

Lysophosphatidic Acid-induced EGFR Transactivation Promotes Gastric Cancer DNA Replication Through Up-Regulation of Geminin

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Abstract

Background

Lysophosphatidic acid (LPA) is one of the simplest active phospholipid molecules. Binding to its receptors on the cell surface, LPA initiates various intracellular signal cascades, involving in numerous biological processes, such as cell proliferation, migration, and apoptosis. If abnormalities occur in the processes of LPA production, receptor expression or signal transduction, it may induce certain diseases and even contribute to the occurrence, development and metastasis of cancer. However, whether the initiation of DNA replication is regulated by LPA has not yet been investigated.

Methods

First, diverse public databases were analyzed to explore the genetic abnormalities affecting geminin. Next, an LPA gradient treatment was performed on gastric cancer cells, followed by detecting geminin expression variation using western blot analysis. Finally, RNAi technology or inhibitors were used to block the biological activity of related factors in the GPCR induced EGFR transactivation signaling pathway to verify whether the effect of LPA evoked gastric cancer DNA replication is dependent on geminin upregulation.

Results

We found that LPA specifically up-regulated expression of an essential replication negative regulator geminin in early S phase in gastric cancer cell lines, and that the deletion of geminin selectively induced DNA re-replication. Neither of these phenomena has been observed in normal gastric epithelial cells, indicating LPA-induced geminin up-regulation is restricted to tumor cells. Using RNAi or specific inhibitors to block the activity of related factors in the signaling pathway, we found that LPA acts through LPAR3 and downstream coupled MMPs signaling to trans-activate EGFR, increasing the expression level of geminin in S phase. On the other hand, LPA stimulation induced the up-regulation of de-ubiquitinating enzyme 3 (DUB3) in a short time and inhibited the ubiquitination degradation of geminin to enhance geminin stability and positively regulate the DNA replication initiation in gastric cancer cells. Taken together, our results suggested that LPA mediated DNA replication and S-phase cell-cycle progression through a LPAR3/MMPs/EGFR/PI3K/mTOR signaling axis in gastric cancer.

Conclusions

Our research is for the first time to study the regulatory effect of LPA-induced EGFR transactivation in DNA replication of tumor cells, and to uncover a novel mechanism for regulating the stability of geminin through LPA and related downstream signaling pathways. All of which will provide potential targets for the development of signaling pathways and tumor cell-specific EGFR transactivation inhibitor for the treatment of gastric cancer.

Introduction

A precise control of DNA replication is crucial to keep genomic stability. To keep genomic stability, both eukaryotic or prokaryotic cells evolve a miscellaneous and sophisticated mechanism to ensure nuclear DNA is completely replicated at right time, right place and only once per cell division[1]. Initiation of DNA replication is highly strictly regulated in eukaryotes. The regulation of replication initiation activation plays an important role in DNA replication, since re-initiation of replication at any starting origin leads to cell death or genomic rearrangement, causing very serious consequences and even cancer. The regulation of initiation of replication mainly includes the following three aspects: inhibition of Cdt1 function mediated by geminin, proteasome mediated Cdt1 degradation in S phase, and cyclin-dependent kinase (CDK)-mediated inhibition of transcriptional initiation activation, all of which play an integral role in maintaining genomic stability[2].

Geminin, a negative factor for DNA replication, directly binds to the licensing factor Cdt1 and inhibits pre-replicative complex formation to maintain chromosomal integrity, prevent aneuploidy and re-replication[3, 4]. The precise control of the levels of geminin throughout the cell cycle mainly dependent on ubiquitin-mediated proteasomal degradation. Geminin protein levels oscillate during the cell cycle, in late mitosis/G1 it is degraded by the anaphase-promoting complex/cyclosome (APC/C) ubiquitin ligase complex to facilitate origin licensing[4]. Conversely, geminin is accumulated in early S, G2 and early M phases due to APC/C ubiquitin ligase complex inhibition, ensuring the inhibition of pre-replicative complex formation[5]. Recently, there are some novel mechanisms were found to be involved in geminin stability regulation. It was reported that in late S-phase, miR-571 could reduce the protein level of geminin independent of the APC/C but through Cdk2-c-Myc-miR-571 axis, inducing efficient DNA replication and S-phase cell-cycle progression[6]. In early M-phase, Aurora-A phosphorylates geminin on Thr25 to prevent APC/C ubiquitin ligase complex-mediated degradation during mitosis[7]. Furthermore, de-ubiquitinating enzymes DUB3 and USP7 could increase replication inhibitor geminin levels through reduced ubiquitination[8].

Geminin is a multi-functional protein, which plays pivotal roles in both DNA replication and transcriptional/epigenetic regulation[9]. Moreover, dysregulation of geminin is often found in various types of human cancers. geminin appeared to be expressed at high level in several malignancies and its upregulation correlated with high proliferative activity, invasion or metastasis of tumor cells[10-18]. Many cancer cells depend on the geminin protein to prevent excess DNA replication from triggering DNA damage-dependent apoptosis. Knockdown of geminin was found to specifically induce DNA re-replication and apoptosis in malignant cancer cells, whereas normal and immortalized cells remain insensitive to geminin depletion[19]. Aberrant expression of geminin was linked to DNA replication damage, abnormal chromosome numbers (aneuploidy) and genomic instability. All of these changes were considered as the basic elements of precancerous condition and malignantly transformed[12]. Therefore, geminin is now regarded as an oncogene but the mechanism of its role in tumorigenesis remains unclear.

The tumor microenvironment (TME) is composed with various cell types and extracellular components, and the communication between tumor cells and the TME mainly include cell–cell and cell–ECM adhesion, as well as soluble molecules mediated reactions[20, 21]. TME plays a pivotal role during tumor initiation, progression, and metastasis. Soluble extracellular components in TME, such as lysophospholipase D autotaxin (ATX) and its product lysophosphatidic acid (LPA), represents two key players in regulating cancer progression. *In vitro* and *in vivo* studies have demonstrated that increased ATX/LPA signaling axis contributes to cancer initiation and progression[22-24]. LPA stimulates cell proliferation, migration and survival by acting through activation of a series of G protein-coupled receptors (GPCR). Both LPA receptor family members and ATX are aberrantly expressed in many human cancers, such as breast, ovarian, prostate, hepatocellular carcinoma and melanoma[22]. The context of LPA biology is complex, as it involves not only several distinct G protein-coupled receptors, but also cross-talk with receptor tyrosine kinase signaling through metalloproteinase activation[25-27]. The EGFR has been shown to activate MAPK pathways, induce gene expression, stimulate DNA synthesis, and regulate cell cycle progression by agonist-mediated EGFR transactivation[28-31]. Importantly, transactivation of EGFR via both LPA and SIP has been identified as a key link to pathophysiologic processes in human cancer cells[25, 32]. However, there is still limited understanding about the pathophysiological role and signaling pathway of LPA in TEM and EGFR transactivation in the development of human cancer.

It is revealed that LPA levels in the the plasma and ascites of gastric cancer patients with peritoneal carcinomatosis were significantly higher than those in healthy people[33]. The submucosal connective tissue-type mast cells is one source of the production of lysophosphatidic acid in gastrointestinal tract[34]. The most notable role of LPA in gastric cancer development is to mediate cell migration and invasion, which acting through diverse downstream effectors[35-39]. Meanwhile, LPA also stimulates gastric cancer cell proliferation through upregulating sphingosine kinase 1 transcription[40]. However, LPA induced DNA replication regulation in gastric cancer has not been defined yet.

In this study, we showed that mutation of geminin is non-predominant in human cancers, which is consistent with the properties of geminin as a regulator protein. Intriguingly, depletion of geminin selectively induces DNA re-replication in gastric cancer cells but not in normal gastric epithelial cells, whereas up-regulation of geminin protein level could be triggered by LPA in gastric cancer cells. Notably, we found that LPA stimulates EGFR transactivation via a metalloprotease-dependent pathway in gastric cancer, which is partly responsible for potentiating geminin stability in early S phase, and in turn promotes DNA replication. Altogether, these data indicate that the cross-talk between LPA and EGFR is a signaling axis to regulate DNA replication by controlling geminin levels, and uncover a novel mechanism in understanding gastric cancer progression.

Materials And Methods

Cell culture

MKN45 and BGC-803 cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (GEMINI) and 1% (vol/vol) penicillin/streptomycin/L-Glutamin (Gibco), GES-1 cells were maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% (vol/vol) fetal bovine serum (GEMINI) and 1% (vol/vol) penicillin/streptomycin/L-Glutamin (Gibco). All cells were cultured at 37°C, 5% CO₂ in a humidified atmosphere. To synchronize cells in G1/S-phase, cells were cultured in serum-free medium for 24 hours.

