

GABA_BR-Induced EGFR Transactivation Promotes Migration of Human Prostate Cancer Cells

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List of nonstandard abbreviations

AR, amphiregulin; CGNs, cerebellar granular neurons; CM, conditioned medium; EGF, epithelial growth factor; EGFR, EGF receptor; ELISA, enzyme-linked immuno-sorbent assay; ERK1/2, extracellular-signal regulated kinase 1/2; HB-EGF,

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heparin-binding EGF; IGF-1R, insulin-like growth factor receptor 1; GABA_BR, metabotropic GABA_B receptor; GPCRs, G-protein coupled receptors; HRP, horse radish peroxidase; MMPs, matrix metalloproteinases; PAMs, positive allosteric modulators; NCE, non-clathrin endocytosis; PCa, prostate cancer; PDGFR, platelet-derived growth factor receptor; PTX, pertussis toxin; RT, room temperature; RTK, receptor tyrosine kinase; SFM, serum free medium; TCL, total cell lysate; TGF- α , transforming growth factor α ; TMB, tetramethylbenzidine.

Abstract

G protein-coupled receptors (GPCRs) and receptor tyrosine kinases (RTKs) act in concert to regulate cell growth, proliferation, survival, and migration. Metabotropic GABA_B receptor (GABA_BR) is the GPCR for the main inhibitory neurotransmitter GABA in the central nervous system. Increased expression of GABA_BR has been detected in human cancer tissues and cancer cell lines, but the role of GABA_BR in these cells is controversial and the underlying mechanism remains poorly understood. Here, we investigated whether GABA_BR hijacks RTK signaling to modulate the fates of human prostate cancer cells. RTKs array analysis revealed that GABA_BR specific agonist, baclofen, selectively induced the transactivation of EGFR in PC-3 cells. EGFR transactivation resulted in the activation of ERK1/2 by a mechanism that is dependent on G_{i/o} protein and that requires matrix metalloproteinases (MMPs) mediated pro-ligand shedding. Positive allosteric modulators (PAMs) of GABA_BR, such as CGP7930, rac-BHFF, and GS39783, can function as PAM agonists to induce EGFR transactivation and subsequent ERK1/2 activation. Moreover, both baclofen and CGP7930 promoted cell migration and invasion through EGFR signaling. In summary, our observations demonstrated that GABA_BR transactivated EGFR in a ligand-dependent mechanism to promote prostate cancer cell migration and invasion, thus providing new insights into developing novel strategy for prostate cancer treatment by targeting neurotransmitter signaling.

Introduction

Prostate cancer (PCa) is the second most frequently diagnosed cancer in men worldwide (Torre et al., 2015). Currently, few therapeutic options are available for patients with advanced PCa. Tyrosine kinase inhibitors (TKIs), being effective in some human malignancies (Rask-Andersen et al., 2014), have been used as single agents or in combination with chemotherapy in clinical trials in patients with castration-resistant PCa; however, the results were not promising (Jakobovits, 2008; Gallick et al., 2012; Molife et al., 2014; Ojemuyiwa et al., 2014; Modena et al., 2016). Therefore, new mechanism-based inhibitors need to be developed to treat PCa patients. Since neurotransmitters have modulatory effects on tumor cells, the potential roles of receptors for neurotransmitters in tumors have attracted more and more research interests (Schuller, 2008a).

GABA is a main inhibitory neurotransmitter in the vertebrate central nervous system. Metabotropic GABA_B receptor (GABA_BR), a G protein-coupled receptor (GPCR) family member, is an obligatory heterodimer composed of GABA_{B1} and GABA_{B2} subunits. The extracellular domain of GABA_{B1} subunit has a ligand binding site for GABA, agonists, or antagonists. Whereas the extracellular domain of GABA_{B2} subunit lacks the ligand binding capacities, GABA_{B2} subunit is responsible for G_{i/o} protein coupling. It has been shown that specific allosteric modulators bind to GABA_{B2} subunit transmembrane domain (Bettler et al., 2004; Pinaud et al., 2010).

Accumulated evidence suggests that GABA_BR is implicated in human cancers. The level of GABA_BR in thyroid and breast cancer specimens is positively correlated with tumor malignancy (Roberts et al., 2009; Jiang et al., 2012); moreover, GABA_BR increases the metastasis of mouse breast 4T1 cancer cells *in vivo* (Zhang et al., 2014). In contrast, the level GABA_{B1} subunit in cholangiocarcinoma tissues is negatively

correlated with the degree of cell differentiation, local invasion, and lymph node metastasis (Huang et al., 2013). Upregulated expression of GABA_{B2} subunit is detected in female lung cancer patients with better outcome (Zhang et al., 2013), while the level of GABA_BR has no obvious correlation with the pathological features of human gastric cancer (Zhu et al., 2004). *In vitro* studies reveal that GABA_BR agonists may inhibit (Fava et al., 2005; Wang et al., 2008; Huang et al., 2013; Zhang et al., 2013; Shu et al., 2016) or display no effect (Abdul et al., 2008; Lodewyks et al., 2011; Zhang et al., 2014) on the proliferation of cancer cells. Activation of GABA_BR may promote (Azuma et al., 2003; Inamoto et al., 2007; Zhang et al., 2014), suppress (Fava et al., 2005; Schuller et al., 2008b; 2008c; Lodewyks et al., 2011), or have no influences (Joseph et al., 2002; Drell et al., 2003; Chen et al., 2012) on the migration and/or invasion of cancer cells. These observations suggest that the roles of GABA_BR in cancer cell proliferation, migration, and tumor malignancy are controversial.

It has been well documented that GPCRs induce the transactivation of various receptor tyrosine kinases (RTKs) including epithelial growth factor receptor (EGFR) family members, insulin-like growth factor receptor 1 (IGF-1R), fibroblast growth factor receptor 1, platelet-derived growth factor receptor, vascular endothelial growth factor receptor 2, TrkA/B, and c-Met (Wetzker and Böhmer, 2003; Tu et al., 2010; Cattaneo et al., 2014; Ryu et al., 2014). We have reported previously that specific activation of GABA_BR induced transactivation of IGF-1R in neurons (Tu et al., 2010; Lin et al., 2012). To investigate whether GABA_BR hijacks RTK signaling to modulate the fates of PCa cells, RTK array analysis was performed. The results showed that a specific GABA_BR agonist, baclofen, selectively induced transactivation of EGFR at multiple sites (Y845, Y1045, Y1068 and Y1086) in PC-3 cells, which was dependent on G_{i/o} protein and pro-ligand shedding mediated by matrix metalloproteases (MMPs).

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Interestingly, positive allosteric modulators (PAMs) of GABA_BR, including CGP7930, rac-BHFF, and GS39783, can function as PAM agonists to induce EGFR transactivation and ERK1/2 activation. Moreover, both baclofen and CGP7930 promoted cell migration and invasion, which were dependent on EGFR transactivation. These observations suggest that GABA_BR mediated EGFR signaling might be the potential targets in the treatment of advanced PCa.

Materials and Methods

Antibodies and Reagents

Primary antibodies including pERK1/2 (T202/Y204), ERK1/2, pEGFR (Y845), pEGFR (Y992), pEGFR (Y998), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), pEGFR (Y1148), pEGFR (Y1173), EGFR, β -actin, EGFR antibody-conjugated sepharose-beads, and horse radish peroxidase (HRP)-conjugated secondary antibodies against mouse and rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-phosphotyrosine antibody 4G10, 4G10-conjugated agarose-beads, and EGFR neutralizing antibody (LA1) were obtained from Millipore (Billerica, MA, USA).

Pertussis toxin (PTX) and MMPs inhibitor GM6001 (Ilomastat or Galardin) were purchased from Millipore (Billerica, MA, USA). GABA_BR agonist baclofen, antagonist CGP54626, PAMs CGP7930, rac-BHFF, and GS39783 were obtained from Tocris Bioscience (Bristol, BS, UK). EGFR inhibitors Tarceva (Erlotinib) and Iressa (Gefitinib) were from Active Biochem (Hongkong, China).

Cell Culture

Human prostate cancer cell line PC-3 was purchased from ATCC. Cells were maintained in F-12 medium (Gibco, China) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Gibco) at 37°C with 5% CO₂. Cells were split every 3-4 days and medium was replaced every 2 days.

Treatment of cells with various compounds

1.5×10^5 cells were seeded into 3.5 cm plates and grown in complete medium for 2 days at 37°C with 5% CO₂. Cells were then serum starved for 24 h followed by incubation with u7 — serum free medium (SFM) for 30 min before being treated with

various compounds as described below.

GABA_BR agonist. Cells were incubated with 200 μ M baclofen at 37°C for 0, 1, 5, 10, 20, 30, 60, and 120 min, or with 0, 50, 100, 150, 200, 250, and 300 μ M of baclofen at 37°C for 5 min.

PAMs of GABA_BR. CGP7930, rac-BHFF, and GS39783 were solubilized in DMSO with stock concentrations of 100 mM, 100 mM, and 10 mM, respectively. Cells were treated with 50 μ M CGP7930, 100 μ M rac-BHFF, or 10 μ M GS39783 at 37°C for 0, 1, 5, 10, 20, 30, 60, and 120 min, or with various concentrations of CGP7930 (0, 3, 6, 12, 25, 50, and 100 μ M), rac-BHFF (0, 25, 50, 75, 100, 125, and 150 μ M), or GS39783 (0, 0.6, 1.2, 2.5, 5, 10, and 20 μ M) at 37°C for 10, 20, or 5 min, respectively. DMSO treated cells were used as control.

GABA_BR antagonist. Cells were incubated with 20 μ M CGP54626 or 0.05% DMSO at 37°C for 30 min followed by incubation with 200 μ M baclofen, 50 μ M CGP7930, 100 μ M rac-BHFF, or 10 μ M GS39783 in the presence or absence of 20 μ M CGP54626 at 37°C for 5, 10, 20, or 5 min, respectively.

G_{i/o} inhibitor. Cells were serum starved for 24 h in the presence or absence of PTX (200 ng/ml) followed by treatment with 200 μ M baclofen in the presence or absence of PTX at 37°C for 5 min.

EGFR inhibitors. Cells were incubated with 5 μ M Tarceva, 5 μ M Iressa, or 0.05% DMSO at 37°C for 8 h. At the end of incubation, cells were treated with 200 μ M baclofen, 50 μ M CGP7930, 100 μ M rac-BHFF, or 10 μ M GS39783 in the presence or absence of Tarceva or Iressa at 37°C for 5, 10, 20, and 5 min, respectively.

MMPs inhibitor. Cells were pre-treated with 10 μ M GM6001 or 0.05% DMSO at 37°C for 1 h followed by incubation with 200 μ M baclofen in the presence or absence of GM6001 at 37°C for 5 min.

EGFR neutralizing antibody. Cells were incubated with or without 2 $\mu\text{g/ml}$ of EGFR neutralizing antibody at 37°C for 1 h followed by treatment with 200 μM baclofen, 50 μM CGP7930, 100 μM rac-BHFF, or 10 μM GS39783 in the presence or absence of neutralizing antibody at 37°C for 5, 10, 20, and 5 min, respectively.

At the end of each treatment, cells were lysed with RIPA buffer containing proteinase inhibitors cocktail on ice for 30 min, sonicated for 30 s and centrifuged at 4°C, 13,000 rpm for 10 min to remove cell debris. The supernatants were transferred to new microtubes and stored at -20°C until use or subjected to SDS-PAGE immediately.

