

## Original Article

# Geranylgeranylation promotes proliferation, migration and invasion of gastric cancer cells through the YAP signaling pathway

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Received October 3, 2019; Accepted August 1, 2020; Epub September 15, 2020; Published September 30, 2020

**Abstract:** Geranylgeranylation (GGylation) is a lipid modification process of signaling proteins. Currently, very little is known about the GGylation signaling for gastric cancer cell proliferation and migration. In this report, we found that inhibition of GGylation by the mevalonate pathway inhibitor atorvastatin and the geranylgeranyltransferase I inhibitor GGTI-298 impairs proliferation and migration of the gastric cancer AGS cells. During searching the signaling pathway for the effect, we observed that YAP, a transcription activator and downstream effector of the hippo pathway, was suppressed by inhibition of GGylation, as evaluated by detection of the mRNA level of its known target genes *CYR61* and *CTGF* and translocation to nuclei. Knockdown of YAP by shRNAs produced a similar effect on proliferation and migration of gastric cancer AGS cells to that of GGylation inhibition, suggesting that GGylation signaling promotes gastric cancer cell proliferation and migration by activation of YAP. Our studies provide a potential new therapeutic targeting pathway for gastric cancer.

**Keywords:** Geranylgeranylation, atorvastatin, YAP, gastric cancer, cellular signaling, cell proliferation, migration and invasion

## Introduction

Gastric cancer (GC) is one of the highest mortality cancer types worldwide [1]. Treatment of GC mainly relies on surgery, chemo- and radio-therapies. Currently, few signaling pathways and proteins have been established for targeted therapy in GC. Identification of the signaling pathways and proteins that drive tumor growth and metastasis of GC and development of novel targeted therapies become urgent for improving the therapeutic efficacy and survival rate of GC patients.

Geranylgeranylation (GGylation) is a lipid modification process of multiple signaling proteins, particularly Rho family GTPases and the  $\gamma$  subunits of trimeric G-proteins [2-5]. Early studies found that treatment with statins, a class of inhibitors of the hydroxymethylglutaryl co-enzyme A (HMG-CoA) reductase, inhibited proliferation and induced apoptosis of cells in multi-

ple types of cancer [6-10]. HMG-CoA reductase is a key enzyme of the mevalonate pathway synthesizing cholesterol and metabolites for prenylation including farnesylation and GGylation [2, 3]. Rescue experiments by supplement of the intermediate metabolites of the mevalonate pathway into the statin-treated culture medium demonstrated that impairment of synthesis of geranylgeranyl pyrophosphate, a lipid donor molecule for GGylation, is the cause for the inhibitory effect of statins on cancer cell proliferation and survival [11, 12]. Many studies have demonstrated that inhibition of GGylation using geranylgeranyltransferase I inhibitors (GGTIs) induces apoptosis of cancer cells [13-15]. However, the role of GGylation signaling in gastric cancer cell proliferation, migration and invasion remains unexplored up to date.

Recent studies discovered a connection of the mevalonate pathway to the YAP/TAZ signaling during breast cancer cell proliferation [16-18].

Our studies have shown that GGylation signaling promotes both proliferation and migration of the triple-negative breast cancer cells through activation of the hippo-YAP/TAZ pathway [18]. These studies first reveal the mechanism underlying the effect of GGylation signaling on breast cancer cell proliferation and migration.

The hippo-YAP/TAZ signaling was also found to play an important role in proliferation and migration in gastric cancer cells [19], and expression of YAP and TAZ is associated with metastasis of gastric tumor [20, 21]. However, the connection between GGylation and the YAP/TAZ signaling in gastric cancer has not been established. Further exploration of the signaling axis of the GGylation/YAP/TAZ in gastric cancer cell proliferation, migration and invasion is important for developing a novel strategy of gastric cancer therapy by using the GGylation inhibitors or targeting the GGylation. Thus, this report investigated the role of GGylation in gastric cancer cell proliferation, migration and invasion and examined whether GGylation transduces cell proliferation, migration and invasion signaling to YAP. The results indicate that GGylation in gastric cancer cells promotes cell proliferation, migration and invasion through the YAP-mediated pathway.

## Materials and methods

### Materials

DMSO, Geranylgeraniol (GGOH) and GGTI-298 (GGTI) were purchased from Sigma. Atorvastatin calcium was from WuXi Sigma. Anti-YAP1 (ab52771) was purchased from Abcam; anti-CYR61 (SC-13100) from Santa Cruz; anti-actin (RLM3028) from Ruiying Biological. The YAP1 and luciferase (control) shRNA oligos were synthesized by ShengGong Company. Transwell dishes were purchased from Corning Inc. Matrigel was purchased from BD Biosciences. The gastric cancer cell line AGS was purchased from the American Type Culture Collection (ATCC).

### Cell culture and treatment

HEK293T cells were cultured and maintained in DMEM (Hyclone) supplemented with 10% FBS at 37°C, 5% CO<sub>2</sub>. The gastric cancer AGS cells were grown in F12K (Boster bio) supplemented with 10% fetal bovine serum (Excell

bio), 100 U/mL penicillin, and 100 mg/mL streptomycin in 5% CO<sub>2</sub> at 37°C. Cells were seeded and incubated for 12 h before treatment, and then treated with DMSO, GGTI (10 μM), atorvastatin (10 μM), atorvastatin plus GGOH (10 μM) for 48 h. The cells were starved in serum-free medium for 12 hrs before the serum stimulation. For serum stimulation, FBS was directly added to the cells in the serum starvation medium at a 10% final concentration and incubated for 30 min at 37°C.

### Cell lysate preparation and immunoblotting

Culture medium was removed and cells were washed with cold PBS once and lysed in pre-cooled mammalian cell lysis buffer (40 mM HEPES, pH 7.4, 1% Triton X-100, 100 mM NaCl, 1 mM EDTA, 25 mM β-glycerolphosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin and 10 μg/ml aprotinin) (0.5 ml/60 mm dish) by rocking at 4°C for 30 min. The cell lysates were collected into an eppendorf tube and cleared by centrifugation at 12,000 rpm for 15 min in a microfuge. The SDS-PAGE samples were prepared by addition of 5 × SDS sample buffer directly to the cleared lysates, followed by rigorous vortex and denatured at 100°C for 10 min. Electrophoresis was run on 10% SDS-PAGE gels, and separated proteins were transferred onto Immobilon PVDF membranes (Millipore). The membranes were incubated with primary antibodies overnight at 4°C, followed by incubating with secondary antibodies for 1 h at room temperature. The protein bands were detected by the Western Lightning ECL Detection Kit (Beytime).

