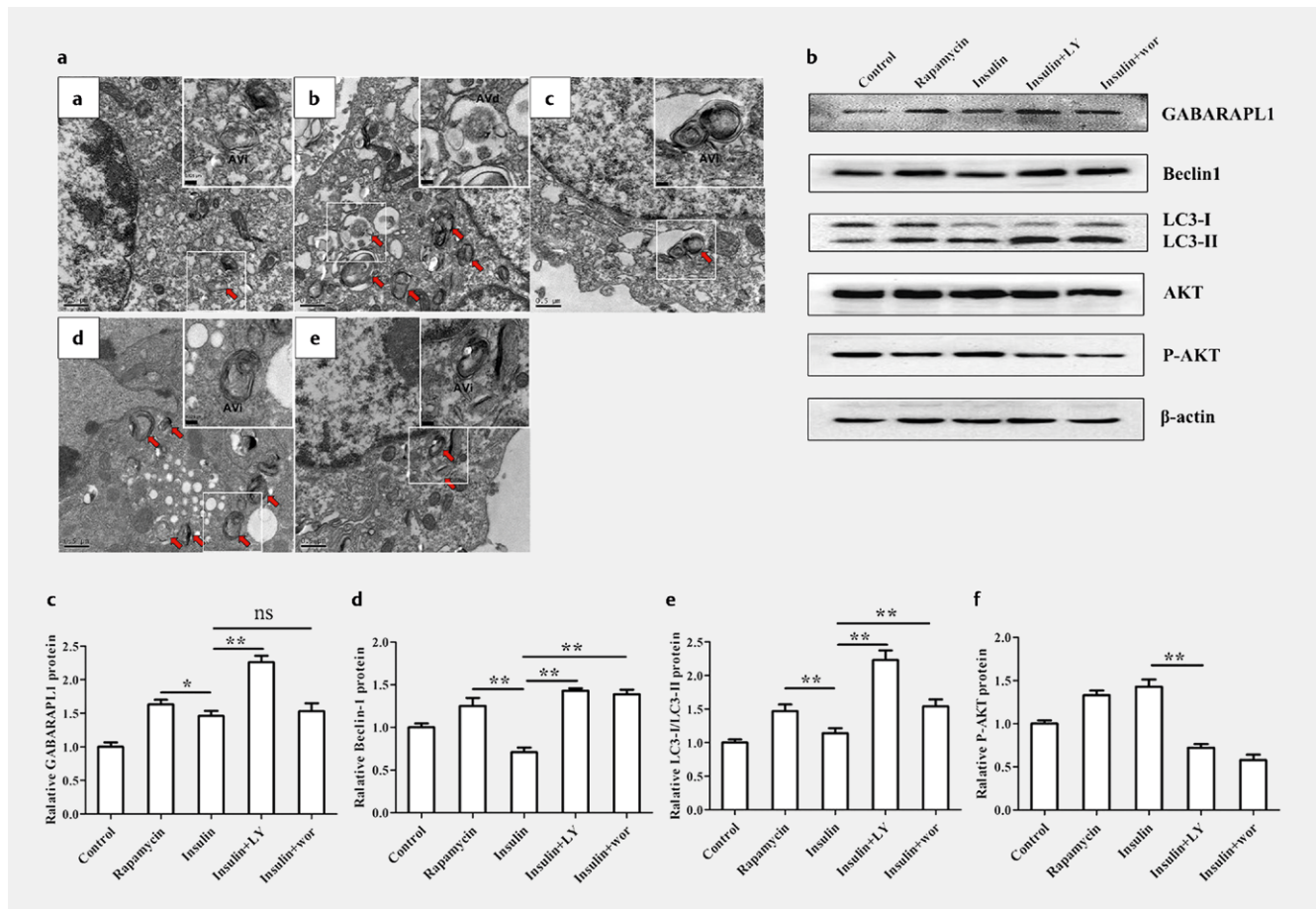
**► Fig. 1** Effects of insulin on autophagy and autophagy-related genes expression in HL-7702 cells: HL-7702 cells were starved in Earle's Balanced Salt Solution for 2 h followed by treatment with different doses of insulin (1, 10, or 100 nM) overnight at 37 °C. **a:** After treatment, cells were incubated with 0.05 mmol/l monodansylcadaverine (MDC) for 45 min at 37 °C. Blue staining represents intracellular autophagic vacuoles. Representative images were shown. Magnification 400 ×. **b:** The fluorescence intensity of MDC was quantified in 5 randomly selected fields using Image J2X. Data are expressed as the mean ± standard deviation (SD). \* p < 0.01 vs. the untreated group; # p < 0.01 vs. the 1 nM group; Δ p < 0.01 vs. the 10 nM group. n = 5. **c:** Western blot assay for gamma-aminobutyric acid A receptor-associated protein-like 1 (GABARAPL1) and beclin1 expression in HL-7702 cells. β-Actin was used as an internal control. **d** and **e:** Quantification of **c**. Data are expressed as mean ± SD. \* p < 0.01 vs. the untreated group; # p < 0.01 vs. the 1 nM group; Δ p < 0.01 vs. the 10 nM group; n = 3. *GABARAPL1*: Gamma-amino butyric acid A receptor-associated protein-like 1.

