

# Obestatin stimulates Akt signalling in gastric cancer cells through $\beta$ -arrestin-mediated epidermal growth factor receptor transactivation

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## Abstract

Obestatin was identified as a gut peptide encoded by the ghrelin gene that interacts with the G protein-coupled receptor, GPR39. In this work, a sequential analysis of its transmembrane signalling pathway has been undertaken to characterize the intracellular mechanisms responsible for Akt activation. The results show that Akt activation requires the phosphorylation of T308 in the A-loop by the phosphoinositide-dependent kinase 1 (PDK1) and S473 within the HM by the mammalian target of rapamycin (mTOR) kinase complex 2 (mTORC2: Rictor, mLST8, mSin1, mTOR kinase) with participation neither of  $G_{i/o}$ -protein nor  $G\beta\gamma$  dimers. Obestatin induces the association of GPR39/ $\beta$ -arrestin 1/Src signalling complex resulting in the transactivation of the epidermal growth factor receptor (EGFR) and downstream Akt signalling. Upon administration of obestatin, phosphorylation of mTOR (S2448) and p70S6K1 (T389) rise with a time course that parallels that of Akt activation. Based on the experimental data obtained, a signalling pathway involving a  $\beta$ -arrestin 1 scaffolding complex and EGFR to activate Akt signalling is proposed.

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## Introduction

Obestatin, a 23-amino acid peptide encoded by the ghrelin gene, was originally isolated from stomach showing to be a circulating peptide whose secretion is pulsatile and displays an ultradian rhythmicity similar to ghrelin and GH secretion (Zhang *et al.* 2005). Obestatin was initially characterized as an anorexigenic peptide being a physiological opponent of ghrelin. This action appeared to be mediated by the orphan receptor GPR39 that belongs to the family of the ghrelin receptor (GH secretagogues receptor type 1a, GHS-R1a) and the motilin receptor (Zhang *et al.* 2005). Since the discovery of obestatin, several observations have decreased the initial enthusiasm about the potential of this molecule putting the effectiveness of obestatin as an anorexigenic peptide

into question. Several studies (Gourcerol *et al.* 2006, Seoane *et al.* 2006, Nogueiras *et al.* 2007, Yamamoto *et al.* 2007, Zizzari *et al.* 2007) with the exception of four reports (Bresciani *et al.* 2006, Carlini *et al.* 2007, Lagaud *et al.* 2007, Tremblay *et al.* 2007) were unsuccessful to reproduce the anorexigenic property of obestatin initially reported. Furthermore, several groups were unable to confirm that obestatin binds to GPR39 or activates signalling in transfected cells, suggesting that obestatin is unlikely to be the cognate ligand for this receptor (Chartrel *et al.* 2007, Holst *et al.* 2007). In spite of it, a recent work demonstrated that obestatin is a metabolic hormone capable of binding to GPR39 to regulate the functions of diverse gastrointestinal and adipose tissues (Zhang *et al.* 2008). Thus, the state-of-knowledge on obestatin leaves

significant unanswered issues, specially the basis for the lack of reproducible biological actions for this ghrelin-associated peptide. Throughout this period, additional actions for this peptide have been reported providing evidence of biological functionality (Dun *et al.* 2006, Szentirmai & Krueger 2006, Carlini *et al.* 2007, Samson *et al.* 2007, Zizzari *et al.* 2007). Among them, it is remarkable that the mitogenic effect described for obestatin in human retinal pigment epithelial cells (hRPE; Camiña *et al.* 2007a), human gastric carcinoma cell line KATO-III (Pazos *et al.* 2007), pre-adipocytes (Zhang *et al.* 2008) and pancreatic  $\beta$ -cells (Granata *et al.* 2008). A signalling pathway involving the consecutive activation of  $G_i$ , phosphatidylinositol 3-kinase, novel protein kinase C $\epsilon$  (PKC $\epsilon$ ), and Src for extracellular signal-regulated kinases 1/2 (ERK1/2) activation mediates this effect in hRPE and KATO-III cells (Camiña *et al.* 2007a, Pazos *et al.* 2007). The fact that obestatin modulates cell proliferation of gastric cells, one of the main sources of this peptide, points to the involvement of this peptide in diverse processes such as repair gastric mucosal damage or as fuel for gastric cancer cell proliferation. It is intriguing that this mitogenic effect is not observed for ghrelin. Because obestatin and ghrelin are derived from the same peptide precursor, this lack of functional correlation supports the concept that obestatin is a biologically relevant peptide and not only a non-functional connective peptide.

The present work addresses the role of obestatin to stimulate Akt signalling, a serine/threonine kinase that acts as a central player in the regulation of metabolism, apoptosis, transcription and cell cycle (Manning & Cantley 2007). As a model, the human gastric carcinoma cell line KATO-III was used to characterize the intracellular signalling pathway. Results were reproduced on the gastric adenocarcinoma cell line AGS, which also endogenously expresses the GPR39 receptor.

## Materials and methods

### Materials

Human obestatin was obtained from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). Pertussis toxin (PTX) was obtained from Sigma Chemical Co. Wortmannin, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-d]pyrimidine (PP2), 4-amino-7-phenylpyrazolo [3,4-d]pyrimidine (PP3), 4-(3-chloroanilino)-6,7-dimethoxyquinazoline (AG1478) and *N*-[(2*R*)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide (GM6001) were purchased

from Calbiochem (Merck KGaA). Rabbit polyclonal IgG antibodies to phospho-p44/42 mitogen-activated protein kinases (MAPK), p44/42 MAPK, phospho-Src(Y416), phospho-Akt HM(S473), phospho-Akt A-loop(T308), Akt, Rictor, phospho-mTOR (S2448), phospho-p70S6K1(T389), phospho-PDK1 (S241), Rictor siRNA and control siRNA were purchased from Cell Signalling Technology (Beverly, MA, USA). Anti-phosphotyrosine rabbit polyclonal IgG antibody was obtained from Upstate Biotechnology (Lake Placid, NY, USA). Goat polyclonal IgG antibodies to  $\beta$ -arrestin 1 and HRP, rabbit polyclonal IgG antibodies to  $\beta$ -actin and EGFR,  $\beta$ -arrestin 1 siRNA and control siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-GPR39 rabbit polyclonal IgG antibody was from Abcam (Cambridge, MA, USA). Anti-rabbit IgG HRP was purchased from Amersham Pharmacia. Prof. R J Lefkowitz (Duke University Medical Center, Durham, NC, USA) kindly provided rabbit anti-rat  $\beta$ -arrestin 1C-terminal (A1CT) antiserum. The cDNA encoding the  $G\beta\gamma$  sequester  $\beta$ -ARK-CT was a gift of Dr P Voigt (Institute of Pharmacology, Charité-Medical University, Campus Benjamin Franklin, Berlin, Germany).