Reagents, antibodies, and inhibitors

Reagents were from the following suppliers: LPA (SIGMA), DMSO (SIGMA), BB94 (SIGMA), DAPI (invitrogen), Ki16425 (SELLECK), AG1478 (SELLECK), LY294002 (SELLECK), Protein G beads (SIGMA), Endo-free Plasmid Mini Kit(OMEGA), Lipofectamine 2000 Reagent (invitrogen), Rapamycin (SELLECK), BSA (SIGMA), OPTI-MEM (Gibco); geminin Rabbit Polyclonal antibody (Proteintech), EGFR-specific Rabbit Polyclonal antibody (Proteintech), Rabbit anti-DUC3 Polyclonal Antibody (Absin), Mouse Anti- β -Tubulin Monoclonal Antibody (Transgen), Anti-phosphotyrosine antibody (abcam), Alexa Fluor 488 Goat anti-Rabbit IgG (H+L) (invitrogen), Goat anti-Rabbit IgG (Transgen), Goat anti-Mouse IgG (Transgen).

Plasmids and siRNA transfections

Plasmids and siRNA transfections were performed using Lipofectamine 2000 (Thermo Fisher Scientific, MA, USA), respectively. Short interfering oligoribonucleotide for luciferase (siGL) and geminin were as previously described[19, 41] and obtained from sangon (Table S1). The transfections were performed according to the manufacturer's protocol.

Flow cytometry analysis of cell cycle

Flow cytometric analysis was performed to monitor the cell cycle progression under different treatment. After treatments, cells were harvested by trypsinization, washed twice with ice-cold PBS, and then fixed overnight at -20°C in 70% ethanol. In the next day, cells were washed with PBS and incubated with 50 μ g/mL propidium iodide and 50 μ g/mL RNase A in PBS on ice for 30 min in the dark. Flow cytometry was performed using a FACSCalibur system (Becton Dickinson, San Jose, CA, USA) with CELLQuest software (Version 3.3, Becton Dickinson) and the cell cycle distributions were calculated by ModFit LT software (Version 3.0, Verity Software House, Topsham, ME, USA).

Quantitative real-time RT-PCR assay

Quantitative real time RT-PCR was performed by TransStart Tip Green qPCR SuperMix(Transgen) using LightCycler[®] 480 II (Roche) according to the manufacturer's instructions. The relative mRNA expression level of target genes was calculated using β -actin as a loading control. The primers were obtained from sangon and listed in Table S2.

Western blotting and immunoprecipitation analysis

Cells were lysed with lysis buffer (250 mM Tris (pH 6.8), 20 mM DL-Dithiothreitol, 150 mM Bromophenol blue, 10% (v/v) glycerol, 1% SDS). Immunoprecipitation was performed as described in article. Cell lysates were subjected to SDS-PAGE and semi-dry film transfer instrument. Membranes were then incubated with antibodies and exposed to Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, MA, USA). The relative amount of geminin and other proteins were quantified by densitometry using Biorad Chemi Doc™ XRS system (Biorad, Hercules, CA, USA) and normalized by β -Tubulin.

Immunofluorescence

Cells were grown on glass coverslips in 12 wells plate, fixed in 4% of paraformaldehyde (PFA) for 15 min, permeabilized with Triton X-100 for 5 min and sodiumborohydride for 10 min. After washed twice in PBS, the glass coverslips were incubated with the primary antibody (Rabbit anti-geminin antibody). Alexa Fluor 488 Goat anti-Rabbit IgG (H+L) (Invitrogen) was used as a secondary antibody. DNA was visualized by DAPI staining. The glass coverslips were visualized by using Laser scanning confocal microscope (CarlZeiss LSM 710, Germany).

Cell proliferation assay

Cells were seeded on 96 wells plate with 2000 cells for each well and grown in 200 μ L serum free medium with 0.1% DMSO, 10 μ M LPA or 10 ng/mL EGF, respectively. Medium was changed every 2 days. 20 μ L of CCK-8 was provided to every well for 1.5 h. The plate was then read using a spectrophotometric microtiter plate reader (EPOCH) set at a dual wavelength of 450 nm. Bromodeoxyuridine (BrdU) is incorporated into newly synthesized DNA strands of actively proliferating cells (abcam, ab126556, BrdU Cell Proliferation ELISA Kit (colorimetric)).

Statistical analyses

All statistical analyses were processed using GraphPad Prism 8.0. Quantitative analysis from three independent experiments (mean \pm SD) is shown, VNOVA Test and Dunnett's Multiple Comparison Test or T test were used for the analysis of data. * P <0.05, ** P <0.01, *** P <0.001 was considered statistical significance.

URLs

The Cancer Genome Atlas (TCGA), <http://cancergenome.nih.gov/>. Human Protein Atlas (HPA), <http://www.proteinatlas.org/>.

Results

Mutation of geminin is non-predominant in human cancers

To explore comprehensively the genetic abnormalities affecting geminin, diverse public databases were analyzed, including The Cancer Genome Atlas (TCGA) and Human Protein Atlas (see Uniform Resource

Locators, URLs). We found that the RNA expression level of geminin is approximately even in all 17 cancer types (Fig. 1a), indicating that geminin expression has no tissue or cell specificity. This is consistent with the properties of geminin as a regulator protein, which is expressed selectively during the proliferative phase of the cell cycle.

In TCGA whole-genome sequencing results, only 57 mutations throughout *GMNN* gene were identified in 13 cancer types. These mutations belonged to 51 variants, and many of them distributed discretely, indicating that mutation of *GMNN* gene is an occasional event in human cancers. Of note, the vast majority of 57 mutations was located at the exon region, while only one mutation was located at 5' UTR region (T>G at the position of chromosome 24777229), and 8 mutations were located at 3'UTR region (Fig. 1b). Moreover, there were only missense or synonymous mutation at N terminus of the gene, while deletion mutation, nonsense mutation and splicing mutation were also found at C terminus (Fig. 1c-d). Significantly, the 54th amino acid Arginine was more susceptible to mutate to Glutamine, and the mutation frequency was higher than other amino acids (Fig. 1c). These data reveal that mutations or deletions of the geminin gene is not predominant in human cancers. In the early tumour progression, cells normally adopt a strategy to manipulate levels of replication factors to conquer replicative stress, and mutations in replication factors are rare in this progress.

Depletion of geminin induces DNA re-replication in gastric cancer cells but not in normal gastric epithelial cells

SiRNA targeted against *GMNN* (siGEM)-induced DNA re-replication was previously shown in colorectal carcinoma, head and neck squamous cell carcinomas (HNSCCs) and breast cancer [19, 41]. However, the sensitivity of cancer cells to geminin depletion was highly cell type dependent. Immortalized cells derived from normal tissues and some cell lines derived from cancers (such as cervix adenocarcinoma cells HeLa, skin melanoma calls A375 and WM-266-4) are resistant to DNA re-replication induced by geminin depletion [19]. To determine the effect of geminin depletion in gastric cancer, the responses of siGEM mediated geminin depletion of three gastric cell lines (MKN45, BGC-803 and GES-1) were observed. In gastric cancer cell lines, geminin expression level was reduced obviously by siGEM transfection (Fig. 2c and f). We found the proportion of cells in S+G2/M-phase increased after 2 days of siGEM transfection (Fig. 2a and d) compared with groups treated with siGL control, and the number of giant nuclei visualized by Laser scanning confocal microscope increased significantly (Fig. 2b and e).

To identify whether siGEM had the same effect on normal gastric epithelial cells, we performed the same assay using GES-1 cells. As expected, siGEM reduced geminin expression level in GES-1 cells obviously (Fig. 2i). However, no changes were detected either in the proportion of cells in S+G2/M-phase, or in the fraction of cells with giant nuclei (Fig. 2g-h).

Taken together, our results indicate that depletion of geminin induces DNA re-replication in gastric cancer cells but not in normal gastric epithelial cells.

LPA selectively triggers the up-regulation of geminin in gastric cancer cells

GPCR agonists LPA mediates multiple downstream signal pathways to activate biological behaviors, such as cell proliferation[25], migration[42] and invasion[43], etc. If abnormalities occur in the processes of LPA signal transduction, it may induce certain diseases and even contribute to the occurrence, development and metastasis of cancer[23]. In contrast, little is known about the influence or molecular mechanism of LPA in DNA replication. To investigate the role of LPA elevation in the properties of gastric cancer cells, we performed an LPA gradient treatment, followed by detecting geminin protein level using western blot analysis. In response to LPA stimulation, the protein level of geminin was increased transiently in early S phase in MKN45 (Fig. 3a) and BGC-803 (Fig. 3d) cells, with a peak of expression at approximate 0.5-1 hour. Whereas, this phenomenon was not detected in GES-1 cells, a normal gastric epithelial cell line (Fig. 3g). These data indicate that LPA selectively promotes up-regulation of geminin protein level in gastric cancer cells.

