

Title: Porcine placenta extract upregulates ceramide synthase 3 expression via the PPAR $\delta$ /ILK/Akt/mTOR/STAT3 pathway

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# Abstract

Porcine placenta extract (PPE) is widely accepted as an ingredient in complementary and alternative medicine and previous studies have reported its availability, however, its underlying action mechanism remains unclear. In this study, we investigated the underlying mechanism of porcine placenta extract-induced ceramide synthase 3 upregulation. PPE enhanced the expression of ceramide synthase 3 at both the mRNA and protein levels in HaCaT cells. Moreover, porcine placenta extract-induced ceramide synthase3 upregulation was suppressed by the Akt inhibitor, suggesting the involvement of Akt in the underlying mechanism. As the PI3K inhibitor did not affect porcine placenta extract-induced ceramide synthase 3 upregulation, the factors upstream of Akt were estimated. Inhibition and small interfering RNA experiments demonstrated that the peroxisome proliferator-activated receptors  $\delta$  (PPAR  $\delta$ ) and integrin-linked kinase (ILK) are involved in the phosphorylation of Akt. Next, we explored the factors downstream of Akt and found that porcine placenta extract induced phosphorylation of the STAT3 while porcine placenta extract-induced upregulation of ceramide synthase 3 was significantly suppressed by the inhibitor of the mTOR, suggesting that the mTOR/STAT3 pathway is involved in the downstream of Akt. These results demonstrate that porcine placenta extract upregulates CerS3 expression via the PPAR $\delta$ /ILK/Akt/mTOR/STAT3 pathway.

**Keywords:** Porcine placenta, ceramide synthase 3, signaling pathway

# Background

The primary function of the epidermis is to construct an indispensable barrier between the organism and the desiccated terrestrial environment preventing invasion of external stimuli and loss of water from the body. As summarized in a previous study, many factors are involved in epidermal barrier function [1, 2]. Lipids, which are stored in lamellar bodies in the stratum granulosum and secreted into the extracellular space, are crucial for epidermal barrier function [3]. Previous studies have reported that lipid synthesis is regulated by various inter- and intracellular signaling pathways. Epidermal barrier disruption induces acute upregulation of the expression of inflammatory cytokines in the epidermis, although a chronic increase in cytokine production could have a harmful effect leading to inflammation and epidermal hyperproliferation [4]. Previous studies have suggested that inflammatory cytokines such as interleukin (IL)-6 and tumor necrosis factor (TNF) are potent stimulators of lipid synthesis to maintain epidermal homeostasis [5, 6]. In contrast, previous studies have demonstrated that peroxisome proliferator-activated receptor (PPAR) activators not only significantly enhance the synthesis of lipids including barrier-specific ceramide species, but also significantly accelerate the lamellar

body formation, secretion, and post-secretory processing [7]. In addition, Mizutani *et al.* reported that activators of PPAR $\beta/\delta$  and PPAR $\gamma$  induce ceramide synthase 3 (CerS3) and that PPAR $\beta/\delta$  small interfering RNA (siRNA) inhibits the induction of CerS3 mRNA during differentiation [8]. These results suggest that PPARs play roles in the maintenance of epidermal barrier homeostasis.

Clinical applications of human and/or porcine placenta extract (PPE) are widely accepted in oriental medicine because the placenta is a rich reservoir of diverse bioactive molecules. A previous study showed that injection of human placenta extract into wound boundaries accelerated the decrease in wound size [9]. Moreover, oral administration of PPE reportedly reduces UVB-induced wrinkle formation in mice, along with the downregulation of matrix metalloproteinase (MMP)-2 mRNA expression [10]. In addition, studies using skin cells have revealed that human placenta extract accelerates keratinocyte and fibroblast proliferation [11, 12]. Recently, we reported that the application of PPE-containing gel on the face alleviated wrinkle formation and improved skin hydration, along with the upregulation of CerS3 in keratinocytes [13]. However, as the underlying mechanism still remains unclear, we explored the signaling pathway of PPE-induced CerS3 induction in this study.