### RNA preparation and reverse transcription (RT)-PCR

After treatment, total RNA was extracted using the Total RNA Extraction Kit (ShengGong Company) and reverse-transcribed into cDNA by the RevertAid First Strand cDNA Synthesis Kit (TaKaRa) according to the manufacturer's instructions. PCR was performed using 2 × PCR Taq plus MasterMix (Applied Biological Materials, Inc). The amplification products were analyzed by agarose gel electrophoresis. The qRT-PCR assay was performed using the comparative cycle threshold (CT) method with SYBR-Green PCR Master Mix (Takara Bio) according to the manufacturer's instruction on a Bio-Rad CFX96 system (Bio-Rad Laboratories, Inc.,

Hercules, CA, USA). The primer pairs used for RT-PCR are: YAP1 (human) forward primer: 5'-ATGGATCCCGGGCAGCAGC-3'; YAP1 (human) reverse primer: 5'-AGCTCCCAACTGCAGAGAAG-3'; CYR61 (human) forward primer: 5'-CCAGTGTACAGCAGCCTGAA-3'; CYR61 (human) reverse primer: 5'-CGCATCTTCACAGTCCTGGT-3'; CTGF (human) forward primer: 5'-ATGGTGCTCCCTGCATCTTC-3'; CTGF (human) reverse primer: 5'-CTGGTACTTGACAGCTGCTCT-3'; GAPDH (human) forward primer: 5'-AACGGATTGGTCGATTG-3'; GAPDH (human) reverse primer: 5'-GGAAGATGGTGATGGGGAT-3'.

## *Construction of the lentiviral shRNA plasmids, package of viral particles and infection of cells*

The synthesized oligos for shRNAs were annealed and inserted into the AgeI/EcoRI-digested pLKO.1 TRC-Cloning Vector. A luciferase shRNA (shLuc) oligo (The targeting sequence: 5'-CGCTGAGTACTTCGAAATGTC-3'), which does not match any known human gene sequence, was used as the control shRNA. The YAP1 shRNA (shYAP1) target sequence is GACCAATAGCTCAGATCCTTT. To produce the lentiviral particles, the pLKO.1 TRC-shRNA was co-transfected with the packaging plasmids ps-PAX2 and pMD2.G into HEK 293 T cells, and the cultured supernatant containing the viral particles was collected at 24, 48 and 72 hrs after transfection. This supernatant was used as the shRNA viral stock solution. For lentiviral infection, the shRNA viral stock solution was added into the AGS cell culture medium for 24 hrs in the presence of 4 µg/mL polybrene. After the infection, cells were cultured in F12K plus 10% FBS and 2 µg/ml puromycin. Selection of puromycin-resistant cell colonies was carried out 72 hrs after transfection. The cell colonies resistant to puromycin were selected and cultured in F12K plus 10% FBS, 2 µg/ml puromycin. The knockdown efficiency was evaluated by detection of the target protein level with immunoblotting or the mRNA level with qRT-PCR.

## *Immunofluorescent staining*

The cells were cultured on a circular microscopic slide glass cover (Nest) to 50-80% confluence. After treatment, the culture medium was aspirated, the cells were rinsed with PBS twice, fixed with 4% paraformaldehyde at 25°C for 30 min, and permeabilized with 0.2% Triton X-100

in PBS at 25°C for 20 min. After washing with TBST buffer, the cells were incubated with a primary antibody overnight at 4°C. Then the cells were washed with TBST buffer three times and incubated with a secondary antibody that was conjugated with a fluorescent dye and DAPI at 37°C for 1 hr. Finally, the cells were washed with TBST buffer three times, and the immunofluorescence staining was visualized under an inverted fluorescent microscope (GE Healthcare).

## *Cell proliferation, migration and invasion assays*

(1) Determination of cell proliferation. The AGS cells were cultured in F12K with 10% FBS at 37°C plus 5% CO<sub>2</sub> for 12 hrs. In a 12 well culture plate, 2 × 10<sup>5</sup> AGS cells were seeded in each well. The cells were treated with the indicated concentrations of treatment reagents. The cells were trypsinized and counted under a phase microscope with a hemocytometer after 48 h. The cell counting was repeated at least three times. The histogram was drawn by Prism5.

(2) Cell migration assays. Cell migration was detected by two methods: the wound-healing assay and the transwell assay. (i) The wound healing assay. The cells (3 × 10<sup>5</sup>) were seeded on 12-well plates for 12 hrs (about 80-90% confluence), which were pretreated with treatment for 12 hrs. Wounds were created by scraping the cell layer with a 10 µl pipette tip. The culture medium was replaced with the medium containing treatment reagents. The wound healing was assessed by photography after 24 hrs. The experiments were repeated at least three times. (ii) The transwell assay. After treatment for 24 hrs with an indicated reagent, the cells (4 × 10<sup>4</sup>) were suspended with 200 µl of the serum-free F12K medium and placed into the upper chamber of the transwell plate (Corning) with the treatment reagent. The lower chamber of the transwell plate was pre-filled with 500 µl of 10% FBS F12K medium with the treatment reagent. After incubation for 24 hrs at 37°C, the cells on the upper side of the chamber membrane were carefully removed, the cells migrated to the bottom side of the membrane were fixed with 4% paraformaldehyde for 30 min and stained with 0.1% crystal violet solution. The stained cells were

washed with PBS three times and visualized by microscopic photography. The migration experiments were repeated three times. For quantification, the cells were counted under a microscope from three randomly selected fields, or the crystal violet dye in the stained cells was extracted with methanol and its absorbance at 540 nm was measured by a spectrometer.