## PI3K/AKT signaling is responsible for the suppressive effect of insulin on hepatic autophagy

Since insulin triggers PI3K signaling pathway [17], we next sought to investigate the role of PI3K signaling in insulin-induced suppression of hepatic autophagy using PI3K-specific inhibitors wortmannin or LY294002. As shown in ► **Fig. 2a**, initial or degradative autophagic vacuoles [18] can be detected in cells. These results strongly suggest that insulin can effectively inhibit autophagy in HL-7702 cells pretreated with rapamycin, a mammalian target of rapamycin (mTOR) inhibitor that has been used as a classical autophagy inducer [19], as evidenced by the decreased number of autophagosomes in HL-7702 cells treated with both insulin and rapamycin, compared with the cells treated with rapamycin alone. However, wortmannin or LY294002 significantly reversed the suppressive effect of insulin on autophagy (► **Fig. 2a**), along with a marked increase in expression of GABARAPL1 and Beclin1 as well as autophagosomal membrane proteins LC3-I and LC3-II which may reflect the number of autophagosomes or the degree of autophagy [20–22] (► **Fig. 2b–e**). On the other hand, wortmannin or LY294002 also inhibited the phosphorylation of AKT (► **Fig. 2b, f**), suggesting that AKT activation is involved in insulin-modulated autophagy. Collectively, our data indicate that activation of PI3K/AKT signaling contributes to insulin-induced suppression of hepatic autophagy.

## Insulin may inhibit GABARAPL1 transcription through blocking IRE2–4 in GABARAPL1 gene promoter

As we have shown above, insulin can inhibit the expression of GABARAPL1, we further investigated how *GABARAPL1* transcription or expression was finely regulated in an insulin-related context. We found that the transcriptional activity of *GABARAPL1* with wild-type (WT) promoter sequence was dose-dependently decreased in response to insulin in HL-7702 cells (► **Fig. 3a**), suggesting that insulin may regulate GABARAPL1 expression at the transcriptional level. To determine the mechanism whereby insulin governs *GABARAPL1* transcription, we identified 15 putative IREs (IRE 1–15) in promoter region of *GABARAPL1* gene, and then obtained 4 deletion mutants as indicated in ► **Fig. 3b**. The results showed that deletion of IRE 2–15 (only 205 bp IRE 1 is remaining) dramatically inhibited the transcriptional activity of *GABARAPL1* gene, compared with the WT *GABARAPL1* promoter (► **Fig. 3c**). However, deletion of IRE 5–15 (551 bp IRE 1–4 is remaining) had no significant effect on transcriptional activity of *GABARAPL1* gene, and similar results were also observed in deletion of IRE 7–15 or IRE 11–15 (► **Fig. 3c**). These data demonstrate that IRE 2–4, but not IRE 5–15, is essential for hepatic *GABARAPL1* transcription.