## Materials and Methods

### Preparation of PPE

PPE was kindly provided by Kasyu Industries (Kitakyushu, Fukuoka, Japan). Briefly, fresh porcine placenta, collected at calving at the farm, was washed with water and then subjected to the freeze (-20°C)-thawing (under 10°C) cycle. After pasteurization at 65°C for 6 h and removal of debris with 0.22 $\mu$ m filter, the obtained freeze-thaw drip was diluted with sterilized water to a nitrogen content of 0.27% to 0.33%, according to the Kjeldahl method described in the Japanese Standards of Food Additive.

### Reagents and antibodies

All inhibitors and an activator were purchased from Selleck Chemicals (Houston, TX, USA). Primary antibodies against  $\beta$ -actin antibody, CerS3, p-Akt (Ser473) and p-STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), Thermo Fisher Scientific (Waltham, MA, USA), and Cell Signaling Technology (Beverly, MA, USA), respectively.

### Cells

To maintain the differentiation stage of HaCaT cells, calcium in fetal bovine serum was depleted by incubation with Chelex 100 resin (BioRad, Hercules, CA, USA) for 1 h at 4°C. HaCaT cells were maintained in Ca<sup>2+</sup>-free Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% Ca<sup>2+</sup>-depleted fetal bovine serum, 4 mM glutamine, 1

mM sodium pyruvate and 2 mM CaCl<sub>2</sub> at 37°C in a 5% carbon dioxide (CO<sub>2</sub>-)-humidified atmosphere.

### **Treatment with PPE and reagents**

HaCaT cells were seeded into 24-well plates at a cell density of  $1 \times 10^5$  cells/well for qPCR and into 6-well plates at a cell density of  $3 \times 10^5$  cells/well for immunoblotting, and then maintained in a 5% CO<sub>2</sub>-humidified atmosphere at 37°C. After cultivation for 24 h, the cells were treated with 5% PPE in the presence or absence of reagents for appropriate periods.

### **siRNA transfection**

HaCaT cells were reverse-transfected with predesigned PPAR $\delta$ , and ILK siRNA (Thermo Fisher Scientific, Waltham, MA), according to the manufacturer's instructions. Cells were seeded into 24-well plates at a cell density of  $1 \times 10^5$  cells/well for qPCR and into 6-well plates at a cell density of  $3 \times 10^5$  cells/well for immunoblotting, and incubated in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C for 24 h, followed by treatment with PPE and reagents for appropriate periods. The harvested cells were subjected to qPCR and immunoblotting assay.

### **RNA isolation and qPCR**

After the treatments, cells were harvested and total RNA was prepared with SV RNA isolation kit (Promega, Madison, WI, USA), according to the manufacturer's instructions, followed by reverse transcription using ReverTra Ace® qPCR RT Master Mix (TOYOBO, Osaka, Japan). PCR amplification and detection were conducted on a CFX96 real-time PCR system (BioRad, Hercules, CA, USA) using a KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Woburn, MA, USA). The following primer pairs were used:  $\beta$ -actin, 5'-GATGAGATTGGCATGGCTTT-3' (sense) and 5'-CACCTTCACCGTTCCAGTTT-3' (antisense); CerS3, 5'-ACATTCCACAAGGCAACCATTTG-3' (sense) and 5'-CTCTTGATTCCGCCGACTCC-3' (antisense); PPAR $\delta$ , 5'-ACTGAGTTCGCCAAGAGCAT-3' (sense) and 5'-TGCACGCCATACTTGAGAAG-3' (antisense); ILK, 5'-AAGGTGCTGAAGGTTTCGAGA-3' (sense) and 5'-ATACGGCATCCAGTGTGTGA-3' (antisense). The expression of target mRNA was quantified using the comparative threshold cycle (Ct) method for relative quantification ( $2^{-\Delta\Delta Ct}$ ), normalized to the geometric mean of the reference gene  $\beta$ -actin.

