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miR-137 suppresses cell growth in ovarian cancer by targeting AEG-1

Jinling Guo, Bairong Xia, Fanling Meng, Ge Lou *

Department of Gynecology, The Affiliated Tumor Hospital of Harbin Medical University, 150 Haping Road, Nan Gang District, Harbin, 150081, Heilongjiang Province, China

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ABSTRACT

Astrocyte elevated gene-1 (AEG-1) is an oncogene overexpressed in multiple types of human cancers including ovarian cancer (OC). However, the underlying mechanism of AEG-1 up-regulation in OC is not well understood. In this study, we showed that miR-137 downregulated AEG-1 expression through interaction with its 3′ untranslated region (3′UTR) and that miR-137 expression was inversely correlated with AEG-1 levels in OC specimens. Similar to the downregulation of AEG-1, overexpression of miR-137 in OC cell lines decreased *in vitro* cell growth, clonogenicity, and also induced G1 arrest. Importantly, miR-137 overexpression suppressed *in vivo* tumor growth in nude mice models. Furthermore, we found that restoring the AEG-1 (without the 3′UTR) significantly rescued miR-137-induced cell growth inhibition and cell-cycle arrest. Taken together, these findings indicate that miR-137 functions as a tumor suppressor by inhibition of AEG-1. These molecules might be targets for prevention or treatment of OC.

1. Introduction

Ovarian cancer (OC) is one of the most common and lethal gynecologic malignancies, accounting for more than 15,000 deaths per year [1,2]. Despite advances in the diagnosis and treatment of this cancer, only 30% of patients survive 5 years after initial diagnosis [3,4]. Therefore, there is an urgent need to discover novel diagnostic biomarkers and therapeutic targets for OC patients.

Astrocyte elevated gene-1 protein (AEG)-1, also known as metadherin (MTDH) [5], is the product of a novel gene induced in primary human fetal astrocytes (PHFA) infected with human immunodeficiency virus type 1 (HIV-1) or treated with tumor necrosis factor-α (TNF-α) [6]. Previous studies have shown that AEG-1 was significantly elevated in several kinds of cancers, including breast cancer, glioma, prostate cancer, and esophageal squamous cell carcinoma [7–10]. Overexpression of AEG-1 promoted tumor cell proliferation, invasion, metastasis and chemoresistance [11–13]. In particular, our team previously detected high expression of AEG-1 in OC and its up-regulation was significantly associated with OC recurrence [14]. However, the functional mechanisms of AEG-1 in OC remains unclear.

MicroRNAs (miRNAs) are a class of single-stranded, small non-coding RNAs, which negatively regulate gene expression through base pairing with the 3′ untranslated region (3′UTR) of target mRNAs, causing mRNA degradation and/or translational repression [15]. miRNAs can direct a wide repertoire of biological mechanisms, such as cell cycle control, cell proliferation, apoptosis, senescence, cell migration and metastasis [16]. In OC, multiple miRNAs including miR-15a, miR-16, miR-31 and miR-125b have been identified as tumor-suppressor genes [17–19]. On the other hand, miR-187 and miR-182 have been shown to act as oncogenes in OC [20,21]. These findings suggest the involvement of miRNAs in OC tumorigenesis.

miR-137 has attracted much attention because it is frequently down-regulated and functions as a tumor suppressor in gastric cancer, glioblastoma, lung cancer, colorectal cancer and neuroblastoma [22–26]. However, whether miR-137 is involved in the progression of OC remains poorly understood. Intriguingly, online bioinformatic analysis showed that miR-137 has a conserved binding site in the AEG-1 3′UTR. Therefore, the objective of the current study was to validate the effect of miR-137 on AEG-1 in OC cells and to explore the role of this mechanism in the tumorigenesis and progression of OC.

2. Materials and methods

2.1. Cell lines and culture conditions

Ovarian cancer cell lines SKOV3, OV2008 and HEK293T were obtained from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultured at 37 °C in a humidified chamber supplemented with 5% CO_2.

* Corresponding author.
E-mail address: lougehrb@126.com (G. Lou).