(3) Cell invasion assay. Matrigel was melted at 4°C overnight. The transwell top chamber membrane was coated with 40 µl of 0.125 mg/ml matrigel in F12K medium and incubated at 37°C for 3 to 5 hrs before use. The AGS cells were pretreated with indicated concentration of treatment reagents for 24 hrs, trypsinized and washed with PBS two times and resuspended in serum-free F12K medium at a density of  $2 \times 10^5$  cells/ml. The cells (200 µl in serum-free F12K medium with the treatment reagents) were added to the top of the matrigel layer. The bottom chamber of the transwell was filled with 500 µl of F12K medium plus 10% FBS and the treatment reagents. The cell invasion was carried out at 37°C and 5% CO<sub>2</sub> for 24 hrs. After the invasion was done, the cells on the upper side of the chamber membrane were removed by cotton balls. The cells on the bottom side of the membrane (invaded cells) were fixed with 4% paraformaldehyde at 25°C for 30 min and stained with 0.1% crystal violet solution at 25°C for 20 min. The stained cells were washed with PBS three times and observed and photographed under a microscope. For quantification, the cells were counted under a microscope from three randomly selected fields.

## Statistical analysis of data

The experimental data are analyzed statistically using Student's t-test for two-treatment comparisons.  $P < 0.05$  is considered as significant.

## Results

### *GGylation is required for gastric cancer AGS cell proliferation*

To determine the effect of GGylation on gastric cancer cell proliferation, we examined the effect of atorvastatin (labeled as AT in figures), an inhibitor of HMG-CoA reductase, on proliferation of gastric cancer AGS cells. As shown in **Figure 1**, continuous treatment with atorvas-

tatin for 48 hrs inhibited more than 90% of the cell proliferation rate. Supplement of geranylgeraniol (GGOH) along with atorvastatin rescued 60-70% of the inhibited proliferation rate by atorvastatin, while GGOH alone did not affect the proliferation rate, indicating that GGylation is the major driving force for the gastric cancer cell proliferation.

### *GGylation is required for gastric cancer cell migration and invasion*

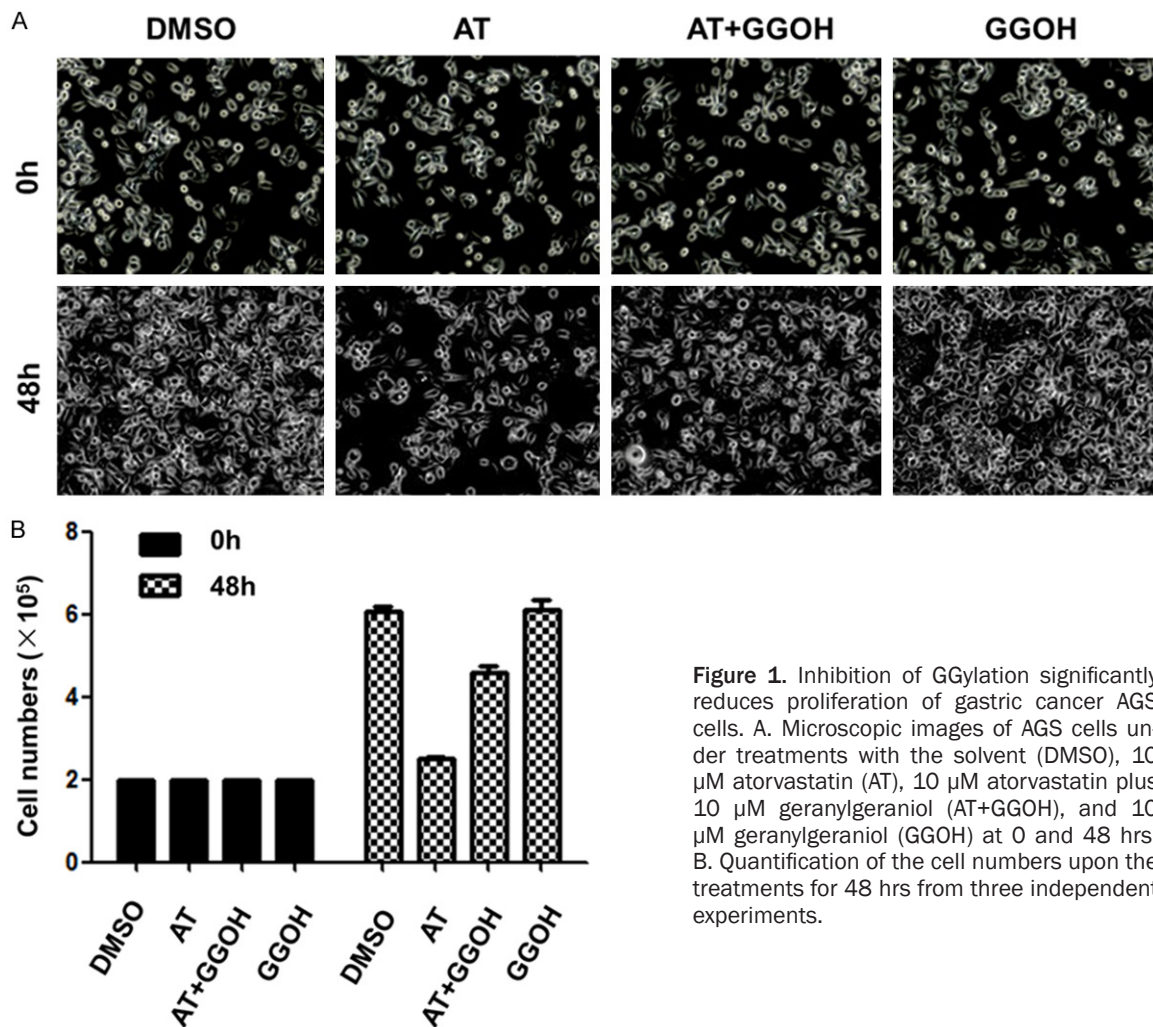
Next, we examined if the GGylation is involved in regulation of gastric cancer cell migration and invasion. The wound healing and the transwell assays were used for determination of cell migration and invasion. As shown in **Figure 2A**, treatment with atorvastatin (AT) significantly inhibited migration of gastric cancer AGS cells to the scratched area, while addition of GGOH to the medium along with atorvastatin recovered the migration ability of the cells, indicating that GGylation plays a decisive role in AGS cell migration. To confirm the role of GGylation in promoting migration of the gastric cancer cells, we performed the transwell assay. Consistent with the result in the wound healing assay, the atorvastatin treatment severely impaired migration ability of AGS cells ( $P < 0.0001$ ), while addition of GGOH in the atorvastatin treatment recovered about 80% of the migration (**Figure 2B**). This result again demonstrates that GGylation is important for the gastric cancer cell migration.

