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MiR-506 Suppresses Proliferation and Induces Senescence by Directly Targeting the CDK4/6-FOXO1 Axis in Ovarian Cancer

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Abstract

Ovarian carcinoma is the most lethal gynecological malignancy. Better understanding of the molecular pathogenesis of this disease and effective targeted therapies are needed to improve patient outcomes. MicroRNAs play important roles in cancer progression and have the potential for use as either therapeutic agents or targets. Studies in other cancers have suggested that miR-506 has antitumor activity, but its function has yet to be elucidated. We found that deregulation of miR-506 in ovarian carcinoma promotes an aggressive phenotype. Ectopic

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overexpression of miR-506 in ovarian cancer cells was sufficient to inhibit proliferation and to promote senescence. We also demonstrated that *CDK4* and *CDK6* are direct targets of miR-506, and that miR-506 can inhibit CDK4/6-FOXM1 signaling, which is activated in the majority of serous ovarian carcinomas. This newly recognized miR-506/CDK4/6-FOXM1 axis provides further insight into the pathogenesis of ovarian carcinoma and identifies a potential novel therapeutic agent.

Keywords

miR-506; ovarian carcinoma; proliferation; senescence; FOXM1

Introduction

Ovarian carcinoma is the most lethal gynecological malignancy in the United States. In 2013, an estimated 22,240 new cases were diagnosed, and 14,030 women died of the disease [1]. Ovarian cancer is frequently referred to as the “silent killer,” as the majority of cases are diagnosed at an advanced stage [2, 3]. Improved understanding of the molecular pathogenesis of this disease and effective targeted therapies are needed to improve patient outcomes [4, 5]. Alteration of microRNA (miRNA) expression has been observed in ovarian cancer, suggesting that a dysfunction of miRNA may be associated with tumorigenesis and progression of this disease [6-8].

A recent study showed that restoration of microRNA 506 (miR-506) in malignant transformed human bronchial epithelial cells led to a decrease in cell proliferation [9]. Streicher *et al.* reported that the miR-506-514 cluster plays an oncogenic role in initiating melanocyte transformation and in promoting melanoma growth [10]. The role of miR-506 in ovarian tumorigenesis and tumor progression, and the molecular mechanisms by which miR-506 exerts its effects, remain largely unknown [9-11]. Recently, through integrated genomic analysis of miRNA regulatory networks from The Cancer Genome Atlas (TCGA) data, we showed that miR-506 augmented E-cadherin expression and prevented TGFβ-induced epithelial-mesenchymal transition (EMT) by targeting *SNAIL2* in ovarian cancer [12].

Herein, we report that deregulation of miR-506 in ovarian cancer is important in the acquisition of an aggressive tumor phenotype. Ectopic overexpression of miR-506 in ovarian cancer cells was sufficient to inhibit proliferation and promote senescence. More importantly, we provide evidence that miR-506 directly targets both *CDK4* and *CDK6*. MiR-506 also inhibited signaling in CDK4/6-FOXM1, the transcription factor network activated in more than 80% of high-grade serous ovarian cancer cases [13]. Collectively, our results provide an experimental basis for investigating miR-506 as a potential therapeutic for ovarian cancer.

Materials and Methods

Cell lines, cell culture, reagents, and miRNA transfection

Ovarian cancer cell lines HeyA8, SKOV3, OVCA432, and OVCA433 and cervical cancer HeLa cells were cultivated with RPMI1640 medium supplemented with 10% fetal bovine serum. The miRNA mimic of miR-506 and negative control (miR-ctrl) were obtained from Dharmacon (Chicago, IL). The antisense miR-506 (anti-miR-506) locked nucleic acid (LNA) and negative control were obtained from EXIQON (Woburn, MA). siRNAs for *CDK4* (SASI_Hs01_00122488, 00122490) and *CDK6* (SASI_Hs01_00048792, 00048790) were obtained from Sigma (St. Louis, MO). Cells were seeded at 2×10^5 per well in 6-well plates and allowed to attach for at least 16 hours. MiR-506 mimic, anti-miR-506 LNA, and miR-ctrl were transfected using Lipofectamine RNAiMAX (Invitrogen, Grand Island, NY) at a final concentration of 20 nM. Total RNA and protein were collected 48 hours after transfection.