### Cell culture

Human gastric cancer cell lines, KATO-III and AGS, were cultured as described by the supplier (ECACC, Wiltshire, UK). Briefly, KATO-III cells were seeded in 100 mm dishes and culture in RPMI-1640 medium supplemented with 20% (v/v) foetal bovine serum (FBS), 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate and 2.5 mM L-glutamine with 5% CO<sub>2</sub> and 37 °C. AGS cells were seeded in 100 mm dishes and cultured in F-12 Ham medium supplemented with 10% (v/v) FBS, 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate and 2.5 mM L-glutamine with 5% CO<sub>2</sub> at 37 °C.

### Cell transient transfection

The cDNA encoding  $G\beta\gamma$  sequester  $\beta$ -ARK-CT was transfected into subconfluent KATO-III cells using Lipofectamine (Invitrogen) following the manufacturer's protocol. The  $\beta$ -ARK-CT incorporation was confirmed by means of intracellular calcium mobilization in transfected cells before and after treatment as previously described (Theodoropoulou *et al.* 2006).

### Immunoblotting analysis

Serum-starved cells were stimulated with obestatin for the indicated time period and doses at 37 °C. The medium was then aspirated and cells were lysed in

ice-cold lysis buffer (RIPA buffer: 50 mM Tris-HCl pH 7.2, 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40, 0.25% (w/v) Na-deoxycholate, protease inhibitor cocktail (1:100, Sigma), phosphatase inhibitor cocktail (1:100, Sigma)). The soluble cell lysates were pre-cleared by centrifuging at 13 000 *g* for 15 min. The protein concentration was evaluated with the Quanti-Pro BCA Assay Kit (Sigma). The same amount of protein of each sample was separated on 10% SDS/polyacrilamide gels and transferred to nitrocellulose membranes (Bio-Rad). The blots were incubated with 5% non-fatty milk in Tris buffered solution/Tween 20 (TBST) (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% (v/v) Tween-20, used for all incubation and washing steps) for 1 h. Then, blots were incubated for 1 h with corresponding antibodies, according to the manufacturer's instructions and were subsequently incubated with the corresponding peroxidase-conjugated IgG antibody. After washing, signals were visualized using ECL plus Western Blotting Detection System (Amersham Pharmacia Biotech). The blots shown are representative of three experiments. Densitometry was performed using IMAGEJ 1.40 g program.

### Immunoprecipitation procedure

Serum-starved KATO-III cells were stimulated with obestatin for the indicated time period at 37 °C and lysed in ice-cold non-denaturing NP-40 solubilization buffer (immunoprecipitation lysis buffer (ILB), Tris-HCl (pH 7.5), 20 mM; NaCl, 150 mM; EDTA, 1 mM; NP-40, 1% (v/v); protease inhibitor cocktail (1:100, Sigma); phosphatase inhibitor cocktail (1:100, Sigma)). Five hundred micrograms of total protein were pre-washed with 20  $\mu$ l of 50% protein A/G-agarose (Santa Cruz) for 30 min at 4 °C, and then, incubated with 1  $\mu$ g corresponding antibody (overnight at 4 °C) followed by addition of 40  $\mu$ l of 50% protein A/G (2 h at 4 °C). After washing two times with ILB, the pelleted beads were resuspended in Laemmli sample buffer. Proteins were analyzed by SDS-PAGE, followed by western blotting.

### Small interfering RNA (siRNA) silencing of gene expression

Chemically synthesized double-stranded siRNA duplexes (with 3' dTdT overhangs) were purchased from Santa Cruz Biotechnology for the following targets:  $\beta$ -arrestin 1 (5'-AAAGCCUUCUGCGCGGA-GAAU-3') and Rictor (5'-CACUUCGAUUAGUCA-GAAA-3', 5'-CGCUUACUUUGCCUAACAA-3', 5'-CCAACUGAGUGCAAUAUGU-3'). A non-silencing

RNA duplex was used as a control for all siRNA experiments. Cells were transfected with Lipofectamine 2000 (Invitrogen) according to manufacturer's instructions. Silencing was quantified by immunoblotting. Only experiments with verified silencing were used.

### Data analysis

Parameters were expressed as mean  $\pm$  S.E.M. Statistical differences among means of percentages were identified with one-way ANOVA followed by Bonferroni *post hoc* test. *P* values of 0.05 or smaller were considered significant. \*Denotes *P* < 0.05 when comparing obestatin-treated with untreated control cells. #Denotes *P* < 0.05 when comparing treatment + obestatin-treated with obestatin-treated cells.