We further examined whether GGylation is involved in the gastric cancer cell invasion by the transwell invasion assay. As shown in **Figure 2C**, treatment with atorvastatin inhibited more than 80% of the invasion ( $P < 0.0001$ ), while addition of GGOH along with atorvastatin rescued the inhibition effect. This result indicates that GGylation signaling mediates the invasion process of the gastric cancer cells. Taken together, GGylation has a pivotal role in both migration and invasion of the gastric cancer AGS cells.

### *YAP is a downstream effector of the GGylation signaling in the gastric cancer cells*

We next searched the effector that might mediate the effect of GGylation on cell proliferation, migration and invasion in the gastric cancer AGS cells. The transcriptional co-activator YAP





**Figure 1.** Inhibition of GGylation significantly reduces proliferation of gastric cancer AGS cells. A. Microscopic images of AGS cells under treatments with the solvent (DMSO), 10  $\mu$ M atorvastatin (AT), 10  $\mu$ M atorvastatin plus 10  $\mu$ M geranylgeraniol (AT+GGOH), and 10  $\mu$ M geranylgeraniol (GGOH) at 0 and 48 hrs. B. Quantification of the cell numbers upon the treatments for 48 hrs from three independent experiments.

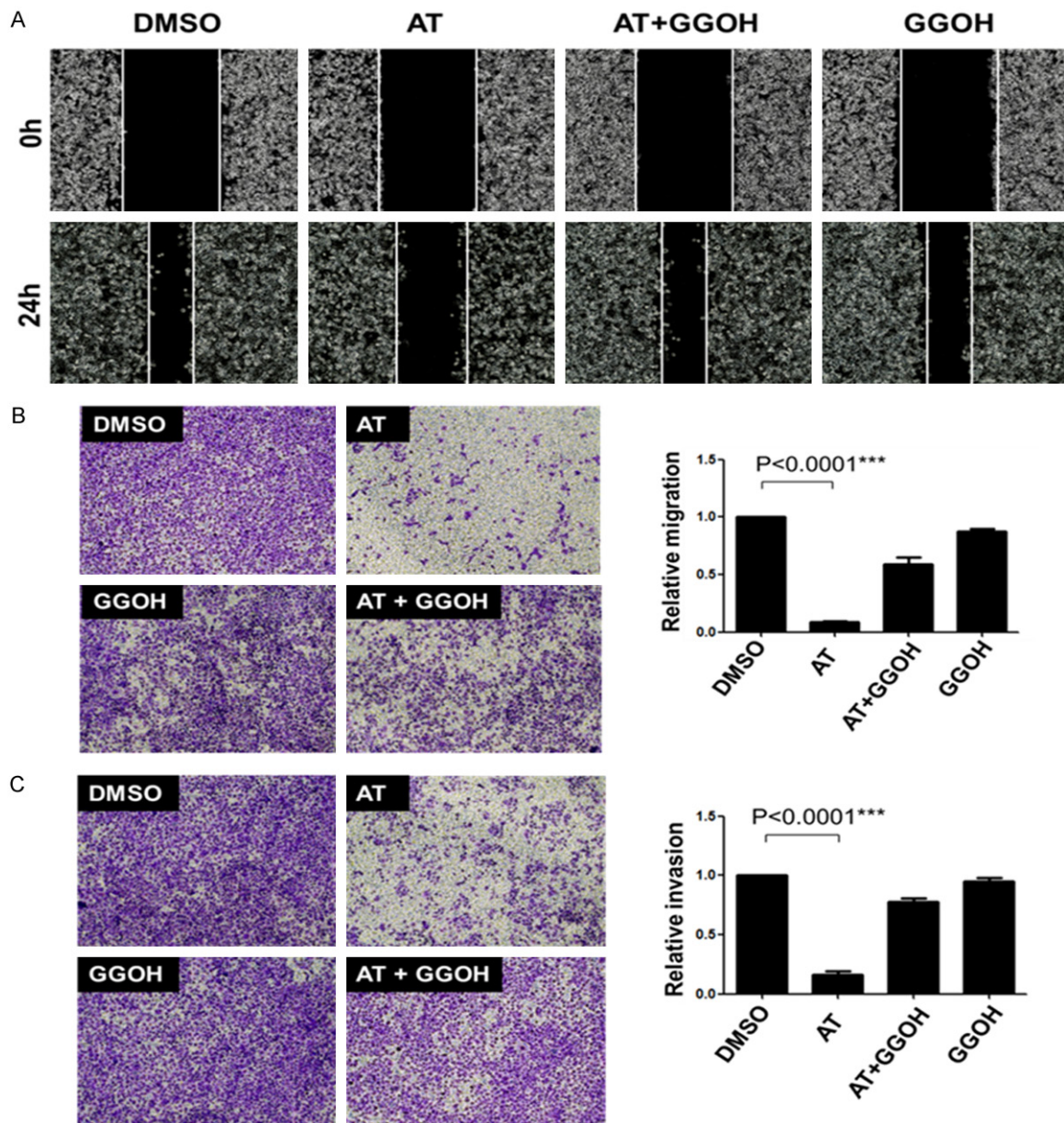
has been found to mediate the GGylation signaling in the estrogen-receptor negative (ER-) breast cancer cells [18]. Thus, we examined the effect of atorvastatin on expression of *CYR61* and *CTGF*, the two known target genes of YAP, in the gastric cancer AGS cells using the quantitative RT-PCR (qRT-PCR) assay. As shown in **Figure 3A** and **3B**, treatment with atorvastatin for 48 hrs significantly inhibited expression of *CYR61* and *CTGF*, while addition of GGOH along with atorvastatin recovered expression of both genes. Furthermore, treatment with GGTI-298, a specific inhibitor of geranylgeranyltransferase I (GGTase I), inhibited expression of *CYR61* and *CTGF* similar to treatment with atorvastatin. These results indicate that GGTase I catalyzed GGylation is required for YAP transcriptional activity.