Real-time RT-PCR analysis and microarray gene expression analysis

Total RNA was isolated with the *miRvana* miRNA isolation kit (Ambion, Grand Island, NY). Reverse transcription was performed by using SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. TaqMan real-time polymerase chain reaction (PCR) assays for miR-506, *CDK4*, and *CDK6* were obtained from Applied Biosciences, Inc. (Grand Island, NY). Cyclophilin and β -actin were used as normalization controls. Data were analyzed by the $-2^{-\Delta\Delta C_t}$ method.

Microarray experiments were carried out using whole human genome oligoarrays with 44k 60-mer probes (Agilent Technologies, Palo Alto, CA), with 500 ng of total RNA starting material, according to the manufacturer's protocol. Hybridized arrays were scanned with Agilent's dual laser-based scanner. Feature Extraction software version 8.0 (Agilent Technologies) was used to link a feature to a design file and to determine the relative fluorescence intensities of the two samples. The microarray data are publicly available at GEO (Accession number GSE50850).

Cell cycle analysis

Forty-eight hours after transfection, cells were harvested, washed with phosphate-buffered saline solution (PBS) and fixed in 70% ethanol at 4°C overnight. After fixation, cells were washed twice with PBS before re-suspension in propidium iodide/RNase A solution (5 μ g/ml propidium iodide and 100 mg/ml RNase A). Cells were incubated with propidium iodide at room temperature in the dark for 1 hour. Stained cells were analyzed by flow cytometry for light-scattering properties and for DNA content using a FACScan flow cytometer (BD Biosciences, Mountain View, CA), and G₀₋₁, S, and G₂-M fractions were determined.

MTT assay

For cell viability assays, cells were transfected with miR-506 or miR-ctrl using Lipofectamine RNAiMAX. Twenty-four hours after transfection, cells were seeded in a 96-well plate at a density of 1×10^3 per well. After incubation for 24, 48, 72, or 96 hours at 37°C in a humidified incubator, 20 μ l of MTT (5mg/ml in PBS) was added to each well, and cells

were incubated for a further 4 hours. After removal of the medium, 150µl of dimethyl sulfoxide was added to each well. The absorbance was recorded on a microplate reader at a wave length of 540nm.

BrdU assay

Cell proliferation was assessed by a fluorescein isothiocyanate (FITC)-bromodeoxyuridine (BrdU) flow kit (BD Pharmingen, San Diego, CA). Briefly, 48 hours after transfection, cells were treated with 10 µM BrdU for 1 hour, harvested, and stained with FITC-conjugated anti-BrdU antibody and 7AAD according to the instructions of the manufacturer.

Colony-formation assay

Cells were harvested 24 hours after transfection with 20nm miR-506 or miR-ctrl. Transfected cells were seeded in a fresh 6-well plate (500 cells/well) and kept in culture undisturbed for 10-14 days, during which time the surviving cells spawned colonies of proliferating cells. Colony formation was analyzed by staining the cells with 0.1% crystal violet. The rate of colony formation was calculated with the following equation: colony formation rate = (number of colonies/number of seeded cells)×100%.

AnnexinV/propidium iodide and Apo-BrdU apoptosis assays

Cell apoptosis was assessed by annexinV/propidium iodide (Invitrogen) and by the Apo-BrdU flow kit (BD Pharmingen). Cells were fixed and treated with terminal deoxynucleotidyl transferase to expose DNA fragments, which were labeled by Br-dUTP and stained with FITC-conjugated anti-BrdU antibody according to the instructions of the manufacturer. Cell samples were then analyzed by FACS to determine proliferating or apoptotic fractions.

