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ORIGINAL RESEARCH

AEG-1 promotes the growth of gastric cancer through the upregulation of eIF4E expression

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Background: AEG-1 has been proven to be tumor enhancer in gastric cancer. However, its mechanism has not yet been fully clarified.

Methods: Gain-of-function and loss-of-function experiments were conducted to determine the role of eIF4E in AEG-1-induced growth of gastric cancer cells and xenografts of a nude mouse model. Western blot analysis and SRB assay were used to determine the protein expression levels and survival cell numbers.

Results: Silencing the expression of AEG-1 inhibited the growth of gastric cancer cells in parallel with a decreased eIF4E and cyclin D1 expression; however, the overexpression of AEG-1 promoted cell growth and increased eIF4E and cyclin D1 expression. Moreover, the overexpression of eIF4E partially reversed the AEG-1 silencing-induced reduction of cyclin D1 and the inhibition of cell growth. An eIF4E knockdown also partially reversed the AEG-1 overexpression-induced upregulation of cyclin D1 and cell growth. Notably, manipulating the expression of eIF4E did not affect the expression of AEG-1. Finally, the silencing of AEG-1 expression inhibited the growth of SGC-7901 xenografts in parallel with the down-regulation of eIF4E and cyclin D1 expression in the nude mouse model.

Conclusion: AEG-1 promoted the growth of gastric cancer through upregulation of eIF4E/cyclin D1 signaling pathway.

Keywords: AEG-1, eIF4E, cyclin D1, gastric cancer

Introduction

Gastric cancer is the fifth most-common cancer and the third leading cause of cancer deaths worldwide.[1] With the development of early detection of tumors and combined therapeutic strategies, the morbidity and the mortality of gastric cancer has decreased significantly in recent years. Although Helicobacter pylori infection is the main risk factor for gastric cancer, factors other than H. pylori are also important. The 5-year survival rate of gastric cancer is approximately 30%.[2] Therefore, extended studies are necessary to identify key molecules in the progression of gastric cancer and develop new targets for cancer therapy.

AEG-1, also known as metadherin (metastasis adhesion protein), or LYRIC, was first reported as a novel late response gene induced in human fetal astrocytes after HIV-1 infection.[3] Then it was found to mediate lung metastasis of breast cancer.[4] To date, AEG-1 has been proven to promote the growth, migration, invasion, survival, autophagy, and angiogenesis, and to play an important role in the development, metastasis, recovery, and chemoresistance of many types of cancers.[5] The PI3K/Akt/c-Myc signaling pathway was reported to upregulated AEG-1 transcription expression.[6] Several microRNAs, such as miR-375 and miR-1297, have been

suggested as a possible downregulator of the expression of AEG-1 in post-transcription levels.[7,8] AEG-1 has been demonstrated to activate several important signaling pathways to promote cancer development, such as PI3K/Akt, NF-κB, Wnt/β-catenin, and MAPK. Its downstream targets include FoxO1, FoxO3a, cyclin D1, AP-1, and c-Myc et al.[5] However, the AEG-1 mechanism in gastric cancer has not yet been fully clarified.

eIF4E is a component of complex eIF4F which regulates translation initiation of a group of mRNAs with a long 5′-UTR and cap structure.[9] These mRNAs include cyclin D1, VEGF, snail, Bcl-2, and Mcl-1 et al which specifically promote malignancy of cancer cells.[10] Therefore, eIF4E is oncogenic in many types of cancers and promotes the transformation, tumorigenesis, metastasis, and chemoresistance.[11] In cancer cells, eIF4E can be activated by PI3K/Akt and MEK/ERK/Mnk pathways.[12,13] It can also be transcriptionally upregulated by c-Myc.[14] Currently, our knowledge of the role and mechanism of eIF4E in gastric cancer is very limited.

We have reported that perifosine, an Akt inhibitor, suppresses the growth of gastric cancer cells and downregulates the expression of AEG-1, eIF4E and cyclin D1.[15,16] The silencing of AEG-1 or eIF4E inhibited the growth of gastric cancer cells and downregulated cyclin D1 expression. However, the mechanisms of AEG-1 and eIF4E affecting cell growth remain unknown. Our previous study revealed that AEG-1 induces the metastasis of gastric cancer through the upregulation of eIF4E mediated MMP-9 and Twist expression.[17] Thus, we speculated that AEG-1 may promote the growth of gastric cancer through the eIF4E-mediated upregulation of cyclin D1. In this study, we confirmed the growth-inhibiting effect of AEG-1 silencing on gastric cancer by both gain-of-function and loss-of-function experiments. Moreover, we examined the role of eIF4E/cyclin D1 signaling pathway in mediating the effects of AEG-1. Finally, we confirmed these findings in a xenograft nude mouse model. Our findings further prove the important role of AEG-1 in gastric cancer and unveil a new mechanism of AEG-1: the upregulation of eIF4E/cyclin D1 signaling pathway.