To confirm the effect, conventional RT-PCR was also performed to detect expression of *CYR61* upon treatment with atorvastatin and GGTI-

298. Consistent with the results in the qRT-PCR assay, expression of *CYR61* was inhibited by treatment with atorvastatin or GGTI-298, and addition of GGOH along with atorvastatin partially rescued the expression (**Figure 3C**). Interestingly, expression of *YAP1* was affected little by treatment with atorvastatin or GGTI-298. Examination of the protein level of *CYR61* and *YAP1* further demonstrated that treatment with atorvastatin or GGTI-298 significantly reduced *CYR61* level, but had few effects on *YAP1* level, suggesting that GGylation signaling promotes expression of the YAP target gene *CYR61* through activating the YAP transcriptional activity, not by elevation of the YAP protein level.

#### *GGylation signaling activates YAP through promoting its nuclear localization*

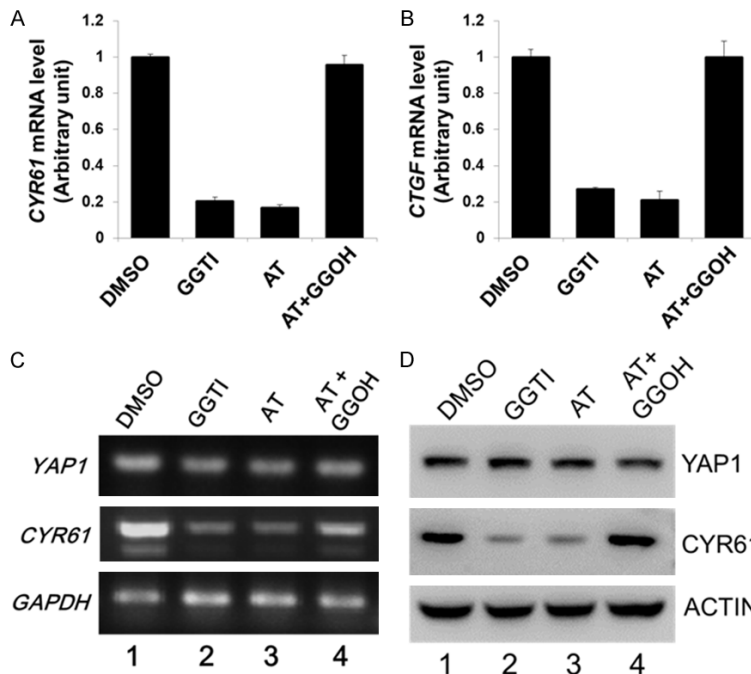
As we described in **Figure 3**, treatment with atorvastatin or GGTI-298 down-regulated ex-



**Figure 2.** Inhibition of GGylation disables migration and invasion of gastric cancer AGS cells. A. The wound-healing assay of AGS cell migration under treatments with the solvent (DMSO), 10  $\mu$ M atorvastatin (AT), 10  $\mu$ M atorvastatin plus 10  $\mu$ M geranylgeraniol (AT+GGOH), and 10  $\mu$ M geranylgeraniol (GGOH). B. The transwell assay of AGS cell migration upon the treatments for 24 hrs. C. The transwell matrigel assay of AGS cell invasion upon the treatments for 24 hrs.

pression of the YAP target gene CYR61 and CTGF, however, had an insignificant effect on expression of YAP in gastric cancer AGS cells. This raises the possibility that GGylation signaling promotes activation of YAP. Translocation from cytoplasm to nucleus is a known activation process for YAP [22, 23]. Thus, we examined the effect of atorvastatin or GGTI-298 on translocation of YAP to nucleus. As shown in

**Figure 4**, under serum starvation, YAP1 was partially localized in nucleus (**Figure 4A**). Upon serum stimulation, YAP1 was entirely localized in nucleus (**Figure 4A'**), indicating that serum stimulation activates YAP1. Treatment with atorvastatin or GGTI-298 completely impaired the ability of YAP1 to translocate into nucleus under both serum starvation and stimulation conditions (**Figure 4D, 4G, 4D'** and **4G'**), while



**Figure 3.** Inhibition of GGylation down-regulates expression of the YAP target gene *CYR61* and *CTGF* in gastric cancer AGS cells. A and B. The quantitative RT-PCR (qRT-PCR) assay of expression of the YAP target genes *CYR61* and *CTGF* in gastric cancer AGS cells upon treatment with the solvent (DMSO), 10  $\mu$ M GGTI-298 (GGTI), 10  $\mu$ M atorvastatin (AT), and 10  $\mu$ M atorvastatin plus 10  $\mu$ M geranylgeraniol (AT+GGOH) for 48 hrs. C. The conventional RT-PCR assay of expression of *YAP1* and *CYR61* upon the treatments for 48 hrs. D. The immuno-blotting assay of the protein level of *YAP1* and *CYR61* upon the treatments for 48 hrs.

supplement of GGOH in the atorvastatin treatment restored the ability of YAP1 to translocate into nucleus (**Figure 4J** and **4J'**). These results demonstrated that GGylase I mediated GGylation signaling is essential for activation of YAP1 (translocation to nucleus) in the gastric cancer cells.

*YAP1 is the downstream effector mediating the effect of GGylation on transcription of *CYR61* and *CTGF* genes in gastric cancer AGS cells*

To confirm the role of YAP in mediating GGylation signaling, we detected the effect of YAP1 knockdown on expression of *CYR61* and *CTGF* genes by the shRNA assay and compared the effect with that of atorvastatin treatment in AGS cells. As shown in **Figure 5A** and **5B**, sh-YAP1 eliminated more than 90% of both mRNA and protein levels of YAP1 in AGS cells. Upon depletion of YAP1, expression of *CYR61* was reduced to about 5% of the control level and expression of *CTGF* to about 25% of the control level, which is comparable to the level inhibited

by treatment with atorvastatin (**Figure 5C** and **5D**), indicating that YAP1 is the downstream effector mediating GGylation signaling for promoting the gene transcription.