### **Immunoblotting**

HaCaT cells were collected in the RIPA buffer supplemented with protease inhibitors and phosphatase inhibitors. Equal amount of protein (10 $\mu$ g) were loaded, resolved via SDS-PAGE and transferred to PVDF membrane, followed by immunoblotting with each antibody. Immunoreactive proteins were visualized using an enhanced

chemiluminescence detection system (Millipore, Bedford, MA, USA). The relative intensity of the blot was measured using the ImageJ software and was normalized by the intensity of  $\beta$ -actin blot.

### **Statistical analysis**

Results of mRNA relative expression and densitometric analysis of western blotting images are expressed as the mean  $\pm$  standard deviation (SD) of at least three independent experiments. Statistical analysis was performed using the Student's t-test. Statistical significance was set  $p < 0.05$ .

## **Results**

### **PPE enhanced CerS3 expression**

To confirm the effect of PPE on the mRNA expression of CerS3, quantitative polymerase chain reaction (qPCR) was performed. The treatment with PPE significantly upregulated the mRNA expression of CerS3 to  $2.40 \pm 0.59$  (Figure 1a). As well, the protein expression of CerS3 was significantly enhanced to  $2.00 \pm 0.58$  (Figure 1b and 1c).

### **Akt was involved in PPE-induced upregulation of CerS3**

GSK690693, an inhibitor of Akt, was employed to evaluate the involvement of Akt in PPE-enhanced CerS3 mRNA and protein expression. Treatment with GSK690693 significantly downregulated PPE-enhanced CerS3 mRNA expression (Figure 2a). In addition, the protein level of CerS3 was significantly decreased by treatment with GSK690693 (Figure 2b and 2c). In contrast, LY294002, an inhibitor of phosphoinositide 3-kinase (PI3K) that phosphorylates Akt, did not affect PPE-induced upregulation of CerS3 expression (Figure 2d).

### **PPAR $\delta$ was involved in PPE-induced upregulation of CerS3**

The PPAR $\delta$  selective inhibitor, GSK3787, significantly downregulated PPE-enhanced CerS3 mRNA expression, whereas GSK9662, a selective inhibitor of PPAR $\gamma$ , did not affect PPE-enhanced CerS3 mRNA expression (Figure 3a). Moreover, GW0742, a PPAR $\delta$  activator, upregulated CerS3 mRNA expression as well as PPE (Figure 3b). While the expression of PPAR $\delta$  was knocked down by siRNA (Figure 3c), the effect of PPE on CerS3 expression was diminished (Figure 3d). In addition, GSK3787 and PPAR $\delta$  knockdown (KD) significantly reduced the upregulation of CerS3 protein levels by PPE treatment (Figure 3e-3h).

### **Integrin-linked kinase (ILK) was involved in PPE-induced upregulation of CerS3**

The ILK inhibitor, OSU-T315, drastically reduced the effect of PPE on CerS3 expression at both the mRNA and protein levels (Figure 4a-4c). While the expression of ILK was knocked down by siRNA (Figure 4d), the upregulation of CerS3 expression by PPE was diminished significantly at both the mRNA and protein levels (Figure 4e-4g). The mRNA

expression of ILK was significantly enhanced after 16 h of PPE treatment, following significant upregulation of PPAR $\delta$  mRNA expression (Figure 4h).

### **mTOR/STAT3 signaling pathway participated in PPE-induced upregulation of CerS3**

The PPE-induced CerS3 expression at the mRNA and protein levels was significantly reduced in the presence of rapamycin, an mTOR inhibitor (Figure 5a-5c). Phosphorylated STAT3 (p-STAT3) was detected in HaCaT cells treated with PPE, as well as phosphorylated Akt (Figure 5d), suggesting the involvement of STAT3 in PPE-induced CerS3 upregulation.