► **Fig. 2** Effects of phosphatidylinositol 3-kinase (PI3K)-specific inhibitor wortmannin or LY294002 on autophagy and autophagy-related genes expression in insulin-treated HL-7702 cells: Cells were exposed to 100 nM rapamycin for 6 h to induce autophagy and then treated with 1  $\mu$ M insulin overnight at 37  $^{\circ}$ C. For PI3K inhibitor treatment, 50  $\mu$ M wortmannin or 100 nM LY294002 was added 30 min before insulin. Untreated cells were used as negative control. **a**: Transmission electron microscopy (TEM) analysis was performed to visualize autophagosomes in HL-7702 cells. Representative images are shown. Red arrows indicate autophagosomes. A: Control group; B: Rapamycin; C: Insulin group; D: Insulin + LY group; E: Insulin + wor group. Close-up of the magnified region associated with autophagosome indicated by white rectangle in A. AVI: Initial autophagic vacuoles; AVd: Degradative autophagic vacuole. Scale bar: 0.125  $\mu$ m. **b**: Western blot assay for GABARAPL1, beclin1, microtubule-associated protein 1 chain 3 (LC3)-I, LC3-II, serine/threonine kinase 1 (Akt) and phosphorylated-Akt expression in HL-7702 cells.  $\beta$ -Actin was used as an internal control. **c-f**: Quantification of **b**. \*  $p < 0.05$ ; \*\*  $p < 0.01$ ; n = 3. GABARAPL1: Gamma-aminobutyric acid A receptor-associated protein-like 1; LY: LY294002; wor: Wortmannin; Akt: Serine/threonine kinase 1; p-Akt: Phosphorylated Akt.