Before the gradient treatment of gastric cells with LPA, cells were serum starved and kept in a quiescent state, and 1% bovine serum albumin (BSA) was added to the medium to maintain the biological activity of LPA. To exclude the effect of BSA on geminin expression, the changes of protein level of geminin stimulated for 0.5 h in the presence or absence of 1% BSA was observed. As expected, BSA did not affect the protein level of geminin in gastric cancer cells (Fig. 3c and f).

Geminin is a DNA replication factor, which shuttle among the nucleus, nucleoplasm and cytoplasm[44]. In normal human gastric tissue, geminin was found to locate in cytoplasm and cytoplasmic membrane of all tissue, and it was distributed in nucleus only in 67% tissue via IHC staining. Whereas in tumor patient tissues, geminin protein can be detected in the nucleus of 81.8% of the samples, and 72.73% of them were only present in the nucleus via IHC staining (data were obtained from HPA, Fig. S1a). These results indicate that compared with normal cells, geminin has a translocation tendency from cytoplasm to nucleus in cancer cells. This is indicating that selective expression of geminin during the DNA replication phase and its nuclear specificity increase its potential to be used as a diagnostic marker of proliferation in cancer patients.

To investigate whether LPA can affect the cytoplasmic-nuclear trafficking of geminin in gastric cancer cells, the localization of geminin after LPA time gradient treatment was observed. geminin was mainly detected in cytoplasm without LPA treatment in quiescent cells, while the proportion of geminin localized in nucleus increased with prolonged LPA treatment. It reveals that LPA stimulation promotes nucleus transfer of geminin (Fig. S1b).

LPA stimulates EGFR transactivation via a metalloprotease-dependent pathway in gastric cancer

According to the previous reports[30, 45], LPA induce an intracellular transactivated mechanism by which it could indirectly play its cellular function through a GPCR-regulated transmembrane MMPs at the cell surface, allowing EGFR transactivation in a classic autocrine manner. To address whether LPA was involved in EGFR transactivation in gastric cancer, we first examined the changes of EGFR phosphotyrosine content to LPA (10 μ M) stimulation. In two gastric cancer cell lines we tested, LPA stimulation resulted in tyrosine phosphorylation of EGFR obviously. In comparison with LPA stimulation,

the vehicle DMSO showed little effect, whereas EGF-induced EGFR autophosphorylation was more pronounced (Fig. 4a). The above results implicate that the cross-talk linking GPCR with EGFR signal pathway are installed in gastric cancer.

Because metalloproteases (MMPs) have been implicated in the cross-talk linking GPCR and EGFR in HEK-293[45] and HNSCC[25] cells, we analyzed whether a MMPs-dependent mechanism is also involved in LPA-induced EGFR transactivation in gastric cancer. In MKN45 and BGC-803 cells, we analyzed the LPA-induced EGFR transactivation in the absence or presence of batimastat (BB94), a potent inhibitor of MMPs. In gastric cancer, BB94 (10 μ M) completely blocked the EGFR transactivation signal by LPA stimulation, whereas EGF-induced EGFR autophosphorylation was not interfered (Fig. 4b). In addition, BB94 also affected the basic EGFR autophosphorylation in gastric cancer, perhaps by influencing the activity of basic EGFR ligand (Fig.. 4b).

Taken together, these results support our hypothesis that a MMPs-dependent mechanism is involved in LPA-induced EGFR transactivation in gastric cancer.

LPA potentiates geminin stability through LPAR3/MMPs/EGFR/PI3K/mTOR signaling axis and de-ubiquitinating enzyme DUB3

As described above, we demonstrated that LPA could upregulate geminin protein level in early S phase of gastric cancer cells. However, the signaling pathway of how LPA regulates geminin protein level in gastric cancer is uncovered. It is possible that LPA up-regulates the gene transcriptional level of *GMNN*, or LPA regulates the protein translation or the post-translational modification to potentiate the stability of geminin protein. To further investigate the underlying mechanism, the mRNA level of geminin under the gradient treatment of LPA was evaluated by RT-qPCR analyses. Unlike the effect of LPA gradient treatment on geminin protein level in gastric cancer cells, the mRNA level of geminin did not respond obviously to LPA treatment (Fig.. 5a and Fig. S2a). These RT-qPCR analyses suggest that LPA stimulation could not affect *GMNN* gene expression.

APC/C is known to control geminin degradation, recent study showed that de-ubiquitinating enzymes DUB3 is also identified as a factor to regulate geminin protein stability[8]. To investigate the relationship between LPA-mediated geminin upregulation pathway and the de-ubiquitinating degradation ability of DUB3, the change of DUB3 protein content after LPA stimulation was observed. In MKN45 and BGC-803 cells, the time course western blot analyses revealed an up-regulation of DUB3 protein, with a peak of expression at approximate 10 min after LPA stimulation (Fig. 5b and Fig. S2b). Owing to the rapid kinetic of DUB3, geminin protein level reached the maximum within 1 h after LPA stimulation (Fig. 3). These results indicate that LPA potentiates geminin stability by upregulating de-ubiquitinating enzyme DUB3 in gastric cancer.

LPA receptors (LPARs) include six different types and the best known receptors are LPAR₁, LPAR₂ and LPAR₃. To test which LPAR was involved in LPA-induced EGFR transactivation in gastric cancer, the mRNA level of diverse LPARs was detected. RT-qPCR analyses indicated that LPAR₃ expression was

significantly higher than other LPARs (Fig. 5c and Fig. S2c). To explore the role of LPAR₃ in LPA-mediated geminin upregulation, we assessed the response of geminin protein level to Ki16425, a specific LPAR_{1/3} inhibitor. The time course western blot analyses indicated that Ki16425 (10 μM) completely abrogated the up-regulation of geminin protein upon LPA stimulation whereas the vehicle DMSO had no effect in gastric cancer cells MKN45 (Fig. 5e-g) and BGC-803 (Fig. S2d and i). To further test the precise effect of LPAR₃, short interfering RNA targeted against the human LPAR₃ gene was transfected into MKN45 cells. When interfering the expression of LPAR₃, a significant decrease in geminin protein content was observed after LPA treatment (Fig. 5d and f). These findings suggest that LPA regulates geminin stability through LPAR₃.

To explore the relationship between LPA-mediated geminin stability enhancement and LPA-induced EGFR transactivation, we screened the changes of geminin protein content with DMSO (vehicle, 0.1%), BB94 (10 μM), EGFR inhibitor AG1478 (250 nM). Gastric cancer cells were synchronized with serum-free medium for 24 h, pretreated with vehicle or inhibitors as described above for 30 minutes, and treated with LPA (10 μM) in a time gradient. The time course western blot analyses indicated that all inhibitors completely abrogated the up-regulation of geminin protein upon LPA stimulation whereas the vehicle DMSO had no effect in gastric cancer cells MKN45 (Fig. 5d, h and i) and BGC-803 (Fig. S2e, f and i). These results suggest that LPA regulates geminin stability through EGFR transactivation signal pathway.

GPCR-induced EGFR transactivation was shown previously to relay signals to the Ras-MAPK pathway in some cell lines such as GT1-7, COS-7, and HEK-293 cells[46]. However, inhibition of MAPK pathway using MEK or ERK inhibitors could not attenuate LPA induced geminin upregulation (data not shown). Next, we investigated which downstream factor of EGFR was involved in LPA-mediated geminin stability. In time course experiments, LY294002 (10 μM, the specific inhibitor of PI3K) and Rapamycin (100 nM, the specific inhibitor of mTOR) notably eliminated the up-regulation of geminin protein upon LPA stimulation whereas the vehicle DMSO had no effect in gastric cancer cells MKN45 (Fig. 5e, j and k) and BGC-803 (Fig. S2g-i).

Taken together, these results suggest that LPA potentiates geminin stability through LPAR₃/MMPs/EGFR/PI3K/mTOR signaling axis and de-ubiquitinating enzyme DUB3 activity in gastric cancer.

LPA mediates S-phase cell-cycle progression through the LPAR₃/MMPs/EGFR/PI3K/mTOR signaling axis

Because we have observed that LPA potentiated geminin stability through LPAR₃/MMPs/EGFR/PI3K/mTOR signaling axis and DUB3 activity in gastric cancer, we next examined whether this signaling pathway affected DNA synthesis and cell-cycle progression of gastric cancer. Gastric cancer cells were synchronized with serum-free medium for 24 h. G1/S-arrested gastric cancer cells were pretreated with vehicle or inhibitors as described above for 30 min and stimulated with LPA (10 μM) in a time gradient. After the time gradient treatment, cells were harvested and stained with propidium iodide (PI) to quantify DNA content by FACS analysis. In both MKN45 and BGC-803 cells, time course

experiment analyses indicated that all groups with inhibitor treatment did not show significantly increase in the percentage of cells in S and G2/M phases, compared with the vehicle DMSO group (Fig. 6 and Fig. S3). These results indicate that LPA could promote S-phase cell-cycle progression in gastric cancer cells through LPAR₃/MMPs/EGFR/PI3K/mTOR signaling axis.