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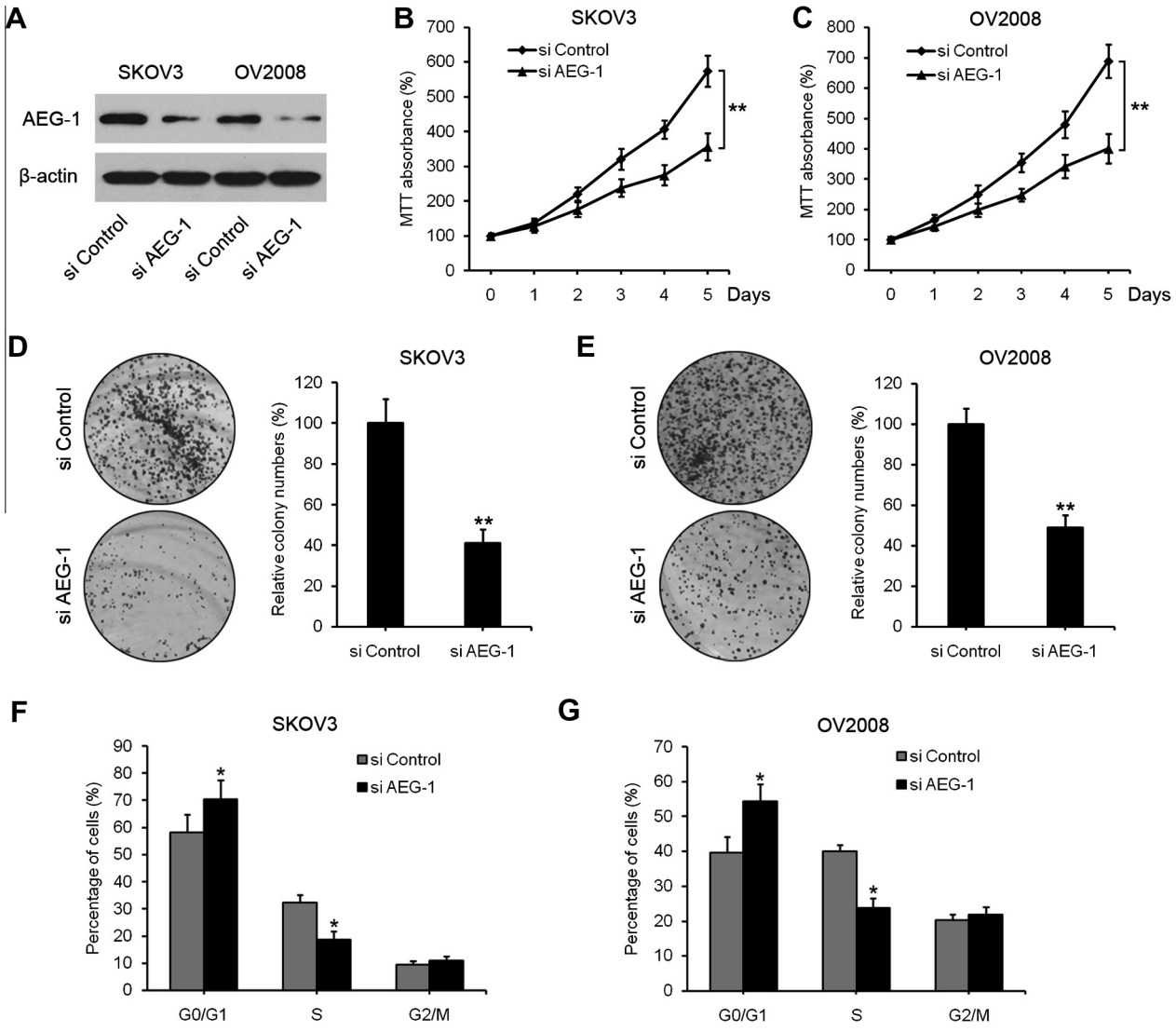

Fig. 1. Downregulation of AEG-1 inhibits OC cell growth. (A) Western blot analysis of AEG-1 expression in SKOV3 and OV2008 cells infected with AEG-1 siRNA (si AEG-1) or negative control (si Control). (B and C) Effect of AEG-1 down-regulation on OC cell growth was measured by MTT assay after si AEG-1 or si Control infection in SKOV3 and OV2008 cells. The line charts showed the relative MTT absorbance, which indicated the cellular viability. (D and E) Effect of AEG-1 down-regulation on the colonigenic ability of SKOV3 and OV2008 cells. The relative percentage of colony numbers from si Control group is designated as 100%. (F and G) Typical results of cell cycle analysis of SKOV3 and OV2008 cells infected with si AEG-1 or si Control. The bar chart represents the percentage of the cells in the G0/G1, S or G2/M phase of the cell cycle. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. si Control group.