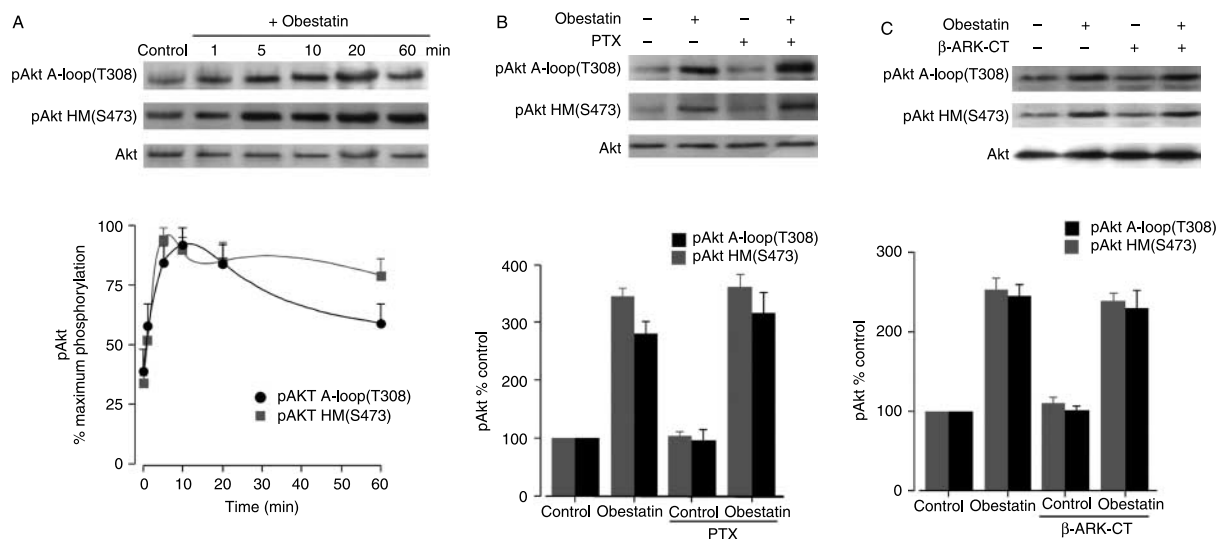
## Results

### Obestatin-induced Akt activity is PTX-insensitive and not mediated by G $\beta\gamma$ dimmers

The dose response curves of Akt phosphorylation, at both the A-loop(T308) and the HM(S473), following stimulation of KATO-III cells with obestatin showed a dose-dependent pattern, being maximal at 200 nM (data not shown), so this concentration was used in subsequent experiments. Figure 1A shows that Akt phosphorylation in both the activation loop within the kinase domain (A-loop(T308)) and the hydrophobic motif in the C-terminal region (HM(S473)) reached maximal levels within 10 min of obestatin stimulation (200 nM), keeping the sustained activity by at least 60 min. The role of G<sub>i/o</sub> protein was evaluated by pre-treatment with PTX, which uncouples G<sub>i/o</sub> protein from receptors (PTX; 100 ng/ml, 4 h). As shown in Fig. 1B, PTX did not modify phosphorylation at both the A-loop(T308) and the HM(S473) within 10 min of obestatin addition (200 nM). Overexpression of a peptide that contains the G $\beta\gamma$  binding domain (G $\beta\gamma$  sequestrers,  $\beta$ -ARK-CT) had no effect on Akt phosphorylation at both residues, ruling out the involvement of the  $\beta\gamma$ -subunit of G proteins (Fig. 1C). Pre-treatment with the PI3K inhibitor wortmannin (1  $\mu$ M, 30 min) completely inhibited the obestatin-induced Akt phosphorylation at both residues (data not shown).

### PDK1 and mTORC2 mediate Akt phosphorylation activated by obestatin

Figure 2A shows that PDK1 phosphorylation at S241 (pPDK1(S241)) reached maximal levels within 5–10 min of obestatin stimulation (200 nM), keeping



**Figure 1** (A) Time-course of the effect of obestatin (200 nM) on Akt phosphorylation at A-loop(T308) and HM(S473). (B) Effect of PTX (100 ng/ml, 4 h) on obestatin-induced Akt phosphorylation (200 nM, 10 min). (C) Obestatin-induced Akt phosphorylation (200 nM, 10 min) in the absence or presence of  $\beta\gamma$  sequester  $\beta$ -ARK-CT. Akt phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation obtained for each residue (A) or as the percentage of the basal phosphorylation obtained in control cells for each residue (B and C) (mean  $\pm$  S.E.M. of three independent experiments). Blots were representative of three independent experiments developed in KATO III cells.

a sustained phosphorylation for 60 min after stimulation. This increase was parallel to the phosphorylation of the Akt A-loop(T308). Pre-treatment of the cells with PTX (100 ng/ml, 4 h) had no effect on PDK1 phosphorylation in response to obestatin (200 nM, 10 min; Fig. 2B). Transient transfection with  $\beta$ -ARK-CT did not inhibit the obestatin-induced PDK1(S241) phosphorylation (Fig. 2C). On the other hand, ablating mTORC2 function by siRNA targeting Rictor (75% reduction in Rictor expression), Akt HM(S473) phosphorylation was reduced by 63% (Fig. 2D).

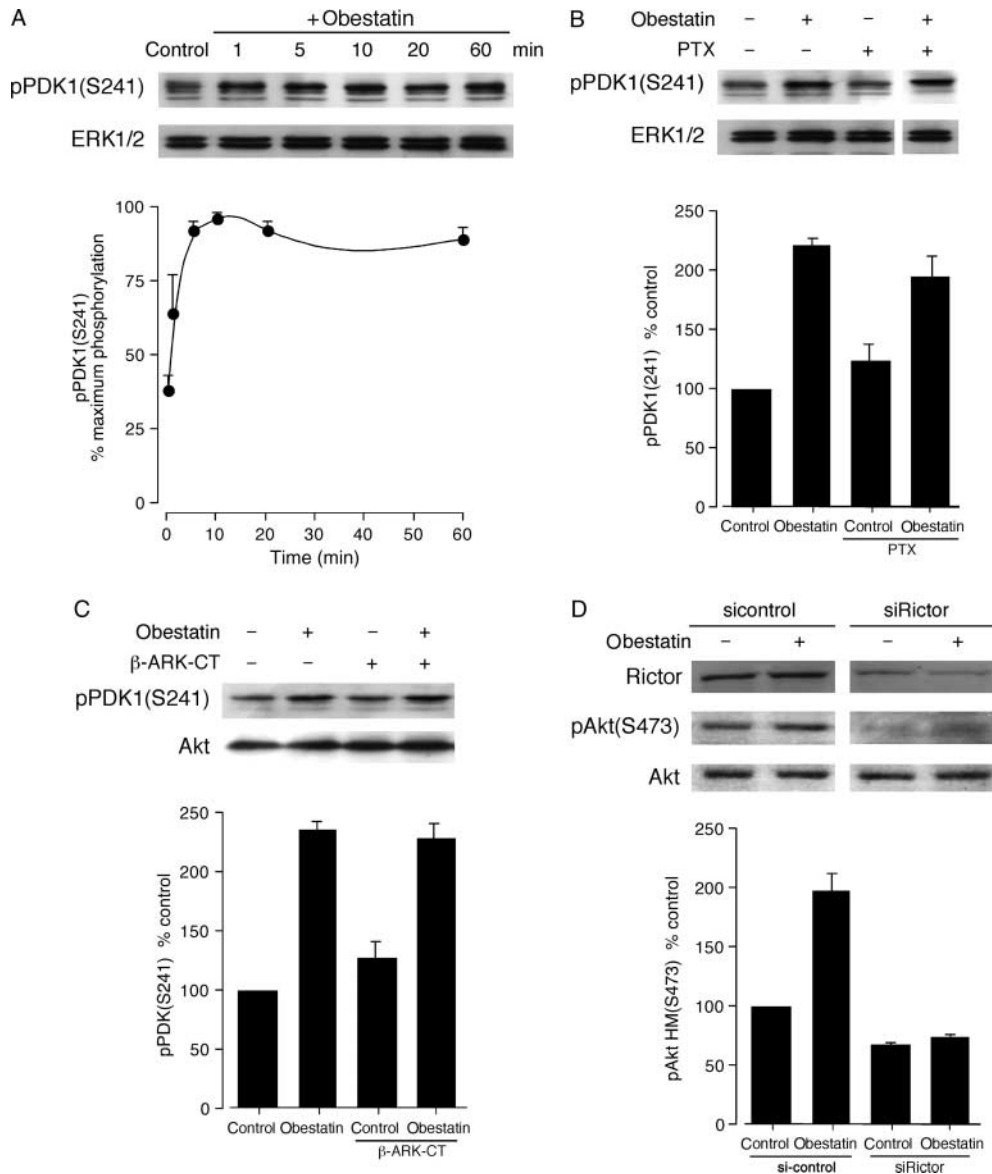
### Src, MMP and EGFR regulate Akt activity in response to obestatin