Receptor Tyrosine Kinase (RTK) Array Assay

Human RTK array was purchased from RayBiotech (Norcross, GA, USA). The experiment was performed according to manufacturer's instructions. Briefly, PC-3 cells grown in 10 cm plates were serum starved for 24 h followed by treatment with or without 200 μM baclofen at 37°C for 5 min. Cells were then washed quickly with cold PBS, and lysed immediately with cold RIPA buffer on ice for 30 min. Total cell lysates (TCLs) were sonicated for 30 s and centrifuged at 4°C, 13,000 rpm for 10 min to remove cell debris. Supernatants were diluted by blocking buffer in a final volume of 1.2 ml (300 $\mu\text{g/ml}$), and incubated with RTK array membranes, which were pre-incubated with blocking buffer overnight at 4°C. The membranes were washed three times (3 min each) with 2 ml of 1×Wash Buffer I at room temperature (RT) with shaking, carefully transferred to a plastic container, and washed with a minimum of 20 ml of 1×Wash Buffer I for three times (5 min each) followed by washing with a minimum of 20 ml of 1×Wash Buffer II for three times (5 min each). The membranes were transferred to an eight-well tray, incubated with 1.2 ml of diluted biotin-conjugated antibody at RT with gentle shaking for 2 h, followed by incubating with 1.5 ml of 1×HRP-conjugated streptavidin for 2 h at RT, washed with 1×Wash

Buffer I and II as described previously. Membranes were developed by using enhanced chemiluminescence reagents (Thermo, China).

Immunoprecipitation

PC-3 cells grown in 10 cm plates were serum starved for 24 h in the presence or absence of PTX (200 ng/ml) followed by treatment with or without 200 μ M baclofen at 37°C for 5 min. Serum starved PC-3 cells were also treated with or without 50 μ M CGP7930, 100 μ M rac-BHFF, or 10 μ M GS39783 at 37°C for 10, 20, or 5 min, respectively. At the end of the treatment, cells were washed quickly with cold PBS, and lysed immediately with cold RIPA buffer on ice for 30 min. TCLs were sonicated for 30 s and centrifuged at 4°C, 13,000 rpm for 10 min to remove cell debris. The supernatants (500 μ g) were mixed with 20 μ l of 4G10-conjugated agarose-beads or EGFR antibody-conjugated sepharose-beads in a final volume of 500 μ l, and incubated at 4°C overnight with continuous agitation. The beads were washed with RIPA buffer for five times (600 μ l each) followed by PBS for 2 times, resuspended in 20 μ l of 2 \times electrophoresis loading buffer, boiled for 5 min, and centrifuged at 12,000 rpm for 5 min. The supernatants were transferred to new microtubes and subjected to immunoblot analysis.

Immunoblot Analysis

Equal amounts of TCLs were resolved by 10% SDS-PAGE gels followed by membrane transfer. Membranes were blocked with TBS containing 5% non-fat dry milk and 0.1% Tween-20 for 2 h at RT, and incubated with primary antibodies against EGFR, pEGFR (Y845), pEGFR (Y992), pEGFR (Y998), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), pEGFR (Y1148), pEGFR (Y1173), ERK1/2, pERK1/2, or β -actin (1:2000-1:3000) overnight at 4°C with continuous agitation. The next day, the membranes were washed and probed with proper HRP-conjugated secondary

antibodies (1:20,000-1:30,000) at RT for 2 h. Membranes were then washed and developed by using enhanced chemiluminescence reagents.

Enzyme Linked-Immunosorbent Assay (ELISA)

Human EGF, heparin-binding EGF (HB-EGF), amphiregulin (AR), and transforming growth factor- α (TGF- α) ELISA kits were purchased from BOSTER (Pleasanton, CA 94566, USA). Serum starved PC-3 cells seeded on 3.5 cm plates were treated with 200 μ M baclofen at 37°C for 0, 5, 10, or 15 min, and conditioned media (CM) were transferred to 1.5 ml microtubes. 100 μ l CM or standards were added into 96-well plates and incubated at 37°C for 90 min followed by extensive washing. 100 μ l biotinylated antibodies were then added to the plates, incubated at 37°C for 60 min followed by 3 washes (1 min each). 100 μ l avidin-biotin-peroxidase complex working solution was added to the plates except the tetramethylbenzidine (TMB) blank well and incubated at 37°C for 30 min followed by five washes (1-2 min each). 90 μ l TMB developing reagent was added to the plate and incubated at 37°C in dark for 15-20 min. 100 μ l TMB stop solution was added to the plates, and OD₄₅₀ was measured in a microplate reader within 30 min. The experiments were performed in triplicates and repeated three times.

Transwell Assay

Modified Boyden chamber (Corning Costar, Rochester, NY, USA) containing a polycarbonate membrane filter (6.5 mm in diameter with pore size of 8 μ m) was used in this assay. The upper chamber was coated with 70 μ l of Matrigel (1 mg/ml; Corning) for invasion assay. 700 μ l F-12 medium supplemented with 10% FBS was placed in the lower chamber. 200 μ l of cell suspension (5×10^4 cells for migration, 1×10^5 cells for invasion) in the presence or absence of baclofen, CGP54626, CGP7930, or EGFR neutralizing antibody was seeded into the upper chamber and incubated for 36 h at

37°C, 5% CO₂. Cells remained on the upper side of the filter membrane were gently removed with a cotton swab. Cells migrated or invaded to the bottom surface of the filter were quickly fixed with methanol for 10 min and stained with 1% Crystal Violet/20% methanol for 20 min. The stained cells from at least 8 random fields were examined under microscope at the magnification of 200×. Images were captured by cellSens software and processed by Adobe Photoshop without changing the original appearance.

Statistical Analysis

Immunoblot results were quantitated by using ImageJ software, and the optical density of each band was normalized to EGFR, ERK1/2, or β-actin. Statistical analyses were performed by using Graph Pad Prism 5 software. Data from three independent experiments were presented as means ± SEM. Statistical comparisons between groups were performed with paired *t*-test or one way ANOVA with *post hoc* test (Tukey). Data obtained from dose response experiments were analyzed by using non-linear curve fitting. Significance levels are indicated as: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Results

GABA_BR agonist induced EGFR transactivation in human PCa cells

The expression of GABA_BR was detected in human PCa cell lines PC-3 and LNCaP (**Fig. S1**). To identify whether activation of GABA_BR hijacks RTKs signaling to modulate the fates of PCa cells, RTK array analysis was performed in PC-3 and LNCaP cells, which were stimulated with a specific GABA_BR agonist, baclofen (**Fig. S2A**), for 5 min. The results showed that baclofen selectively upregulated the phosphorylation level of EGFR but not other RTKs in PC-3 cells (**Fig. 1A**).

To confirm the results of RTK array assay, PC-3 cell lysates prepared from untreated or baclofen treated cells were immunoprecipitated with pTyr antibody 4G10, and immunoblotted with EGFR antibody. As shown in **Figs. 1B and 1C**, baclofen increased the total phosphorylation level of EGFR for 2.063 ± 0.627 ($p < 0.05$, $n=3$) folds. Upon agonist stimulation, Y845 in the kinase domain and 8 tyrosine residues in the C-tail of EGFR (Y974, Y992, Y1045, Y1068, Y1086, Y1101, Y1148, and Y1173) could be phosphorylated (Conte and Sigismund, 2016). Analysis of single site phosphorylation of EGFR revealed that baclofen significantly upregulated the levels of pY845, pY1045, pY1068, and pY1086 for 0.490 ± 0.101 ($p < 0.05$, $n=3$), 0.481 ± 0.052 ($p < 0.01$, $n=3$), 0.883 ± 0.221 ($p < 0.05$, $n=3$), and 1.070 ± 0.356 ($p < 0.05$, $n=3$) folds respectively, but showed no obvious influence on the phosphorylation of Y1148 and Y1173 (**Figs. 1B and 1C**). Phosphorylation of Y992 and Y998 was not detected in the presence of baclofen (data not shown), and phosphorylation of Y974 and Y1101 was not analyzed due to lack of antibodies.

GABA_BR agonist induced activation of ERK1/2 in human PCa cells

GPCR-induced EGFR transactivation can activate ERK1/2 signaling cascades (Xiao et al., 2003; Zhou et al., 2012; George et al., 2013; Lai et al., 2016). To examine

whether EGFR transactivation by GABA_BR induced ERK1/2 activation, PC-3 cells were treated with baclofen or GABA for various period of time. The results showed that both baclofen and GABA induced a fast and transient phosphorylation of ERK1/2 in a time dependent manner, which peaked at 5 min, and returned to basal level after 60 min (**Figs. 2A and S3**). Baclofen also displayed dose-dependent effect on ERK1/2 activation, with the minimum concentration of 200 μ M (**Fig. 2B**).

Pre-treatment of PC-3 cells with CGP54626 (**Fig. S2B**), a high affinity and highly selective GABA_BR antagonist (Filip and Frankowska, 2008), abrogated baclofen induced ERK1/2 phosphorylation (**Fig. 2C**), indicating that ERK1/2 activation was specifically mediated by GABA_BR.

Tarceva (also named erlotinib) and Iressa (also named gefitinib) are small molecule inhibitors of EGFR (Sharma et al., 2007). Pre-treatment of PC-3 cells with Tarceva or Iressa completely blocked baclofen-induced ERK1/2 phosphorylation (**Figs. 2E and 2F**), suggesting that baclofen induced ERK1/2 activation was dependent on EGFR transactivation by GABA_BR.

EGFR transactivation by GABA_BR agonist was mediated by G_{i/o} protein and ligand-dependent pathway

GABA_BR is coupled to G_{i/o} protein (Filip and Frankowska, 2008). Pre-treatment of cells with pertussis toxin (PTX), an inhibitor of G_{i/o} protein, suppressed baclofen induced total and site-specific phosphorylation of EGFR and ERK1/2 phosphorylation (**Figs. 3A-3C**), implicating that baclofen induced EGFR transactivation and subsequent ERK1/2 activation was dependent on G_{i/o} protein.

EGFR transactivation by GPCRs is mediated by either ligand-dependent or ligand-independent pathway. Ligand-dependent RTKs transactivation by GPCRs is dependent on the activation of MMPs (Cattaneo et al., 2014). EGFR ligands,

including EGF, HB-EGF, AR, TGF- α , epiregulin, betacellulin, and epigen, are synthesized as transmembrane pro-ligands that are later processed by MMPs (Harris et al., 2003; Cattaneo et al., 2014). GM6001, a pan-MMPs inhibitor, abolished baclofen induced multisite phosphorylation of EGFR and ERK1/2 activation (**Figs. 3D-3F**), suggesting that baclofen induced EGFR transactivation was dependent on MMPs.

MMPs has been reported to mediate the shedding of pro-HB-EGF, -AR, or -TGF- α from cell surface and subsequent EGFR transactivation (Zhao et al., 2004; Kamanga-Sollo et al., 2014; Moody et al., 2014; Light and Hammes, 2015; Overland and Insel, 2015). EGFR neutralizing antibody, which blocked the ligand binding site of EGFR, abrogated baclofen-induced ERK1/2 phosphorylation in PC-3 cells (**Fig. 3G**). To identify which EGFR pro-ligand(s) was/were released by MMPs from cell surface, ELISA experiments were performed. As shown in **Fig. 3H**, the basal EGF level in control group was 3.177 ± 0.292 pg/ml ($n=3$), baclofen treatment for 5, 10, or 15 min increased EGF level to 4.008 ± 0.214 pg/ml ($p < 0.05$, $n=3$), 4.888 ± 0.361 pg/ml ($p < 0.05$, $n=3$) and 6.056 ± 0.235 pg/ml ($p < 0.05$, $n=3$) respectively. Baclofen had no significant influence on HB-EGF level until fifteen-minute-treatment, while no upregulation of TGF- α and AR levels was detected in the presence of baclofen (Data not shown). These data indicated that production of EGF but not HB-EGF was upstream of ERK1/2 phosphorylation upon GABA_BR activation, therefore, MMPs-mediated shedding of pro-EGF might be involved in baclofen induced EGFR signaling in PC-3 cells.