2.2. Plasmid construction

Human miR-137 precursor and AEG-1 siRNA were purchased from GeneChem (Shanghai, China). The pre-miR-137 sequences were cloned into the lentiviral vector pCDH-CMV-MCS-EF1-GFP (System Biosciences, California, USA). AEG-1 siRNA sequences were subcloned into the pLKO.1 vector (Addgene, Cambridge, MA, USA). The coding sequences of AEG-1 (OriGene Technologies, Rockville, MD, USA) were cloned into pcDNA3.1 (+) to generate AEG-1 expression vector. The wild-type 3′UTR segment of AEG-1 was cloned into the *Not*I/*Xho*I sites downstream of the stop codon of Renilla luciferase in the psi-check2 vector (Promega, Madison, WI, USA). The corresponding mutant constructs were created by mutating the seed regions of the miR-137-binding sites. All constructs were verified by direct sequencing.

2.3. Lentivirus production and transduction

Virus particles were harvested 48 h after pCDH-miR-137 or pLKO.1-AEG-1 siRNA transfection with the packaging plasmid into HEK-293T cells as previously described [27,28]. OC cell lines SKOV3 and OV2008 were infected with recombinant lentivirus-transducing units plus 10 mg/ml Polybrene (Sigma–Aldrich, St. Louis, Missouri, USA). An empty lentiviral vector was used as negative control.

2.4. Western blot analysis

Proteins were extracted with RIPA buffer (PBS, 1% NP40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate) with protease inhibitors and quantified using a Bradford reagent (Bio-Rad, Hamburg, Germany). Equivalent amounts of protein were subjected to 10% SDS–PAGE separation and then electrotransferred to polyvinylidene difluoride membranes (Millipore, Schwalbach, Germany). After blocking, the membranes were immunoblotted overnight at 4 °C with anti-AEG-1 or anti-β-actin antibody (Abcam, Cambridge, MA, UK), followed by their respective horseradish peroxidase-conjugated secondary antibodies. Signals were detected using an enhanced chemiluminescence.

2.5. Quantitative RT-PCR

For qRT-PCR analysis, total RNA was extracted using TRIzol reagent (Invitrogen). miR-137 expression levels were quantified by stem-loop qRT-PCR with U6 as the internal control (GenePharm, Shanghai, China). For AEG-1, 100 ng of total RNA was reverse transcribed and quantified with a SYBR Green Real-Time PCR Master Mix kit (TaKaRa, Dalian, China); β-actin was used as an endogenous control. The relative expression of each gene was calculated and normalized using the $2^{-\Delta\Delta Ct}$ method relative to U6 or β-actin. Each assay was performed in triplicate.

2.6. Cell viability assay and cell cycle analysis

Cells (2000 per well) were plated in 96-well plates. At the completion of incubation, cell viability was assessed by MTT assay as described previously [29]. For cell cycle analysis, cells were seeded in 6-well plates. At 48 h after transfection, cells were fixed in 70% ethanol and then stained with 20 μL propidium iodide (PI). Cell cycle analysis was performed using a FACSAria cell sorting system (BD Biosciences, San Jose, CA, USA). The experiment was repeated at least three times.

2.7. Colony formation assay

The 1×10^3 cells were plated into 10 cm dish and maintained in complete medium for 2 weeks. Colonies were washed twice with PBS, fixed with methanol and stained with 0.1% crystal violet in 20% methanol. The number of colonies was counted using a clono-counter program as previously described [30].