## Insulin inhibits binding of transcription factor FoxO1 to IRE1 in GABARAPL1 gene promoter

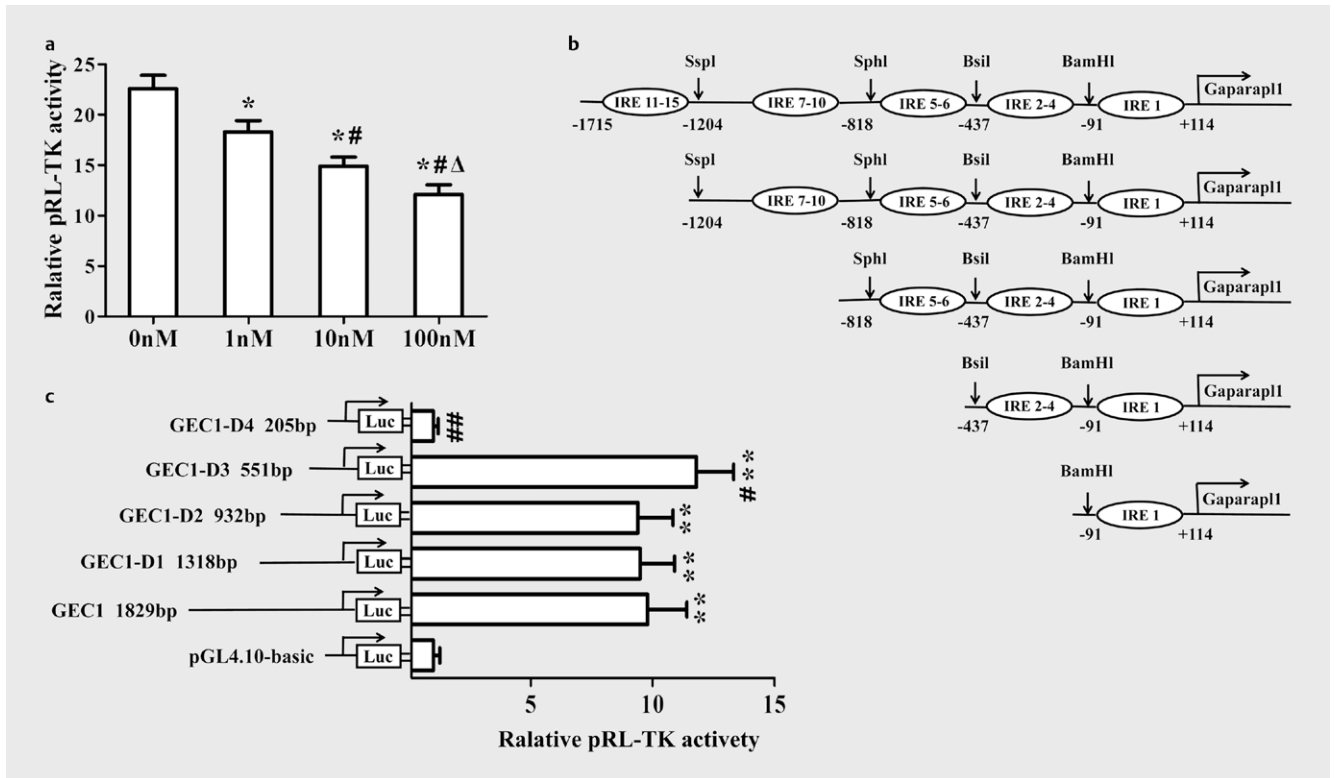
FoxO1 is a key transcription factor majorly involved in *GABARAPL1* transcription and insulin signaling [23, 24]. To further determine whether insulin suppresses *GABARAPL1* transcription by blocking binding of FoxO1 to IREs of *GABARAPL1* gene promoter, we designed 5 IRE site-specific mutations in *GABARAPL1* gene promoter as indicated in ► **Fig. 4a**, in order to identify the binding site of FoxO1. Our previous results have demonstrated that IRE 2–4 is essential for hepatic *GABARAPL1* transcription. The results showed that mutations in IRE 1 and 2 greatly decreased *GABARAPL1* transcription activity, compared with mutation in IRE 1, 2, 3 or 4 alone (► **Fig. 4b**). Interestingly, *GABARAPL1* transcription activity was significantly increased when IRE4 was deleted alone (► **Fig. 4b**). It indicates that IRE4 functions as an inhibitory element for the transcription of the *GABARAPL1* gene and suggests that IRE1 or 2 are possible binding sites for FoxO1 on the promoter of *GABARAPL1* gene. We

