Reciprocal Negative Regulation between EGFR and DEPTOR Plays an Important Role in the Progression of Lung Adenocarcinoma

Xuefeng Zhou1, Jialong Guo2, Yanmei Ji3, Gaofeng Pan1, Tao Liu2, Hua Zhu4, and Jinping Zhao1

Abstract

The epidermal growth factor receptor (EGFR) activates downstream mTOR phosphorylation to promote the progression of many different tumor types, thus making it a prime therapeutic target. However, the role of DEP domain-containing mTOR-interacting protein (DEPTOR), a natural mTOR inhibitor, remains unclear in this process. Here, it is reported that EGFR expression is significantly increased in tumors of lung adenocarcinoma patients and is negatively correlated with the expression of DEPTOR. Activation of EGFR signaling, by EGF, in A549 lung adenocarcinoma cells (overexpressing EGFR) significantly enhanced the function of the mTOR autoamplification loop, consisting of S6K, mTOR, CK1ε, and TRCP1, which resulted in downregulation of DEPTOR expression. Gefitinib, a specific EGFR inhibitor, stimulated DEPTOR accumulation by downregulating the function of the mTOR autoamplification loop. Furthermore, a series of assays conducted in DEPTOR knockout or ectopic expression in A549 cells confirmed that DEPTOR inhibited proliferation, migration, and invasion as well as the in vivo tumor growth of lung adenocarcinoma. Importantly, tumor progression mediated by EGFR ectopic expression was diminished by transfection with DEPTOR. This study uncovers the important inhibitory role of DEPTOR in lung adenocarcinoma progression and reveals a novel mechanism that EGFR downregulates DEPTOR expression to facilitate tumor growth.


Introduction

As one of the most common cell-surface receptors that specifically bind with extracellular protein ligands of the epidermal growth factor (EGF) family, the epidermal growth factor receptor (EGFR) belongs to the ErbB family of receptors and is involved in different types of diseases, including age-related diseases, autism spectrum disorders, and cancers (1–3). In light of the confirmed evidence that EGFR plays a pivotal role in tumorigenesis, drug resistance, relapse, and the metastasis of various cancers (4–7), some of EGFR tyrosine kinase inhibitors (TKIs) or monoclonal antibodies have been developed and entered clinical trials as cancer therapeutics, such as afatinib, gefitinib, and erlotinib (www.fda.gov; refs. 8, 9).

In lung adenocarcinoma, EGFR is overexpressed in more than 39% and has emerged as a leading target for the treatment of patients with adenocarcinoma (10–12). Some TKIs, like gefitinib, significantly improved survival and prognosis of patients with lung adenocarcinoma, while some patients acquired resistance after initial response to TKIs or even had intrinsic resistance without EGFR mutations (13). To date, the possible mechanisms that underlie the primary resistance of these drugs have been supposed as follows: (i) a result of somatic mutations occurring within the tyrosine kinase domain of EGFR (14, 15), the phosphorylation of EGFR triggered several downstream signal transduction cascades, such as the MAPK, Akt/mTOR, and JNK pathways, which almost totally inhibited by gefitinib, while mutated receptors sustain a hyperactivated downstream signaling (16–18); (ii) activation of other signaling pathways alternative to EGFR, such as the insulin-like growth factor 1 receptor (IGF-1R) and vascular endothelial growth factor receptor (VEGFR; ref. 19), and resulted in the upregulation of downstream signaling pathways such as AKT/mTOR (20–23). Therefore, it is of great significance to further explore the downstream pathways of EGFR and thus develop novel therapeutic strategies for patients with lung adenocarcinoma.