2.8. Luciferase reporter assays

SKOV3 and OV2008 cells (3.5×10^4) were seeded in 48-well plates and allowed to settle for 24 h. Then cells were co-transfected with 100 ng of psi-check2-AEG1-3′UTR or psi-check2, plus 1 ng of

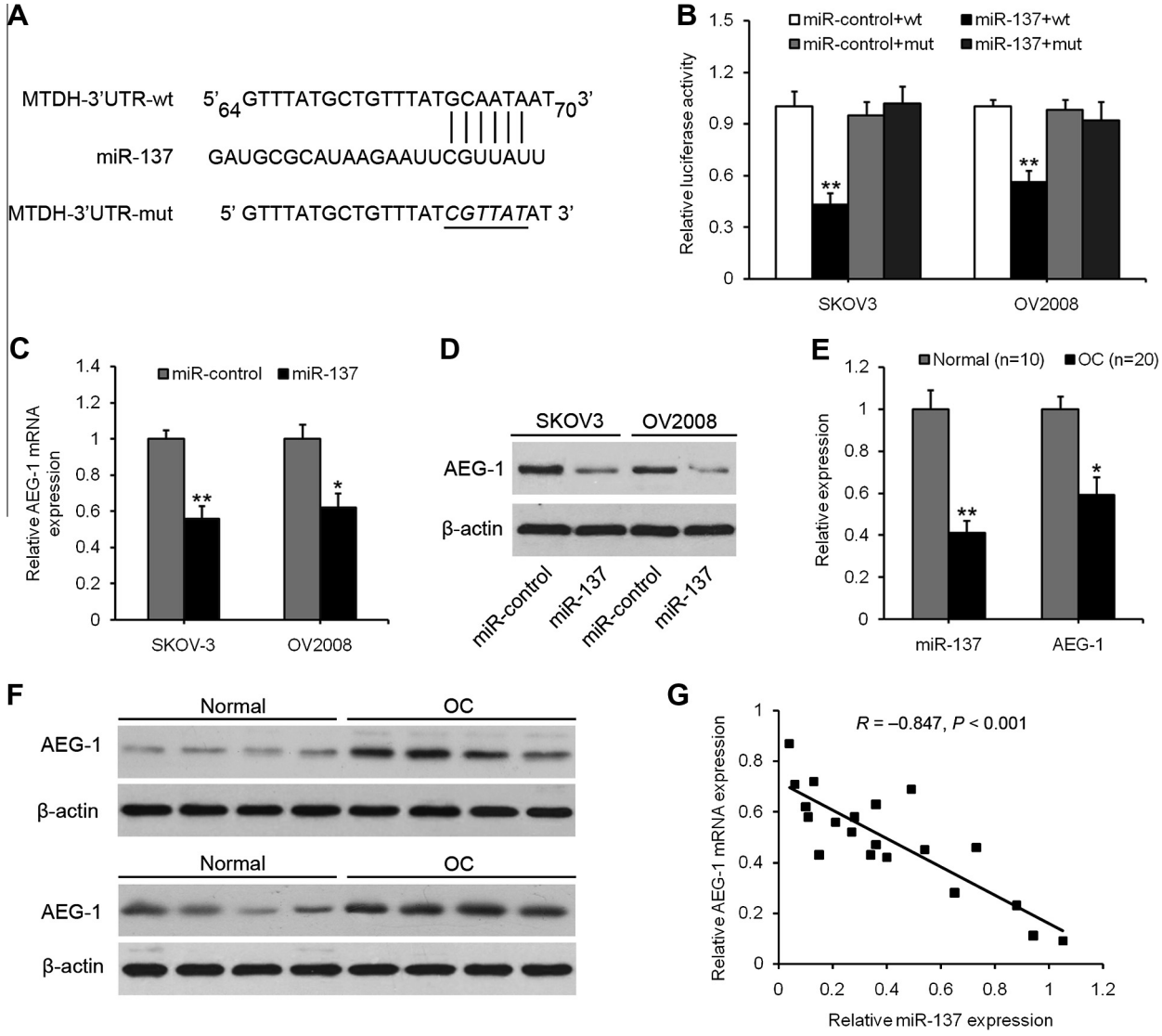

Fig. 2. AEG-1 is a target of miR-137. (A) miR-137 and its putative binding sequences in the 3′UTR of AEG-1. Mutations were generated in the complementary site that binds to the seed region of miR-137. (B) Luciferase reporter assay of the interaction between the 3′UTR of AEG-1 and miR-137 in SKOV3 and OV2008 cells. (C) qRT-PCR analysis revealed miR-137 overexpression significantly reduced AEG-1 mRNA (MTDH) expression in SKOV3 and OV2008 cells. (D) Western blot analysis revealed miR-137 overexpression significantly reduced AEG-1 expression in SKOV3 and OV2008 cells. (E) qRT-PCR analysis showed significantly lower miR-137 and higher AEG-1 mRNA expression in human OC specimens compared with normal ovaries. (F) Western blot analysis showed significantly higher AEG-1 protein expression in human OC specimens compared with normal ovaries. (G) Spearman's correlation analysis showed that miR-137 expression was inversely correlated with AEG-1 mRNA expression in OC specimens. $^*P < 0.05$, $^{**}P < 0.01$ vs. miR-control or normal group.