GABA_BR agonist promoted migration and invasion of PC-3 cells

Baclofen has been shown to enhance the invasive ability of human prostate C4-2 cancer cells (Azuma et al., 2003). To analyze the effects of baclofen on migration and

invasion of PC-3 cells, transwell experiments were performed. In the control group, the number of migrated cells was 105.8 ± 9.176 per field of view (FV) ($n=8$), which increased to 167.6 ± 7.573 ($p < 0.001$, $n=8$), 204.5 ± 5.292 ($p < 0.001$, $n=8$), and 220.3 ± 9.080 ($p < 0.001$, $n=8$) in the presence of 50, 100, and 200 μM baclofen respectively (**Figs. 4A and 4B**). GABA_BR antagonist CGP54626 (5 μM) suppressed baclofen (100 μM) induced migration and invasion (**Figs. 4C-4D and 5A-5B**). Transwell experiments were then performed in the presence or absence of EGFR neutralizing antibody to explore the role of GABA_BR-induced ligand-dependent EGFR transactivation in migration and invasion. The results showed that EGFR neutralizing antibody remarkably suppressed baclofen induced migration and invasion (**Figs. 4E-4F and 5C-5D**), suggesting that ligand-dependent transactivation of EGFR by baclofen was essential for migration and invasion of PC-3 cells.

As a control, Baclofen displayed no effect on the survival of PC-3 cells after 96 h treatment. However, CGP54626 inhibited cell survival in a time- and dose-dependent manner, and 40 μM CGP54626 significantly inhibited cell survival after 48 h treatment (**Fig. S4**).

PAMs of GABA_BR directly induced ERK1/2 activation as PAM agonists

CGP7930, rac-BHFF, and GS39783, the PAMs of GABA_BR (**Fig. S2C**), are capable of modulating the efficacy of GABA_BR induced signal transduction (Urwyler et al., 2001; 2003; 2005; Malherbe et al., 2008). PAM agonist activities of these three compounds have been reported previously (Binet et al., 2004; Tu et al., 2007; 2010; Malherbe et al., 2008; Gjoni and Urwyler, 2009; Koek et al., 2010; Gannon and Millan, 2011; Sun et al., 2016). To analyze whether these PAMs have intrinsic activity in cancer cells, PC-3 cells were incubated with PAMs without addition of agonist. As shown in **Figs. 6A and 6B**, CGP7930 can act as a PAM agonist to induce ERK1/2

phosphorylation in a time and dose-dependent manner. Compared to baclofen induced transient activation of ERK1/2, CGP7930 induced a sustained phosphorylation of ERK1/2, which peaked at 10 min, and lasted for more than 2 h. 12 μM of CGP7930 was sufficient to activate ERK1/2, and 50 μM of CGP7930 resulted in maximum activation of ERK1/2 with an EC₅₀ value of 20.56 μM . The profile of ERK1/2 phosphorylation induced by rac-BHFF was similar to that induced by CGP7930, which was sustained, peaking at 20 min, and lasting for more than 2 h. The minimum concentration of rac-BHFF to activate ERK1/2 was 75 μM (**Figs. 6D and 6E**). ERK1/2 activation induced GS39783 was similar to that induced by baclofen, which was transient, peaked at 5 min, and returned to basal level after 30 min. 0.6 μM of GS39783 was sufficient to induce ERK1/2 phosphorylation, and 5 μM GS39783 resulted in maximum phosphorylation of ERK1/2 with an EC₅₀ value of 4.85 μM (**Figs. 6G and 6H**).

It has been documented that PC-3 cells may synthesize endogenous GABA (Ippolito and Piwnicka-Worms, 2014). To clarify whether ERK1/2 activation induced by PAMs was due to a direct agonistic effect or the potentiation of endogenously produced GABA, PC-3 cells were pre-treated with GABA_BR antagonist CGP54626 followed by PAMs. The results showed that CGP54626 did not abrogate ERK1/2 activation induced by CGP7930 and rac-BHFF (**Figs. 6C and 6F**), suggesting that CGP7930 and rac-BHFF had a direct agonistic effect on ERK1/2 activation. GS39783 induced activation of ERK1/2 was suppressed by CGP54626 (**Fig. 6I**), indicating that GS39783 may function as a pure PAM in PC-3 cells.

PAMs of GABA_BR stimulated EGFR signaling in PC-3 cells

To investigate whether CGP7930, rac-BHFF, and GS39783 can activate EGFR signaling in PC-3 cells, immunoprecipitation experiments were performed. The results

showed that these three PAMs significantly increased the total phosphorylation level of EGFR (**Figs. 7A-7C**). Further analysis showed that ERK1/2 phosphorylation induced by CGP7930, rac-BHFF, and GS39783 was suppressed by EGFR inhibitors Tarceva (**Figs. 7D-7F**), Iressa (**Figs. 7G-7H**), and EGFR neutralizing antibody (**Figs. 7J-7L**). These results suggested PAMs of GABA_BR functioned similarly as baclofen to induce EGFR transactivation and EGFR ligand-dependent ERK1/2 activation.

CGP7930 promoted migration and invasion of PC-3 cells

Based on the time and dose effects of CGP7930 on cell survival (data not shown), transwell experiments were performed in the presence of maximal 4 μ M CGP7930. As shown in **Figs. 8A and 8B**, CGP7930 promoted migration of PC-3 cells in a dose-dependent manner, and 1 μ M CGP7930 was sufficient to enhance cell migration. Transwell experiments were also performed in the presence or absence of EGFR neutralizing antibody to explore the role of CGP7930 induced EGFR transactivation in migration and invasion. The results showed that EGFR neutralizing antibody blocked CGP7930 induced migration (**Figs. 8C and 8D**) and invasion (**Figs. 8E and 8F**), suggesting that ligand-dependent transactivation of EGFR by CGP7930 was important for migration and invasion of PC-3 cells.

Discussion

EGFR family has received much attention in pharmacological research due to their strong association with malignant proliferation (Gallick et al., 2012). In addition to direct activation by specific ligands, EGFR family members could also be transactivated by a variety of GPCR agonists (Daub et al., 1996; Zhou et al., 2012; Cattaneo et al., 2014; Muñoz-Moreno et al., 2014; Lai et al., 2016; Wang, 2016). In this study, we demonstrated for the first time that specific activation of GABA_BR, a neurotransmitter receptor belonging to GPCR family, selectively induced EGFR transactivation in PC-3 cells. Published data revealed that EGFR ligands can induce multisite phosphorylation of EGFR at Y845, Y974, Y992, Y1045, Y1068, Y1086, Y1101, Y1148, and Y1173 (Conte and Sigismund, 2016). Single site phosphorylation of EGFR at Y845 or Y992 has been detected in PCa cells treated with neuropeptides (GPCR agonists) (Amorino et al., 2007; DaSilva et al., 2013). Dual site phosphorylation of EGFR at Y845 and Y1068 was observed in rat-1 cells stimulated with bombesin and lysophosphatidic acid (GPCR agonists) (Santiskulvong et al., 2003). We identified that baclofen selectively induced the multisite phosphorylation of EGFR at Y845, Y1045, Y1068, and Y1086. Phospho-Y1045, -Y1068, and -Y1086 in EGFR are involved directly or indirectly in the recruitment of Cbl to EGFR, leading to ubiquitination of EGFR. Extensively ubiquitinated EGFR might be internalized through non-clathrin endocytosis (NCE) and degraded in lysosomes (Conte and Sigismund, 2016). It is of particular interest that baclofen selectively induced multisite phosphorylation of tyrosine residues that are involved in EGFR ubiquitination. It is worth to know whether baclofen induces massive ubiquitination and NCE biased internalization of EGFR (compared to classical clathrin-mediated endocytosis) in PC-3 cells.

EGFR transactivation by GPCRs occurs mainly through ligand-dependent and ligand-independent mechanisms. In the ligand-dependent mechanism, MMPs mediate the shedding of pro-ligands from cell surface and subsequent RTK transactivation by GPCRs (Cattaneo et al., 2014). It has been reported that MMPs mediated the shedding of pro-HB-EGF and subsequent EGFR transactivation in PC-3 cells treated with neurotensin, bombesin, tetradecanoyl-phorbol-13-acetate, or calcium-sensing receptor stimulus (increased level of extracellular Ca^{2+}) (Prenzel et al., 1999; Madarame et al., 2003; Hassan et al., 2004; Yano et al., 2004). Our study suggested that MMPs may promote the cleavage of pro-EGF from cell surface and EGFR transactivation in PC-3 cells stimulated with baclofen. A significant increase of HB-EGF level was detected 10 min later than that of pro-EGF. Therefore, EGF might be the main ligand that induces the fast transactivation of EGFR. AR and TGF- α was not detected in this study, probably due to lack of expression or shedding of these two ligands, or the concentration of these two ligands were too low to be detected.

We have reported previously that GABA_BR induced transactivation of IGF-1R in neurons in a ligand-independent pathway, which was mediated by phospholipase C, cytosolic Ca^{2+} , and focal adhesion kinase 1 (Tu et al., 2010), while this study demonstrated that GABA_BR induced EGFR transactivation in a ligand-dependent pathway, indicating that a single GPCR may transactivate two RTKs by different mechanisms, which might be tissue specific. So far, most studies suggested one GPCR to one RTK transactivation pathway (Cattaneo et al., 2014; Wang, 2016). However, similar to our results, several other studies suggested a single GPCR to two RTKs pathway. In PC-3 and LNCaP cells, neuropeptides induced Src- and MMP-dependent EGFR transactivation (pY992) and subsequent IGF-1R phosphorylation (DaSilva et al., 2013). In PC-3 cells, growth hormone-related

hormone stimulated a rapid (30 s) ligand-independent activation of EGFR and HER2, and a slow (30 min) ligand-dependent activation of EGFR and HER2 (Muñoz-Moreno et al., 2014). In primary cultures of Müller cells of the guinea pig, P2Y receptor agonist ATP induced transactivation of PDGFR and EGFR in a ligand-dependent pathway (Milenkovic et al., 2003). Therefore, GPCR agonist(s) may induce a more global RTK response depending on cellular context.

ERK1/2 signaling cascade, one of the overlapping signaling pathways downstream of GPCRs and RTKs, is involved in the development and progression of various human cancers (Osborne et al., 2012). This study revealed that both agonist and PAMs of GABA_BR can induce ERK1/2 activation in an EGFR ligand-dependent pathway. Interestingly, CGP7930 and rac-BHFF induced ERK1/2 activation was not antagonized by GABA_BR antagonist CGP54626, suggesting a direct agonist effect of these two compounds, which is consistent with data published by us and other research groups (Binet et al., 2004; Tu et al., 2007; 2010; Malherbe et al., 2008; Koek et al., 2010; Sun et al., 2016). ERK1/2 activation induced by GS39783 was blocked by CGP54626, indicating that GS39783 may function as a pure PAM in PC-3 cells. But we didn't exclude the possibility that GS39783 may have a direct agonist activity. As described in Materials and Methods, serum starved PC-3 cells were washed with SFM before PAMs treatment, so the endogenous level of GABA in PC-3 cells might be too low to be effective. The different effects of CGP54626 on ERK1/2 activation induced by CGP7930, rac-BHFF, and GS39783 suggested that GABA_BR PAMs may have distinct binding sites on the transmembrane domain of GABA_{B2} subunit. Upon CGP54626 binding, both GABA_{B1} and GABA_{B2} subunits undergo a conformational change, which may distort the binding site for GS39783 but retain the binding capacity for CGP7930 and rac-BHFF.

It has been reported that GABA simulated proliferation of PC-3 cells (Wu et al., 2014). In this study, baclofen displayed no effect on the survival of PC-3 cells, but GABA_BR antagonist CGP54626 inhibited cell survival. Therefore, the effect of baclofen on the survival of PC-3 cells was probably masked by endogenous GABA; when the effect of GABA was blocked by GABA_BR antagonist, cell survival was suppressed.