## **Discussion**

Ceramides, which are the predominant lipids comprising 30 to 50 % of the intercellular lipid content in the stratum corneum, are synthesized by the enzymatic actions of ceramide synthases (CerS). CerS3, which is one of the six distinct isoforms of CerSs, is highly expressed in keratinocytes, synthesizes ceramides containing  $\alpha$ -hydroxy fatty acids, and is involved in maintaining skin barrier function [14-16]. We have recently reported that PPE, which is widely used in alternative and traditional medicine, enhances CerS3 expression [13]. However, because the regulation of CerS3 expression is largely uncharacterized, we explored the underlying mechanism of the PPE-induced upregulation of CerS3 in this study. The expression levels of CerS3 mRNA and protein were significantly upregulated in the cells treated with PPE (Figure 1). The upregulation was diminished by Akt inhibitor (Figure 2a and 2b), although LY294002, a PI3K inhibitor, did not affect the effect of PPE (Figure 2c). This suggests that some signaling pathways other than PI3K/Akt are involved in the mechanism underlying the induction of CerS3. To elucidate the upstream and downstream pathways of Akt, we first examined the involvement of PPAR $\delta$  in the signaling pathway. As shown in Figure 3, PPAR $\delta$  is involved in the PPE-induced upregulation of CerS3 expression. This corresponds to a previous study demonstrating that PPAR $\beta/\delta$  is involved in CerS3 upregulation during keratinocyte differentiation [8]. However, to the best of our knowledge, the binding site of PPAR $\delta$  does not exist in the promoter region of the *CerS3* gene, suggesting that PPAR $\delta$  contributes indirectly to CerS3 upregulation. As described above, Akt is crucial in the signaling pathway involved in PPE-induced CerS3 upregulation, as well as PPAR $\delta$ . A previous study demonstrated that ILK, which is ubiquitously expressed, phosphorylates Akt at serine 473, leading to the activation of Akt [17, 18]. The inhibition of ILK and ILK KD diminished PPE-induced CerS3 upregulation (Figure 4a-g), suggesting that ILK also plays a pivotal role in the signaling pathway of the effect of PPE. Di-Poï *et al.* reported that PPAR $\beta/\delta$  modulates Akt activation via the upregulation of ILK [19]. As shown in

Figure 4h, PPE treatment enhanced PPAR $\delta$  mRNA expression, followed by the upregulation of ILK mRNA expression, suggesting that enhancement of the expression of PPAR $\delta$  and ILK contributes to maintaining the upregulated level of CerS3 expression during the experimental period. Collectively, the PPE treatment regulates Akt phosphorylation via ILK whose expression is enhanced by PPAR $\delta$  upstream of the signaling pathway of PPE-induced CerS3 upregulation. An important effector of Akt via tuberous sclerosis (TSC)-1/2 and the small GTPase Rheb is the mTOR signaling pathway [20, 21]. Therefore, we elucidated the involvement of mTOR in the signaling pathway involved in PPE-induced CerS3 upregulation. Rapamycin, an mTOR inhibitor, suppressed PPE-upregulated CerS3 expression at both the mRNA and protein levels (Figure 5a-c), suggesting the involvement of mTOR in the signaling pathway. STAT3 is a crucial regulator of various cell processes. STAT3 is selectively activated by stimulation with cytokines and growth factors [22, 23]. In keratinocytes, STAT3 plays a crucial role in keratinocyte migration and skin remodeling [24]. mTOR also positively regulates STAT3 activation, similar to cytokines and growth factors [25]. Collectively, the mTOR/STAT3 signaling pathway participates downstream of Akt phosphorylation in PPE-upregulated CerS3 expression. In conclusion, we found that PPE upregulated CerS3 expression via the PPAR $\delta$ /ILK/Akt/mTOR/STAT3 signaling pathway (Figure 6). In particular, PPE induced the persistent expression of PPAR $\delta$ , while ILK contributed to the enhanced expression of CerS3.

## Conclusion

In this study, we demonstrated, for the first time, the underlying mechanism of PPE function and provided scientific evidence for the application of PPE in alternative medicine.