The mammalian target of rapamycin (mTOR) is a highly conserved serine-threonine kinase that is located in the PI3K/AKT pathway and is frequently hyperactivated in tumors by mutations in upstream signaling factors (e.g., EGFR, VEGFR, and PI3K regulators; refs. 23, 24). Various mTOR inhibitors have been used in trials for cancer therapeutic (25, 26). DEP domain-containing mTOR-interacting protein (DEPTOR), as a naturally occurring inhibitor of mTOR, usually acts as a tumor suppressor by blocking
the activation of mTOR to inhibit cell proliferation, invasion, and survival effect of AKT (27, 28). However, under certain circumstances, DEPTOR could act as an oncogene by relieving the feedback inhibition of mTOR, activation of mTOR enhances the downstream S6K expression, which could negatively regulate AKT via downregulating expression of IRS-1/2 (27, 29). More importantly, mTOR could also cooperate with S6K or CK1ε to enhance βTrCP to degrade the expression of DEPTOR and thus generate an autoamplification loop to promote its own full activation (27, 30).

Although the potential role of DEPTOR as an oncogene or a tumor suppressor has been investigated in different types of tumors (28, 31–33), and DEPTOR could inhibit proliferation of lung adenocarcinoma cells (34), it has not been previously tested whether and how DEPTOR plays a role in EGFR-induced tumor progression of lung adenocarcinoma. In this study, we showed that DEPTOR expression is significantly reduced in patients with lung adenocarcinoma and negatively correlated with EGFR expression. Activation of EGFR signaling with EGF in EGFR overexpressing A549 cells dramatically inhibited DEPTOR expression by enhancing the function of the mTOR autoamplification loop, which mainly responds to the feedback degradation of DEPTOR. Furthermore, DEPTOR knockout by using the CRISPR/Cas9 system significantly promoted tumor growth both in vivo and in vitro. And, more importantly, tumor progression induced by EGFR ectopic expression was significantly inhibited by simultaneously transfected with DEPTOR-overexpressing plasmid. Taken together, our results reveal a novel mechanism that EGFR used to facilitate tumor growth and DEPTOR may be a novel therapeutic option for the treatment of human lung adenocarcinoma.

**Materials and Methods**

**Patients and tissue samples**

The study was approved by the Ethical Review Board for research in the Zhongnan Hospital of Wuhan University. Fifty-one patients diagnosed as lung adenocarcinoma were given

<table>
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<tr>
<th>Characteristic</th>
<th>Cases (%)</th>
<th>mRNA expression</th>
<th>DEPTOR</th>
<th>P</th>
<th>mRNA expression</th>
<th>EGFR</th>
<th>P</th>
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<td>Total</td>
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<td>0.9667</td>
<td>0.52 ± 0.28</td>
<td>0.8607</td>
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<td>Male</td>
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<td>0.52 ± 0.29</td>
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<td>Female</td>
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<td>3.34 ± 1.94</td>
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<td>Median age (years, range)</td>
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<td>0.7153</td>
<td>3.33 ± 1.85</td>
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informed consent according to institutional guidelines. Biopsy specimens and paired adjacent normal tissues of all patients were acquired and immediately stored at liquid nitrogen until use. The patients' characteristics are summarized in Table 1.

Cell culture
The normal human lung epithelial cell line BEAS-2B and lung adenocarcinoma cell line A549 and NCI-H1975 were obtained from Shanghai Bogoo Biotechnology Limited, and cultured in RPMI-1640 medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) at 37°C in a humidified atmosphere containing 5% CO2. BEAS-2B cells were cultured in BEBM medium according to ATCC protocols.

Immunohistochemistry
Biopsy samples were fixed in 4% formalin and embedded in paraffin. Tissue slices were cut into 5-μm thick for H&E staining and examined under a microscope. Immunohistochemistry was performed using the Vectastain ABC Kit (Rabbit IgG, Vector Laboratories; ref. 34). Rabbit primary antibodies for DEPTOR (Abcam) and phospho-EGFR (Tyr1068; Abcam), EGFR (Abcam), EGFR (Tyr1068; Abcam), mTOR, mTOR (Ser2448), CK1α, βTrCP1, S6K, S6K (Thr389), and β-actin (all from Cell Signaling Technology). Signals were detected using ECL detection reagent (Millipore) following the manufacturer’s instructions.