Materials and methods

Reagents

Lipofectamine 2000 transfection reagent (11,668–019) was purchased from Life Technologies Co. Invitrogen (Carlsbad, CA, USA). AEG-1/MTDH antibodies (13,860–1-AP) were purchased from Zhongshan Goldenbridge Biotechnology, Inc. (Beijing, China). eIF4E (9472) and cyclin D1(2922) were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). Actin antibody (AP0060) was purchased from Bioworld Technology, Inc. (Louis Park, MN, USA).

Cell lines and cell culture

Human gastric cancer cell lines, SGC-7901 and MGC-803 were purchased from Shanghai Institute for Biological Science, Chinese Academy of Sciences, China. The AEG-1 stable silencing cell lines were established by infecting SGC-7901 and MGC-803 cells with lentivirus carrying AEG-1 shRNA (5'-CAGAAGAAGAA GAACCGGA-3') and the subsequent selection by puromycin as previously described.[17] Cells were cultured in RPMI-1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) at 37°C in a humidified atmosphere consisting of 5% CO_2.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total-RNA were prepared for and subjected to qRT-PCR assay as previously described.[18]

Western blot analysis

Whole-cell protein lysates were prepared and subjected to Western blotting as previously described.[18]

Sulforhodamine B assay

Survival cell numbers were determined by Sulforhodamine B staining as previously described.[18]

Overexpression or knockdown of genes by transient transfection

Two sequences of eIF4E siRNA (5′- GGACGAUG GCUAAUUACAU - 3′; 5′- AAGCAAACCUGCGGCU GAUCUTT - 3′) and control siRNA were designed and synthesized as previously reported.[16] The AEG-1 overexpression plasmid was a gift from Dr. Kunmei Liu of Ningxia Medical University. The coding sequences of AEG-1 were inserted to the pcDNA vector.[17] The eIF4E overexpression plasmid was provided by Dr. Shi-Yong Sun of Emory University. The coding sequences of eIF4E were constructed to the p3×flag-CMV-14 vector.[12] Cells were transfected with 100 nmol/L siRNAs or plasmids for 48 hrs using Lipofectamine 2000 and subjected to

Western blot analysis. After 24 hrs' transfection, cells were reseeded to 96-well plates and observed for 5 days, before being subjected to the sulforhodamine B (SRB) assay.

The xenograft nude mouse model

Animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Athymic nude mice (BALB/C-nu/nu, 4–5 weeks old, female) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). 1×10^7 cells were inoculated subcutaneously to the nude mice and observed for 24 days (n=6 for each group). Tumor sizes and weights were recorded, and xenografts were subjected to Western blotting as previously described.[17] Briefly, tumor volume (mm^3)= $\pi/6$ (width^2×length).

Statistical analysis

The two-sided unpaired Student's *t*-test was used to analyze the statistical differences between treated or control groups. $P<0.05$ was considered to be statistically significant.

Results

Silencing of AEG-1 inhibited the growth of gastric cancer cells and downregulated the expression of eIF4E

To identify the mechanism of AEG-1 in gastric cancer, we first established stable AEG-1 silencing cell lines in SGC-7901 and MGC-803 cells. Results show that AEG-1 mRNA (Figure 1A) and protein (Figure 1B) levels were significantly decreased in AEG-1 knockdown cell lines, compared with control cells. We then found, via Western blot analysis, that eIF4E and cyclin D1 expression were decreased in AEG-1 silencing cells (Figure 1B). Moreover, AEG-1 silencing cells grew more slowly than control cells, as demonstrated by SRB assay (Figure 1C). These results suggest that the knockdown of AEG-1 expression inhibited cell growth in parallel with the downregulation of eIF4E and cyclin D1 expression in gastric cancer cell lines.

Overexpression of AEG-1 promoted the growth of gastric cancer cells in parallel with the upregulation of eIF4E expression

Next, we examined the effect of AEG-1 overexpression on eIF4E expression and cell growth. SGC-7901 and MGC-803