*YAP1 is the downstream effector mediating the effect of GGylation signaling on gastric cancer cell proliferation, migration and invasion*

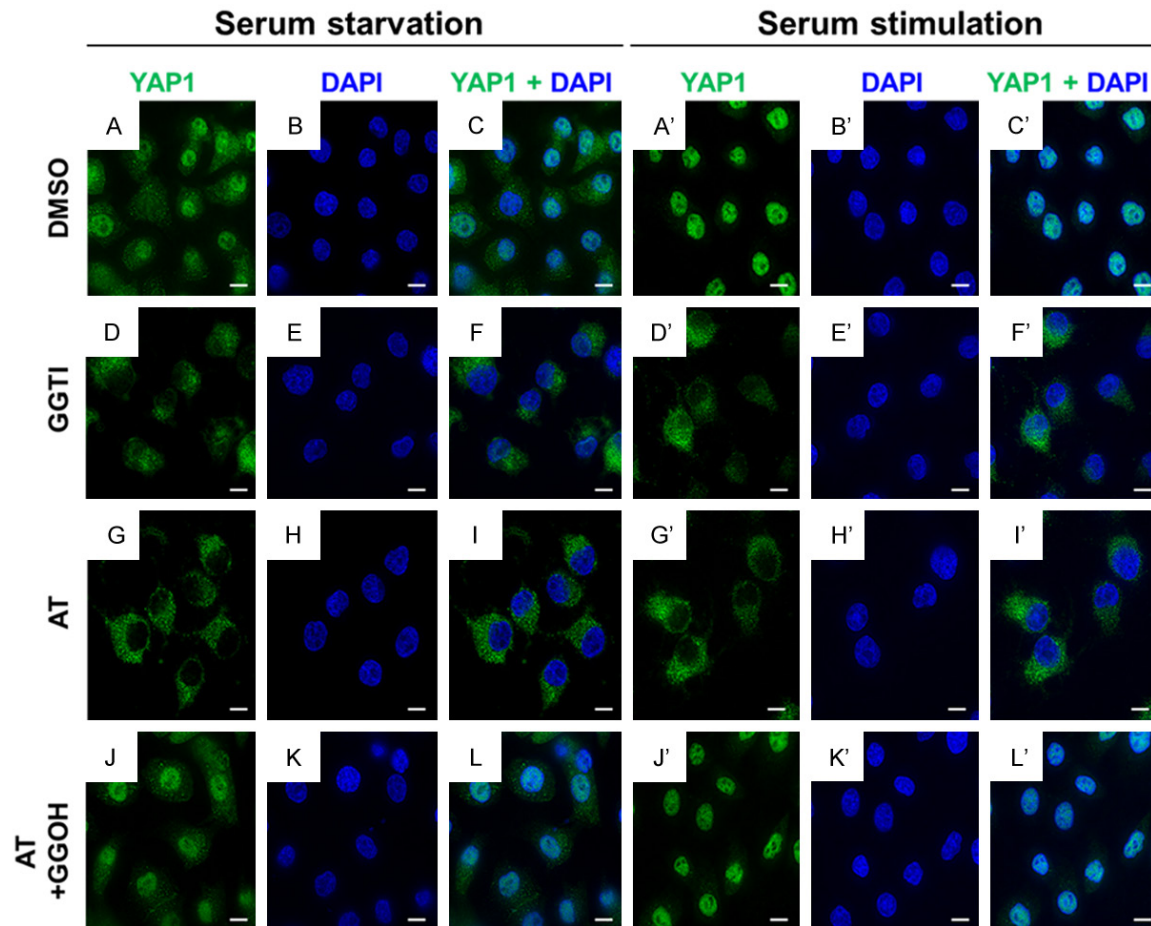
The above studies have demonstrated that YAP1 is the downstream signaling effector. Next, we determined if YAP1 mediates the effects of GGylation on the gastric cancer cellular functions. We first detected the effect of depletion of YAP1 by the shRNA assay on AGS cell proliferation. As shown in **Figure 6A** and **6B**, knockdown of YAP1 by shYAP1 in AGS cells, similar to treatment with atorvastatin, caused a significant inhibition of cell proliferation when cells were cultured 2 or 3 days.

We then examined the effect of YAP knockdown on cell migration and invasion. Similar to the effect of atorvastatin, depletion of YAP1 in AGS cells significantly inhibited the cell migration upon EGF or no EGF stimulation detected by both the wound healing and transwell assays (**Figure 7A** and **7B**). As expected, knockdown of YAP1 in AGS cells caused a remarkable inhibition of the cell invasion (**Figure 7C**), which is similar to the effect of atorvastatin. These results indicate that YAP1 is the protein mediating the signaling of GGylation to promote gastric cancer cell proliferation, migration and invasion.

## Discussion

In this report, we have shown that GGylation signaling plays an essential role in proliferation, migration and invasion of gastric cancer AGS cells, and found that GGylation signaling drives gastric cancer cell proliferation, migration and invasion via activating YAP. Our work suggests