Metalloprotease-dependent transactivation of the EGFR is critical for LPA-induced efficient DNA synthesis and cell proliferation in gastric cancer

In order to quantification the efficiency of DNA synthesis in response to LPA stimulation, we measured the rate of DNA synthesis by measuring BrdU incorporation using ELISA assay. In both MKN45 and BGC-803 cells, BB94 and AG1478 notably abrogated DNA synthesis upon LPA stimulation (Fig. 7a-b). Furthermore, AG1478 notably eliminated DNA synthesis upon exogenous EGF stimulation (Fig. 7a-b). It is surprised to observe that BB94 also reduced DNA synthesis induced by exogenous EGF in gastric cancer cells, this suggested that exogenous EGF stimulation may result in enhanced shedding of endogenous EGFR ligands in gastric cancer as observed in HNSCCs[25, 47]. This result indicated that cross-talk between GPCRs signal pathways and the EGFR signal pathways were relevant for DNA synthesis in gastric cancer. Taken together, these data strongly indicate that LPA-induced efficient DNA synthesis is dependent on metalloprotease function in gastric cancer.

Besides DNA synthesis and S-phase cell-cycle progression, cell proliferation is another critical parameter in cancer pathobiology. To investigate whether metalloprotease or EGFR inhibition influences LPA-induced efficient cell proliferation in gastric cancer cells cultured in serum-free medium, we assessed cell proliferation using CCK-8 assay. As shown in Fig. 7c-d, we firstly confirmed the influence of LPA and EGF on cell proliferation. LPA notably induced efficient cell proliferation in both MKN45 and BGC-803 cells, so did EGF. As shown in Fig. 7e-f, Ki16425 notably blocked LPA-induced cell proliferation. Similarly, both BB94 and AG1478 notably eliminated LPA-induced cell proliferation in MKN45 and BGC-803 cells (Fig. 7g-j). Moreover, BB94 notably abrogated LPA-induced cell proliferation whereas it had no effect in EGF-induced cell proliferation (Fig. 7g-h). Based on the above results, we demonstrate that LPAR₃, metalloprotease and EGFR activities are required for LPA-induced efficient cell proliferation in gastric cancer.

Discussion

In this study, we demonstrated that LPA specifically up-regulated of geminin expression in early S phase in gastric cancer cell lines, and that the deletion of geminin selectively induced DNA re-replication. Neither of these phenomena has been observed in normal gastric epithelial cells, indicating LPA-induced geminin up-regulation is restricted to tumor cells. Using RNAi or specific inhibitors to block the activity of related factors in the signaling pathway, we found that LPA acts through LPAR₃ and downstream coupled MMPs signaling to transactivate EGFR, increasing the expression level of geminin in S phase. On the other hand, LPA stimulation induced the up-regulation of de-ubiquitinating enzyme 3 (DUB3) in a short time and

inhibited the ubiquitination degradation of geminin to enhance geminin stability and positively regulate the DNA replication initiation in gastric cancer cells (Fig. 8).

The survival of tumor cells depends on the interaction with surroundings. Apart from individual cancers, the components of tumor microenvironment (TME) include extracellular matrix (ECM), surrounding vasculature, other non-malignant cells and signaling molecules[48]. TME components are highly plastic. Any perturbation of TME not only promotes cancer cell transformation and tissue invasion, but also helps to alter TME that further facilitates cancer progression[49]. Recently, the increasing evidences indicate that many cancers have an up-regulation of LPA, which is also located in ECM[50, 51]. LPA is now emerging as an important factor in TME, which could promote tumor growth, migration, invasion, metastasis and angiogenesis. LPA markedly increased cell migration and invasion mainly via acting through LPAR₁₋₃[35, 37], however, the role of LPA/LPAR in DNA replication has not been discussed before. Here, we for the first time reveal that LPA promotes DNA replication through upregulating geminin protein expression in gastric cancer cells. As an oncogene, geminin levels are upregulated in human tumors and its expression is prognostic in several cancers, including colon, rectal, and breast cancers[8, 10, 52]. It is indicated that expression of geminin is cell-cycle dependent, and the newly synthesized geminin is turned over during S phase with a half-life of 3-4 h[53]. Our results demonstrate that LPA selectively upregulates geminin expression in early S phase in gastric cancer cells, with a peak at approximate 0.5-1 hour. The LPA-induced upregulation of geminin is cell context dependent. LPA treatment could trigger geminin upregulation in gastric and ovarian cancer cells, but not in breast cancer cell line MCF-7 which frequently has geminin overexpression (data not shown), the underlying mechanism worth further investigation. Deregulation of geminin is often found in different human cancers and is associated with metastasis. It is revealed in other research that the dual roles of geminin in DNA replication and in metastasis are separate[8, 10]. Our data establish a novel link between geminin-regulated DNA replication and tumor progression, that is the elevated level of LPA in TME enhances the stability of geminin in early S phase, and in turn keeps the high speed of replication of gastric tumor cells and promotes tumor cell progression. We speculate that the raising LPA and altered TME might contribute to diverse cancer phenomena. Targeting the TME alternation may provide a unique perspective on tumorigenesis or cancer therapy for cancer prevention and treatment.

In recent years, medical oncology has focused on personalizing therapeutic approaches with the aim of identifying patient subpopulations, which would benefit from the specific and personalizing targeted therapeutics. Genomic mutations present in human cancers has great influence on oncogenic signaling and proliferative potential. Our cohort data analyzed genomic abnormalities of geminin, demonstrating that mutations or deletions of the geminin gene is not predominant in human cancers. R54Q mutation was occurred more frequently than other amino acids in geminin. R54 seemed to be an important site, as well as Thr25, S32, S60[7] and S45, S49[53] which could be phosphorylated under different circumstances, although the physiological relevance of these modification remains to be clarified. Further studies are needed, especially proper *in vitro* and *in vivo* models for analyzing mutations in geminin.

It is indicated that mutations in replication factors are rare in cancer, they also adopted other strategies to manipulate levels of replication factors to induce replicative stress. Previous studies demonstrate the expression of geminin protein is strictly periodic-mediated in cell cycle. As a DNA replication regulator, geminin is down-regulated in the G0/G1 phases through APC/C E3 ubiquitin ligase[44], including Polycomb-group complex 1, which is a Ring type E3 ubiquitin ligase, and RDCOX complex (containing Scmh1Hoxb4/Hoxa9), which are associated with E3 ubiquitin ligase core complex Roc1/Ddb1/Cul4a[54]. Recent study certifies that DUB3 and USP7 can control geminin protein stability in breast cancer[8]. As the previous study, we also provided DUB3 as a factor to mediate geminin protein stability in gastric cancer. We also identified a novel signaling axis to manipulate geminin expression through LPA induced EGFR transactivation in gastric cancer cells. It is well known that EGFR initiates a signaling pathway that leads to DNA synthesis and cell proliferation, EGFR and PI3K initiate malignant transformation via activating other pathways such as Myc and Rb-E2F, in turn induces the formation of functional cyclinD/Cdk4 and cyclinE/Cdk2 complexes[55]. It is reported recently that in the early S phase, Cdk2 phosphorylates c-Myc, promotes its association with promoter of miR-571 and reduces miR-571 expression. miR-571 could specifically target geminin mRNA, thus the downregulation of miR-571 is associated with accumulation of geminin protein[6]. This may elucidate one possible downstream signaling pathway of LPA-induced geminin upregulation. It is also showed that Aurora-A phosphorylates geminin on Thr25 for it from protecting APC/C-dependent proteolysis during early M phase[7]. We also attempt to investigate whether LPA stimulation could lead to geminin phosphorylation. Unfortunately, we unable to identify the phosphorylation site using mass spectrometric analysis (data not shown), further investigations are necessary to find downstream kinase of EGFR, which could perhaps phosphorylate geminin and increase its stability. G protein-coupled receptors (GPCR) and EGFR are two pivotal family of drug targets, and the GPCR induced transactivation of EGFR is a major mechanism in cancers[27], which has been shown to be conducted in the activation of MAPK pathways, cell migration, and the regulation of cell cycle progression. In this study, we implicated efficient DNA synthesis, S-phase cell cycle progression and cell proliferation induced by LPA-induced EGFR transactivation in gastric cancer cells. Our results elucidate that interruption of the cross-talk between the two receptors might be potential a therapeutic approach and realistic addition to the treatment of gastric cancer.