Quantitative real-time PCR (qRT-PCR)
Total RNA of tumor tissues or cells was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was reversely transcribed into cDNA using the First-Strand cDNA Synthesis Kit (Toyobo). The mRNA expression of EGFR and DEPTOR was quantified using a real-time RT-PCR with the SYBR Green real-time PCR Master Mix kit (Toyobo). The following primers were used: EGFR, 5'-TGATAGCAGCATATGTGCCC-3' and 5'-TCGAGGCCACGGTTAGAGTTC-3' (35); DEPTOR, 5'-TTTGTTGCGAGGAAGTAA-3' and 5'-CATTTGTTGGTCATTGTCATTCGG-3'; GAPDH, 5'-CITCCTGCATCCCTCGTGAC-3' and 5'-TGAGGAGTGTGGCTCCGGCT-3' (31). The ABI StepOne Plus (Applied Biosystems) was used to perform the amplification reaction. Each experiment was performed in triplicate. And the date was analyzed by the 2−DDΔΔCt method.

Western blotting
Total proteins of tissue samples were extracted by using the Total Protein Extraction Kit (Millipore). As for cultured tumor cells, the cells were lysed with RIPA Lysis Buffer (Beyotime). Equivalent proteins (30 μg per sample) were electrophoresed in SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). The PVDF membranes were blocked with 5% nonfat milk in TBST buffer for 1 hour and incubated overnight at 4°C with primary antibodies. The following rabbit antibodies were used: DEPTOR (Abcam), EGFR (Abcam), EGFR (Tyr1068; Abcam), mTOR, mTOR (Ser2448), CK1α, βTrCP1, S6K, S6K (Thr389), and β-actin (all from Cell Signaling Technology). Signals were detected using ECL detection reagent (Millipore) following the manufacturer’s instructions.

Establishment of stably transfected cells
Full-length EGFR and DEPTOR were cloned into the vehicle vector pcDNA3.1 to generate EGFR and DEPTOR overexpressing plasmids, as we previously described (1, 27, 31). A549 cells were transiently transfected with the above three plasmids using Lipofectamine 2000 according to the manufacturer’s instructions (Invitrogen) and retained in a medium containing 10% FBS and 1 μg/mL puromycin to select the stable transfected cells.

DEPTOR CRISPR/Cas9 Knockout
A549 cells (2 × 10^5) in 3 mL antibiotic-free medium were plated in 6-well plate. Twenty-four hours later, after cells reach 80% confluency, DEPTOR CRISPR/Cas9 KO Plasmid and DEPTOR HDR Plasmid (Cat. No. sc-402253 and sc-402253-HDR from Santa Cruz Biotechnology, Inc.) were mixed at equivalent ratio and then cotransfected into A549 cells using Lipofectamine.
EGFR and DEPTOR in Lung Cancer Progression

Figure 3. EGFR activation enhances the function of the mTOR autoamplification loop to inhibit DEPTOR expression. A, protein expression of EGFR, pEGFR, and DEPTOR was detected in EGFR stable overexpressing A549 cells. EGFR overexpressing A549 cells was constructed as described in Materials and Methods, and then was subjected to Western blotting analysis with indicated antibodies. B, EGFR overexpressing A549 cells were stimulated with EGF (20 ng/mL) for 1 hour, gefitinib (5 μmol/L) for 4 hours, DMSO used as a solvent control. Then, cell lysates were subjected to Western blotting analysis with indicated antibodies. The treatment of C and D was the same as in B, the expression of proteins that consist of the mTOR autoamplification loop was detected by Western blotting.

Cell proliferation assay
A cell proliferation assay was carried out with a Cell Counting Kit-8 (Beyotime; ref. 36). A549 or stable transfected cells were plated in 96-well plates at approximately 2 × 10^3 cells per well. The numbers of cells per well were detected by the absorbance (450 nm) of reduced WST-8 at the indicated time points. The absorbance (450 nm) was measured by using SpectraMax i3x microplate reader (Molecular Devices).