Akt phosphorylation at both residues was strongly inhibited by the selective non-receptor tyrosine kinase Src inhibitor PP2 (5  $\mu$ M, 30 min; Fig. 3A) in response to obestatin (200 nM, 10 min). This inhibition was specific, since pre-treatment with PP3 (5  $\mu$ M, 30 min), a negative control for PP2, had no effect on the obestatin-induced Akt phosphorylation at both residues. Pre-treatment with GM6001 (1  $\mu$ M, 1 h), an inhibitor of  $Zn^{2+}$ -activated metalloproteinases that catalyzed the shedding of the EGF-like factors, inhibited Akt phosphorylation at both residues (Fig. 3A). Pre-treatment with AG1478 (1  $\mu$ M, 30 min),

the potent and specific inhibitor of the EGFR, also inhibited the Akt phosphorylation by obestatin (200 nM, 10 min; Fig. 3B). By contrast, GM6001 (1  $\mu$ M, 1 h) and AG1478 (1  $\mu$ M, 30 min) had no effect on ERK1/2 phosphorylation in response to obestatin (Fig. 3A and B respectively). Obestatin-stimulated EGFR phosphorylation was assessed by immunoprecipitation of the EGFR followed by immunoblotting with anti-phosphotyrosine antibody (pY). As shown in Fig. 3C, obestatin induced a rapid EGFR phosphorylation that resembled with the dynamic of Akt activation. Furthermore, pre-treatment with PP2 (5  $\mu$ M, 30 min) or GM6001 (1  $\mu$ M, 1 h), inhibited the increase in tyrosine phosphorylation of EGFR in response to obestatin (200 nM, 5 min; Fig. 3D).

### Regulation of Akt phosphorylation by $\beta$ -arrestin 1

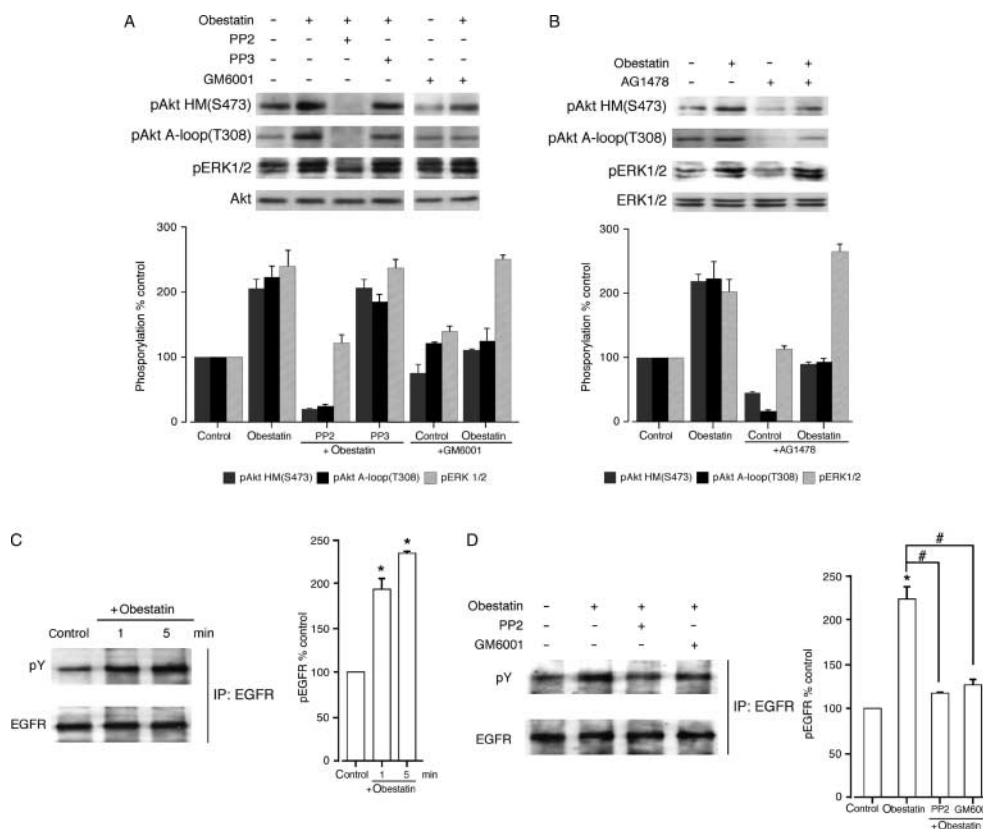
Immunoprecipitation assays of  $\beta$ -arrestin 1 showed the association with the activated form of Src (pSrc(Y416)) and GPR39 in response to obestatin (200 nM, 10 min; Fig. 4A), indicating that obestatin does induce the association of GPR39/ $\beta$ -arrestin 1/Src complex. Next, the role of  $\beta$ -arrestin 1 on Akt phosphorylation was evaluated by siRNA technique to down-regulate the expression of endogenous  $\beta$ -arrestin 1 in KATO III cells. siRNA experiments targeting  $\beta$ -arrestin 1 reduced its expression by



**Figure 2** (A) Time-course of the effect of obestatin (200 nM) on phosphorylation of PDK1. Cell extracts were analyzed by SDS-PAGE using specific antibody against pPDK1(S241). PDK1 phosphorylation was quantified by densitometry and expressed as a percentage of the maximal phosphorylation (mean  $\pm$  S.E.M. of three independent experiments). (B) PDK1 phosphorylation induced by obestatin (200 nM, 10 min) in the absence or presence of PTX (100 ng/ml, 4 h). PDK1 phosphorylation was quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (means  $\pm$  S.E.M.). (C) Obestatin-induced PDK1 phosphorylation (200 nM, 10 min) in the absence or presence of  $\beta\gamma$  sequester  $\beta$ -ARK-CT. (D) Effect of siRNA depletion of Rictor on obestatin-induced Akt HM(S473) phosphorylation. KATO III cells transfected with Rictor siRNA were stimulated with obestatin (200 nM, 10 min) at 37 °C. After stimulation, cell extracts were prepared as described in Experimental Procedures. Equal amounts of protein in each sample were used to assess the expression of Rictor and Akt HM(S473) phosphorylation by western blotting. Akt phosphorylation was quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (means  $\pm$  S.E.M.). In A, B, C and D, blots were representative of three independent experiments developed in KATO III cells.