Declarations

Authors' contributions

Haile Zhao, Gezi, Xiaoxia Tian and Peijun Jia were responsible for conducting experiments, acquisition of data and analysis. Haile Zhao carried out Western blot analysis and molecular biological studies *in vitro*. Xiaoxia Tian performed the statistical analysis and some functional experiments. Peijun Jia provided technical and material support for immunofluorescent staining and some functional experiments. Lifei Fan and Morigen were responsible for designing the experiments, research supervision and drafted the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The databases analyzed during the current study are available.

Ethics approval and consent to participate

No ethical approval are required for this meta-analysis.

Consent for publication

Not applicable.

Competing interests

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

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Figures

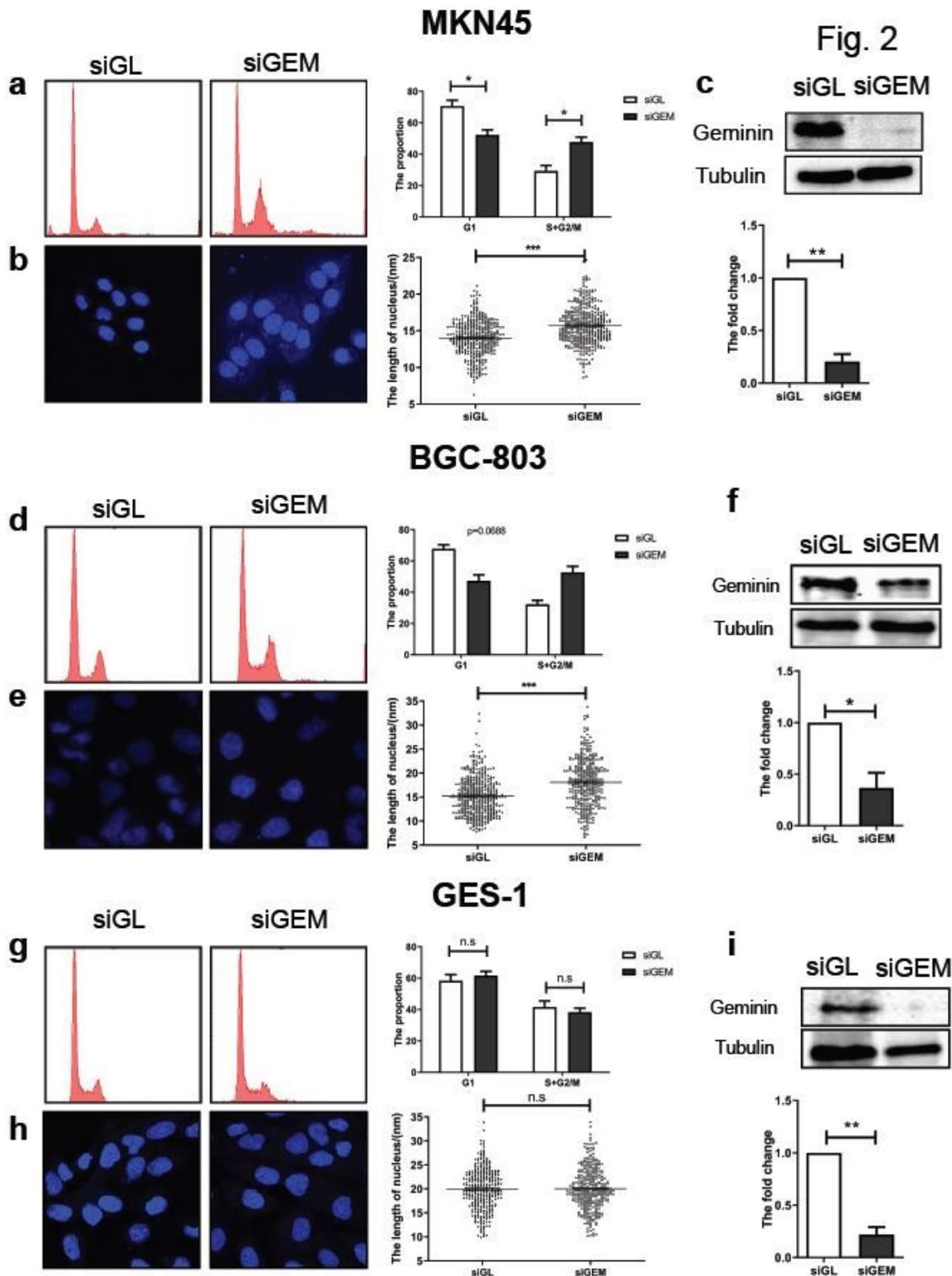


Figure 2

Depletion of geminin induces DNA re-replication in gastric cancer cells but not in normal gastric epithelial cells. The indicated cells were transfected with short interfering RNA (siGL or siGEM) oligos using Lipofectamine 2000. After 48 h post-transfection, cells were harvested and stained with DAPI to visualize nuclei by Laser scanning confocal microscope or with propidium iodide to quantify DNA content by FACS analysis. Geminin and tubulin proteins were detected by Western blot. siRNA oligos sequences were

shown in materials and methods. Quantitative analyses from three independent experiments (mean±SD) are shown, *P<0.05, **P<0.01, ***P<0.001, T test was used for data analysis.

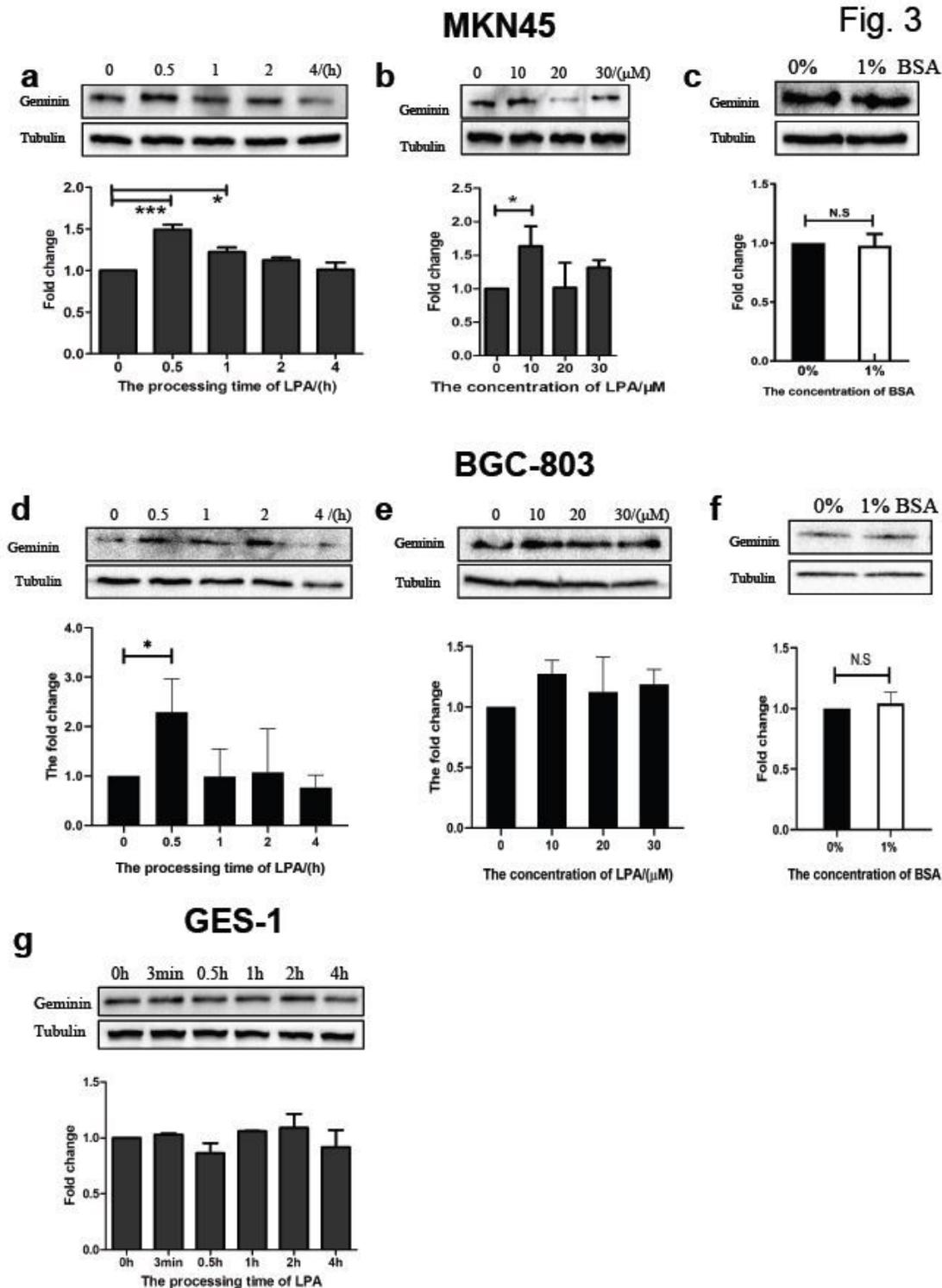


Figure 3

LPA selectively triggers the up-regulation of geminin in gastric cancer cells. The indicated cells were synchronized with serum-free medium for 24 h. G1/S-arrested cells were treated with LPA time gradient (a, d) or concentration gradient (b, e). G1/S-arrested MKN45 (c) and BGC-803 (f) cells were under the

treatment with or without 1% BSA. After the treatment, geminin and tubulin proteins were detected by Western blot. (g) Western blot analysis of GES-1 cells treated with 10 $\mu\text{mol/L}$ LPA in a time gradient experiment. After the treatment, geminin and tubulin proteins were detected by Western blot. Quantitative analysis from three independent experiments (mean \pm SD) is shown, * P <0.05, *** P <0.001, VNOVA Test and Dunnett's Multiple Comparison Test were used for data analysis.