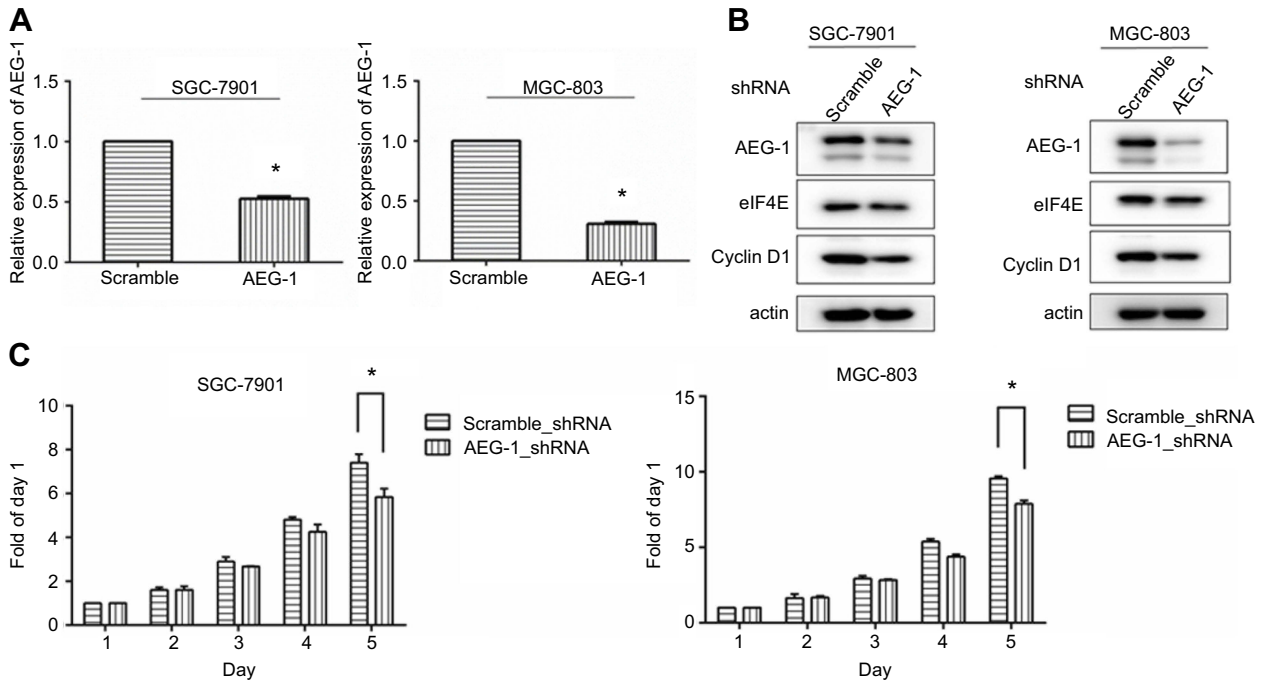

Figure 1 Silencing of AEG-1 expression inhibited the growth of gastric cancer cells and downregulated the expression of eIF4E. (**A**) qRT-PCR assays, (**B**) Western blot analysis and (**C**) 5-day SRB assays for AEG-1 stable silencing SGC-7901 and MGC-803 cells. Columns, means of three (**A**) or four (**C**) replicated determinations; bars, SD. *$P<0.05$.

cells were transiently transfected with plasmids carrying AEG-1 coding sequences. Western blot analysis showed that eIF4E and cyclin D1 expression levels were increased in AEG-1 overexpression cells (Figure 2A). Further, AEG-1 overexpression cells grew faster than control cells, as indicated by the SRB assay (Figure 2B). These results suggest that the overexpression of AEG-1 promoted cell growth in parallel with the upregulation of eIF4E and cyclin D1 expression.

Overexpression of eIF4E partially attenuated the AEG-1 silencing-induced growth inhibition of gastric cancer cells

To identify the role of eIF4E in AEG-1-induced cell growth, we first examined the rescue effect of eIF4E overexpression in AEG-1 knockdown cells. We found that the expression of eIF4E and cyclin D1 were decreased in AEG-1 silencing cells; however, overexpression of eIF4E partially reversed cyclin D1 expression level as indicated by Western blotting (Figure 3A). Moreover, the growth-inhibiting effect of AEG-1 silencing was partially reversed by eIF4E overexpression, as indicated by the SRB assay (Figure 3B). These results suggest that eIF4E played an important role in silencing of AEG-1 induced decreases of cyclin D1 expression and growth inhibition.