## List of abbreviations

Cers3: ceramide synthase 3; DMEM: Dulbecco's modified Eagle's medium; IL-6: interleukin-6; ILK: integrin-linked kinase; MMP-2: matrix metalloproteinase-2; mTOR: mammalian target of rapamycin; PI3K: phosphoinositide 3-kinase; PPAR: peroxisome proliferator-activated receptor; PPE: porcine placenta extract; PVDF: poly vinylidene difluoride; qPCR: quantitative polymerase chain reaction; RIPA: radio-immunoprecipitation assay; siRNA: small interfering RNA; SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis; STAT3: signal transducer and activator of transcription 3; TNF: tumor necrosis factor

## Declarations



## Availability of data and materials

All data generated or analyzed during this study are included in this published article.

## Conflict of interests

The authors declare that they have no conflict of interest.

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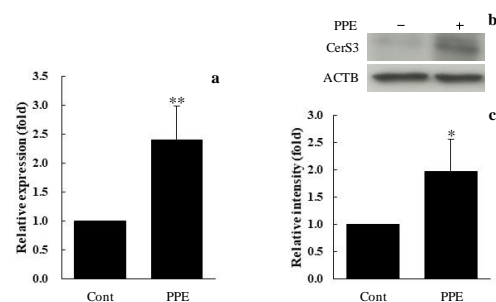


Figure 1. Porcine placenta extract (PPE) enhanced ceramide synthase 3 (CerS3) expression. (a) CerS3 mRNA expression was upregulated in PPE-treated cells. (b) Representative western blotting image of equal amounts of protein extracts from the control and PPE-treated cells. (c) Relative intensity of CerS3 blots was measured using the ImageJ software and was normalized by the intensity of the  $\beta$ -actin blot. Statistical analyses were performed with a Student's t-test. \* p-value < 0.05, \*\* p-value < 0.01 were considered to be significantly different from the control.

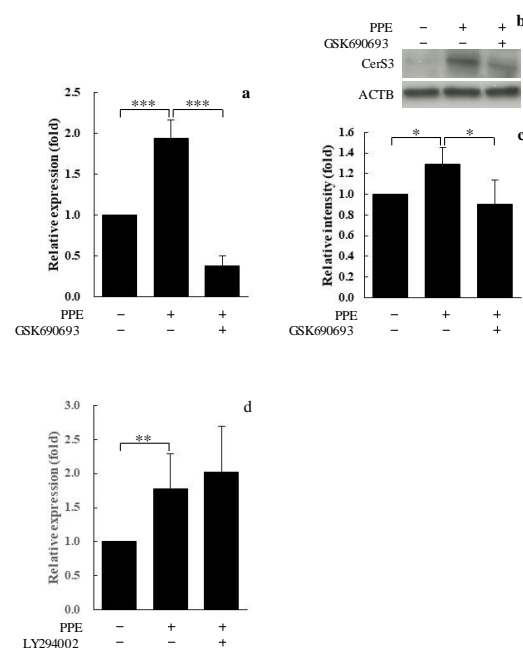


Figure 2. Akt was involved in PPE-induced upregulation of CerS3 expression, but not PI3K. (a) GSK690693 suppressed CerS3 mRNA expression upregulated by PPE treatment. (b) Representative western blotting image of equal amounts of protein extracts from the control and PPE-treated cells in the absence and presence of GSK690693. (c) Relative intensity of CerS3 blots was measured using the ImageJ software and was normalized by the intensity of the  $\beta$ -actin blot. (d) LY294002 did not affect PPE-upregulated CerS3 mRNA expression. Statistical analyses were performed with a Student's t-test. \* p-value < 0.05, \*\* p-value < 0.01 and \*\*\* p-value < 0.001 were considered to be significantly different from the control.