67  $\pm$  2%. In the presence of a non-targeting control siRNA, obestatin-activated Akt phosphorylation was identical to that observed without any transfection (data not shown). Under these conditions,  $\beta$ -arrestin 1 siRNA decreased both the A-loop(T308) and the

HM(S473) phosphorylation after 10 min of obestatin stimulation (200 nM) with respect to siRNA control (90  $\pm$  1% at S473; 90  $\pm$  3% at T308). Furthermore,  $\beta$ -arrestin 1 siRNA significantly reduced the phosphorylation of Src(Y416) by 55  $\pm$  5% (Fig. 4B).



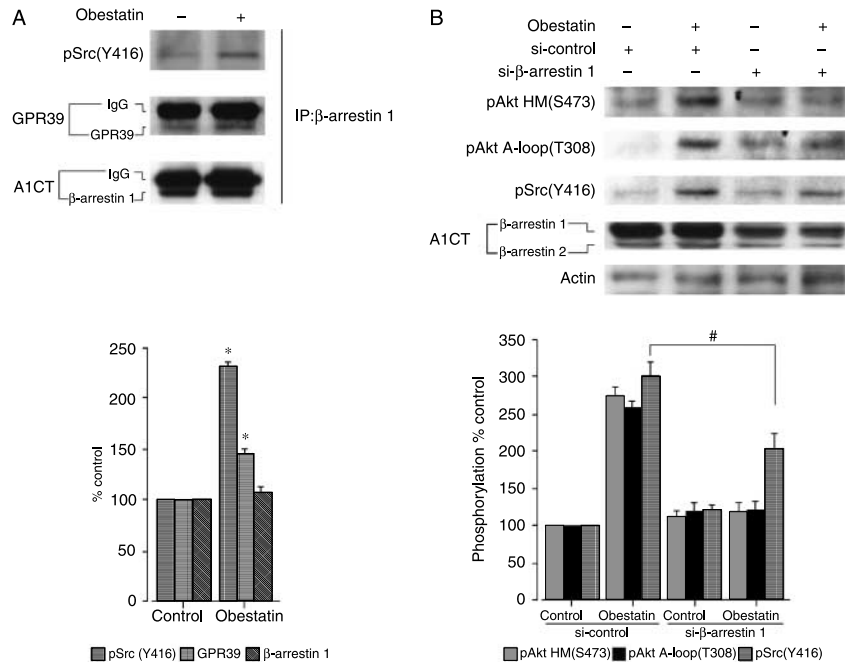
**Figure 3** (A) Effects of the Src inhibitor PP2 and the Zn<sup>2+</sup>-activated metalloproteinases inhibitor GM6001 on the obestatin-induced Akt and ERK1/2 phosphorylations. (B) Effect of the EGFR inhibitor AG1478 on the obestatin-induced Akt and ERK1/2 phosphorylations. Serum-starved cells were pre-treated with PP2 (5 μM, 30 min), PP3 (5 μM, 30 min), GM6001 (1 μM, 1 h) or AG1478 (1 μM, 30 min) previous to obestatin stimulation (200 nM, 10 min). Akt and ERK1/2 phosphorylations were quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (mean ± s.e.m. of three independent experiments). (C) Effect of obestatin on EGFR phosphorylation. Cells were incubated with obestatin (200 nM) at 37 °C, lysed and immunoprecipitated (IP) with antibodies to EGFR, and then analyzed by western blotting with pY and EGFR antibodies. (D) Effects of the Src inhibitor PP2 and the Zn<sup>2+</sup>-activated metalloproteinases inhibitor GM6001 on the obestatin-induced EGFR phosphorylation. Cells were incubated with obestatin (200 nM, 5 min) at 37 °C, immunoprecipitated (IP) with antibodies to EGFR and then analyzed by western blotting with pY and EGFR antibodies. In (C) and (D) EGFR phosphorylation was quantified by densitometry and expressed as the percentage of untreated cells (control; mean ± s.e.m. of three independent experiments; \* denotes *P* < 0.05 when comparing obestatin-treated with untreated control cells; # denotes *P* < 0.05 when comparing obestatin-treated with PP2/GM6001 + obestatin-treated cells). In A, B, C and D, blots were representative of three independent experiments developed in KATO III cells.

### Obestatin regulates mTOR and p70S6K1 phosphorylation

Obestatin (200 nM) promoted rapid increases (maximal in 5–10 min) in mTOR phosphorylation at S2448 (Fig. 5A). Furthermore, obestatin (200 nM) evoked an increase in p70S6K1 phosphorylation at T389 (Fig. 5B). The dynamic of this phosphorylation showed a maximum at 10 min that was followed by a 50% reduction over 20 min period post-stimulus. Pre-treatment of cells with rapamycin (50 nM, 30 min) inhibited the p70S6K1(T389) phosphorylation in response to obestatin (200 nM, 10 min), implicating mTOR as an upstream mediator in this pathway (Fig. 5C).

### Regulation of Akt phosphorylation by β-arrestin 1, Src and EGFR in AGS cells

Obestatin activated Akt phosphorylation at both the A-loop(T308) and the HM(S473) in a dose-dependent manner being maximal at 200 nM and 10 min post-stimulation, keeping the sustained activity by at least 60 min (data not shown). Akt phosphorylation was inhibited by GM6001 (1 μM, 1 h), AG1478 (1 μM, 30 min) and PP2 (5 μM, 30 min) pre-treatments in response to obestatin (200 nm, 10 min; Fig. 6A and B). Furthermore, siRNA experiments targeting β-arrestin 1 decreased both the Akt (84 ± 9% at S473; 77 ± 3% at T308; reduction of β-arrestin 1 expression 77 ± 1%)