This study showed that both baclofen and CGP7930 significantly promoted migration and invasion of PC-3 cells, but EGFR neutralizing antibody abolished such effects, indicating that GABA_BR induced EGFR transactivation was essential for enhanced migration and invasion of PC-3 cells. Similar results were obtained in prostate cancer cells C4-2, renal cell carcinoma cells, and mouse 4T1 cells treated with GABA or baclofen (Azuma et al., 2003; Inamoto et al., 2007; Zhang et al., 2014).

Taken together, our study revealed that GABA_BR agonist selectively induced multisite phosphorylation of EGFR at Y845, Y1045, Y1068 and Y1086, which was dependent on G_{i/o} protein and MMPs mediated cleavage of pro-EGF, resulting in ERK1/2 activation. PAMs of GABA_BR (CGP7930, rac-BHFF, and GS39783) can function as PAM agonists to induce EGFR transactivation and subsequent ERK1/2 activation. Baclofen and CGP7930 induced EGFR transactivation played an important role in migration and invasion of PC-3 cell (Fig. 9). To our knowledge, this is the first time to identify the mechanism of GABA_BR induced EGFR signaling in cancer cells. Since clinical trials of EGFR inhibitors in patients with castration-resistant PCa produced disappointing results (Jakobovits, 2008; Gallick et al., 2012; Molife et al., 2014; Ojemuyiwa et al., 2014), neurotransmitter GABA and its receptor GABA_BR might be potential targets in the clinical treatment of PCa patients.

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Authorship contributions

Participated in research design: XJ, JL

Conducted experiments: SX, XJ, CH, YZ, SW, HL, ZZ

Performed data analysis: SX, XJ, CH, YZ, SW, HL

Wrote or contributed to the writing of the manuscript: XJ, JL, SX, CH

References

- Abdul M, Mccray SD, and Hoosein NM (2008) Expression of gamma-aminobutyric acid receptor (subtype A) in prostate cancer. *Acta Oncol* **47**: 1546-1550.
- Amorino GP, Deeble PD, and Parsons SJ (2007) Neurotensin stimulates mitogenesis of prostate cancer cells through a novel c-Src/Stat5b pathway. *Oncogene* **26**: 745-756.
- Azuma H, Inamoto T, Sakamoto T, Kiyama S, Ubai T, Shinohara Y, Maemura K, Tsuji M, Segawa N, Masuda H, Takahara K, Katsuoka Y, and Watanabe M (2003) Gamma-aminobutyric acid as a promoting factor of cancer metastasis; induction of matrix metalloproteinase production is potentially its underlying mechanism. *Cancer Res* **63**: 8090-8096.
- Bettler B, Kaupmann K, Mosbacher J, and Gassmann M (2004) Molecular structure and physiological functions of GABA_B receptors. *Physiol Rev* **84**: 835-867.
- Binet V, Brajon C, Le Corre L, Acher F, Pin JP, and Prézeau L (2004) The heptahelical domain of GABA_{B2} is activated directly by CGP7930, a positive allosteric modulator of the GABA_B receptor. *J Biol Chem* **279**: 29085-29091.
- Cattaneo F, Guerra G, Parisi M, De Marinis M, Tafuri D, Cinelli M, and Ammendola R (2014) Cell-surface receptors transactivation mediated by g protein-coupled receptors. *Int J Mol Sci* **15**: 19700-19728.
- Chen ZA, Bao MY, Xu YF, Zha RP, Shi HB, Chen TY, and He XH (2012) Suppression of human liver cancer cell migration and invasion via the GABA_A receptor. *Cancer Biol Med* **9**: 90-98.
- Conte A and Sigismund S (2016) Chapter Six - The ubiquitin network in the control of EGFR endocytosis and signaling. *Prog Mol Biol Transl Sci* **141**: 225-276.

- DaSilva JO, Amorino GP, Casarez EV, Pemberton B, and Parsons SJ (2013) Neuroendocrine-derived peptides promote prostate cancer cell survival through activation of IGF-1R signaling. *Prostate* **73**: 801-812.
- Daub H, Weiss FU, Wallasch C, and Ullrich A (1996) Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. *Nature* **379**: 557-560.
- Drell TL IV, Joseph J, Lang K, Niggemann B, Zaenker KS, and Entschladen F (2003) Effects of neurotransmitters on the chemokinesis and chemotaxis of MDA-MB-468 human breast carcinoma cells. *Breast Cancer Res Treat* **80**: 63-70.
- Fava G, Marucci L, Glaser S, Francis H, De Morrow S, Benedetti A, Alvaro D, Venter J, Meininger C, Patel T, Taffetani S, Marzioni M, Summers R, Reichenbach R, and Alpini G (2005) gamma-Aminobutyric acid inhibits cholangiocarcinoma growth by cyclic AMP-dependent regulation of the protein kinase A/extracellular signal-regulated kinase 1/2 pathway. *Cancer Res* **65**: 11437-11446.
- Filip M and Frankowska M (2008) GABA_B receptors in drug addiction. *Pharmacol Rep* **60**: 755-770.
- Gallick GE, Corn PG, Zurita AJ, and Lin SH (2012) Small-molecule protein tyrosine kinase inhibitors for the treatment of metastatic prostate cancer. *Future Med Chem* **4**: 107-119.
- Gannon RL and Millan MJ (2011) Positive allosteric modulators at GABA_B receptors exert intrinsic actions and enhance the influence of baclofen on light-induced phase shifts of hamster circadian activity rhythms. *Pharmacol Biochem Behav* **99**: 712-717.

- George AJ, Hannan RD, and Thomas WG (2013) Unravelling the molecular complexity of GPCR-mediated EGFR transactivation using functional genomics approaches. *FEBS J* **280**: 5258-5268.
- Gjoni T and Urwyler S (2009) Changes in the properties of allosteric and orthosteric GABA_B receptor ligands after a continuous, desensitizing agonist pretreatment. *Eur J Pharmacol* **603**: 37-41.
- Harris RC, Chung E, and Coffey RJ (2003) EGF receptor ligands. *Exp Cell Res* **284**: 2-13.
- Hassan S, Dobner PR, and Carraway RE (2004) Involvement of MAP-kinase, PI3-kinase and EGF-receptor in the stimulatory effect of Neurotensin on DNA synthesis in PC3 cells. *Regul Pept* **120**: 155-166.
- Huang Q, Zhu CL, Liu CH, Xie F, Zhu K, and Hu SY (2013) Gamma-aminobutyric acid binds to GABA_B receptor to inhibit cholangiocarcinoma cells growth via the JAK/STAT3 pathway. *Dig Dis Sci* **58**: 734-743.
- Inamoto T, Azuma H, Sakamoto T, Kiyama S, Ubai T, Kotake Y, Watanabe M, and Katsuoka Y (2007) Invasive ability of human renal cell carcinoma cell line Caki-2 is accelerated by gamma-aminobutyric acid, via sustained activation of ERK1/2 inducible matrix metalloproteinases. *Cancer Invest* **25**: 574-583.
- Ippolito JE and Piwnica-Worms D (2014) A fluorescence-coupled assay for gamma aminobutyric acid (GABA) reveals metabolic stress-induced modulation of GABA content in neuroendocrine cancer. *PLoS One* **9**: e88667.
- Jakobovits A (2008) Monoclonal antibody therapy for prostate cancer. *Handb Exp Pharmacol* **181**: 237-256.

- Jiang X, Su L, Zhang Q, He C, Zhang Z, Yi P, and Liu J (2012) GABA_B receptor complex as a potential target for tumor therapy. *J Histochem Cytochem* **60**: 269-279.
- Joseph J, Niggemann B, Zaenker KS, and Entschladen F (2002) The neurotransmitter gamma-aminobutyric acid is an inhibitory regulator for the migration of SW 480 colon carcinoma cells. *Cancer Res* **62**: 6467-6469.
- Kamanga-Sollo E, Thornton KJ, White ME, and Dayton WR (2014) Role of G protein-coupled estrogen receptor-1, matrix metalloproteinases 2 and 9, and heparin binding epidermal growth factor-like growth factor in estradiol-17 β -stimulated bovine satellite cell proliferation. *Domest Anim Endocrinol* **49**: 20-26.
- Koek W, France CP, Cheng K, and Rice KC (2010) GABA_B receptor-positive modulators: enhancement of GABA_B receptor agonist effects in vivo. *J Pharmacol Exp Ther* **335**: 163-171.
- Lai X, Ye L, Liao Y, Jin L, Ma Q, Lu B, Sun Y, Shi Y, and Zhou N (2016) Agonist-induced activation of histamine H3 receptor signals to extracellular signal-regulated kinases 1 and 2 through PKC-, PLD-, and EGFR-dependent mechanisms. *J Neurochem* **137**: 200-215.
- Light A and Hammes SR (2015) LH-induced steroidogenesis in the mouse ovary, but not testis, requires matrix metalloproteinase 2- and 9-mediated cleavage of upregulated EGF receptor ligands. *Biol Reprod* **93**: 65.
- Lin X, Li X, Jiang M, Chen L, Xu C, Zhang W, Zhao H, Sun B, Xu X, Nan F, and Liu J (2012) An activity-based probe reveals dynamic protein-protein interactions mediating IGF-1R transactivation by the GABA_B receptor. *Biochem J* **443**: 627-634.

- Lodewyks C, Rodriguez J, Yan J, Lerner B, Lipschitz J, Nfon C, Rempel JD, Uhanova J, and Minuk GY (2011) GABA-B receptor activation inhibits the in vitro migration of malignant hepatocytes. *Can J Physiol Pharmacol* **89**: 393-400.
- Madarame J, Higashiyama S, Kiyota H, Madachi A, Toki F, Shimomura T, Tani N, Oishi Y, and Matsuura N (2003) Transactivation of epidermal growth factor receptor after heparin-binding epidermal growth factor-like growth factor shedding in the migration of prostate cancer cells promoted by bombesin. *Prostate* **57**: 187-195.
- Malherbe P, Masciadri R, Norcross RD, Knoflach F, Kratzeisen C, Zenner MT, Kolb Y, Marcuz A, Huwyler J, Nakagawa T, Porter RH, Thomas AW, Wettstein JG, Sleight AJ, Spooen W, and Prinssen EP (2008) Characterization of (R,S)-5,7-di-tert-butyl-3-hydroxy-3-trifluoromethyl-3H-benzofuran-2-one as a positive allosteric modulator of GABA_B receptors. *Br J Pharmacol* **154**: 797-811.
- Milenkovic I, Weick M, Wiedemann P, Reichenbach A, and Bringmann A (2003) P2Y receptor-mediated stimulation of Müller glial cell DNA synthesis: dependence on EGF and PDGF receptor transactivation. *Invest Ophthalmol Vis Sci* **44**: 1211-1220.
- Modena A, Massari F, Ciccarese C, Brunelli M, Santoni M, Montironi R, Martignoni G, and Tortora G (2016) Targeting Met and VEGFR Axis in Metastatic Castration-Resistant Prostate Cancer: 'Game Over'? *Target Oncol* **11**: 431-446.
- Molife LR, Omlin A, Jones RJ, Karavasilis V, Bloomfield D, Lumsden G, Fong PC, Olmos D, O'Sullivan JM, Pedley I, Hickish T, Jenkins P, Thompson E, Oommen N, Wheatley D, Heath C, Temple G, Pelling K, and de Bono JS (2014) Randomized Phase II trial of nintedanib, afatinib and sequential combination in castration-resistant prostate cancer. *Future Oncol* **10**: 219-231.