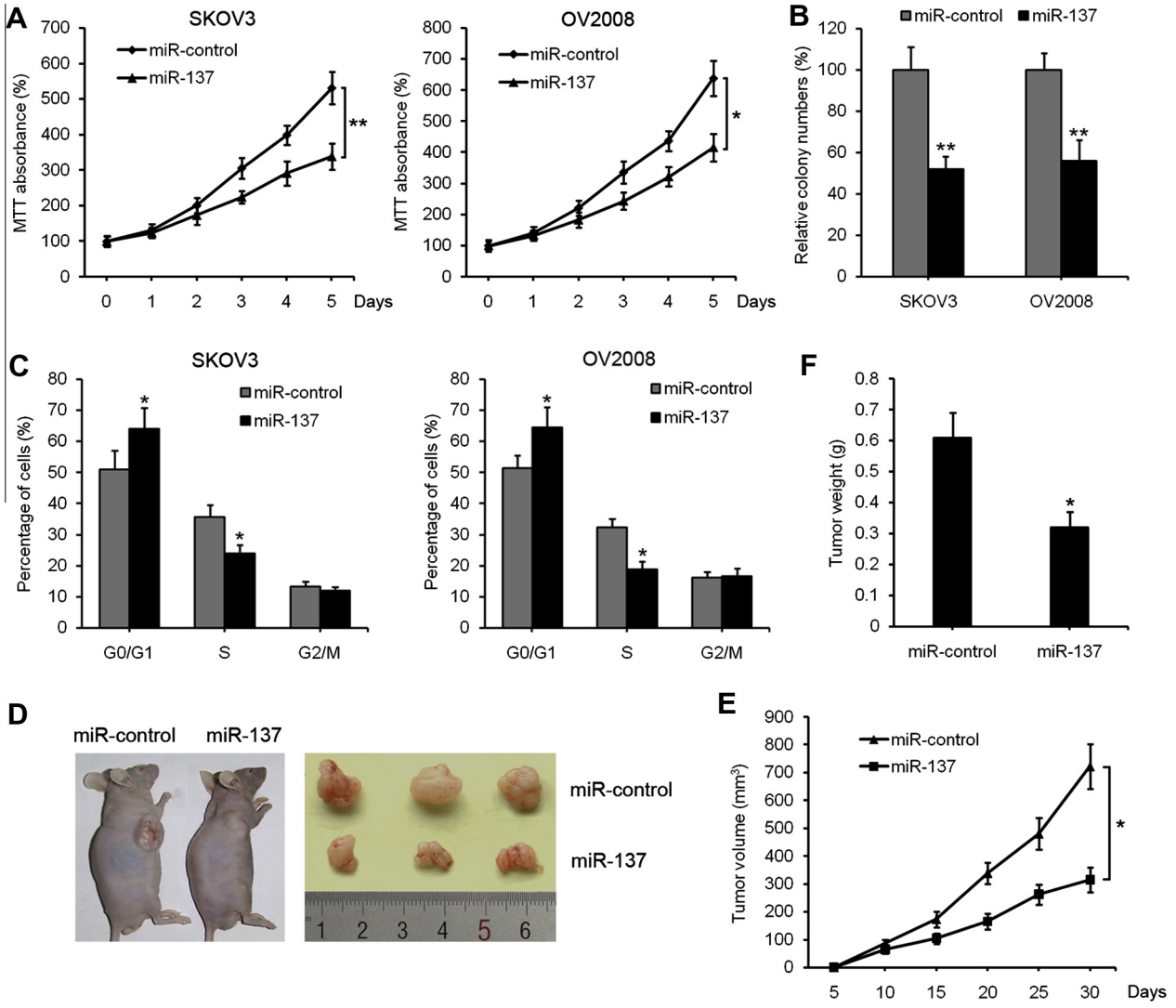

Fig. 3. miR-137 inhibits OC cell growth *in vitro* and *in vivo*. (A) MTT assays of SKOV3 and OV2008 cells stably expressing miR-137 or miR-control. (B) Colonogenic assays. (C) Cell cycle analysis. (D) miR-137 overexpressing or control SKOV3 cells were injected subcutaneously into nude mice ($n = 6$). The mice were killed and the tumors were recovered 30 days after implantation. Representative graph of tumors are shown. (E) Growth curve of tumor volumes. (F) Tumor weight. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. miR-control group.

pRL-TK plasmid (Promega, Madison, WI, USA) in the presence of either miR-137 or miR-control. Luciferase and Renilla signals were measured 48 h after transfection using the Dual-Luciferase Reporter Assay kit (Promega). The experiments were performed independently in triplicate.

2.9. Clinical specimens

A total of 30 snap-frozen normal and malignant ovarian specimens were collected at the Affiliated Tumor Hospital of Harbin Medical University (Heilongjiang, China). The tissue collection included 10 normal ovarian tissue sections and 20 malignant tissues, including 12 ovarian serous cystadenocarcinomas, 4 ovarian endometrioid carcinomas, 4 ovarian clear cell adenocarcinomas. Written informed consent was obtained from all study participants and this study was approved by the Ethical Committee of the Affiliated Tumor Hospital of Harbin Medical University.

2.10. In vivo tumorigenesis assay

2×10^6 SKOV-3 cells stably overexpressing miR-137 or miR-control were injected subcutaneously into the flanks of nude mice ($n = 6$). Tumor growth rate was monitored by measuring tumor diameters every 5 days and the tumor growth curve was recorded accordingly. Tumor volume was calculated according to the formula: volume = 0.5 × length × width^2. Thirty days after inoculation, the mice were euthanized and tumour weights were assessed. All animal procedures were performed in accordance with institutional guidelines.

2.11. Statistical analysis

All of the experiments were performed at least three times. The results were expressed as mean ± SD. The differences between groups were analyzed using two-sided Student's *t*-tests. The relationship between miR-137 and AEG-1 expression was explored by Spearman's correlation. All statistical analyses were conducted