Western blot analysis

Primary antibody for β-actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). CDK4 and CDK6 antibodies were purchased from Cell Signaling Technology (Boston, MA). CyclinD1 and cyclinD3 antibodies were obtained from Thermo Scientific (Waltham, MA) and Sigma, respectively. Western blotting was performed by loading whole-cell lysate (30 µg) from each sample onto a 10% polyacrylamide gel for electrophoresis. The membrane was blocked in 5% non-fat milk in 1× Tris-buffered saline solution (pH 7.4) containing 0.05% Tween-20 and probed with primary antibodies at a concentration of 1:1000 (for β-actin, CDK4, CDK6, cyclinD3, and β-tubulin) or 1:200 (for cyclinD1). The secondary antibodies were used at a concentration of 1:10,000 to 1:20,000. The proteins were visualized using the SuperSignal West Pico or SuperSignal Femtochemiluminescent substrate from Pierce Chemical (Rockford, IL).

Luciferase reporter assay

The 3'-untranslated regions (3'-UTRs) of *CDK4* and *CDK6*, which contained the predicted binding sites of miR-506, were amplified separately from normal fetal genomic DNA by PCR using the following specific primers: *CDK4* forward, GGCGAGCTCTGAGCAATGGAGTGGCTGCC, *CDK4* reverse,

GGCCTCGAGAAGACCATTATTTCTTTGTTTTGTTTTTCCTG; *CDK6*-U1 forward, GCGAGCTCTCGCAGTCTCAGCTTATG, reverse, GGCCTCGAGCCTTGAATGCTGTGGATTC; and *CDK6*-U2 forward, GCGAGCTCAACTGAAGGCAGAAAGTGTTAG, reverse, GGCCTCGAGCTGTATGTGACAGTCTTCCTG. The PCR products were cloned into the pmirGLO-control vector between the *SacI* and *XhoI* sites or the *SacI* and *XbaI* sites in the correct direction. The consensus miR-506 binding sites in the 3'-UTRs of *CDK4* and *CDK6* were deleted by PCR using a QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following primers were used: *CDK4* forward, CTACAGAGATTACTTTGCAATGACATTCCCCTC; reverse, GAGGGGAATGTCATTGCAAAGTAATCTCTGTAG; *CDK6* forward 1, GTGCTTATTTCTAGTGCTCACTCCTGCTCTC, reverse 1, GAGAGCAGGAGTGAGCACTAGAAATAAGCAC; forward 2, CCTTTATATTTTCATACCATCTCTAAGTGGTTTAGTGCTCAG, reverse 2, CTGAGCACTAAACCACTTAGAGATGGTATGAAATATAAAGG; forward 3, GTAGAAAAGCATTTTAGATAATTTAGAAACTGTGCCAGGTG, reverse 3, CACCTGGCACAGTTTCTAAATTATCTAAAATGCTTTTCTAC; and forward 4, GAACTGTCAGCTTGAGGCTTCCAGAACG, reverse 4, CGTTCTGGAAGCCTCAAGCTGACAGTTC. All clones were verified by DNA sequencing.

For the luciferase reporter assay, subconfluent HeLa cells in 24-well plates were transfected with a triplicate repeat of pmirGLO reporter plasmid (0.5 µg) wild-type or mutant 3'-UTR construct, miR-506 mimic or miR-ctrl (50 nM), and Lipofectamine 2000 (2 µl) (Invitrogen). Forty-eight hours after transfection, cells were harvested, and luciferase activities of lysates were determined using a dual luciferase assay reporter system (Promega, Fitchburg, WI) according to the manufacturer's instructions.

Senescence assay

Senescence assays were performed according to the manufacturer's protocol for the SA-β-Galstaining kit (Cell Signaling Technology). Forty-eight hours after transfection of miR-506 or miR-ctrl, cells were rinsed with PBS, fixed for 15 minutes, and stained with β-Gal overnight. Blue color indicated β-Gal-positive cells. The β-Gal-positive rate was calculated through a microscope (200× magnification).