Wound-healing assay
Cell migration was determined by a wound-healing assay, and cell monolayers were carefully scratched using a sterile 200-μL pipette tip, and the cellular debris was subsequently removed by washing with PBS to form wound gaps (37). The wound location was marked, and cells were photographed at 0 and 24 hours to measure the cell migration ability by using an IX70 microscope (Olympus GmbH).

Invasion assay
Cell invasion was determined by the Transwell assay. A total of 3 × 10^4 cells in 0.5% FBS medium were transferred on the top of the Matrigel-coated invasion chambers (8-μm pore; Corning Costar), and 10% FBS medium was added in the lower chamber. After 48 hours of incubation, noninvasion cells were removed by wiping with a cotton swab. The invading cells that had invaded the bottom side of the membrane were then fixed with 4% formaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI; ref. 38). The numbers of invasive cells were obtained by counting five fields (×100) per membrane and represented the average of three independent experiments.

Results
mRNA expression of EGFR negatively correlates with DEPTOR in lung adenocarcinoma tissues
The abnormal hyperactivity of EGFR and its downstream mTOR pathways is the leading cause of resistance to TKIs; furthermore, mTOR inhibitors could effectively control the growth of lung adenocarcinoma even after acquiring resistance to TKIs (13, 41, 42). In order to further unveil the downstream regulatory molecular mechanisms involved in EGFR/mTOR activation, we simultaneously analyzed mRNA expression of EGFR and DEPTOR, a naturally occurring inhibitor of mTOR, in tumor tissues of patients with lung adenocarcinoma. As shown in Fig. 1A, the

Xenograft mouse model
Six-to-eight-week-old female BALB/c nude mice were purchased from the Laboratory Animal Center of Wuhan University (Wuhan, China) and maintained in cages under sterile conditions with a specific pathogen-free environment. A549 cells, EGFR-overexpressing A549 cells, or EGFR/DEPTOR double overexpressing cells (1 × 10^6) were injected subcutaneously within a volume of 100 μL in the flank at the time of inoculation (39). Tumor size was measured by external caliper measurement every three days after injection, and tumor volume was calculated as 1/2 × (tumor length) × (tumor width)^2 (40). Mice were sacrificed, and tumors were dissected and measured on day 21 after inoculation.

Statistical analysis
All data are presented as mean ± SD and analyzed by using Graphpad Prism V.5.00 software (GraphPad Software). A Spearman correlation test was used to assess the association between mRNA expression of EGFR and DEPTOR in tumor tissues. A comparison between two groups for statistical significance was performed with an unpaired Student t test. For more groups, one-way ANOVA followed by Neuman–Keuls post hoc test was used. P < 0.05 was considered statistically significant.
mRNA level of EGFR is significantly upregulated in lung carcinoma tissues than in adjacent normal compartment, and DEPTOR is downregulated in tumor tissues (Fig. 1B). In addition, this result is further confirmed by Western blotting (Fig. 1D) and immunohistochemistry analysis (Fig. 2A). Next, we then examined potential correlation between aberrant EGFR and DEPTOR expression. Pearson correlation assay revealed that mRNA expression of EGFR significantly and negatively correlated with DEPTOR expression in tumor tissues ($r^2 = 0.6247$, $P < 0.0001$; Fig. 1C). As DEPTOR has been reported to inhibit tumor cell growth of lung adenocarcinoma (34), these findings suggest that there may be a link between EGFR and DEPTOR to regulate tumor growth.

EGFR activation inhibits DEPTOR expression via enhancing the function of the mTOR autoamplification loop

Next, we detected EGFR and DEPTOR expression in three lung adenocarcinoma cell lines, including A549, PC-9, and NCI-H1975. Compared with normal human lung epithelial cells, BEAS-2B, protein (Fig. 2B) and mRNA (Fig. 2C) expression of EGFR were significantly increased in these tumor cells, and DEPTOR expression was decreased at different levels (Fig. 2B and D). As A549 cells occupy the lowest level of DEPTOR expression and a relative higher level of EGFR expression, we then used A549 cells in our following experiments.