then performed EMSA using IRE1 sequence as a probe. As shown in ► **Fig. 4c**, rapamycin profoundly promoted IRE1-FoxO1 binding (lane a), which was reversed by insulin treatment (lane b). Insulin inhibitors restored the IRE1-FoxO1 binding affinity that was decreased by insulin (lane c). Importantly, incubation with FoxO1 antibody abolished IRE1-FoxO1 binding (lane d), compared with non-specific IgG (lane e). Taken together, our results suggest that insulin may inhibit *GABARAPL1* transcription through blocking binding of FoxO1 to IRE1 of *GABARAPL1* gene promoter, which is likely due to the occupation of IRE2–4 by insulin.

## Discussion

Hepatic autophagy is known to be suppressed in the presence of hyperinsulinemia and insulin resistance, leading to intracellular accumulation of misfolded proteins and dysfunctional organelles which contributes to the development of HCC [11]. However, how



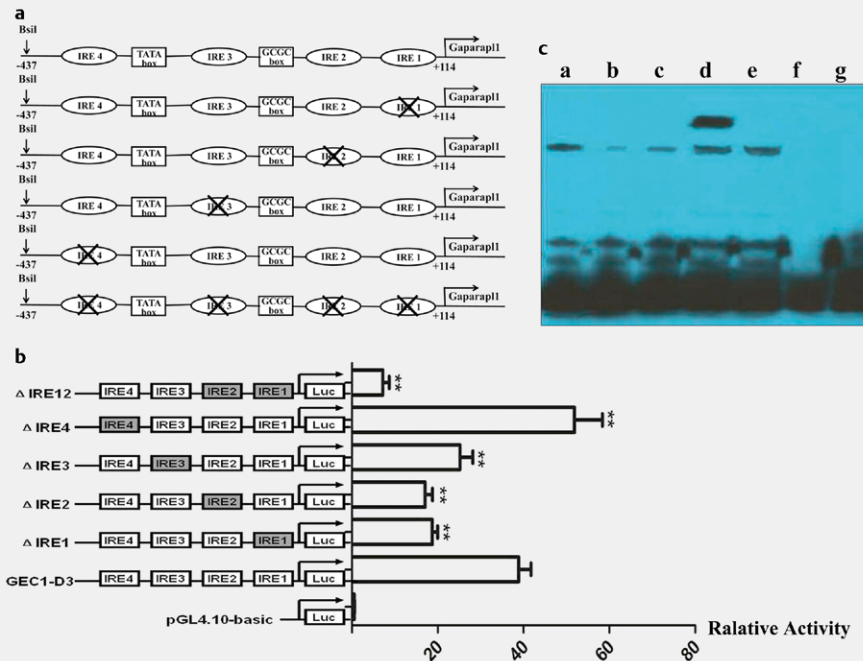


**Fig. 3** Insulin inhibits *GABARAPL1* transcription through blocking insulin response element (IRE) 2–4 in *GABARAPL1* gene promoter: **a**: A recombinant luciferase reporter plasmid *GABARAPL1* promoter/pGL4.10 or GEC1 expressing the entire 1829-bp mouse *GABARAPL1* promoter region spanning –1715 bp to +114 bp was transfected into HL-7702 cells. Renilla luciferase reporter plasmid pRL-TK was used as an internal control. After 48 h of transfection, the cells were treated with different doses of insulin as indicated overnight and the luciferase activity was immediately determined after cell lysis using a luminometer. The results were normalized to corresponding pRL-TK activity. **b**: Prediction of 15 putative IRE in the promoter region of mouse *GABARAPL1* gene. The diagram of sequential deletion mutants was shown. GEC1-D1 (–1204/+114), GEC1-D2 (–818/+114), GEC1-D3 (–437/+114), and GEC1-D4 (–91/+114), expressing 1318-, 932-, 551-, and 205-bp fragments of mouse *GABARAPL1* promoter region, respectively, were generated using GEC1 (–1715/+114) as a template, and were subcloned into pGL4.10 vector to generate plasmids GEC1-D1-pGL4.10, GEC1-D2-pGL4.10, GEC1-D3-pGL4.10, and GEC1-D4-pGL4.10, respectively. **c**: Each deletion mutant was transfected into HL-7702 cells. The empty pGL4.10 vector was used as a blank control. The luciferase activity was determined after 48 h of transfection. \*\*  $p < 0.01$  vs. negative control; #  $p < 0.05$ , ##  $p < 0.01$  vs. GEC1-transfected cells;  $n = 3$ . *GABARAPL1*: Gamma-aminobutyric acid A receptor-associated protein-like 1; *IRE*: Insulin response element.

autophagy is suppressed by insulin in hepatocytes remains largely unknown. In this study, we used a rapamycin-induced autophagy model to explore the mechanism of insulin-induced autophagy suppression in cultured human hepatocytes. Our results indicated that autophagy was significantly inhibited in insulin-treated HL-7702 cells, which is associated with the downregulation of a panel of autophagy-related genes, including *GABARAPL1*, *Beclin1*, *LC3-I*, and *LC3-II*. We then further explored the molecular mechanisms by which insulin participates in the modulation of *GABARAPL1* expression. Our findings revealed that insulin could inhibit hepatocyte autophagy by transcriptional silencing of *GABARAPL1*, which suggests that overexpression of *GABARAPL1* may be a potential preventive strategy against insulin-associated T2D and HCC.

The PI3K/Akt pathway is the major signaling pathway responsible for metabolic actions of insulin [25]. Activation of the PI3K/Akt cascade was initiated by the interaction of insulin receptors with their ligands, which in turn allows the phosphorylation and activation of many downstream targets. mTOR is one of these downstream components of the PI3K/AKT pathway, which is critical for autophagy [26]. It has been reported that insulin activates the PI3K/