- Moody TW, Chan DC, Mantey SA, Moreno P, and Jensen RT (2014) SR48692 inhibits non-small cell lung cancer proliferation in an EGF receptor-dependent manner. *Life Sci* **100**: 25-34.
- Muñoz-Moreno L, Arenas MI, Carmena MJ, Schally AV, Prieto JC, and Bajo AM (2014) Growth hormone-releasing hormone antagonists abolish the transactivation of human epidermal growth factor receptors in advanced prostate cancer models. *Invest New Drugs* **32**: 871-882.
- Ojemuyiwa MA, Madan RA, and Dahut WL (2014) Tyrosine kinase inhibitors in the treatment of prostate cancer: taking the next step in clinical development. *Expert Opin Emerg Drugs* **19**: 459-470.
- Osborne JK, Zaganjor E, and Cobb MH (2012) Signal control through Raf: in sickness and in health. *Cell Res* **22**: 14-22.
- Overland AC and Insel PA (2015) Heterotrimeric G proteins directly regulate MMP14/membrane type-1 matrix metalloprotease: a novel mechanism for GPCR-EGFR transactivation. *J Biol Chem* **290**: 9941-9947.
- Pinard A, Seddik R, and Bettler B (2010) GABA_B receptors: physiological functions and mechanisms of diversity. *Adv Pharmacol* **58**: 231-255.
- Prenzel N, Zwick E, Daub H, Leserer M, Abraham R, Wallasch C, and Ullrich A (1999) EGF receptor transactivation by G-protein-coupled receptors requires metalloproteinase cleavage of proHB-EGF. *Nature* **402**: 884-888.
- Rask-Andersen M, Zhang J, Fabbro D, and Schiöth HB (2014) Advances in kinase targeting: current clinical use and clinical trials. *Trends Pharmacol Sci* **35**: 604-620.

- Roberts SS, Mendonça-Torres MC, Jensen K, Francis GL, and Vasko V (2009) GABA receptor expression in benign and malignant thyroid tumors. *Pathol Oncol Res* **15**: 645-650.
- Ryu JM, Baek YB, Shin MS, Park JH, Park SH, Lee JH, and Han HJ (2014) Sphingosine-1-phosphate-induced Flk-1 transactivation stimulates mouse embryonic stem cell proliferation through S1P1/S1P3-dependent β -arrestin/c-Src pathways. *Stem Cell Res* **12**: 69-85.
- Santiskulvong C and Rozengurt E (2003) Galardin (GM 6001), a broad-spectrum matrix metalloproteinase inhibitor, blocks bombesin- and LPA-induced EGF receptor transactivation and DNA synthesis in rat-1 cells. *Exp Cell Res* **290**: 437-446.
- Schuller HM (2008a) Neurotransmission and cancer: implications for prevention and therapy. *Anticancer Drugs* **19**: 655-671.
- Schuller HM, Al-Wadei HA, and Majidi M (2008b) GABA_B receptor is a novel drug target for pancreatic cancer. *Cancer* **112**: 767-778.
- Schuller HM, Al-Wadei HA, and Majidi M (2008c) Gamma-aminobutyric acid, a potential tumor suppressor for small airway-derived lung adenocarcinoma. *Carcinogenesis* **29**: 1979-1985.
- Sharma SV, Bell DW, Settleman J, and Haber DA (2007) Epidermal growth factor receptor mutations in lung cancer. *Nat Rev Cancer* **7**: 169-181.
- Shu Q, Liu J, Liu X, Zhao S, Li H, Tan Y, and Xu J (2016) GABA_BR/GSK-3 β /NF- κ B signaling pathway regulates the proliferation of colorectal cancer cells. *Cancer Med* **5**: 1259-1267.

- Sun B, Chen L, Liu L, Xia Z, Pin JP, Nan F, and Liu J (2016) A negative allosteric modulator modulates GABA_B-receptor signalling through GB2 subunits. *Biochem J* **473**: 779-787.
- Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, and Jemal A (2015) Global cancer statistics, 2012. *CA Cancer J Clin* **65**: 87-108.
- Tu H, Rondard P, Xu C, Bertaso F, Cao F, Zhang X, Pin JP, and Liu J (2007) Dominant role of GABA_{B2} and Gbetagamma for GABA_B receptor-mediated-ERK1/2/CREB pathway in cerebellar neurons. *Cell Signal* **19**: 1996-2002.
- Tu H, Xu C, Zhang W, Liu Q, Rondard P, Pin JP, and Liu J (2010) GABA_B receptor activation protects neurons from apoptosis via IGF-1 receptor transactivation. *J Neurosci* **30**: 749-759.
- Urwyler S, Gjoni T, Koljatić J, and Dupuis DS (2005) Mechanisms of allosteric modulation at GABA_B receptors by CGP7930 and GS39783: effects on affinities and efficacies of orthosteric ligands with distinct intrinsic properties. *Neuropharmacology* **48**: 343-353.
- Urwyler S, Mosbacher J, Lingenhoehl K, Heid J, Hofstetter K, Froestl W, Bettler B, and Kaupmann K (2001) Positive allosteric modulation of native and recombinant gamma-aminobutyric acid_B receptors by 2,6-Di-tert-butyl-4-(3-hydroxy-2,2-dimethyl-propyl) -phenol (CGP7930) and its aldehyde analog CGP13501. *Mol Pharmacol* **60**: 963-971.
- Urwyler S, Pozza MF, Lingenhoehl K, Mosbacher J, Lampert C, Froestl W, Koller M, and Kaupmann K (2003) N,N'-Dicyclopentyl-2-methylsulfanyl-5-nitro-pyrimidine-4,6-diamine (GS39783) and structurally related compounds: novel allosteric enhancers of gamma-aminobutyric acid_B receptor function. *J Pharmacol Exp Ther* **307**: 322-330.

- Wang T, Huang W, and Chen F (2008) Baclofen, a GABA_B receptor agonist, inhibits human hepatocellular carcinoma cell growth in vitro and in vivo. *Life Sci* **82**: 536-541.
- Wang Z (2016) Transactivation of epidermal growth factor receptor by G protein-coupled receptors: recent progress, challenges and future research. *Int J Mol Sci* **17**: E95.
- Wetzker R and Böhmer FD (2003) Transactivation joins multiple tracks to the ERK/MAPK cascade. *Nat Rev Mol Cell Biol* **4**: 651-657.
- Wu W, Yang Q, Fung KM, Humphreys MR, Brame LS, Cao A, Fang YT, Shih PT, Kropp BP, and Lin HK (2014) Linking γ -aminobutyric acid A receptor to epidermal growth factor receptor pathways activation in human prostate cancer. *Mol Cell Endocrinol* **383**: 69-79.
- Xiao D, Qu X, and Weber HC (2003) Activation of extracellular signal-regulated kinase mediates bombesin-induced mitogenic responses in prostate cancer cells. *Cell Signal* **15**: 945-953.
- Yano S, Macleod RJ, Chattopadhyay N, Tfelt-Hansen J, Kifor O, Butters RR, and Brown EM (2004) Calcium-sensing receptor activation stimulates parathyroid hormone-related protein secretion in prostate cancer cells: role of epidermal growth factor receptor transactivation. *Bone* **35**: 664-672.
- Zhang D, Li X, Yao Z, Wei C, Ning N, and Li J (2014) GABAergic signaling facilitates breast cancer metastasis by promoting ERK1/2-dependent phosphorylation. *Cancer Lett* **34**: 100-108.
- Zhang X, Zhang R, Zheng Y, Shen J, Xiao D, Li J, Shi X, Huang L, Tang H, Liu J, He J, and Zhang H (2013) Expression of gamma-aminobutyric acid receptors on

neoplastic growth and prediction of prognosis in non-small cell lung cancer. *J Transl Med* **11**: 102.

Zhao D, Zhan Y, Koon HW, Zeng H, Keates S, Moyer MP, and Pothoulakis C (2004) Metalloproteinase-dependent transforming growth factor- α release mediates neurotensin-stimulated MAP kinase activation in human colonic epithelial cells. *J Biol Chem* **279**: 43547-43554.

Zhou Q, Li G, Deng XY, He XB, Chen LJ, Wu C, Shi Y, Wu KP, Mei LJ, Lu JX, and Zhou NM (2012) Activated human hydroxy-carboxylic acid receptor-3 signals to MAP kinase cascades via the PLC-dependent PKC and MMP-mediated EGFR pathways. *Br J Pharmacol* **166**: 1756-1773.

Zhu R, Qin S, He X, and Wang F (2004) GABA_B receptor expression in human gastric cancer. *Med J Chin PLA* **29**: 958-960.

Footnotes

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- SX and CH contributed equally to this work.

Figure Legends

Fig. 1. GABA_BR induced transactivation of EGFR in PC-3 cells. PC-3 cells grown in 10 cm plates with 80% confluence were serum starved for 24 h and incubated with or without 200 μ M baclofen at 37°C for 5 min. (A) 300 μ g of TCL were subjected to RTK array assay as described in Materials and Methods. (B) 500 μ g of TCL were immunoprecipitated with 4G10-conjugated agarose-beads and probed with EGFR antibody. The levels of total EGFR, pEGFR (Y845), pEGFR (Y992), pEGFR (Y998), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), pEGFR (Y1148), pEGFR (Y1173), and β -actin were assessed by immunoblot analysis (B) and quantitated by ImageJ (C). Data were normalized to total EGFR, expressed as the fold change (means \pm SEM; n=3), and analyzed by paired *t*-test. * p <0.05, ** p <0.01 (*v.s.* control).

Fig. 2. Baclofen induced ERK1/2 activation in a time- and dose-dependent manner. PC-3 cells seeded in 3.5 cm plates with 80% confluence were serum starved for 24 h, replaced with SFM and incubated at 37°C for 30 min. (A) Cells were treated with 200 μ M baclofen at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 min. (B) Cells were incubated with 0, 50, 100, 150, 200, 250, or 300 μ M baclofen at 37°C for 5 min. (C) Cells were pre-treated with 20 μ M CGP54626 or 0.05% DMSO at 37°C for 30 min followed by incubation with or without 200 μ M baclofen at 37°C for 5 min. (D, E) Cells were incubated with 5 μ M Taceva (D) or 5 μ M Iressa (E) at 37°C for 8 h and then treated with 200 μ M baclofen at 37°C for 5 min. Cells treated with 0.05% DMSO were used as controls. At the end of incubation, equal amounts of TCLs were subjected to immunoblot analysis. The levels of pERK1/2 were normalized to total ERK1/2 and expressed as the fold change (means \pm SEM; n=3). Data were analyzed using non-linear curve fitting (B) or one way ANOVA with *post hoc* test (Tukey) (C-E). *** p <0.001. ns: not significant.

Fig. 3. EGFR transactivation induced by baclofen was dependent on G_{i/o} protein and MMPs. (A) PC-3 cells grown in 10 cm plates with 80% confluence were serum starved for 24 h in the presence or absence of 200 ng/ml PTX. Cells were then incubated with or without 200 μ M baclofen at 37°C for 5 min. 500 μ g of TCL were immunoprecipitated with EGFR-conjugated agarose-beads and probed with EGFR antibody. The levels of total EGFR, pEGFR (Y845), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), and β -actin were assessed by immunoblot analysis (A) and quantitated (B). (C) The levels of pERK1/2, ERK1/2, and β -actin in samples prepared in (A) were analyzed by immunoblot assay. (D) Serum starved cells grown in 3.5 cm plates were pre-incubated with 20 μ M GM6001 (GM) or 0.05% DMSO at 37°C for 1 h, and then treated with 200 μ M baclofen at 37°C for 5 min. The levels of total EGFR, pEGFR (Y845), pEGFR (Y1045), pEGFR (Y1068), pEGFR (Y1086), and β -actin were assessed by immunoblot analysis (D) and quantitated (E). (F) The levels of pERK1/2, ERK1/2, and β -actin in samples prepared in (D) were analyzed by immunoblot assay. (G) Serum starved cells grown in 3.5 cm plates were pre-incubated with or without EGFR neutralizing antibody (2 μ g/ml) at 37°C for 1 h, and then treated with 200 μ M baclofen at 37°C for 5 min. Equal amounts of TCLs were subjected to immunoblot analysis to assess the levels of pERK1/2, ERK1/2, and β -actin. (H) Serum starved cells grown in 3.5 cm plates were treated with 200 μ M baclofen at 37°C for 0, 5, 10, or 15 min, CM were then collected and the levels of EGF and HB-EGF were analyzed by ELISA, which were performed in triplicates and repeated three times. Data were expressed as the fold change (means \pm SEM; n=3), and analyzed by one way ANOVA with *post hoc* test (Tukey) (B-C, E-G) or paired *t*-test (H). * p <0.05, ** p <0.01, *** p <0.001. ns: not significant.