Fig. 4

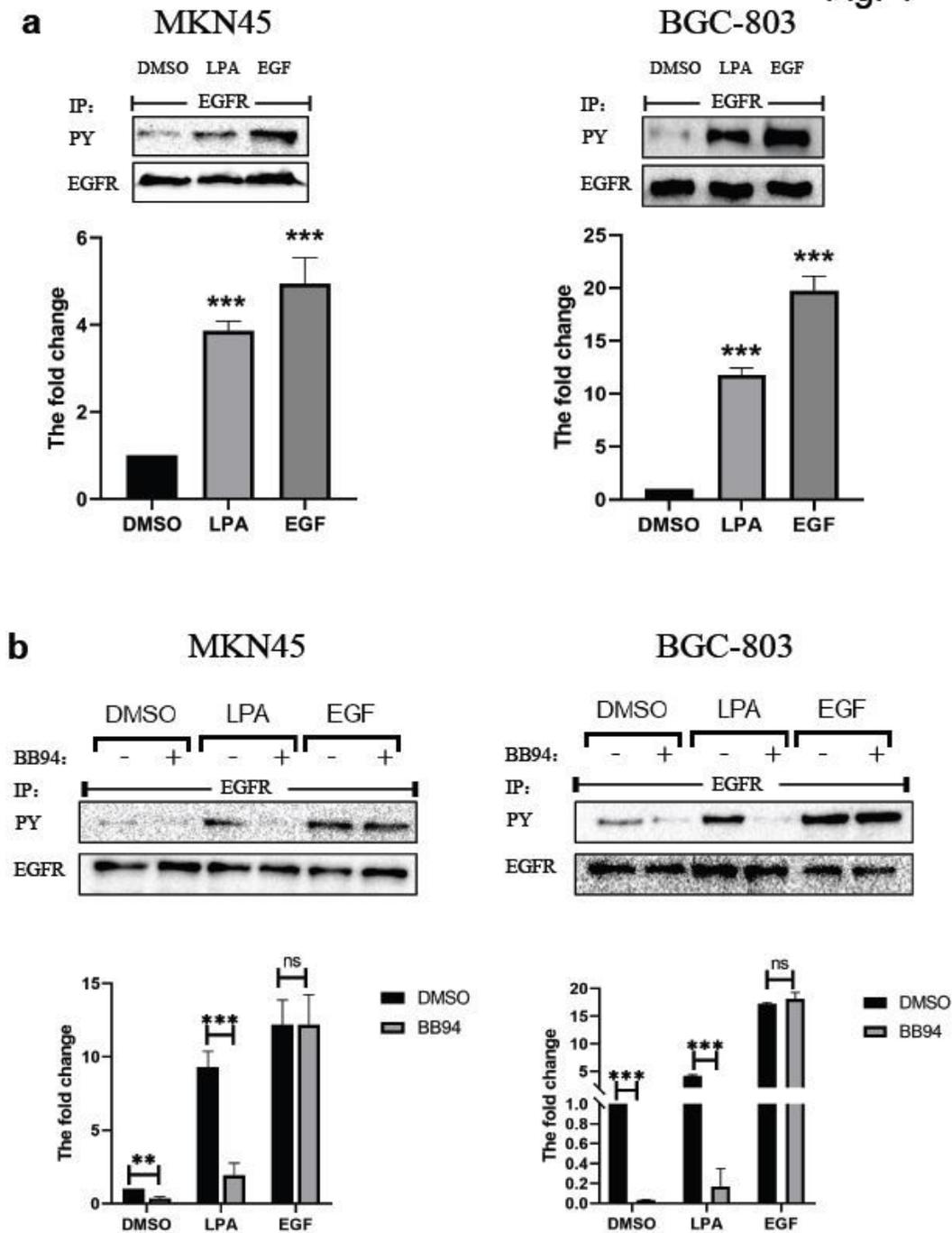


Figure 4

LPA stimulates EGFR transactivation in gastric cancer. (a) Quiescent MKN45 and BGC-803 cells were serum starved for 24 h and treated with 0.1% DMSO, 10 μ M LPA or 10 ng/mL EGF for 3 min. Cells were then lysed, and EGFR was immunoprecipitated (IP) using monoclonal anti-EGFR antibody. Tyrosine-phosphorylated EGFR was detected by immunoblotting (IB) with monoclonal anti-phosphotyrosine (PY) antibody. (b) Quiescent MKN45 and BGC-803 cells were serum starved for 24 h, pretreated with DMSO (vehicle, 0.1%) or BB94 (10 μ M) for 30 min, and stimulated with 10 μ M LPA. Cell lysate were analyzed as described in FIG.URE 4a. Quantitative analysis from three independent experiments (mean \pm SD) is shown. **P<0.01, ***P<0.001, VNOVA Test and Dunnett's Multiple Comparison Test were used for data analysis.