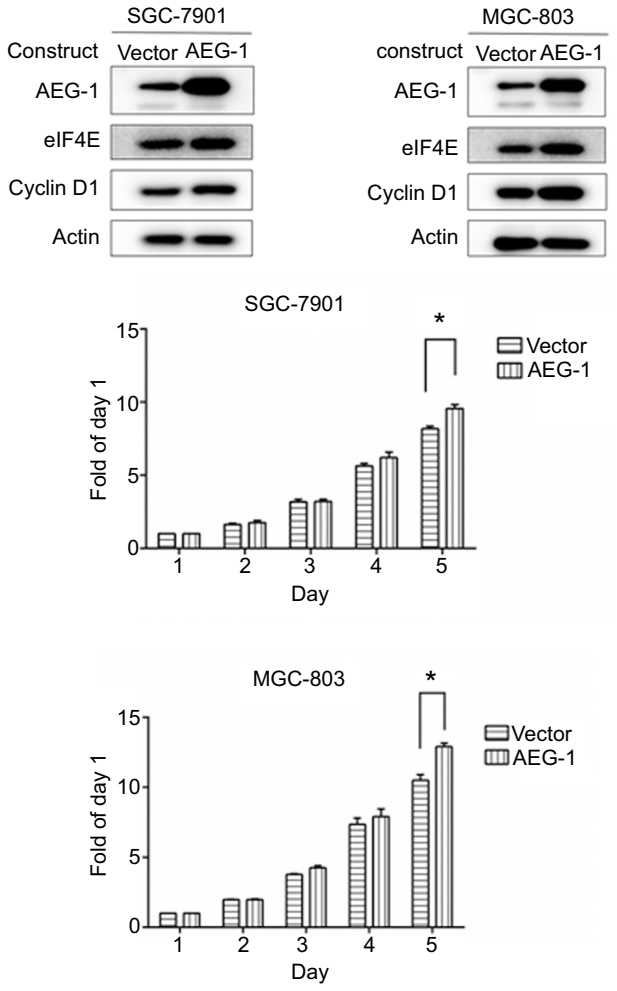

Figure 2 Overexpression of AEG-1 induced the growth of gastric cancer cells and upregulated the expression of eIF4E. (**A**) Western blot analysis and (**B**) 5-day SRB assays for SGC-7901 and MGC-803 cells transfected with AEG-1 plasmids for 48 h or 5-day respectively. Columns, means of four replicated determinations; bars, SD. *$P<0.05$.

Knockdown of eIF4E expression partially attenuated AEG-1-induced cell growth

Next, we conducted experiments opposite to those previous detailed, examining the rescue effect of eIF4E knockdown on AEG-1 overexpression cells. Gastric cancer cell lines were co-transfected with AEG-1 overexpression plasmids, with or without eIF4E siRNAs. Western blot analysis showed that AEG-1 overexpression upregulated eIF4E and cyclin D1 expression levels; however, knockdown eIF4E partially abrogated the AEG-1-induced upregulation of cyclin D1 (Figure 4A). As we speculated, eIF4E knockdown partially reversed AEG-1-induced cell growth (Figure 4B). These findings suggest that eIF4E plays an important role in mediating the AEG-1-induced upregulation of cyclin D1 and cell growth.

eIF4E upregulated cyclin D1 expression to promote the growth of gastric cancer cells

Although we have previously reported that eIF4E transiently knockdown using siRNAs downregulated cyclin D1 expression and inhibited the growth of gastric cancer cells, we has not yet examined the effect of eIF4E overexpression on gastric cancer cells.[16] In SGC-7901 and MGC-803 cells, we found that the overexpression of eIF4E increased cyclin D1 expression and promoted cell growth (Figure 5A, B); however, the knockdown of eIF4E expression showed the opposite effects (Figure 5C, D). It should be noted that neither the overexpression nor silencing of eIF4E affected AEG-1 expression level, indicating that eIF4E located downstream from AEG-1. These results suggest that AEG-1 regulated the eIF4E/cyclin D1 signaling pathway to inhibit the growth of gastric cancer cells.