Immunohistochemical staining

Immunohistochemical staining was performed on the tumor tissues from the mouse orthotopic model and tissue microarrays that included samples from 92 patients with serous ovarian cancer, both of which were assembled for our previous study [12]. Rabbit anti-human monoclonal antibody against Ki-67 (1:1500, ab15580; Abcam, Cambridge, MA), rabbit anti-human polyclonal antibody against CDK4 (1:250, c-22; Santa Cruz Biotechnology), rabbit anti-human polyclonal antibody against CDK6 (1:100, ab-13; Sigma), rabbit anti-human polyclonal antibody against FOXM1 (1:400, c-20; Santa Cruz Biotechnology), and EnVision+ System-HRP rabbit (Dako, Carpinteria, CA) were used. Ki-67-, CDK4-, and CDK6-positive cells were defined as those with brown staining in the

nucleus, and the expression of these proteins was evaluated using the percentage of positive tumor cells in 1000 tumor cells. FOXM1-positive cells were defined as those with immunoreactivity in both cytoplasm and nucleus and were quantified using a scoring system from 0 to 9, multiplied by intensity of signal, and classified by percentage of positive cells (0 = no signal, 1 = weak signal, 2 = intermediate signal, and 3 = strong signal; percentage: 0 = 0%, 1 = <25%, 2 = 25%-50%, and 3 = >50%). miRNA *in situ* hybridization and RT-PCR had been performed in our previous study, and low and high miR-506 expression were defined as scores of <6 and ≥6, respectively [12].

Statistical analysis

Data are expressed as mean ± standard deviation (SD) of three independent experiments. All analyses were performed using GraphPad Prism version 6 software for Windows, and $P < 0.05$ by either the Student *t*-test or two-way ANOVA was considered to be statistically significant.

Results

MiR-506 directly targeted *CDK4* and *CDK6* expression through their 3'-untranslated regions

To identify genes potentially regulated by miR-506, microarray analysis was performed on HeyA8 ovarian cancer cells transfected with either miR-506 mimic (miR-506) or a scrambled negative miRNA control (miR-ctrl). Analysis revealed that *CDK4* and *CDK6* mRNA were decreased by miR-506 overexpression (Figure 1A). This result was confirmed with western blotting, which showed that miR-506 overexpression decreased CDK4 and CDK6 protein levels (Figure 1D). Conversely, HeyA8, SKOV3, and OVCA433 ovarian cancer cells transfected with miR-506 antisense oligonucleotides (anti-miR-506 LNA) had higher levels of CDK4 and CDK6 proteins than cells transfected with anti-miR-ctrl (Figure 1E).

The miRNA target prediction algorithm TargetScan 6.0 predicted that the 3'-UTRs of *CDK4* and *CDK6* mRNA contain putative miR-506 binding sites (Figure 1F). The potential binding site and the flanking sequences of CDK4 are highly conserved across mammals (Supplementary Figure 1). To determine whether miR-506 regulates *CDK4* and *CDK6* through binding to their 3'-UTRs, we cloned the *CDK4* and *CDK6* 3'-UTRs into the pmirGLO luciferase reporter vector and transfected one of these pmirGLO vectors or the parent luciferase expression vector, with miR-506 mimic or miR-ctrl, into HeLa cells. Co-transfection of pmirGLO-*CDK4*-3'-UTR or -*CDK6*-3'-UTR-F1 and miR-506 mimic resulted in lower luciferase activity than in cells co-transfected with miR-ctrl (Figure 1B,C), suggesting that miR-506 directly targets these two genes.

To confirm that miR-506 specifically regulates these two genes, we generated the constructs pmirGLO-*CDK4*-3'-UTR-mutant and -*CDK6*-3'-UTR-mutant in which the miR-506 binding site sequence on the 3'-UTR was deleted. These constructs were then co-transfected with miR-506 mimic or miR-ctrl into HeLa cells. Deletion of the miR-506 binding site from the 3'-UTRs of these two genes (*CDK4* and *CDK6*-F1) abolished the effects of miR-506 on

luciferase activity. Taken together, these results provide evidence that miR-506 specifically targets the 3'-UTR regions of *CDK4* and *CDK6* and thus inhibits the expression of these genes.