Fig. 5

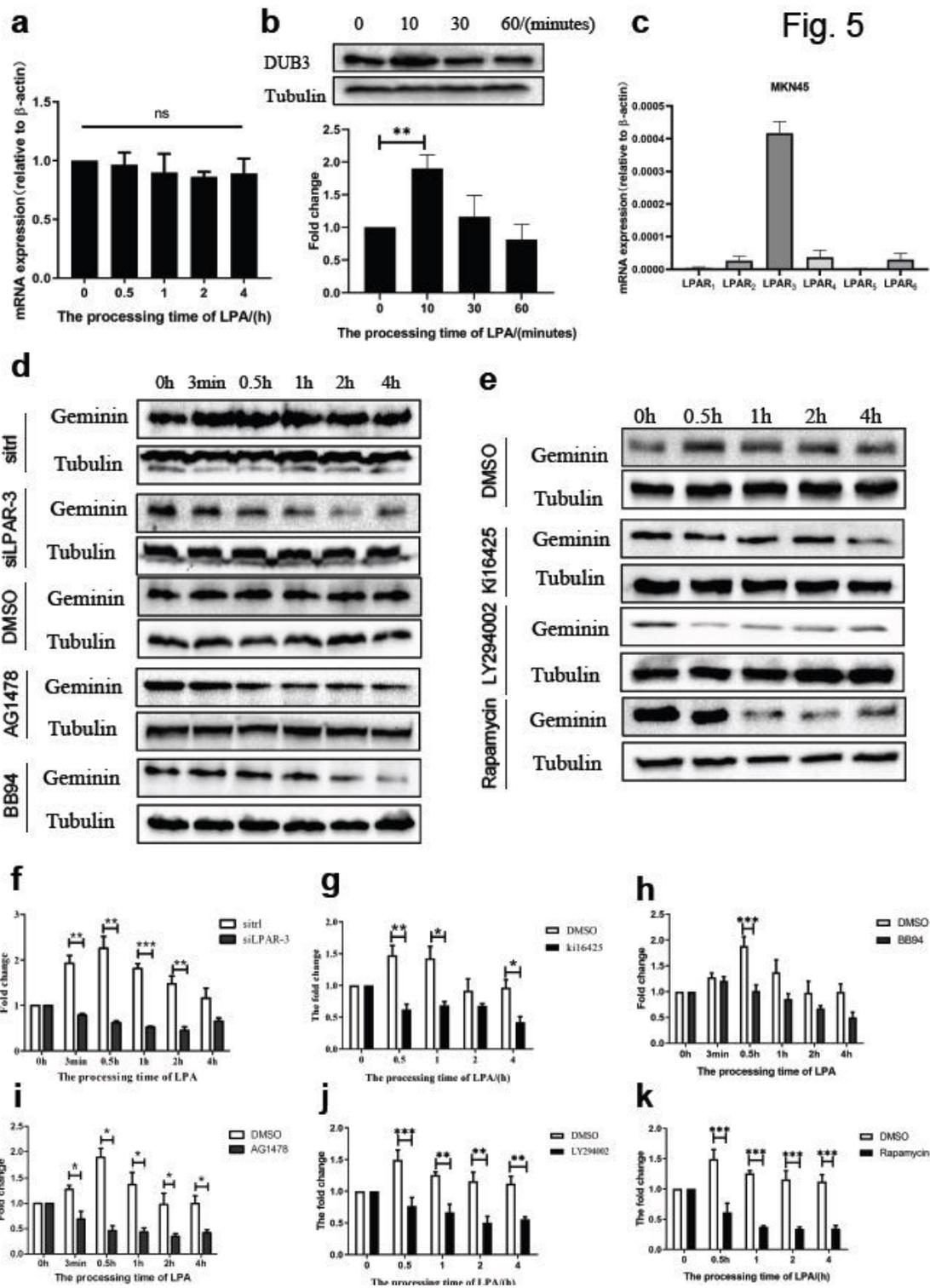


Figure 5

LPA potentiates geminin stability through LPAR-3/MMPs/EGFR/PI3K/mTOR signaling axis and deubiquitinating enzyme DUB3 in MKN45 cells. (a) MKN45 cells were synchronized with serum-free medium for 24 h and treated with LPA time gradient. Real Time PCR technology was used to detect the mRNA expression of geminin. (b) MKN45 cells were synchronized with serum-free medium for 24 h. G1/S-arrested cells were treated with 10 μ M LPA time gradient. After the treatment, DUB3 and tubulin were

detected by Western blot, respectively. (c) The mRNA expression of LPAR1-6 in MKN45 cells was detected by Real Time PCR. (d-e) G1/S-arrested MKN45 cells were serum starved for 24 h, then cells were pretreated with DMSO (vehicle, 0.1%), Ki16425 (10 μ M), BB94 (10 μ M), AG1478 (250 nM), LY294002 (10 μ M) or Rapamycin (100 nM) for 30 min, and stimulated with 10 μ M LPA. In siRNA silencing experiments, cells were transfected with either siGL or siGEM for 6 h after cultured for 2 h with OPTI-MEM medium, and then cultured for another 48 h with total medium until harvested. After the treatment, geminin and tubulin proteins were detected by Western blot, respectively. (f-k) Quantification results of geminin protein level in MKN45 cells treated in d and e. Data represent mean \pm SD from three independent experiments. *P<0.05, **P<0.01, ***P<0.001, VNOVA Test and Dunnett's Multiple Comparison Test were used for data analysis.

Fig. 6

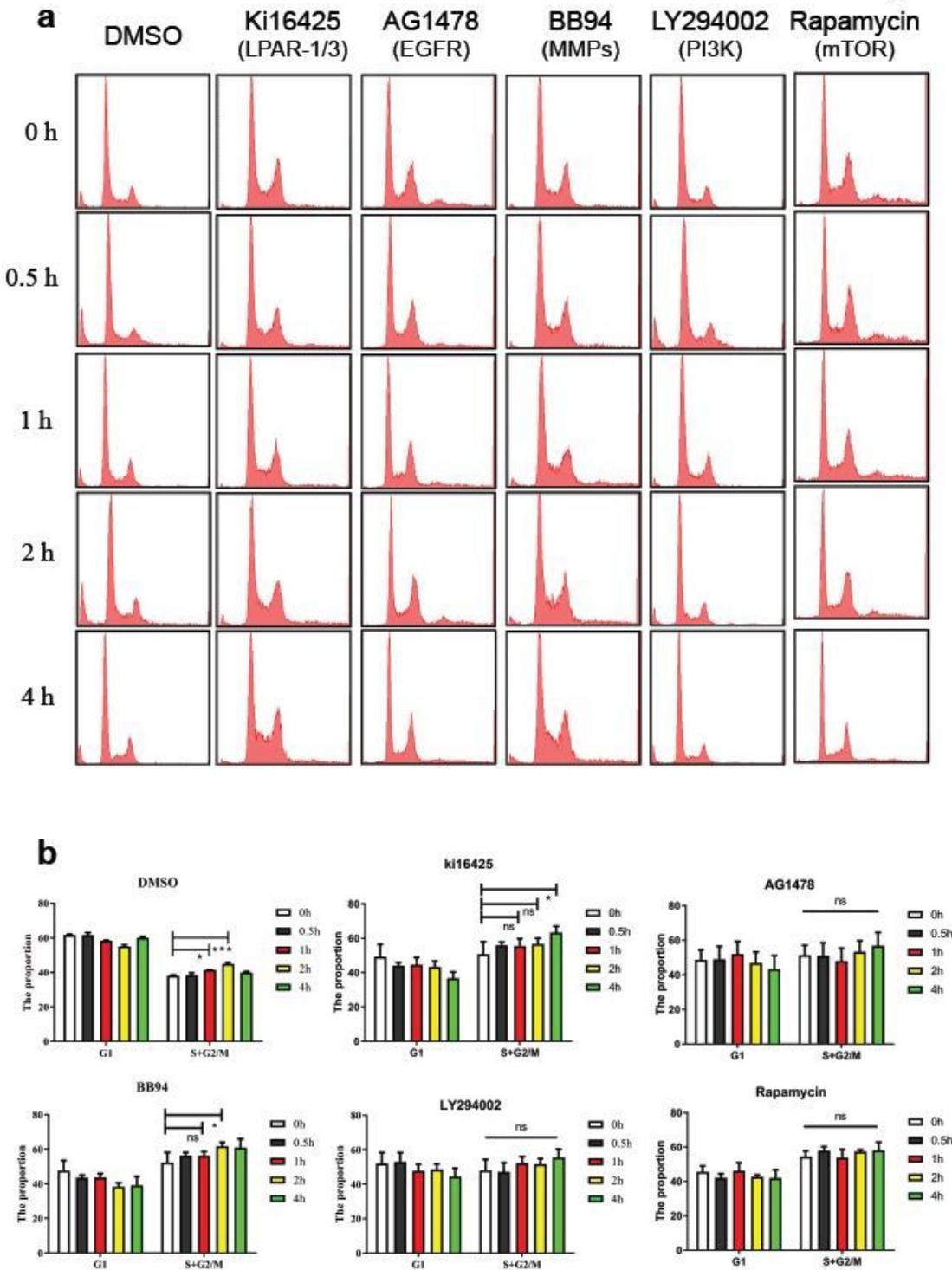


Figure 6

LPA induces S-phase cell-cycle progression through LPA3/MMPs/EGFR/PI3K/mTOR signaling axis in MKN45 cells. (a) G1/S-arrested MKN45 cells were serum starved for 24 h, and then pretreated with DMSO (vehicle, 0.1%), Ki16425 (10 μ M), BB94 (10 μ M), AG1478 (250 nM), LY294002 (10 μ M) or Rapamycin (100 nM) for 30 min, and stimulated with 10 μ M LPA up to 4h. After the treatment, cells were harvested and stained with propidium iodide (PI) to quantify DNA content by FACS analysis. (b) Quantification results of

DNA content by FACS analysis in MKN45 cells treated as described in a. Data represent mean±SD from three independent experiments. *P<0.05, ***P<0.001, VNOVA Test and Dunnett's Multiple Comparison Test were used for data analysis.