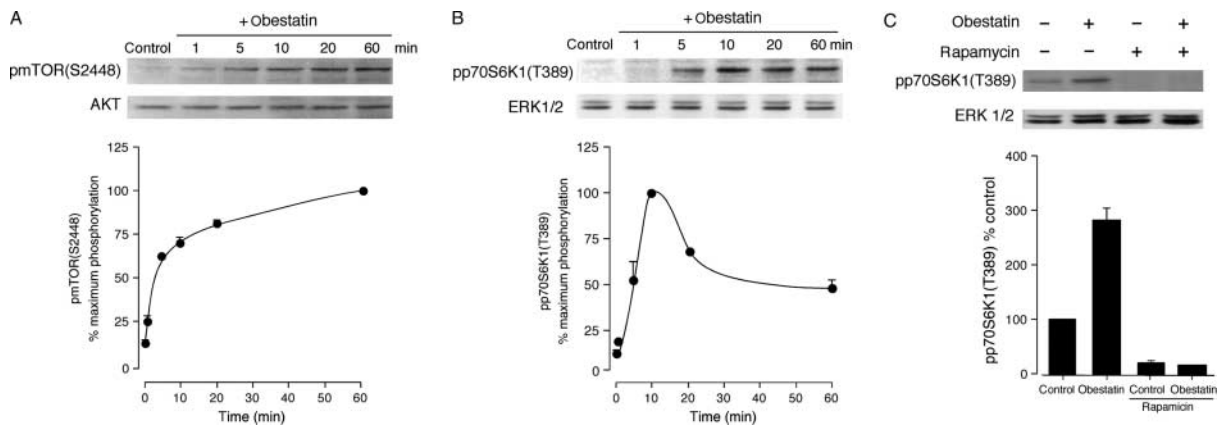
**Figure 4** (A) Effect of obestatin on the assembly of complexes containing pSrc(Y416), GPR39 and  $\beta$ -arrestin 1. Cells were incubated with obestatin (200 nM, 10 min) at 37 °C, lysed and immunoprecipitated (IP) with antibodies to  $\beta$ -arrestin 1, and then analyzed by western blotting with pSrc(Y416), GPR39 and  $\beta$ -arrestin (A1CT) antibodies. pSrc(Y416), GPR39 and  $\beta$ -arrestin 1 were quantified by densitometry in IP and expressed as the percentage of the proteins obtained in control cells (mean  $\pm$  S.E.M. of three independent experiments; \*denotes  $P < 0.05$  when comparing obestatin-treated with untreated control cells). (B) Effect of siRNA depletion of  $\beta$ -arrestin 1 on obestatin-induced Akt and Src phosphorylations. Cells transfected with  $\beta$ -arrestin 1 siRNA were serum-starved for 12 h and then stimulated with obestatin (200 nM, 10 min) at 37 °C. After stimulation, equal amounts of protein in each sample were used to assess Akt A-loop(T308) and HM(S473) phosphorylation, pSrc(Y416) and  $\beta$ -arrestins by western blotting. Akt and Src phosphorylations were quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control siRNA cells (mean  $\pm$  S.E.M.; # denotes  $P < 0.05$  when comparing  $\beta$ -arrestin 1 siRNA obestatin-treated with si-control + obestatin-treated cells). In A and B, blots are representative of three independent experiments developed in KATO III cells.

and Src ( $63 \pm 13\%$  at Y416) phosphorylations at 10 min after obestatin stimulation (200 nM) with respect to siRNA control (Fig. 6C).

## Discussion

The present study offers three major findings related to the activation of Akt in response to obestatin. First, obestatin-induced Akt phosphorylation requires EGFR transactivation and MMP activity through a mechanism that does involve neither  $G_{i/o}$ -proteins nor  $G\beta\gamma$  dimers. Second, obestatin induces the association of GPR39/ $\beta$ -arrestin 1/Src signalling complex resulting in the transactivation to the EGFR and downstream Akt signalling. Third, PDK1 and mTORC2 are essential for A-loop(T308) and HM(S473) phosphorylation of Akt respectively. Thus, results shown provide support for the notion that obestatin activates in parallel the EGFR/Akt and the  $G_{i/o}$ /MEK/ERK pathway.

The protein kinase Akt exerts a key signalling node that regulates the control of cell proliferation, survival, metabolism and nutrient uptake in a cell-type-specific manner through a variety of down-stream targets (Manning *et al.* 2007). From the results presented so far, obestatin activates Akt by two distinct phosphorylation events, both of which depend on PI3K. Obestatin receptor activates PI3K to regulate several downstream signalling pathways through the generation of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 allows membrane translocation of proteins containing PH domain such as PDK1 and Akt. Then, PDK1 autophosphorylates at S241 leading to its own activation and consequently phosphorylates Akt A-loop(T308) (Casamayor *et al.* 1999, Storz & Toker 2002). Finally, the full activation of Akt involves the phosphorylation of HM(S473). Our study shows that ablating mTOR complex 2 function by siRNA targeting Rictor, impaired obestatin-stimulated HM(S473) phosphorylation of Akt. This fact supports the model by which both Akt and PDK1



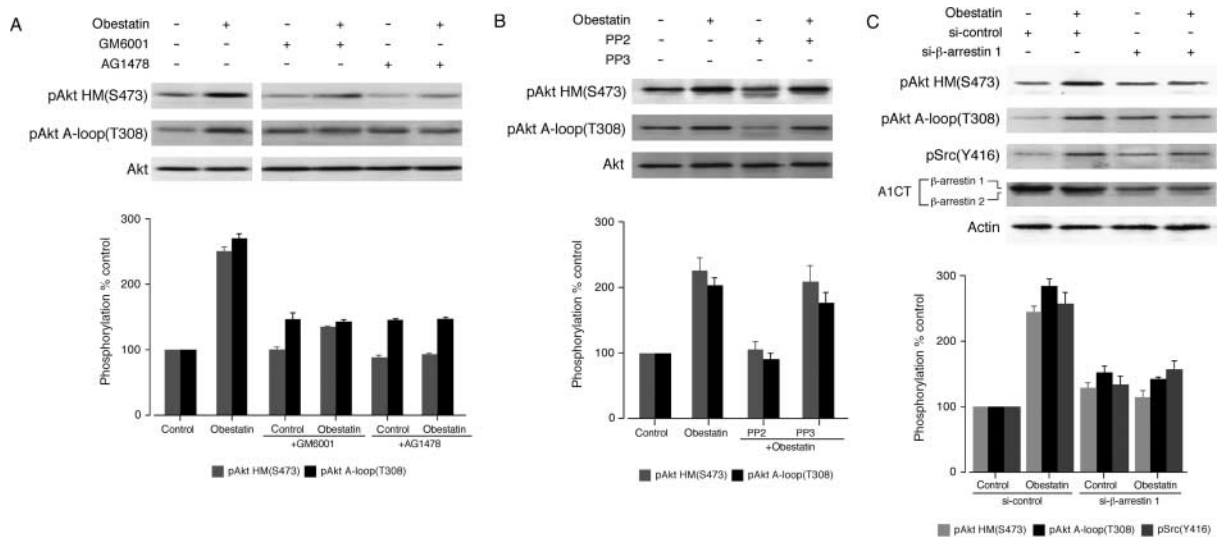
**Figure 5** Time-course of the effect of obestatin (200 nM) on mTOR (A) and p70S6K1 (B) phosphorylations. pmTOR(S2448) and pp70S6K1(T389) were quantified by densitometry and expressed as a percentage of the maximal phosphorylation (mean  $\pm$  S.E.M. of three independent experiments). (C) Effect of rapamycin (50 nM, 30 min) on obestatin-induced p70S6K1 phosphorylation (200 nM, 10 min). pp70S6K1(T389) was quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (mean  $\pm$  S.E.M. of three independent experiments). In A, B and C, blots are representative of three independent experiments developed in KATO III cells.