Fig. 4. Baclofen promoted migration of PC-3 cells. 700 μ l of medium containing 10%

FBS was added in the lower chamber of the transwell. PC-3 cells grown in 10 cm plates were serum starved for 24 h and resuspended in SFM. (A) 5×10^4 cells/200 μ l in the presence of 0, 50, 100, or 200 μ M baclofen were added into the upper chamber and incubated for 36 h at 37°C, 5% CO₂. (C) 5×10^4 cells/200 μ l in the presence or absence of 100 μ M baclofen (Bac) or 5 μ M CGP54626 (CGP) were transferred to the upper chamber and incubated for 36 h at 37°C, 5% CO₂. (E) 5×10^4 cells/200 μ l in the presence or absence of 100 μ M baclofen or EGFR neutralizing antibody (Ab) (0.5 μ g/ml) were added to the upper chamber and incubated for 36 h at 37°C, 5% CO₂. At the end of incubation, cells remained on the upper side were fixed, stained, and observed under microscope. Images from at least 8 random fields were captured by cellSens. The numbers of migrated cells in panels A, C, and E were counted, and expressed as means \pm SEM (n=8) in panels B, D, and F respectively. The experiments were performed three times and the results were reproducible. One representative experiment was shown in each panel. Data were analyzed by paired *t*-test (B) or one way ANOVA with *post hoc* test (Tukey) (D, F). *** $p < 0.001$. Scale bar: 200 μ m.

Fig. 5. Baclofen promoted invasion of PC-3 cells. 700 μ l of medium supplemented 10% FBS was added in the lower chamber, and the filter of the transwell was coated with 70 μ l of Matrigel (1 mg/ml). PC-3 cells grown in 10 cm plates were serum starved for 24 h and resuspended in SFM. (A) 1×10^5 cells/200 μ l in the presence or absence of 100 μ M baclofen or 5 μ M CGP54626 (CGP) were transferred to the upper chamber and incubated for 36 h at 37°C, 5% CO₂. (C) 1×10^5 cells/200 μ l in the presence or absence of 100 μ M baclofen or EGFR neutralizing antibody (Ab) (0.5 μ g/ml) were added to the upper chamber and incubated for 36 h at 37°C, 5% CO₂. Cells remained on the upper side of the filters were fixed, stained, and observed under microscope. Images from at least 8 random fields were captured by cellSens. The

numbers of invasive cells in panels A and C were counted and expressed as means \pm SEM (n=8) in panels B and D respectively. The experiments were performed three times and the results were reproducible. One representative experiment was shown in each panel, and statistical analysis was performed using one way ANOVA with *post hoc* test (Tukey).*** $p < 0.001$. Scale bar: 200 μm .

Fig. 6. GABA_BR PAMs induced ERK1/2 activation in PC-3 cells. Cells seeded in 3.5 cm plates with 80% confluence were serum starved for 24 h, replaced with SFM and incubated at 37°C for 30 min. (A) Cells were treated with 50 μM CGP7930 at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 min. (B) Cells were incubated with 0, 3, 6, 12, 25, 50, or 100 μM CGP7930 at 37°C for 10 min. (C) Cells were pre-treated with 20 μM CGP54626 or 0.05% DMSO at 37°C for 30 min followed by incubation with or without 50 μM CGP7930 37°C for 10 min. (D) Cells were treated with 100 μM rac-BHFF at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 min. (E) Cells were incubated with 0, 25, 50, 75, 100, 125, or 125 μM rac-BHFF at 37°C for 20 min. (F) Cells were pre-treated with 20 μM CGP54626 or 0.05% DMSO at 37°C for 30 min followed by incubation with or without 100 μM rac-BHFF at 37°C for 20 min. (G) Cells were incubated with 10 μM GS39783 at 37°C for 0, 1, 5, 10, 20, 30, 60, or 120 min. (H) Cells were treated with 0, 0.6, 1.2, 2.5, 5, 10, or 20 μM GS39783 at 37°C for 5 min. (I) Cells were pre-treated with 20 μM CGP54626 or 0.05% DMSO at 37°C for 30 min followed by incubation with or without 10 μM GS39783 at 37°C for 5 min. At the end of incubation, equal amounts of TCLs were resolved by SDS-PAGE followed by immunoblot analysis; the levels of pERK1/2 were normalized to total ERK1/2 and expressed as the fold change (means \pm SEM; n=3). Statistical analysis was performed using non-linear curve fitting (B, E, H) or one way ANOVA with *post hoc* test (Tukey) (C, F, I). ** $p < 0.01$, *** $p < 0.001$. ns: not significant.

Fig. 7. GABA_BR PAMs induced EGFR signaling in PC-3 cells. (A-C) PC-3 cells grown in 10 cm plates with 80% confluence were serum starved for 24 h and incubated with or without 50 μ M CGP7930 (A), 100 μ M rac-BHFF (B), or 10 μ M GS39783 (C) at 37°C for 10, 20, or 5 min, respectively. 500 μ g of TCL were immunoprecipitated with 4G10-conjugated agarose-beads and probed with EGFR antibody. The levels of total EGFR, pERK1/2, ERK1/2, and β -actin were analyzed by immunoblot assay. (D-L) Cells seeded in 3.5 cm plates with 80% confluence were serum starved for 24 h and pre-treated with 5 μ M Taceva (D-F), 5 μ M Iressa (G-I), or 2 μ g/ml EGFR neutralizing antibody (J-L) at 37°C for 8, 8, or 1 h, respectively. Cells were then incubated with 50 μ M CGP7930 (D, G, J), 100 μ M rac-BHFF (E, H, K), or 10 μ M GS39783 (F, I, L) at 37°C for 10, 20, or 5 min, respectively. The levels of pERK1/2, ERK1/2, and β -actin were analyzed by immunoblot assay and quantitated. Data were normalized to total EGFR or ERK1/2, expressed as the fold change (means \pm SEM, n=3), and analyzed by paired *t*-test (A-C) or one way ANOVA with *post hoc* test (Tukey) (D-L). * p <0.05, ** p <0.01, *** p <0.001. ns: not significant.

Fig. 8. CGP7930 promoted migration and invasion of PC-3 cells. 700 μ l of medium containing 10% FBS was added in the lower chamber of the transwell. PC-3 cells grown in 10 cm plates were serum starved for 24 h and resuspended in SFM. (A) 5×10^4 cells/200 μ l in the presence of 0, 1, 2, or 4 μ M CGP7930 were transferred to the upper chamber and incubated for 36 h at 37°C, 5% CO₂. (C) 5×10^4 cells/200 μ l in the presence or absence of 4 μ M CGP7930 or 0.5 μ g/ml EGFR neutralizing antibody were added to the upper chamber and incubated for 36 h at 37°C, 5% CO₂. (E) The filter of the transwell was coated with 70 μ l of Matrigel (1 mg/ml). 1×10^5 cells/200 μ l in the presence or absence of 4 μ M CGP7930 or 0.5 μ g/ml EGFR neutralizing antibody were added to the upper chamber and incubated for 36 h at 37°C, 5% CO₂.

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Images of cell migration from at least 8 random fields were captured by cellSens. The numbers of migrated or invaded cells in panels A, C, and E were counted and expressed as means \pm SEM (n=8) in panels B, D, and F respectively. The experiments were performed three times and the results were reproducible. One representative experiment was shown in each panel. Statistical analysis was performed using paired *t*-test (B) or one way ANOVA with *post hoc* test (Tukey) (D, F). ** $p < 0.01$, *** $p < 0.001$. ns: not significant. Scale bar: 200 μ m.

Fig. 9. Diagram of GABA_BR induced EGFR transactivation in PC-3 cells. Upon agonist binding, GABA_BR activates G_{i/o} protein, and MMPs mediated cleavage of pro-EGF from cell surface leads to multisite phosphorylation of EGFR at Y845, Y1045, Y1068, Y1086, and subsequent ERK1/2 activation. In addition to baclofen, PAMs of GABA_BR (CGP7930, rac-BHFF, and GS39783) can function as PAM agonists to induce EGFR transactivation and increase ERK1/2 activity. Both baclofen and CGP7930 promote cell migration and invasion in an EGFR transactivation dependent pathway.