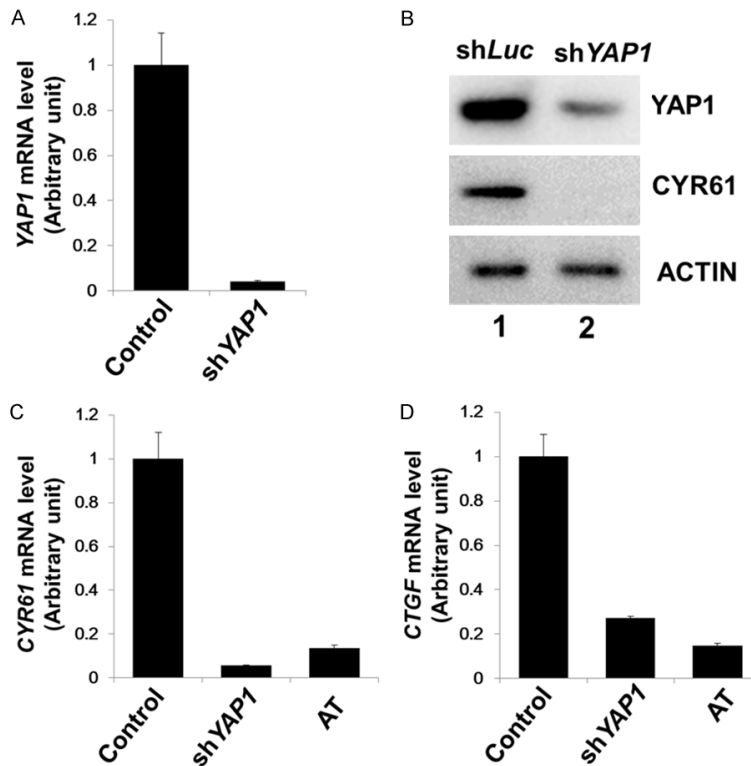
**Figure 4.** Inhibition of GGylation impairs translocation of YAP1 to nucleus in gastric cancer AGS cells. The cells were treated with the solvent (DMSO), 10  $\mu$ M GGTI-298 (GGTI), 10  $\mu$ M atorvastatin (AT), and 10  $\mu$ M atorvastatin plus 10  $\mu$ M geranylgeraniol (AT+GGOH) for 36 hrs, followed by serum starvation for 12 hrs. For serum stimulation, FBS was directly added to the cells in serum starvation medium and incubated at 37 °C for 30 min. The cells were fixed and immuno-stained, and the fluorescently stained cells were observed under a fluorescent microscope. Bar, 10  $\mu$ m.

that the GGylation signaling might be a major driving force for tumor growth and metastasis of gastric cancer, thus an important target for gastric cancer therapy, particularly for the advanced stage gastric cancer therapy.

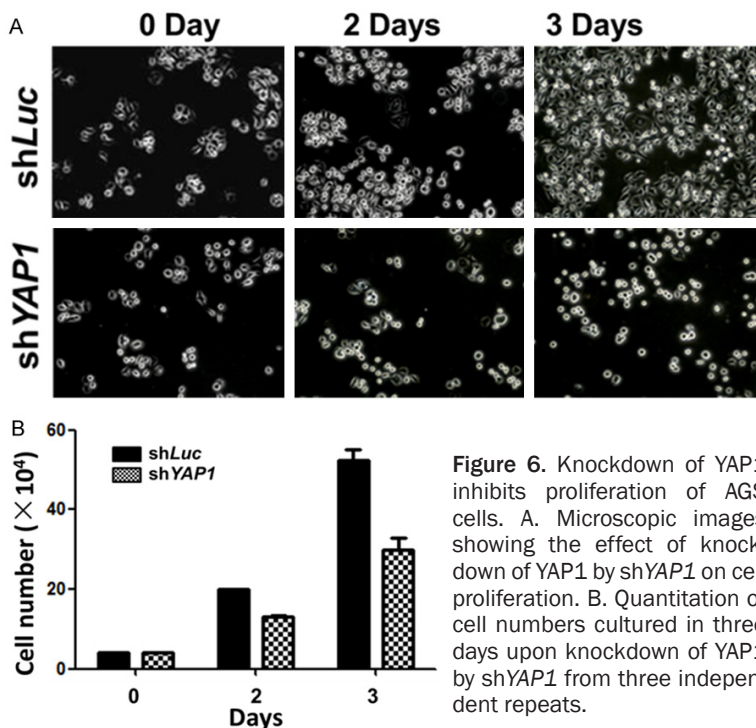
Currently, GGylation signaling in promoting gastric cancer initiation and progression has been poorly investigated. However, GGylation signaling in cell proliferation and survival has been well studied in other types of cancers, such as leukemia and breast cancer [6-10]. Three types of signaling molecules, including Rho family GTPases, Rab family GTPases, and gamma subunits of trimeric GTPases, are known to be modified by geranylgeranylation [2-5]. Rho GTPase, known to promotes formation of actin stress fibers [24, 25], is a direct effector mole-

cule downstream of geranylgeranylation involved in cancer cell proliferation and survival. Recent studies found that Rho might be a major GGylation signaling molecule mediating activation of YAP/TAZ [16-18]. Our previous studies have shown that inhibition of either Rho or the beta/gamma subunit of heterotrimeric GTPase produced the same effect on breast cancer cell proliferation and migration as that of inhibition of GGylation or knockdown of YAP/TAZ [18], suggesting that GGylation of Rho and the gamma subunit may mediate the activation of YAP/TAZ. In this report, although we showed that proliferation, migration and invasion of gastric cancer cells, similar to breast cancer cells, are dependent on the GGylation signaling and its subsequent activation of YAP, the molecular linkage of GGylation signaling to YAP





**Figure 5.** Knockdown of YAP1 by shRNA produces a similar down-regulation effect on expression of CYR61 and CTGF to that by treatment with atorvastatin in gastric cancer AGS cells. A. The knockdown effect of shYAP1 on the mRNA level of YAP1 determined by the qRT-PCR assay. B. The knockdown effect of shYAP1 on the protein level of YAP1 and CYR61 determined by immunoblotting. C and D. The mRNA level of CYR61 and CTGF upon expression of shYAP1 or treatment with atorvastatin determined by the qRT-PCR assay. The qRT-PCR data were obtained from three independent samples.