interact by colocalization at the plasma membrane, where Akt is further phosphorylated by mTORC2 (Guertin & Sabatini 2007). Despite being a key element in the Akt activation, the way mTORC2 is activated remains unknown. Based on the PH-like domain of mSIN1, mTORC2 and Akt may interact as consequence of colocalization at plasma membrane when PI3K is activated (Schroder *et al.* 2007). Other possibility would be that other upstream signals regulate the activation of the mTORC2 complex, for instance such Ras as mSIN1 contains a Ras-binding domain (Lee *et al.* 2005, Schroder *et al.* 2007). In addition to mTORC2 activation, obestatin mediates the phosphorylation of mTOR at S2448, an important site for regulation of mTOR function (Kenerson *et al.* 2002, Reynolds *et al.* 2002), and the phosphorylation of p70S6K1 at T389, an event blocked by rapamycin, thereby implicating mTOR complex 1 (mTORC1: Raptor, mLST8, PRAS40, mTOR kinase). Therefore, obestatin regulates the activity of mTORC1 and mTORC2, and this fact involves this peptide in cell growth and G1 cell cycle progression through activation of p70S6K1 (Pullen & Thomas 1997).

We first described that obestatin stimulates cell proliferation by MEK/ERK1/2 phosphorylation in the KATO-III cells (Pazos *et al.* 2007). Although obestatin activates ERK1/2 through  $G_{i/o}$ -protein dependent signalling pathway, the lack of effect of PTX and the  $G\beta\gamma$  sequester on Akt phosphorylation rules out  $G_{i/o}$ -protein and  $G\beta\gamma$  dimers as upstream signals for Akt activation. The blocking effect of AG1478 and GM6001 indicate that obestatin-mediated Akt

activation requires EGFR transactivation and MMP activities. Indeed, obestatin induced a striking increase in EGFR tyrosine phosphorylation, an effect inhibited by GM6001 pre-treatment. This is congruent with EGFR transactivation through the proteolytic release of EGF-like ligands at the cell surface that activate EGFR by autocrine or paracrine stimulation (Carpenter 2000, Olayioye *et al.* 2000, Prenzel *et al.* 2001, Ohtsu *et al.* 2006, Higashiyama *et al.* 2008). Little is known regarding the detailed upstream mechanisms that link G protein-coupled receptors and their effectors for MMPs activation. Besides phosphorylation, MMPs are regulated through protein-protein interactions. In fact, several kinases such as PKC, PYK2 and Src were identified as direct MMP interacting proteins (Ohtsu *et al.* 2006). Our results showed that Src acts upstream of Akt, as PP2 inhibited the obestatin-induced Akt phosphorylation at both A-loop(T308) and HM(S473). Because Src contains a SH3 domain, it is proposed that Src interacts with the PXXP motif of MMPs to transactivate EGFR (Seals & Courtneidge 2003). This model is supported by the fact that coimmunoprecipitation assays show that  $\beta$ -arrestin 1 recruits Src through the formation of a  $\beta$ -arrestin complex. Furthermore,  $\beta$ -arrestin 1 mediates Src and Akt activation, as shown by the inhibition of Src phosphorylation at Y416 and Akt phosphorylation at both residues after depletion of  $\beta$ -arrestin 1 by siRNA. Src activation by  $\beta$ -arrestin might result from a conformational change induced by  $\beta$ -arrestin binding, as reported for the  $\beta_2$  adrenergic receptor (Luttrell *et al.* 1999), neurokinin 1 receptor (DeFea *et al.* 2000) and





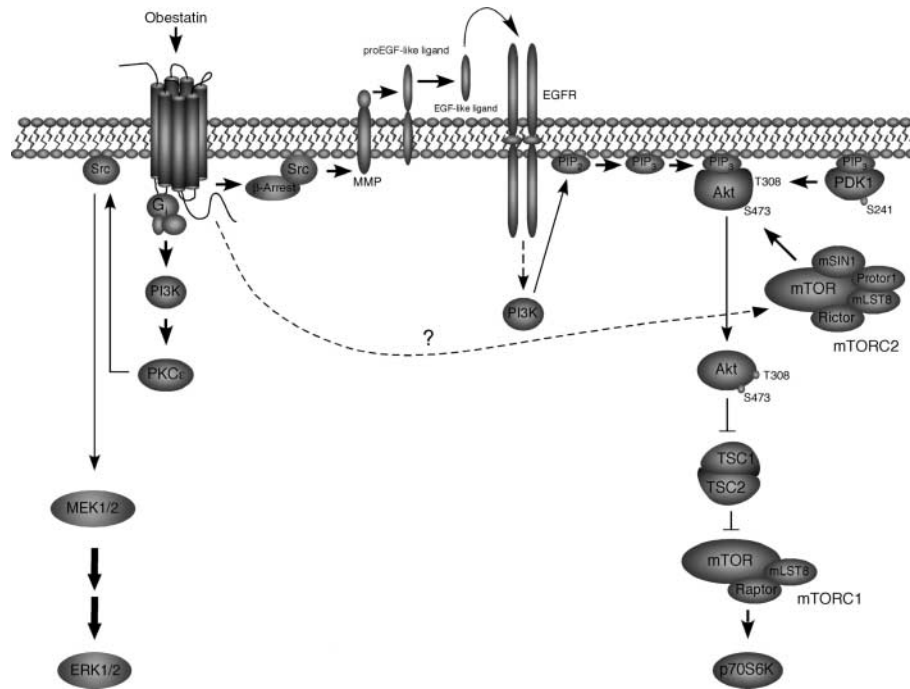
**Figure 6** Effects of the  $Zn^{2+}$ -activated metalloproteinases inhibitor GM6001, EGFR inhibitor AG1478 (A) and Src inhibitor PP2 (B) on the obestatin-induced Akt phosphorylation in AGS cells. Serum-starved AGS cells were pre-treated with GM6001 (1  $\mu$ M, 1 h), AG1478 (1  $\mu$ M, 30 min), PP2 (5  $\mu$ M, 30 min) or PP3 (5  $\mu$ M, 30 min) previous to obestatin stimulation (200 nM, 10 min). (C) Effect of siRNA depletion of  $\beta$ -arrestin 1 on obestatin-induced Akt and Src phosphorylations in AGS cells. After siRNA transfections, cells were stimulated with obestatin (200 nM, 10 min) at 37  $^{\circ}$ C and then equal amounts of protein in each sample were used to assess Akt A-loop(T308) and HM(S473) phosphorylation, pSrc(Y416) and  $\beta$ -arrestins (A1CT) by western blotting. In A, B and C, phosphorylations were quantified by densitometry and expressed as the percentage of the basal phosphorylation obtained in control cells (mean  $\pm$  s.e.m). Blots are representative of three independent experiments.