Fig. 1

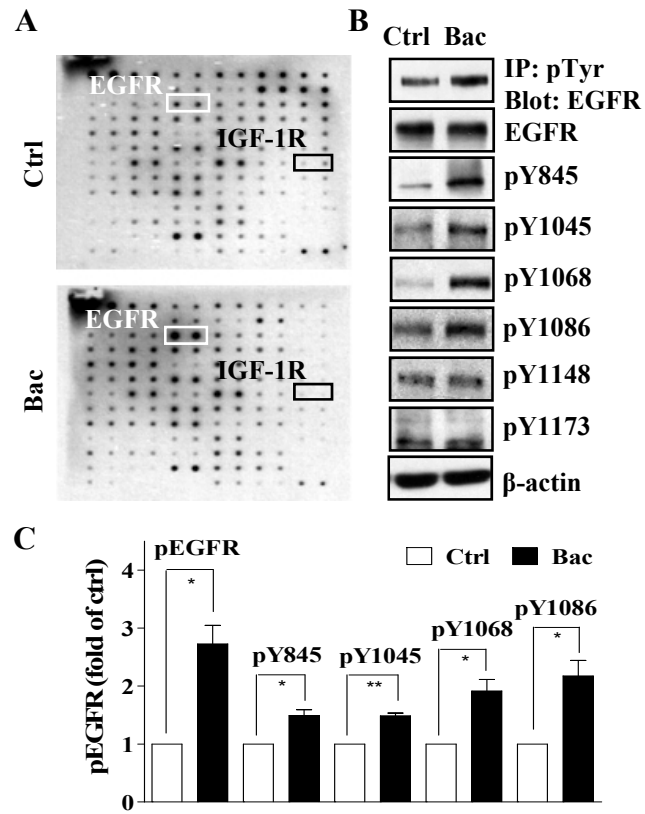


Fig. 2

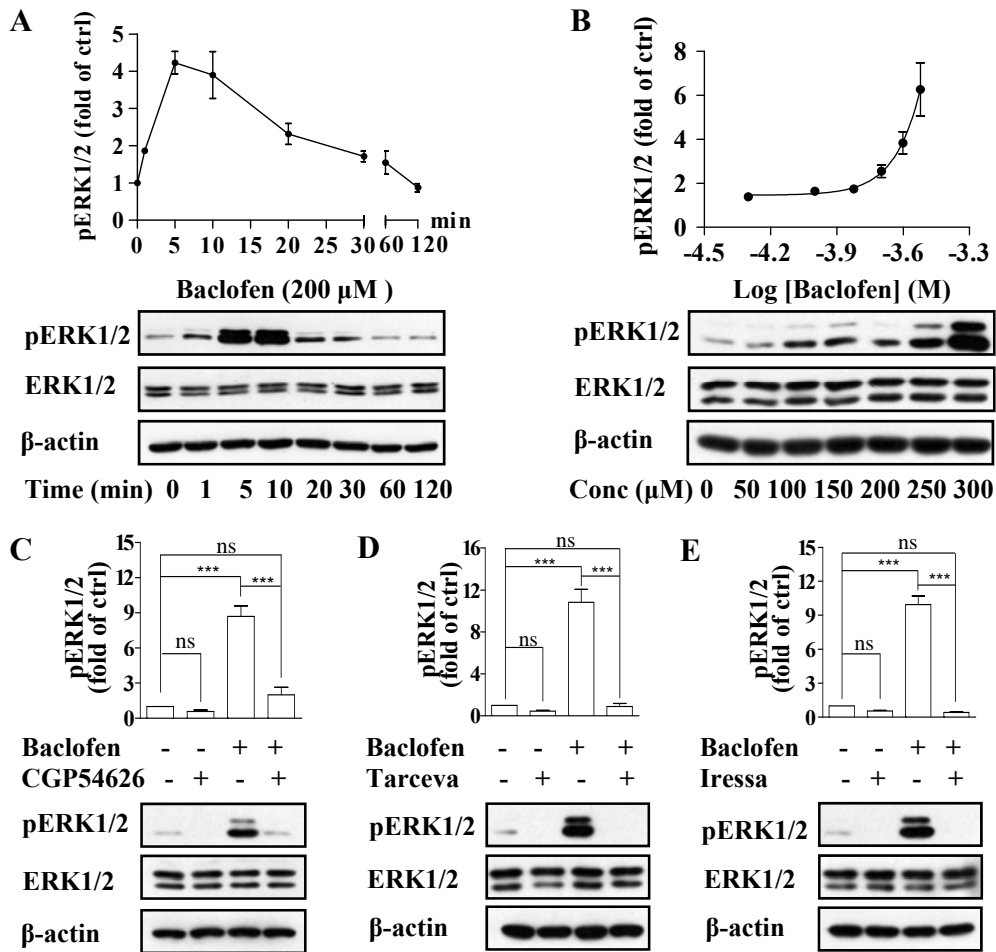


Fig. 3

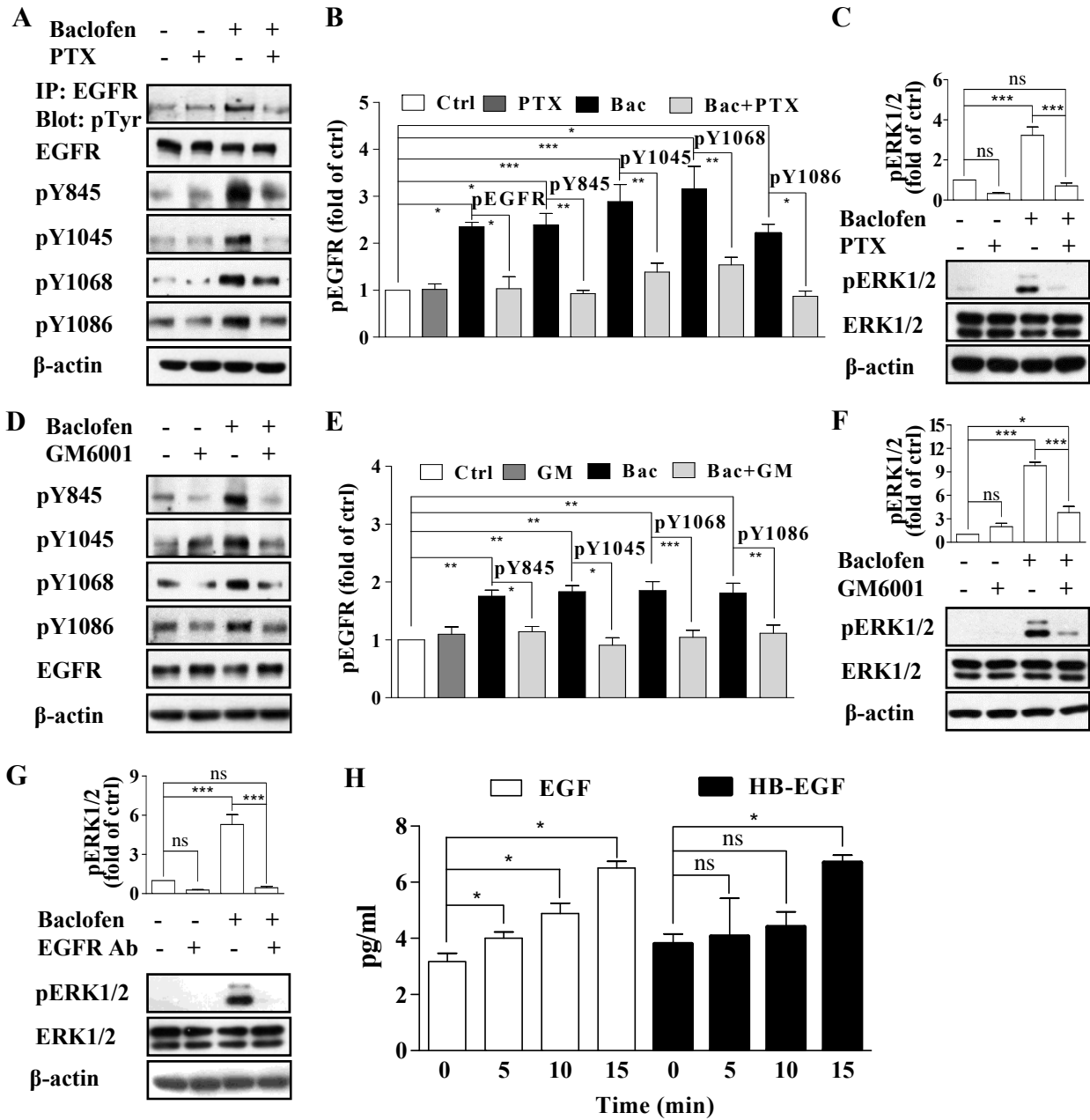


Fig. 4

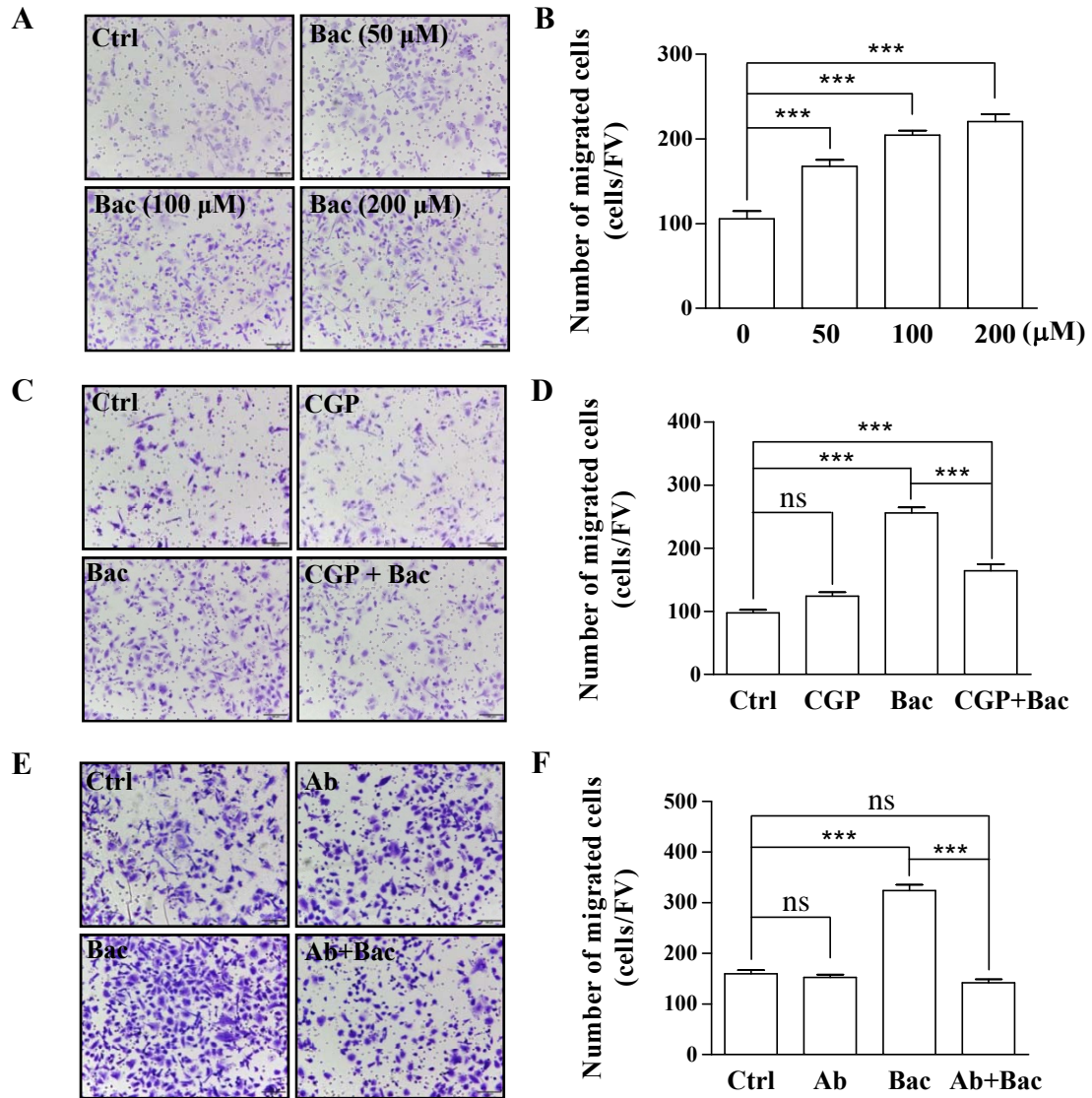