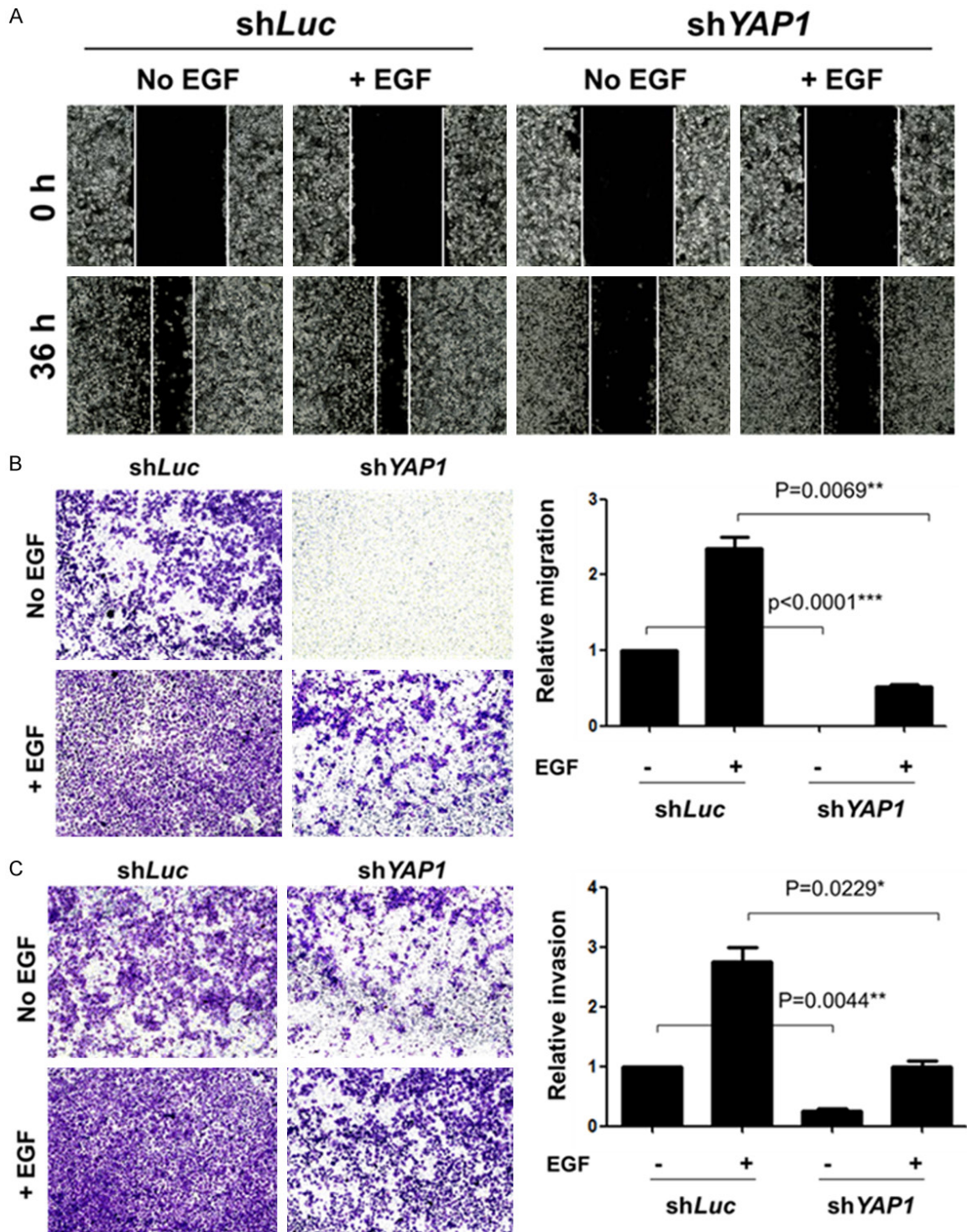


**Figure 6.** Knockdown of YAP1 inhibits proliferation of AGS cells. A. Microscopic images showing the effect of knockdown of YAP1 by shYAP1 on cell proliferation. B. Quantitation of cell numbers cultured in three days upon knockdown of YAP1 by shYAP1 from three independent repeats.

activation is still missing. We speculate that Rho GTPase and gamma subunits are the major mediators for transducing GGylation signaling to YAP activation, thus promoting cell proliferation, migration and invasion in gastric cancer. We will confirm this speculation and further investigate the molecular mechanism of YAP activation by GGylation in gastric cancer cells in future studies.

Although the role of YAP in gastric cancer has been investigated and is known to promote gastric cancer progression [20, 21], the downstream effectors remain largely elusive. Our previous studies have shown that the YAP/TAZ target gene CYR61 is overexpressed in gastric cardia adenocarcinoma (GCA), correlated with GCA metastasis, and inversely associated with the overall survival of GCA patients [26]. Knockdown of CYR61 by shRNA dramatically inhibited migration and invasion of gastric cancer AGS cells [26]. These results suggest that the effects of the GGylation/YAP signaling on the gastric cancer cell migration and invasion found in this study might be largely mediated by CYR61. However, the molecular mediator for the effects of the GGylation/YAP signaling on the gastric cancer cell proliferation in this study is still unidentified. It has been reported that YAP/TAZ activates the transcription and protein level of MYC, a known cancer cell proliferation driver gene, in AGS cells [27]. Thus, MYC might be the mediator in GGylation/YAP signaling-promoted gastric cancer cell proliferation.

Targeted therapy in gastric cancer currently has few eff-



**Figure 7.** Knockdown of YAP1 disables migration and invasion of gastric cancer AGS cells. (A and B) The effect of YAP1 knockdown by *shYAP1* on cell migration determined by the wound-healing assay (A) and the transwell assay (B). (C) The effect of YAP1 knockdown by *shYAP1* on cell invasion determined by the transwell matrigel assay (B).

ective targeting drugs due to lacking information about dominant driving signaling pathways for tumor growth and metastasis in gastric can-

cer. Our previous studies examined expression of the YAP/TAZ target gene product CYR61 in tumor samples from 214 GCA cases and found

that 44% of GCA tumor samples overexpress CYR61 [26], indicating that YAP/TAZ is activated in a large portion of GCA population. Besides, epidemiological studies found that statin usage significantly reduced the risk of gastric cancer [28-30]. These studies suggest that GGylation/YAP signaling might be a promising druggable target for gastric cancer therapy. We will utilize a mouse model further to investigate the application of GGylation/YAP signaling for gastric cancer therapy in future studies.

## Acknowledgements

This work is supported by National Natural Science Foundation of China (No.81372208 and No. 81871888 to Q. L. and No. 81472558 to W.Y.) and the Innovation and Entrepreneurship Team Program of Jiangsu Province (to H. L. and Q. L.).

## Disclosure of conflict of interest

None.

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