A549 cells were transfected with pcDNA3.1-EGFR to construct stable EGR overexpressing cell line, and in comparison with empty vector, overexpression of EGFR significantly decreased DEPTOR expression in A549 cells (Fig. 3A). Previous studies revealed that EGF could induce the activation of EGFR kinase activity, and Gefitinib is selective EGFR TKI that usually used to block the EGFR signaling (43, 44). Herein we treated EGFR overexpressing A549 cells with these two reagents to test the effect of EGFR activation on DEPTOR expression. Our data showed that EGF (20 ng/mL) strongly stimulated the phosphorylation of EGFR and inhibited the expression of DEPTOR, while gefitinib (5 μmol/L) inhibited the phosphorylation of EGFR and significantly enhanced DEPTOR expression (Fig. 3B).

As studies have identified that S6K, CK1α, and βTrCP1 could be cooperated with mTOR to generate an autoamplification loop and thus trigger the degradation of DEPTOR (27, 30), we then detected the expression of these proteins with the presence of EGF or gefitinib. EGF significantly enhanced the phosphorylation of mTOR and S6K, as well as the expression of CK1α and βTrCP1 (Fig. 3C). Furthermore, blocking the activation of EGFR with gefitinib showed the opposite function on these four proteins' expression with EGF (Fig. 3D). Thus, we confirmed that EGFR activation enhances the function of mTOR autoamplification loop to downregulate DEPTOR expression.

DEPTOR suppresses cellular proliferation, migration, and invasion of lung adenocarcinoma cells

Given that EGFR inhibited DEPTOR expression in A549 cells, we next investigated the potential roles of DEPTOR in lung adenocarcinoma development. First, we constructed two stable A549 cell lines, DEPTOR overexpressing cells and DEPTOR knockout cells,
DEPTOR ectopic expression suppresses EGFR-mediated tumor-promoting effects in human lung adenocarcinoma cells. A, DEPTOR and EGFR double overexpressing A549 cells were constructed, and then cell lysates were subjected to Western blotting analysis with indicated antibodies; β-actin was used as a loading control. B, cell proliferation of three cell lines was examined with a Cell Counting Kit-8 assay. C, cellular migration was examined by using a wound-healing assay, and migration was assessed after 24 and 48 hours. D, cellular invasion was evaluated in the Transwell chamber that precoated with Matrigel, and then stained with DAPI after 24 hours of incubation. E, quantification of wound closure. Histogram represents the wound width as the mean ± SD of the percentage of the closure of original wound in triplicate plates. F, quantification of invaded cells. Histogram represents the invaded cells of six random fields. Data presented as Mean ± SD, and similar results were obtained in three experiments. **P < 0.01; ***P < 0.0001. Scale bar, 50 μm.

DEPTOR ectopic expression suppresses EGFR-driven tumorigenesis

Because EGFR has been found to widely overexpress and promote tumor progress in lung adenocarcinoma (15, 17), we consider whether DEPTOR ectopic expression could reverse the tumor-promoting effects of EGFR. EGFR overexpressing A549 cells were then further stably transfected with pcDNA-DEPTOR plasmid. As shown in Fig. 5A, pcDNA-DEPTOR plasmid transfection significantly elevated DEPTOR expression in EGFR overexpressing A549 cells. Simultaneously enforcing DEPTOR expression in EGFR ectopic expression A549 cells markedly decreased EGFR expression and its phosphorylation level. Analysis of tumor-related activities further revealed that DEPTOR inhibited the cell proliferation (Fig. 5B), migration (Fig. 5C and D), and invasion (Fig. 5E and F) of EGFR ectopic expression in A549 cells. Thus, these results demonstrated that ectopic expression of DEPTOR reverses EGFR-driven tumorigenesis in lung adenocarcinoma cells.