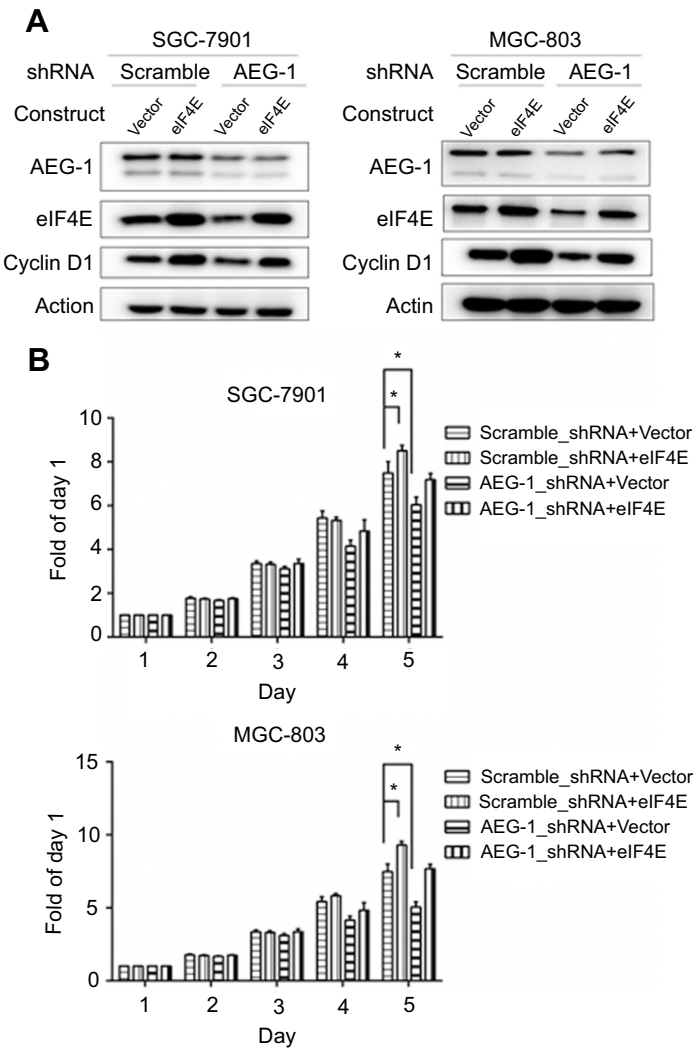

Figure 3 Overexpression of eIF4E partially reversed the downregulation of cyclin D1 and the growth inhibition induced by the silencing of AEG-1. AEG-1 stable silencing cells transfected with eIF4E plasmids for 48 h or 5-day were subjected to (**A**) Western blot analysis and (**B**) 5-day SRB assays respectively. Columns, means of four replicated determinations; bars, SD. *P<0.05.

Silencing of AEG-1 expression inhibited the growth of gastric tumors in parallel with the downregulation of eIF4E and cyclin D1 expression in a xenograft mouse model

We further confirmed the effect of AEG-1 on the growth of gastric cancer in vivo, using a xenograft mouse model. Stable AEG-1 silencing SGC-7901 cells (SGC-7901-AEG-1_shRNA) and control (SGC-7901-scramble_shRNA) cells were injected subcutaneously to the nude mouse for 24 days. Figure 6A is a photo of the mice when we collected the tumors. We found that the average tumor size (Figure 6B) and tumor weight (Figure 6C) were significantly decreased in the AEG-1 silencing tumors compared with control tumors. Moreover, eIF4E and cyclin D1 expression levels were significantly decreased in AEG-1 silencing tumors compared with control tumors (Figure 6D). These results suggest that the silencing of AEG-1 expression inhibited the growth of gastric cancer and downregulated the expression of the eIF4E/cyclin D1 signaling pathway in vivo.

Discussion

We have reported that the transient knockdown of AEG-1 expression using siRNAs inhibited the growth of gastric cancer cells.[15] In this study, we further confirmed the role of AEG-1 in gastric cancer, both in vitro and in vivo. Through gain-of-function and loss-of-function studies, we demonstrated that AEG-1 promoted the growth of gastric cancer cell lines SGC-7901 and MGC-803. Moreover, stably silencing AEG-1 expression inhibited the growth of gastric tumors in a xenograft nude mouse