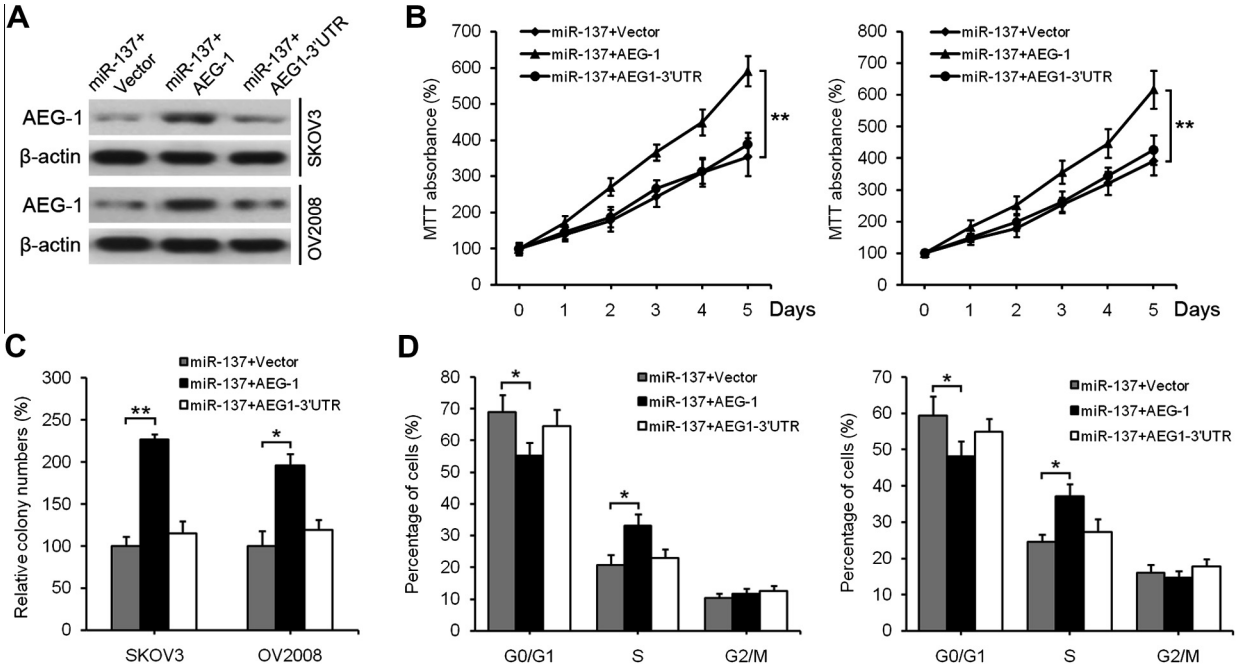

Fig. 4. Restoration of AEG-1 rescues the miR-137-induced OC cell growth inhibition. (A) AEG-1 plasmid containing either with or without 3′UTR was transfected into miR-137 overexpressing SKOV3 and OV2008 cells, and western blot was performed 48 h after transfection. (B) MTT assays. (C) Colonogenic assays. (D) Cell cycle analysis.

using SPSS 15.0 software. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Downregulation of AEG-1 inhibits OC cell growth in vitro

To explore the function of AEG-1 in OC cell growth, two OC cell lines SKOV3 and OV2008, were infected with lentiviral constructs containing AEG-1 siRNA or the negative control (Fig. 1A). We found that knockdown of AEG-1 significantly inhibited the growth of SKOV3 and OV2008 cell lines and reduced cologinic ability in these cells (Fig. 1B–D). We then sought to determine whether AEG-1 knockdown has any impact on cell cycle progression of OC cells. The cell cycle distribution of SKOV3 and OV2008 cells showed that the cell number in G0/G1 phase was significantly increased in the AEG-1 knockdown cells when compared to their corresponding controls, whereas the cell population in S phase decreased sharply (Fig. 1E and F). Together, these results indicate that downregulation of AEG-1 is able to inhibit cell growth and induce G1 phase arrest in OC cells.

3.2. miR-137 downregulates AEG-1 through interaction with its 3′UTR

Bioinformatics analysis by using TargetScan showed that AEG-1 was a potential target of miR-137 (Fig. 2A). To confirm AEG-1 as a molecule directly targeted and regulated by miR-137 in OC cells, we cloned AEG-1 3′UTR and the mutant counterpart into luciferase reporter vectors. These vectors were used to co-transfect SKOV3 and OV2008 cells. Increased expression of miR-137 upon infection, significantly