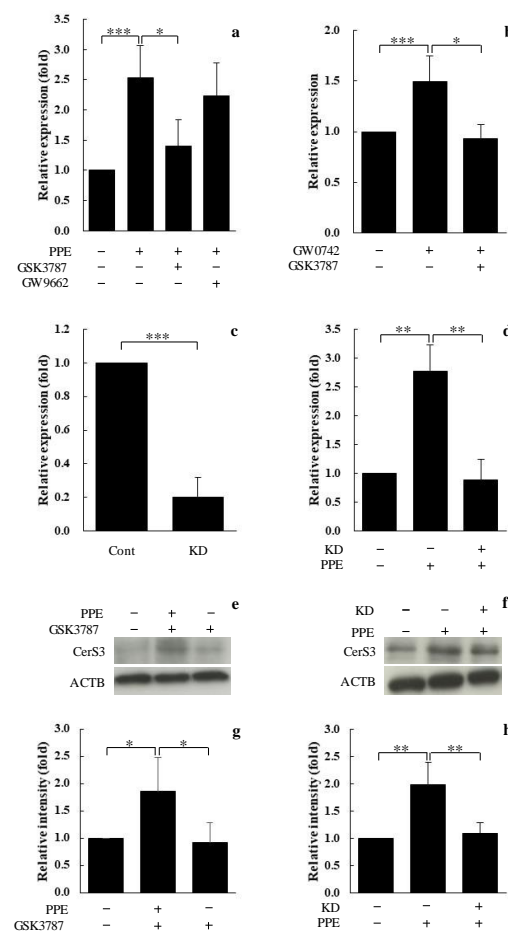


Figure 3. PPAR $\delta$  was involved in PPE-induced upregulation of CerS3 expression, but not PPAR $\gamma$ . (a) GSK3787 suppressed CerS3 mRNA expression upregulated by PPE treatment, while GW9662 did not affect the upregulation. (b) GW0742 enhanced CerS3 mRNA expression and its upregulation was suppressed by GSK3787. (c) Expression of PPAR $\delta$  mRNA was reduced by the small interfering RNA (siRNA). (d) PPAR $\delta$  knockdown (KD) suppressed CerS3 mRNA expression upregulated by PPE treatment. (e) Representative western blotting image of equal amounts of protein extracts from the control and PPE-treated cells in the absence and presence of GSK3787. (f) Representative western blotting image of equal amounts of protein extracts from the control and PPAR $\delta$  KD cells. (g and h) Relative intensity of CerS3 blots was measured using the ImageJ software and was normalized by the intensity of the  $\beta$ -actin blot in inhibition and siRNA experiments. Statistical analyses were performed with a Student's t-test. \* p-value < 0.05, \*\* p-value < 0.01 and \*\*\* p-value < 0.001 were considered to be significantly different from the control.

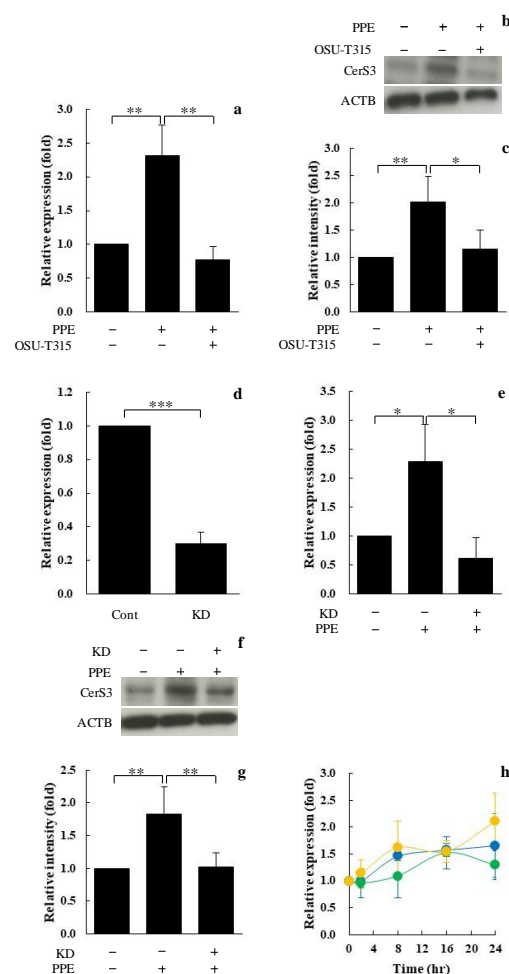
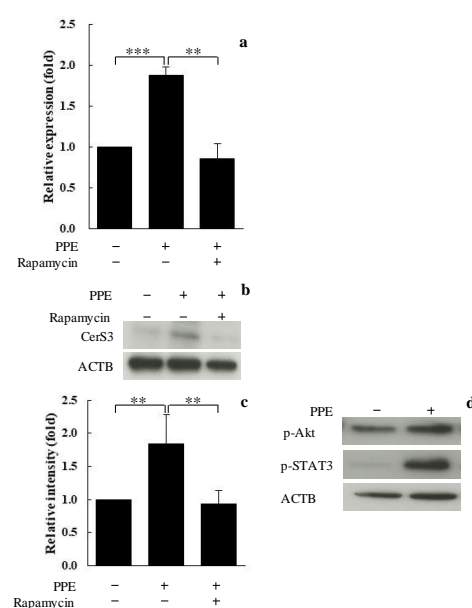