ghrelin receptor (Camiña *et al.* 2007b). To date, this  $\beta$ -arrestin mechanism was not suggested to be EGFR dependent. Thus,  $\beta$ -arrestin 1 functions as an adaptor that recruits Src to GPR39, leading to the activation of MMP through a GPR39/ $\beta$ -arrestin 1/Src complex, and ultimately, EGFR transactivation. This model is further supported by the fact that PP2 pre-treatment inhibited EGFR tyrosine phosphorylation in response to obestatin. The interplay between G-protein and  $\beta$ -arrestin to transactivate EGFR was described for prostaglandin E2 receptor and  $\beta$ 1-adrenergic receptor transactivation of EGFR (Buchanan *et al.* 2006, Noma *et al.* 2007). It seems reasonable to speculate that MMP activation by obestatin involves receptor endocytosis and compartmentalization determining spatial regulation for specific activation of Akt signalling.

Adding to the complexity and obvious cell type specificity of EGFR transactivation pathways, we found that the activation of both ERK1/2 and Akt signalling pathways act in parallel in KATO III cells. This is supported by the fact the suppression of EGFR tyrosine kinase by AG1478 or MMP activity by GM6001 treatment did not inhibit the obestatin-stimulated ERK1/2 phosphorylation. Furthermore, PTX treatment had no effect on obestatin-induced Akt and PDK1 activation. In this way, EGFR transactivation is the link between obestatin receptor

and Akt signalling pathway, whereas  $G_{i/o}$  proteins regulates ERK1/2 pathway. A similar signalling network was described for ANG II-stimulated mitogenesis in intestinal epithelial cells (Chiu *et al.* 2005) where ErbB/PI3K/Akt/mTOR/p70S6K1 and  $G_q$ /PLC/PKC/MEK/ERK pathways act in parallel. It is becoming clear that the signalling specificity depends not only on the presence of a specific ErbB receptor from EGFR family, but also on the biochemical characteristics of the individual EGF-like ligand (Olayioye *et al.* 2000, Ohtsu *et al.* 2006). These ligands are bivalent, a property that determines which homo- or heterodimer combinations are formed and the downstream signalling to be activated (Olayioye *et al.* 2000). Furthermore,  $\beta$ -arrestin-scaffolded complex presumably places the different components in close proximity ensuring substrate specificity or MMP activity. Such elaborate mechanisms reflect the importance of this transactivation process in obestatin signalling.

Recent studies have shown that  $Zn^{2+}$  induces EGFR phosphorylation through the extracellular release of EGF-like ligands that are mediated by MMPs (Wu *et al.* 2004, Hwang *et al.* 2005).  $Zn^{2+}$  exposure has been shown to activate MAPKs and PI3K/Akt pathways through activation of EGFR in various cell types (Wu *et al.* 2002, 2005, Samet *et al.* 2003).



**Figure 7** Proposed model of signalling pathway for Akt and ERK1/2 activation in response to obestatin. Translocation of  $\beta$ -arrestins 1 to obestatin receptor (GPR39) allows its association with Src.  $\beta$ -arrestin 1 activates Src (phosphorylation at Y416) that initiates the transactivation of EGFR and subsequent downstream Akt signalling.

These findings also suggested that there are marked cell-type-specific differences in the mechanism of EGFR activation induced by  $Zn^{2+}$  exposure. In view of this fact, the stimulatory effects of  $Zn^{2+}$  on GPR39 signalling might be due to the activation of MMP-EGFR since obestatin requires EGFR transactivation and MMP activities. It remains to determine the function of  $Zn^{2+}$  on GPR39 signalling lacking MMP and/or EGFR to clearly define its function as ligand or ago-allosteric modulator on this receptor (Storjohann *et al.* 2008).

Taken together, our data in gastric cancer cells (KATO-III and AGS) are consistent with a model in which Akt signalling pathway is activated by EGFR transactivation. Once obestatin receptor is activated, a signalling pathway is mediated by  $\beta$ -arrestin 1 involving the recruitment and activation of Src into a  $\beta$ -arrestin-scaffolded complex. Thus, Src functions as a switch that activates MMPs to initiate the proteolytic release of EGF-like ligands at the cell surface and then bind to EGFR. Ligand binding drives receptor dimerization, leading to activation of the intrinsic kinase and autophosphorylation of specific docking sites, among them for PI3K. Activation of PI3K generates the second messenger PIP3 that allows the translocation of Akt to the plasma membrane through the binding of its PH domain. Akt is phosphorylated at

Akt A-loop(T308) and HM(S473) by PDK1 and mTORC2 respectively. Then, activated Akt inactivates the heterodimer TSC1/TSC2 leading the activation of mTORC1 and the phosphorylation of downstream targets, p70S6K1 among them (Fig. 7). mTORC1 mediates phosphorylation of p70S6K1(T389) within the hydrophobic motif, whereas PDK1 is responsible for phosphorylation of the p70S6K1 at the T loop. This signalling network adds a new component to the intracellular signalling targets regulated by obestatin. Furthermore, obestatin is added to the group of MMPs regulator factors, which have been implicated in diverse human diseases, such as inflammatory diseases and cancer (Seals & Courtneidge 2003, Huovila *et al.* 2005). It is quite likely that obestatin-induced EGFR transactivation could be a key mechanism by which MMPs regulate these diseases. In support of this hypothesis, numerous studies demonstrate that altered expression and/or mutations in EGFR/ErbB receptor family members are observed in tumours and the cell lines derived from these tumours, and these alterations may contribute to cancer progression (Normanno *et al.* 2006, Ohtsu *et al.* 2006, Bhola & Grandis 2008). Elucidation of the detailed activation/regulation mechanism of EGFR transactivation by obestatin and the pathophysiological significance of the signalling events are intriguing areas for further research.

## Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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