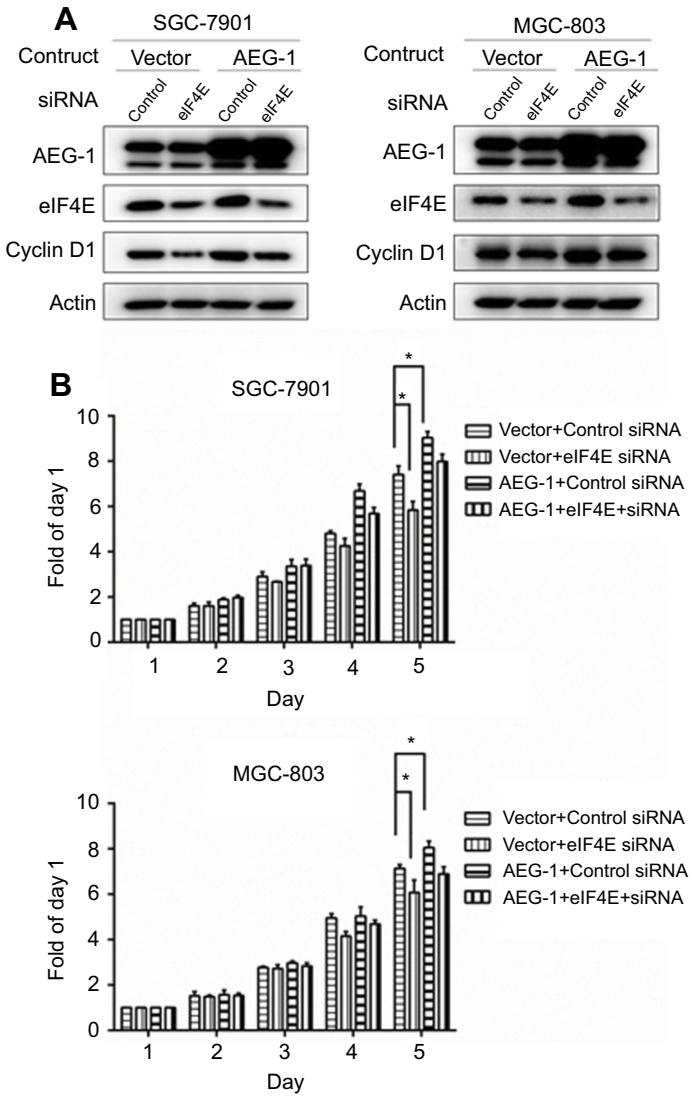

Figure 4 Knockdown of eIF4E expression partially abrogated the AEG-1-induced upregulation of cyclin D1 and cell growth. (**A**) SGC-7901 and MGC-803 cells were transiently transfected with AEG-1 constructs or control vector for 24 h, then reseeded and transfected with eIF4E siRNA or control siRNA for another 24 h. Next, the whole cell lysates were prepared and subjected to Western blot assay. (**B**) Cells with transfection as aforementioned were reseeded in 96-well plates and subjected to a 5-day SRB assay. Columns, means of four replicated determinations; bars, SD. *$P<0.05$.
Abbreviation: SRB, sulforhodamine B.

model. These findings suggest that AEG-1 plays an important role in the progression of gastric cancer and could be developed as a new therapeutic target.

The mechanisms of AEG-1 in gastric cancer has not yet been fully clarified. In 2011, Xu et al first reported the elevated expression of AEG-1 in gastric cancer and indicated its correlation with the poor prognosis of gastric cancer. They also found that the knockdown of AEG-1 expression decreased the phosphorylation of Akt and GSK3β, downregulated β-catenin, LEF1, and cyclin D1 expression, indicating that AEG-1 was involved in Wnt/β-catenin-mediated cancer progression.[19] Our group has revealed that the knockdown of AEG-1 downregulates cyclin D1 expression and the inhibition of Akt/GSK3β signaling pathway, as perifosine decreases AEG-1 and its downstream target cyclin D1 expression.[15] In another study by our group, we have reported that eIF4E expression is elevated in human gastric cancer tissues. and perifosine downregulates eIF4E expression to inhibit the growth of gastric cancer cells.[16] Therefore, we suspect that eIF4E may be a downstream target of AEG-1 in gastric cancer. In this study, we found that the silencing of AEG-1 downregulated the eIF4E and cyclin D1 expression, both in gastric cancer cell lines and in the xenograft mouse model. In contrast, the overexpression of AEG-1 upregulated eIF4E and cyclin D1 expression. Importantly, the overexpression of eIF4E partially reversed the silencing