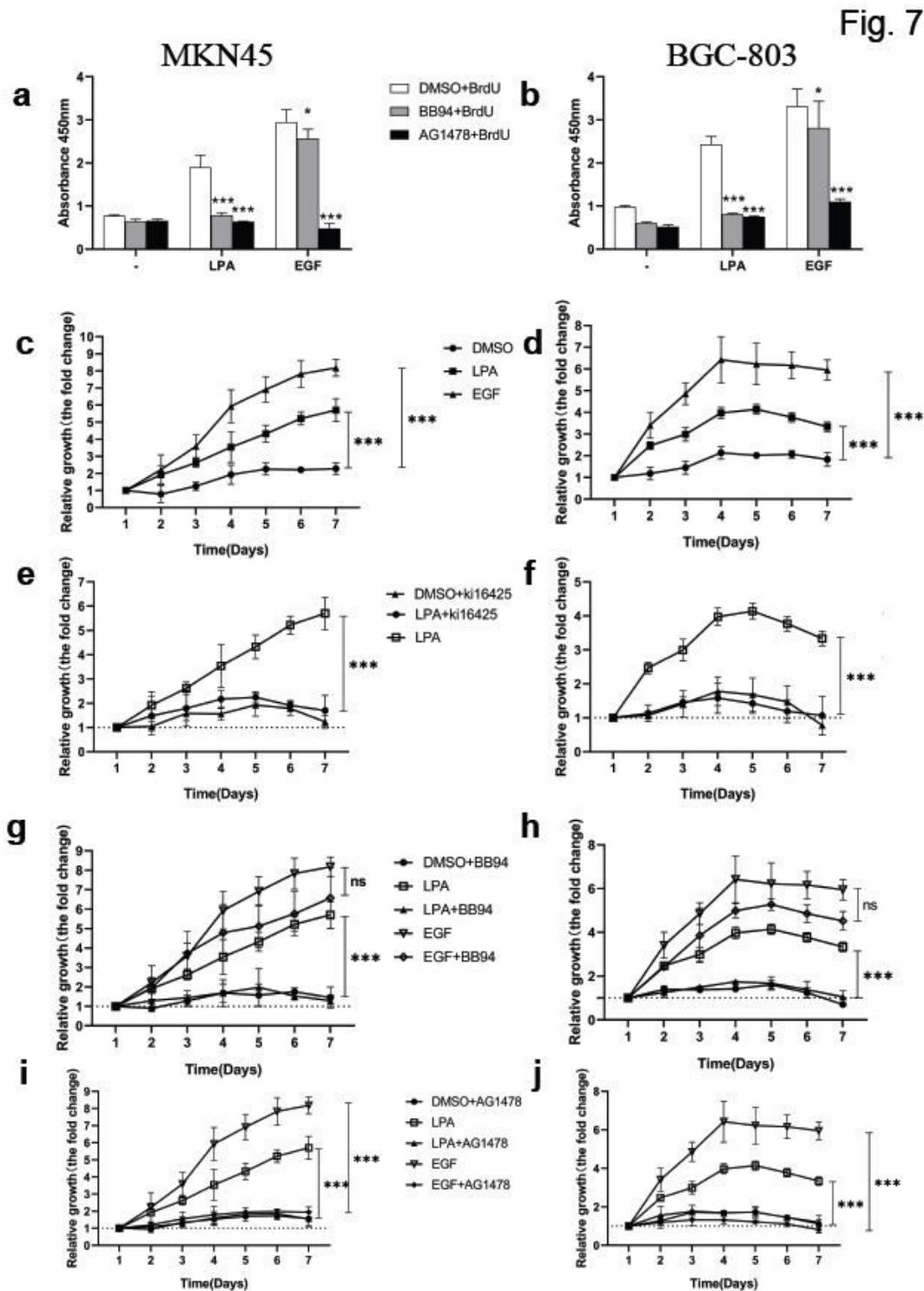


Figure 7

Ki16425, BB94 and AG1478 inhibit LPA-induced efficient DNA synthesis and cell proliferation in gastric cancer. (a-b) Bromodeoxyuridine (BrdU) is incorporated into newly synthesized DNA strands of actively

proliferating cells (abcam, ab126556 – BrdU Cell Proliferation ELISA Kit (colorimetric)). Quiescent MKN45 and BGC-803 cells were serum starved for 24 h and pretreated with DMSO (vehicle, 0.1%), Ki16425 (10 μ M), BB94 (10 μ M), or AG1478 (250 nM) for 30 min and grown in the absence or presence of ligands (10 μ M LPA or 10 ng/mL EGF) for 4 days. The plate was read using a spectrophotometric microtiter plate reader setting at a dual wavelength of 450 nm. (c-d) Quiescent MKN45 and BGC-803 cells were serum starved for 24 h and grown in 200 μ L serum free medium with 0.1% DMSO, 10 μ M LPA or 10 ng/mL EGF. Medium was changed every 2 days. 20 μ L of CCK-8 was provided to every well for 1.5 h. The color of positive wells will change to yellow. The plate was read using a spectrophotometric microtiter plate reader setting at a dual wavelength of 450 nm. (e-j) Quiescent MKN45 and BGC-803 cells were serum starved for 24 h and pretreated with DMSO (vehicle, 0.1%), Ki16425 (10 μ M), BB94 (10 μ M), or AG1478 (250 nM) for 30 min and grown in the absence or presence of ligands (10 μ M LPA or 10 ng/mL EGF). Medium was changed every 2 days. 20 μ L of CCK-8 was provided to every well for 1.5 h. Cell growth was measured as described above. Quantitative analysis from three independent experiments (mean \pm SD) is shown. *P<0.05, ***P<0.001, VNOVA Test and Dunnett's Multiple Comparison Test were used for data analysis.

Fig. 8

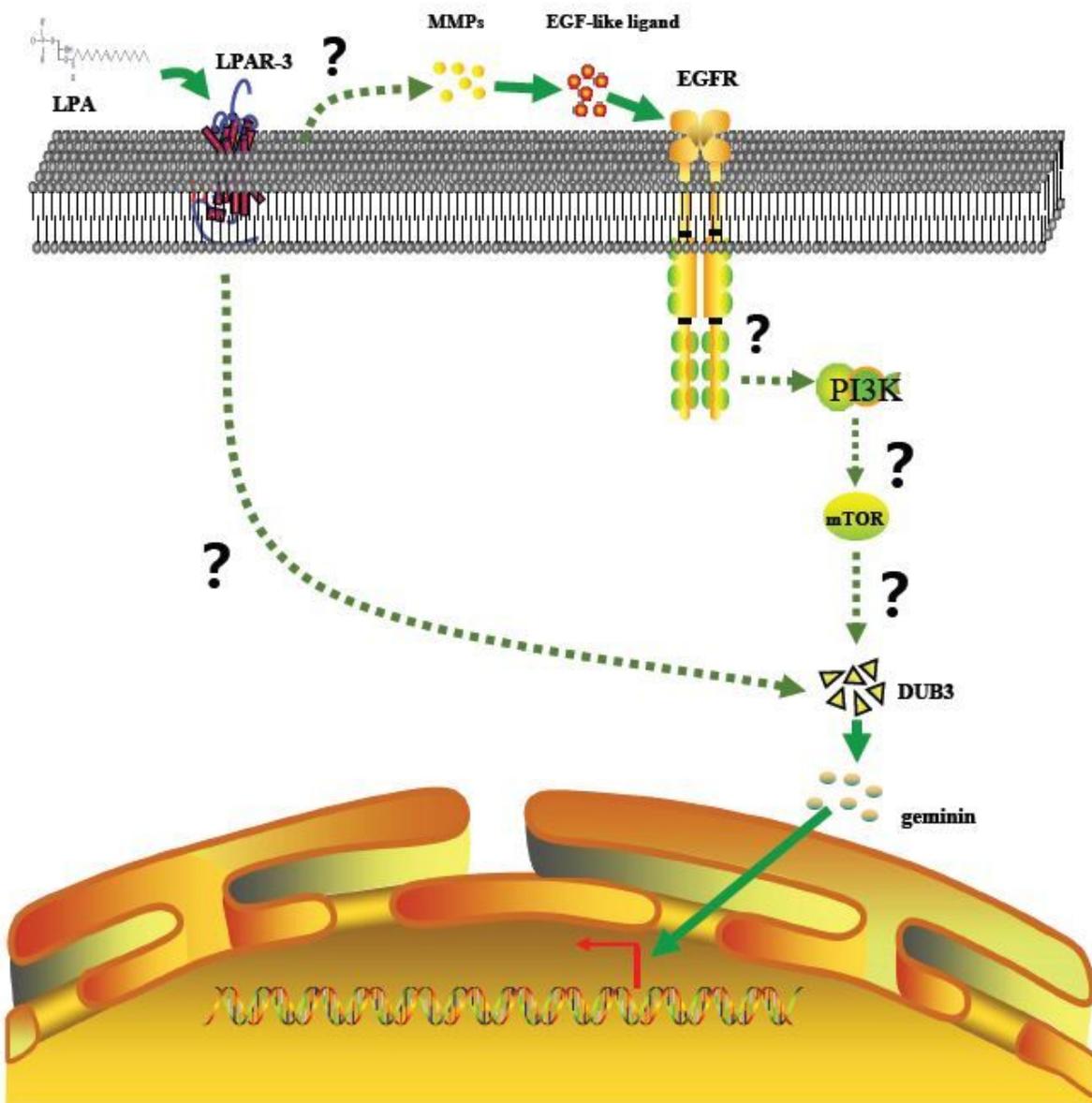


Figure 8

The signaling pathway of LPA-mediated DNA replication initiation. LPA worked through LPAR3 to transactivate EGFR by MMPs, and to increase the expression of geminin protein level in S phase through PI3K/mTOR signaling pathway. Meanwhile, LPA stimulation induced the up-regulation of deubiquitinating enzymes 3 (DUB3) in a short time, inhibiting the ubiquitination degradation of geminin

protein and enhancing its stability, and then positively regulating the DNA replication initiation of gastric cancer cells.

Supplementary Files

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