DEPTOR provides feedback promotion on protein degradation of EGFR

We noted that ectopic expression of DEPTOR significantly suppressed EGFR expression and its phosphorylation (Figs. 4A and 5A). To determine if protein synthesis was involved in DEPTOR-induced suppression of EGFR, cycloheximide (5 μg/mL) was used to inhibit protein synthesis, which at this dose has been shown to have an inhibitory effect on protein translation (45, 46). As shown in Fig. 6A and B, ectopic expression of DEPOR significantly promoted the degradation of EGFR protein. To further confirm the role of DEPTOR on mRNA expression of EGFR, the mRNA expression levels of EGFR in DEPTOR overexpression or knockout cells were detected, and there is no detectable difference in EGFR mRNA expression in response to DEPTOR expression (Fig. 6C). Next, actinomycin D (5 μg/mL; refs. 47, 48) was used to block mRNA transcription and then EGFR mRNA half-life was evaluated. Followed with actinomycin D treatment, no significant difference was seen in the rate of EGFR mRNA decay in response to DEPTOR expression (Fig. 6D). These results revealed that DEPTOR provides a feedback promotion on protein degradation of EGFR and does not influence EGFR mRNA transcription. So there is a reciprocal negative regulation between DEPTOR and EGFR expression.
DEPTOR ectopic expression suppresses EGFR-driven tumor growth in vivo

The inhibitory effect of DEPTOR on the growth of lung adenocarcinoma cells in vivo was evaluated by using nude mouse models. As pcDNA empty vector stable transfection showed no detectable influence on EGFR and DEPTOR expression, as well as the proliferation, migration and invasion of A549 cells (Fig. 5), we used normal A549 cells as a control. EGFR overexpressing A549 cells and EGFR/DEPTOR double overexpressing A549 cells were injected into the flank of nude mice. The volume of tumor xenografts was monitored every 3 days. At day 21, mice were sacrificed, and the tumor xenograft of each mouse was dissected (Fig. 7A). As compared with normal control (214 ± 66 mm³), EGFR overexpressing A549 cells significantly promoted the growth of xenografts in nude mice (570 ± 132 mm³), while simultaneously transfected with EDPTOR overexpressing (169 ± 65 mm³) almost totally abrogated EGFR-enhanced tumor growth (Fig. 7B), as well as tumor weights (Fig. 7C). Taken together, these in vivo tumor growth results were in agreement with our in vitro results and indicated that DEPTOR is a pivotal tumor suppressor that was downregulated by the EGFR signaling pathway to promote tumor growth of lung adenocarcinoma.

Discussion

The EGFR signaling pathway plays a critical role in promoting cancer cell growth and survival, which serves as an attractive therapeutic target, is attracting even more and more attention (49). EGFR tyrosine kinase inhibitors (TKI), such as gefitinib, which operate by repression of EGFR oncogenic signaling, have proven to provide a significant response and survival benefit for patients with lung cancer. However, all responders eventually acquire chemotherapy resistance (15, 50), and lung cancer is still estimated to be responsible for nearly one in five (1.59 million deaths, 19.4% of the total) of death from cancer worldwide (http://globocan.iarc.fr/Default.aspx). Because we found DEPTOR upregulated in response to gefitinib treatment (Fig. 3B), it would be interesting to test the role of DEPTOR in gefitinib resistance, the exact molecular mechanisms of which we are still unclear about.
One of the downstream pathways controlled by EGFR involves mTOR, a proto-oncogene activated in various cancers (51). The kinase mTOR is a critical target of EGFR signaling, linking growth factor abundance to cell growth and proliferation (52). DEPTOR, as a natural negative regulator of mTOR, has been reported to involve in sensitive to apoptosis gene (SAG)-induced lung tumor formation and may inhibit cell growth of lung adenocarcinoma (34); furthermore, loss of DEPTOR increased drug resistance to EGFR TKIs of lung adenocarcinoma cells (53). Thus, we questioned whether DEPTOR serves as a tumor suppressor and EGFR downregulates DEPTOR expression in lung adenocarcinoma.