suppressed the luciferase activity of AEG-1 containing a wild-type 3′UTR but did not suppress activity of AEG-1 with a mutant 3′UTR (Fig. 2B). Then, we determined the expression of AEG-1 regulated by miR-137. We found that miR-137 overexpression decreased the levels of AEG-1 mRNA and protein (Fig. 2C and D). To further determine whether miR-137-mediated repression of AEG-1 is of clinical relevance, we measured the expression of miR-137 and AEG-1 in 20 OC specimens and 10 normal ovaries. Compared with normal ovaries, OC showed lower miR-137 expression and higher AEG-1 expression (Fig. 2E and F). Furthermore, a statistically significant inverse correlation was revealed by Spearman's correlation analysis between mRNA levels of miR-137 and AEG-1 ($R = -0.847$, $P < 0.001$; Fig. 2G). Together, these results suggest that AEG-1 is a target of miR-137 in OC.

3.3. miR-137 inhibits OC cell growth in vitro and in vivo

We further evaluated the effects of miR-137 on OC cell growth. Similarly to the effect of AEG-1 knockdown, overexpression of miR-137 in SKOV3 and OV2008 cells significantly inhibited cell growth rate, reduced colony formation activity, and induced G0/G1 phase cell cycle arrest (Fig. 3A–C). To further explore the relationship between miR-137 and tumorigenesis *in vivo*, SKOV3 cells stably overexpressing miR-137 or negative control were injected subcutaneously into nude mice. Tumor volume was measured every 5 days, and mice were sacrificed 30 days after tumor cell implantation. As shown in Fig. 3D, miR-137 overexpressing tumors were significantly smaller than tumors in the control group. The average volume and weight of the miR-137 overexpressing tumors were reduced significantly (Fig. 3E and F). Together, these results indicate that miR-137 has a strong ability to suppress OC cell growth *in vitro* and *in vivo*.