Akt/mTOR signaling in mouse fibroblast cells, which promotes necrotic cell death via autophagy suppression [27]. Likewise, the results of this study demonstrated that the upregulation of autophagy and autophagy-related gene expression in hepatocytes induced by the mTOR inhibitor rapamycin was significantly counteracted by insulin but restored in the presence of the specific PI3K inhibitor wortmannin or LY294002, but the expression level of *GABARAPL1* remains stable upon wortmannin treatment, it differs from *Beclin1*, *LC3-I*, and *LC3-II*. Although both wortmannin and LY294002 are PI3K-specific inhibitors, their inhibitory effects on PI3K signaling may vary. It may be the main cause of the diversity in the expression levels of *GABARAPL1*. In addition, the expression of *GABARAPL1*, *Beclin1*, *LC3-I*, and *LC3-II* induced by 100 nM LY294002 is higher than that induced by rapamycin. This may be due to the excessive inhibitory effect of LY294002 on the PI3K/Akt pathway. Along with the deactivation of Akt by these inhibitors in insulin-treated hepatocytes, these findings suggest that the suppressive effect of insulin suppresses hepatic autophagy and the expression of related genes via the PI3K/Akt signaling pathway.



**▶ Fig. 4** Insulin inhibits binding of transcription factor forkhead box protein O1 (FoxO1) to IRE1 in *GABARAPL1* gene promoter: **a**: The diagram of 5 IRE site-specific mutations in *GABARAPL1* gene promoter was shown. **b**: HL-7702 cells were transfected with each mutant as indicated for 48 h and then treated with 1  $\mu$ M insulin overnight at 37 °C. The luciferase activity was determined immediately after cell lysis. The empty pGL4.1 vector and GEC1-D3 mutant were used as controls. \*\*  $p < 0.01$  vs. GEC1-D3 transfected cells.  $n = 3$ . **c**: Nuclear extracts from HL-7702 cells treated with rapamycin, rapamycin + insulin, or rapamycin + insulin inhibitor + insulin was subjected to electrophoretic mobility shift assay (EMSA) by incubating with a probe of IRE1 sequence. Lane a: rapamycin + probe; lane b: insulin + probe; lane c: insulin inhibitor + insulin + probe; lane d: rapamycin + probe + FoxO1 antibody; lane e: rapamycin + probe + nonspecific IgG; lane f: rapamycin + probe + unlabeled cold probes; lane g: probe. *GABARAPL1*: Gamma-aminobutyric acid A receptor-associated protein-like 1; *IRE*: Insulin response element.

Autophagy pathway genes *GABARAPL1*, *Beclin1*, and *LC3* are involved in multiple stages of the autophagosome formation [13]. The results of the present study showed that these genes were all altered at the translational level in response to insulin. FoxO transcription factors FoxO1 and FoxO3 have been implicated in regulating autophagy in cultured rat cardiomyocytes by nuclear localization and binding to the regulatory sequences of these autophagy-related genes [28]. Based on the fact that FoxO possesses a forkhead box-type DNA binding domain and recognizes IRE on the promoter of a gene [29], we sought to find the essential regulatory region in *GABARAPL1* promoter by sequentially deleting the predicted IREs. The results showed that IRE 2–4, but not IRE 5–15, is required for hepatic *GABARAPL1* transcription, suggesting that insulin may interact with IRE 2–4 of *GABARAPL1* gene promoter to suppress *GABARAPL1* transcription. However, the results of subsequent site-directed mutations and EMSA demonstrated that *GABARAPL1* transcription might be inhibited by insulin through blocking the binding of FoxO1 to IRE1 instead of IRE 2–4 of *GABARAPL1* gene promoter, which is likely due to the steric effect caused by occupation of insulin in IRE 2–4. The regulatory mechanisms of insulin-induced Beclin1 and LC3 suppression need further investigation.

In conclusion, our study examined the effect of insulin on autophagy and autophagy-related genes in human hepatocytes. The results demonstrated that insulin inhibits hepatic autophagy and autophagy-related proteins expression in a PI3K/Akt-dependent

manner through hampering the binding of transcription factor FoxO1 to IRE1 in the promoter region of *GABARAPL1* gene, which suggest that overexpression of *GABARAPL1* might be a preventive strategy against increased risk of HCC in hyperinsulinemia-related T2D patients.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

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#### Notice

This article was changed according to the following Erratum on September 25<sup>th</sup> 2023.

#### Erratum

In the above-mentioned article, the authors Tao Hong and Jie Wen contributed equally. This was corrected in the online version.