To answer this question, we first detected the mRNA expression of EGFR and DEPTOR in clinical samples from patients with lung adenocarcinoma. Our results showed that EGFR expression increases with TNM stage and lymph node metastasis, while DEPTOR shows a contrary tendency (Table 1), and there is significant negative correlation between EGFR and DEPTOR expression (Fig. 1). In the EGFR signaling cascade, ligand stimulation promotes EGFR phosphorylation and then leads to receptor internalization and downstream signaling activation (54). In lung adenocarcinoma patients, we also observed that EGF/pEGFR is partially expressed in the cytoplasm that DEPTOR mainly located (Fig. 2A).

Next, we chose human lung adenocarcinoma A549 cell lines to conduct our study and cloned EGFR overexpressing A549 cells. Compared with parental cells, EGFR ectopic expression significantly decreased DEPTOR expression; this could be further enhanced with the presence of EGF, EGFR ligand. More importantly, DEPTOR expression was enhanced with the stimulation of gefitinib. The following molecular analysis further identified that EGFR activation enhanced mTOR and S6K phosphorylation, CK1α and ßTrCP1 expression. Of these four molecules, S6K is a direct downstream molecule of mTOR (55) and consists of the autophosphorylation loop of mTOR to degrade DEPTOR expression (27, 30). CK1α and ßTrCP1 are involved in promoting tumor progression and have been found to act synergistically with EGFR to inhibit cell expansion of lung cancer (56, 57), but how EGFR activation stimulates CK1α and ßTrCP1 expression is still unknown and needs to be identified in our future study.

The role of DEPTOR expression in the progression of human malignancies is still controversial; this may be because the regulation of DEPTOR is complicated in different individuals and tissues (28, 31), as two opposite results were obtained in the same myeloma cells (31, 58). In lung adenocarcinoma, there is still no direct evidence that DEPTOR regulates tumor progression. By constructing two stable A549 cell lines, namely, DEPTOR overexpression or knockout, we first demonstrated that DEPTOR acts as a tumor suppressor in the tumorigenesis of lung adenocarcinoma, as DEPTOR inhibited cell proliferation, migration, invasion, and in vivo tumor growth. Furthermore, simultaneous knockin DEPTOR in EGFR overexpressing A549 cells suppressed EGFR expression and markedly abolished EGFR-driven tumorigenesis of lung adenocarcinoma. Accompanied with the observation that DEPTOR expression provides a feedback promotion on EGFR protein degradation, more detailed molecular mechanisms need to be investigated in the future.

Of course, there are still some potential problems with our current study. For example, it is well known that CRISPR/Cas9-mediated gene editing has off-target effects. Although we believe that the guide RNA sequence has been tested by the commercial provider for our study, there might still be chances that our guide RNA can target non-DEPTOR sequence in human genome. We will take this into consideration in future studies. Inspired by two elegant studies from leading scientists in the CRISPR/Cas9
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research field (59, 60), we will try to use modified Cas9 nucleases with high fidelity in our future studies, with the hope of minimal off-target effects.

In conclusion, our study reveals a novel link between EGFR and DEPTOR, and that EGFR promotes DEPTOR degradation by enhancing the function of the mTOR autoamplification loop, including increased mTOR and S6K phosphorylation, as well as Cki1 and BtrCP1 expression. Furthermore, we also provide solid experimental evidence that DEPTOR plays a tumor suppressive role in lung adenocarcinoma cells, and ectopic expression of DEPTOR could suppress EGFR-driven tumor progression.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: X. Zhou, J. Guo, H. Zhu, J. Zhao
Development of methodology: X. Zhou, J. Guo, H. Zhu

References

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Zhou, J. Guo, G. Pan, T. Liu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Zhou, J. Guo, Y. Ji, H. Zhu, J. Zhao
Writing, review, and/or revision of the manuscript: X. Zhou, J. Guo, G. Pan, T. Liu, J. Zhao

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Zhou, J. Guo, J. Zhao

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