3.4. Restoration of AEG-1 inhibits miR-137-mediated OC cell growth

We further investigated whether the restoration of AEG-1 could rescue the growth-suppressive effect of miR-137. As shown in Fig. 4A, overexpression of AEG-1 efficiently restored AEG-1 expression of miR-137 overxpressing SKOV3 and OV2008 cells. AEG-1 overexpression significantly increased the growth rate and colony formation activity, and rescued miR-137-induced cell cycle arrest (Fig. 4B–D). However, overexpressing AEG1–3′UTR cells had no significant impact on the antitumor effects induced by miR-137

(Fig. 4B–D). These results demonstrated that AEG-1 is a functional target of miR-137.

4. Discussion

In this study, we showed that miR-137 downregulated AEG-1 expression through interaction with its 3′UTR and that miR-137 expression was inversely correlated with AEG-1 levels in OC specimens. Overexpression of miR-137 markedly suppressed OC cell growth and clonogenicity, induced G1 arrest *in vitro* and restrained tumor growth *in vivo*. Moreover, we demonstrated that knockdown of AEG-1 recapitulated the growth-suppressive effect of miR-137, whereas restoring the AEG-1 (without 3′UTR) expression attenuated the function of miR-137 in OC cells. These findings suggest that miR-137 acts as a novel tumor suppressor in OC and that downregulated miR-137 contributes to tumor progression in OC patients.

Up-regulation of AEG-1 is a frequent event and it is involved in the development and progression of various types of cancers [7–10]. Lee and co-workers previously reported that astrocyte elevated gene-1 (AEG-1) was a downstream target molecule of Ha-ras and c-myc, mediating their tumor-promoting effects [31]. They also found that overexpression of AEG-1 enhanced proliferation and expression of the transformed state in less aggressive neuroblastoma cells through activation of the PI3K/Akt signaling pathway and stabilization of MYCN [32]. Yoo and colleagues showed that AEG-1 were up-regulated in human HCC and activated Wnt/beta-catenin signaling to regulate HCC development and progression [33]. In ovarian cancer, our team previously detected high expression of AEG-1 in OC specimens and its up-regulation was significantly associated with OC recurrence [14]. In this study, we confirmed these results and found that knockdown of AEG-1 significantly inhibited OC cell growth and clonogenicity, and induced G1 arrest. Furthermore, AEG-1 was identified as a direct and functional target of miR-137. There was a significant inverse correlation between miR-137 expression and AEG-1 mRNA levels in OC specimens. Indeed, AEG-1 could be regulated by several molecules, such as CPEB1, miR-136 and miR-375 [13,34,35]. Our results added to our understanding of how AEG-1 was elevated in OC, as well as the biological function of miR-137 in OC.

It is well known that miR-137 was downregulated in a variety of cancers [22–26]. Several studies have reported the tumor-suppressor role of miR-137 in cancers. Chen and colleagues found that miR-137 deregulation is common in glioma, and restoration of its function inhibits cell proliferation and invasion [23]. Liang et al. recently found that miR-137 suppresses colorectal cancer cell invasion and metastasis in mice by directly targeting FMNL2 [24]. Consistent with these studies, in the present study, we found that the expression of miR-137 was reduced in OC tissues compared with normal ovarian tissues. Overexpression of miR-137 significantly suppressed OC cell growth *in vitro* and tumor growth *in vivo*. Furthermore, restoration of AEG-1 in miR-137 overexpressing OC cells reversed the effects of miR-137, suggesting that the growth-suppressive effect of miR-137 was mediated by repression of AEG-1 in OC cells.

In conclusion, the present study provides novel evidence that miR-137 negatively regulates oncogene AEG-1 and suppresses OC cell growth *in vitro* and *in vivo*. Our findings may, at least partially, explain the up-regulation of AEG-1 in OC and provide novel therapeutic targets for future prevention and treatment of this disease.

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