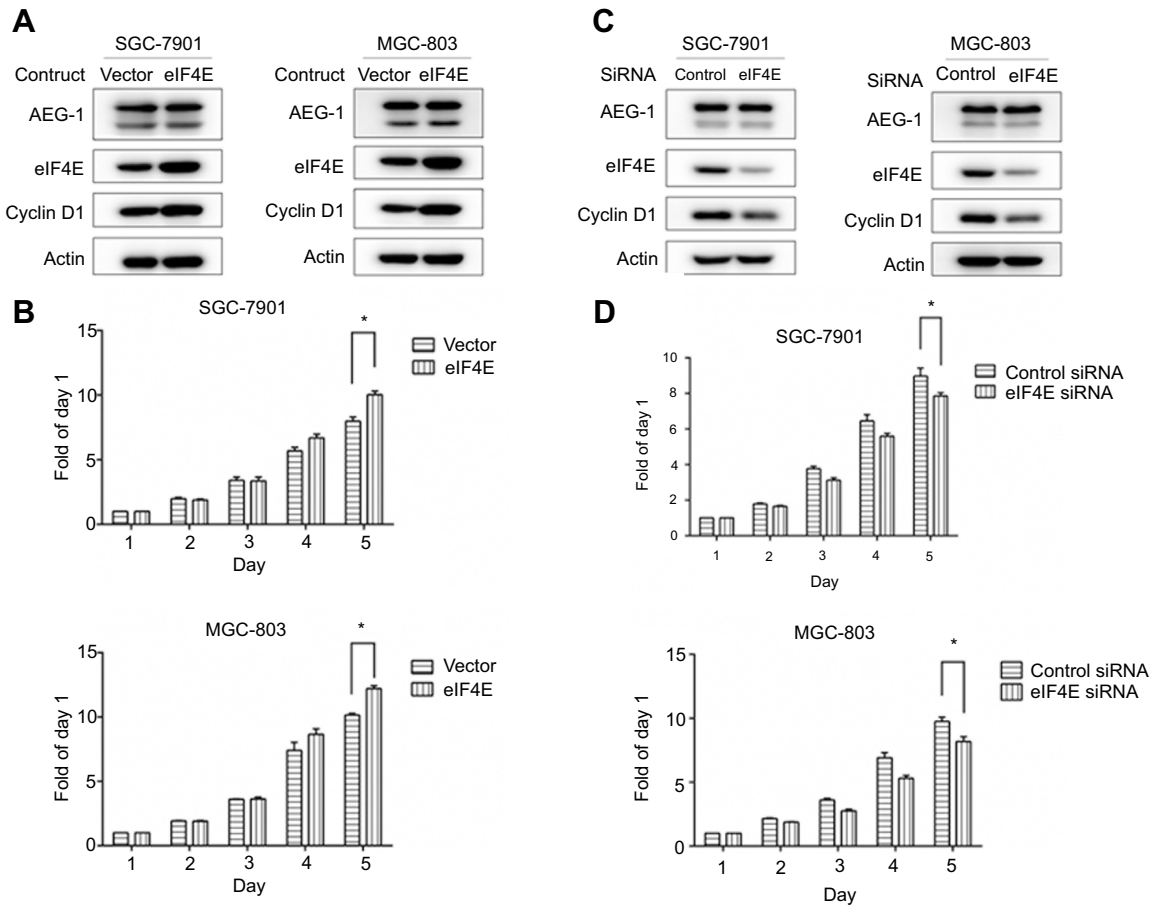

Figure 5 eIF4E upregulated cyclin D1 expression and promoted cell growth. Cells transiently transfected with eIF4E constructs (**A** and **B**) or siRNAs (**C** and **D**), as indicated were, subjected to Western blot (**A** and **C**) analysis and SRB assays (**B** and **D**). Columns, means of four replicated determinations; bars, SD. *P<0.05.).

AEG-1-induced downregulation of cyclin D1 and growth inhibition. Moreover, the overexpression of AEG-1 induced the upregulation of cyclin D1 and cell growth were partially attenuated by eIF4E knockdown (Figure 6E). Notably, manipulating eIF4E expression did not affect AEG-1 expression levels, indicating that eIF4E is regulated by AEG-1. These findings suggest that AEG-1 promotes the growth of gastric cancer through the upregulation of the eIF4E/cyclin D1 signaling pathway. However, we have not clarified how eIF4E is regulated by AEG-1. Currently, the mechanism of AEG-1 has not yet been fully clarified. It can regulate eIF4E transcription by directly functioning as a transcription factor, or by indirectly regulating NF-κB activity. Additionally, the epigenetic regulation of eIF4E transcription through DNA methylation, histone methylation or acetylation, are also possible. This is worthy of further study.

Cyclin D1, as a cell cycle checkpoint signal, when upregulated, indicates a fast cell cycle progression; however, when downregulated, it indicates a G1 phase arrest. It has been proven to be regulated by most of the oncogenic signaling pathways in cancer cells. We previously identified that cyclin D1, but not c-Myc, is located downstream of AEG-1 to mediate its effect on cell growth in gastric cancer; however, we have not clarified how it was regulated by AEG-1.[15] The mRNA of cyclin D1 belongs to a group of mRNAs that possesses long 5′-UTR and which forms a spatial cap structure. Most products of these types of mRNAs are oncogenic signals that are involved in cell growth (c-Myc and cyclin D1), anti-apoptosis (Bcl-2 and Mcl-1), metastasis (snail), and angiogenesis (VEGF).[9,14,20] It has been proven that the eIF4F complex is necessary for the translation of this type of mRNA.[10] eIF4E is a component of the eIF4F complex that mediates the association of eIF4F with a cap structure and promotes the recruitment of ribosome to the 5′ end of mRNA.[21] eIF4E has been proven to be the rate-limiting step in regulating these types of mRNAs' translation.[11] Considering our aforementioned findings, we suspect that the eIF4E-induced translation of cyclin D1 mediates the effect of AEG-1 on cell growth in gastric cancer. Recently,