Fig. 5

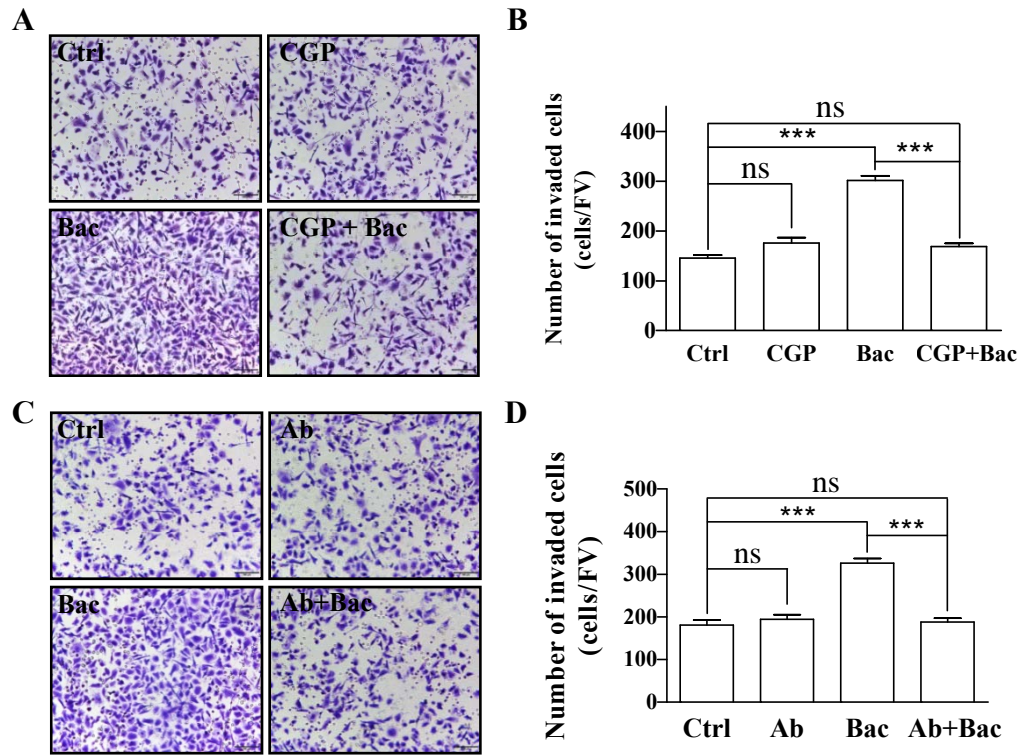


Fig. 6

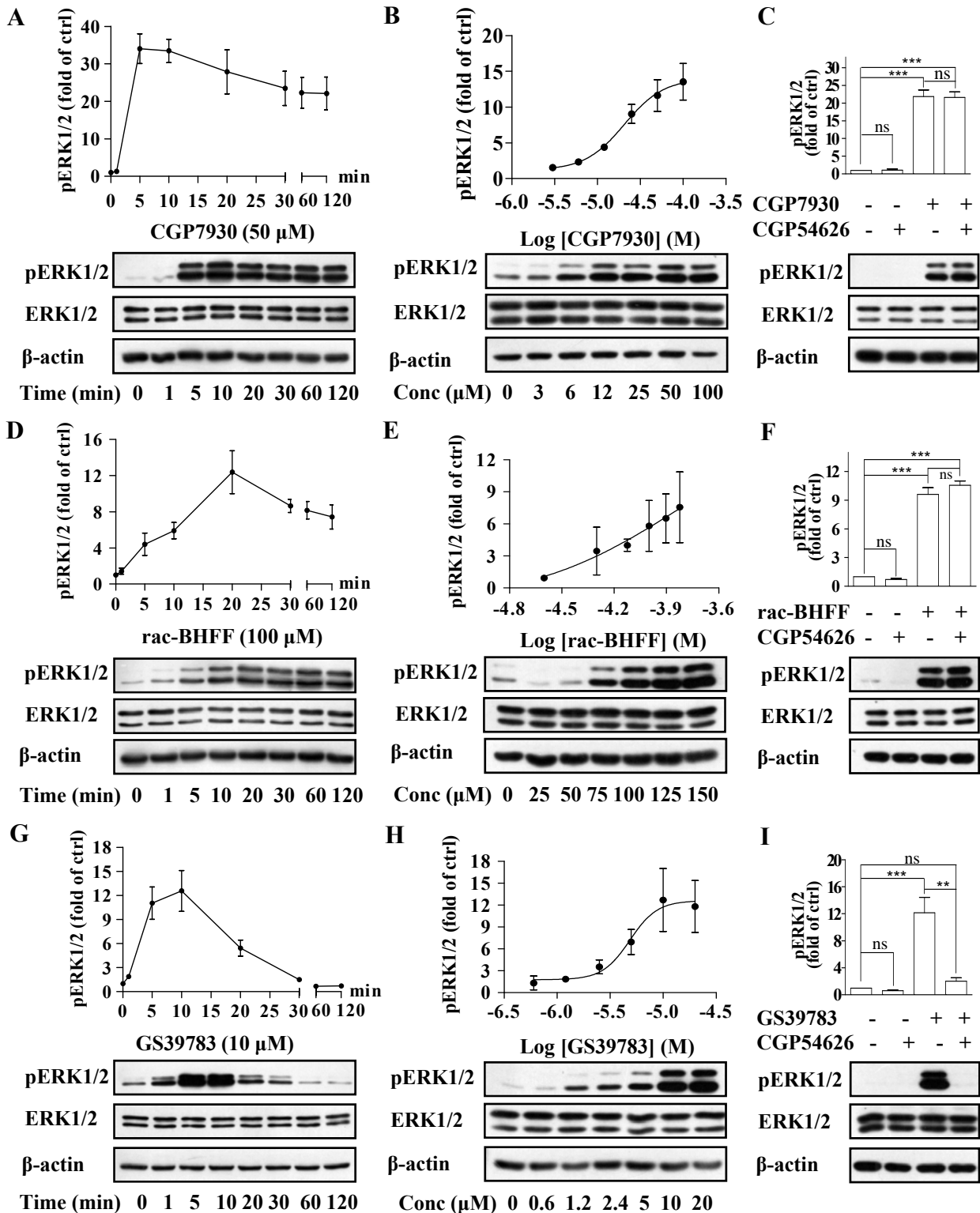


Fig. 7

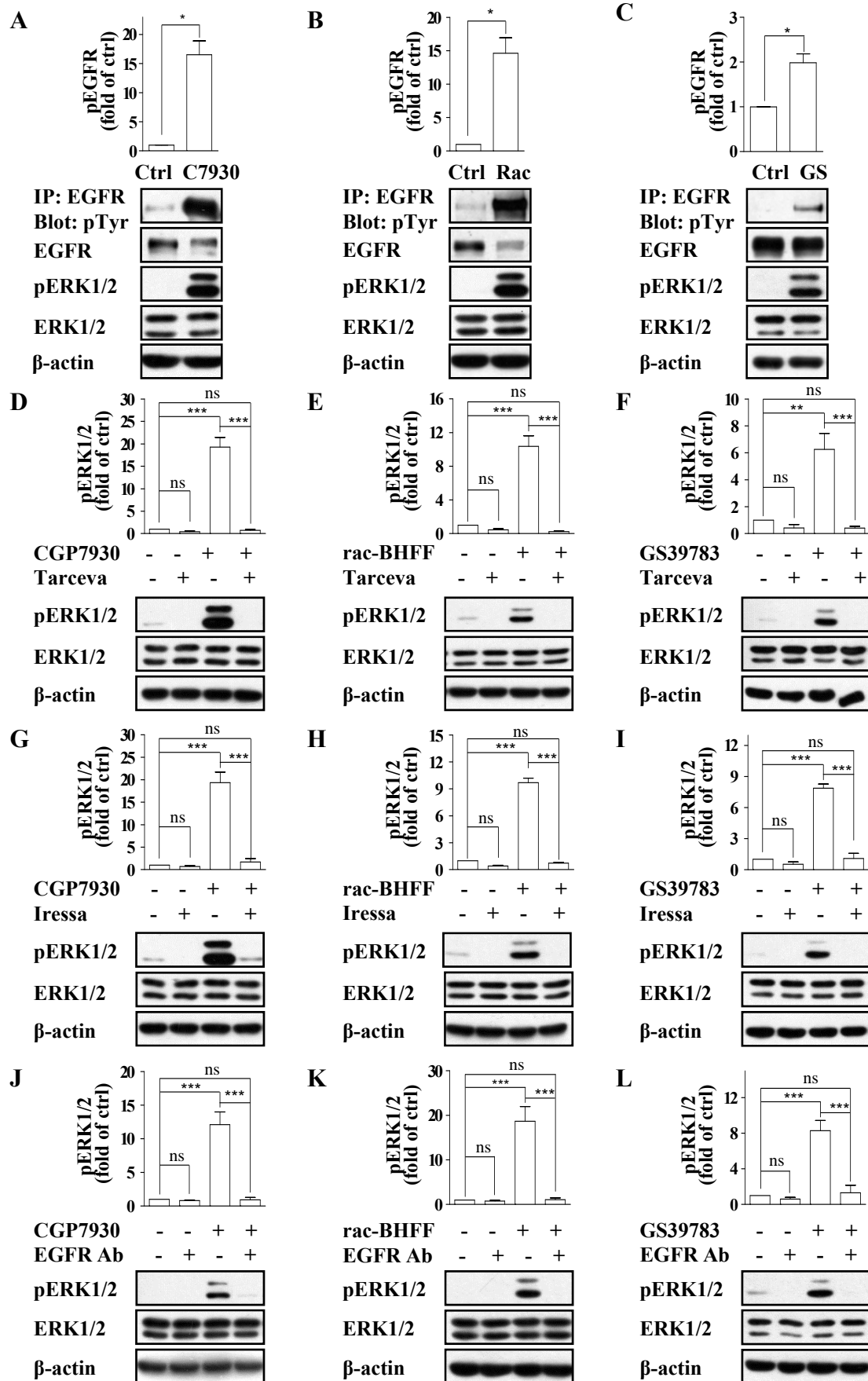


Fig. 8

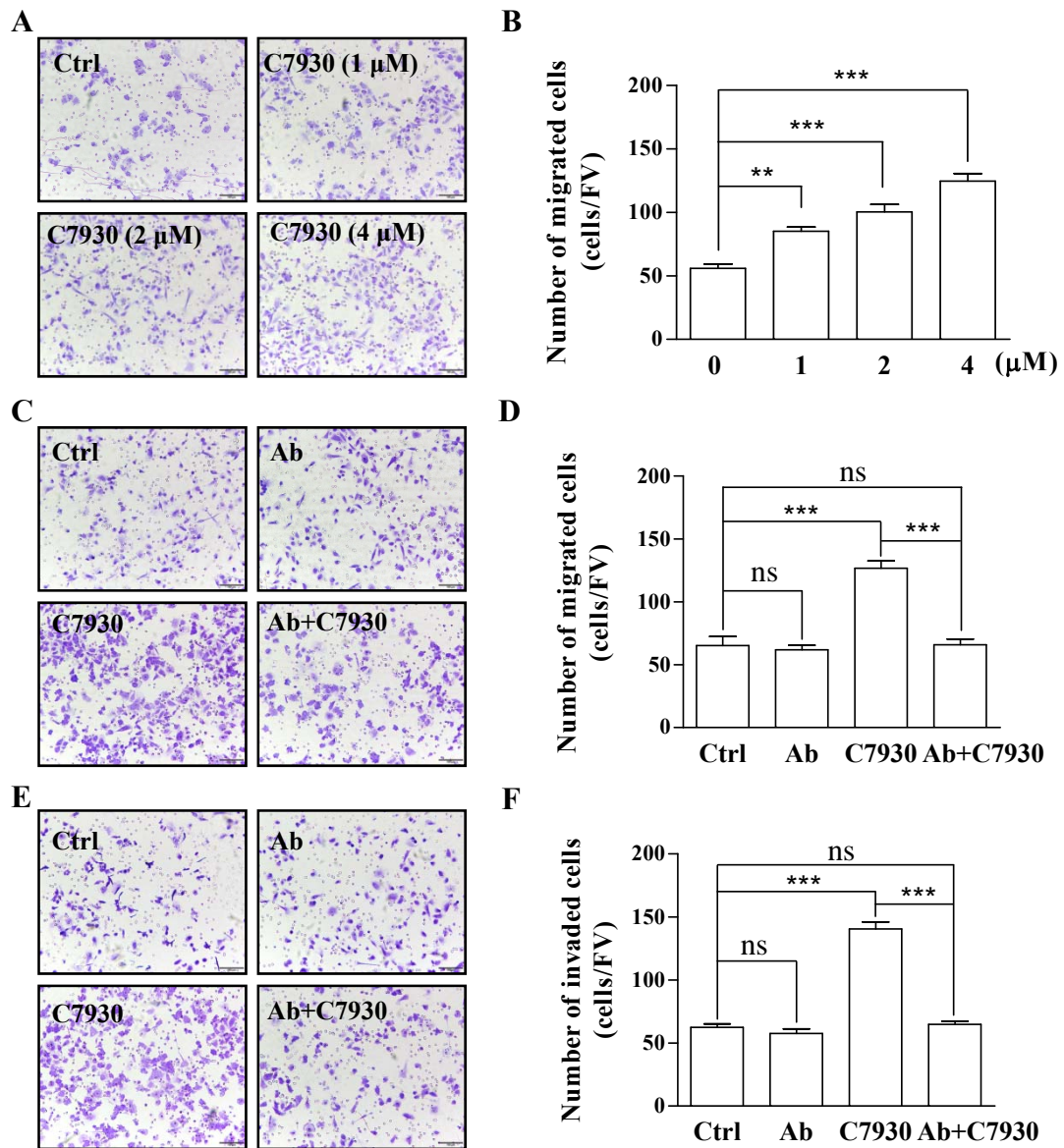


Fig. 9