Figure 4

ILK was involved in PPE-induced upregulation of CerS3 expression. (a) OSU-T315 suppressed CerS3 mRNA expression upregulated by PPE treatment. (b) Representative western blotting image of equal amounts of protein extracts from the control and PPE-treated cells in the absence and presence of OSU-T315. (c) Relative intensity of CerS3 blots was measured using the ImageJ software and was normalized by the intensity of the  $\beta$ -actin blot in the inhibition experiments. (d) Expression of ILK mRNA was reduced by siRNA. (e) ILK KD suppressed CerS3 mRNA expression upregulated by PPE treatment. (f) Representative western blotting image of equal amounts of protein extracts from the control and the ILK KD cells. (g) Relative intensity of CerS3 blots was measured using the ImageJ software and was normalized by the intensity of the  $\beta$ -actin blot in the siRNA experiments. (h) CerS3 mRNA expression (yellow circle) arose in the acute phase (after 2hr) and was maintained throughout the experimental period. PPAR $\delta$  mRNA expression (blue circle) arose after 8hr, followed by ILK mRNA expression (green circle). Statistical analyses were performed with a Student's t-test. \* p-value < 0.05, \*\* p-value < 0.01 and \*\*\* p-value < 0.001 were considered to be significantly different from the control.



**Figure 5**

mTOR)/STAT3 signaling pathway was involved in PPE-induced upregulation of CerS3 expression. (a) Rapamycin, an mTOR inhibitor, suppressed CerS3 mRNA expression upregulated by PPE treatment. (b) Representative western blotting image of equal amounts of protein extracts from the control and PPE-treated cells in the absence and presence of rapamycin. (c) Relative intensity of CerS3 blots was measured using the ImageJ software and was normalized by the intensity of the  $\beta$ -actin blot in inhibition experiments. (d) Representative western blotting image of p-Akt and p-STAT3 in the cells treated with PPE for 30 min. Statistical analyses were performed with a Student's t-test. \* p-value < 0.05, \*\* p-value < 0.01 and \*\*\* p-value < 0.001 were considered to be significantly different from the control.

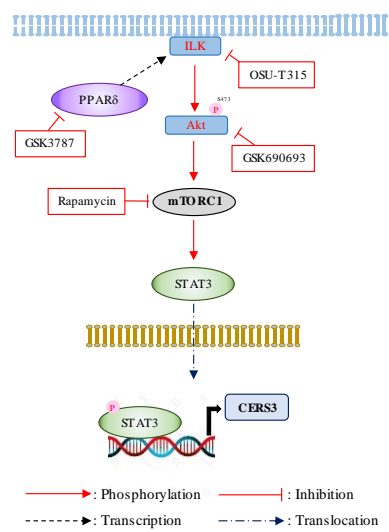


Figure 6  
Potential signaling pathway underlying the PPE-induced upregulation of CerS3.