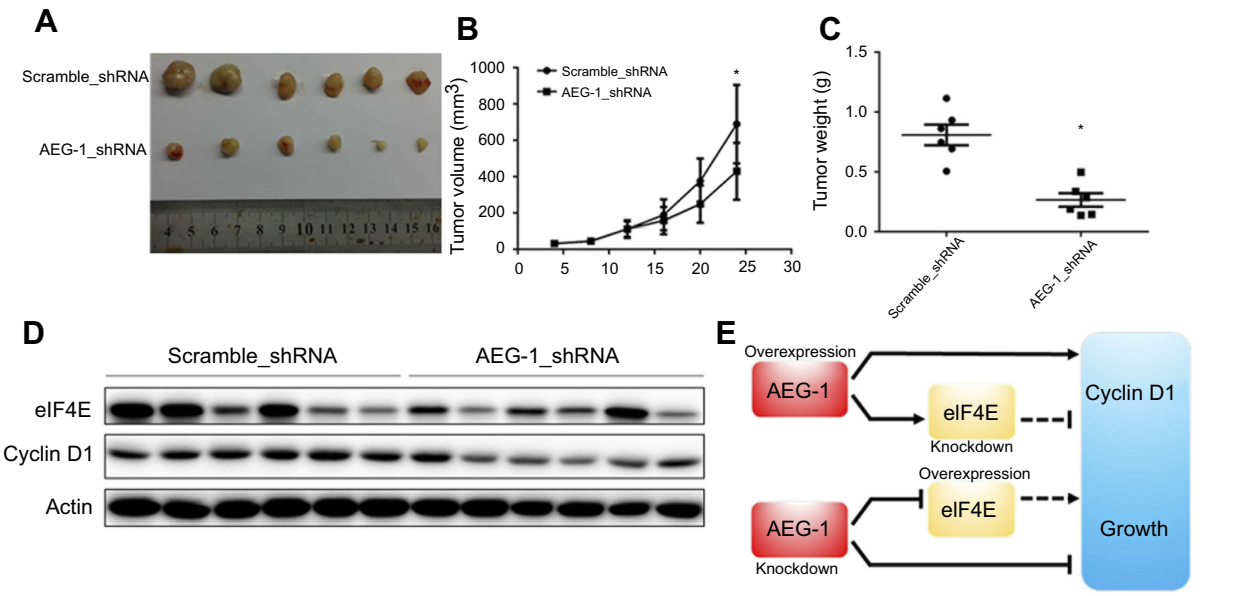

Figure 6 Silencing of AEG-1 inhibited the growth of gastric tumors in parallel with the downregulation of eIF4E and cyclin D1 expression in a xenograft mouse model. (**A**) A photo of mice burden with subcutaneous xenografts. (**B**) Tumor sizes and (**C**) tumor weights for nude mice injected with AEG-1 stable silencing cells. Horizontal lines, means of 6 tumors; points, tumor weight of each mouse; SD. *P<0.05. (**D**) Western blot analysis of tumors. (**E**) The schematic diagram illustrated the experiments we designed to reveal the mechanism of AEG-1 in the growth of gastric cancer.

we reported that AEG-1 promoted the migration and invasion of gastric cancer cells through the upregulation of eIF4E-mediated MMP-9 and Twist expression. Moreover, the knockdown of AEG-1 expression decreased lymph node and peritoneal metastasis of gastric cancer in parallel with the downregulation of eIF4E, MMP-9, and Twist expression in an orthotopic nude mouse model.[17] In this study, we revealed that AEG-1 upregulated the eIF4E/cyclin D1 signaling pathway to promote the growth of gastric cancer. These findings suggest that AEG-1-regulated eIF4E expression plays an important role in gastric cancer, and different downstream signals account for the different functions of AEG-1.

In summary, we have revealed that AEG-1 promotes gastric cancer growth through the upregulation of eIF4E-mediated cyclin D1 expression. Our findings clarify a new mechanism of AEG-1-driven gastric cancer growth and suggest new therapeutic targets for gastric cancer therapy.

Conclusion

In conclusion, our study revealed that the silencing of AEG-1 downregulated the eIF4E and cyclin D1 expression, both in gastric cancer cell lines and in the xenograft mouse model. In contrast, the overexpression of AEG-1 upregulated eIF4E and cyclin D1 expression. Importantly, the overexpression of eIF4E partially reversed the silencing AEG-1-induced downregulation of cyclin D1 and growth inhibition. Moreover, the overexpression of AEG-1 induced the upregulation of cyclin D1 and cell growth were partially attenuated by eIF4E knockdown. Notably, manipulating eIF4E expression did not affect AEG-1 expression levels, indicating that eIF4E is regulated by AEG-1. These findings suggest that AEG-1 promotes the growth of gastric cancer through the upregulation of the eIF4E/cyclin D1 signaling pathway.

Availability of data and materials

The study's data and materials are available from the corresponding author.

Compliance with ethical standards

Animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (Approval No.: IACUC 1,601,054), complied with the guidelines by the Animal Care and Ethical Committee of Nanjing Medical University.

Acknowledgments

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Ningxia Medical University for providing pcDNA3.1-AEG-1/MTDH plasmid and Dr. Shi-Yong Sun of Emory University for providing p3×flag-eIF4E plasmid.

Disclosure

The authors report no conflicts of interest in this work.

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