MiR-506 inhibited viability of ovarian cancer cells

To explore the effect of miR-506 on cell viability, an MTT cell assay was used. HeyA8 and SKOV3 cells were transiently transfected with miR-506 mimic, miR-ctrl/anti-miR506, or anti-miR-ctrl. At 96 hours after transfection, as shown in Figure 2A, miR-506 had inhibited HeyA8 cell viability by 35% ($P<0.01$) and SKOV3 viability by 40% ($P<0.01$) compared to miR-ctrl, whereas anti-miR506 had increased HeyA8 cell viability by 155% ($P<0.01$) and SKOV3 viability by 81% ($P<0.01$) compared to anti-miR-ctrl (Supplementary Figure 2).

The effect of miR-506 on proliferation was further evaluated by BrdU proliferation assay. As shown in Figure 2B, miR-506 mimic significantly decreased the BrdU-positive rate in both HeyA8 and SKOV3 cells. Consistently with that finding, anti-miR506 LNA increased the BrdU-positive rate (Supplementary Figure 3). Furthermore, miR-506 dramatically impaired the colony-forming capacity of ovarian cancer cells (Figure 2C). The colony-formation capacity of miR-506-transfected cells was much lower than that of cells transfected with miR-ctrl in several cell lines, and cells transfected with anti-miR-506 displayed greater colony-formation capacity than cells transfected with anti-miR-ctrl.

Because CDK4 and CDK6 play important roles in $G_{0/1}$ -S phase transition by associating with the D-type cyclins, we examined the cell cycle distribution of cells overexpressing miR-506. Both HeyA8 and SKOV3 cells transfected with miR-506 displayed a greater percentage of cells in $G_{0/1}$ phase and fewer cells in S phase than their counterparts transfected with miR-ctrl (Figure 3A). The same phenomenon was observed in other ovarian cancer cells (Supplementary Figure 4). Conversely, anti-miR506 transfection led to mild decreases (nonsignificant) in the percentage of $G_{0/1}$ phase cells. These results suggest that the growth-suppressive effect of miR-506 is at least partly due to a $G_{0/1}$ phase arrest.

To examine whether the growth-inhibitory effect of miR-506 involved apoptosis, we performed Apo-BrdU and annexinV apoptosis assays after transfection of miR-506 or miR-ctrl. HeyA8 cells displayed a mild but nonsignificant increase in apoptosis rates 48 hours after transfection of miR-506 compared to miR-ctrl ($P>0.05$; Figure 3 B,C). These results suggest that apoptosis was not a key factor in the miR-506-mediated decrease in ovarian cancer cell growth.

MiR-506 inhibited CDK4/6-FOXM1 signaling and promoted senescence

The effects of miR-506 on levels of cyclinD1, cyclinD3, and forkhead box M1 (FOXM1), key CDK4/6 substrates, were then examined. Both HeyA8 and SKOV3 cells transfected with miR-506 had lower FOXM1 protein levels, but no significantly different cyclinD1 or cyclinD3 levels, than cells transfected with miR-ctrl (Figure 3D). Because recent studies showed that CDK4/6-FOXM1 signaling mediated suppression of senescence, the activity of Senescence-associated beta-galactosidase (SA- β -Gal), a marker of senescence, was examined in cells transfected with miR-506. As expected, both HeyA8 and SKOV3 cells

transfected with miR-506 displayed a significantly greater percentage of SA- β -Gal-positive cells than controls (from 7.79% to 54.84% and from 1.06% to 27.37%, respectively, $P < 0.01$; Figure 3E).

CDK4/6 contributed to the growth suppression and cell senescence induced by miR-506

To elucidate whether the growth-suppressive and senescence-inducing effects of miR-506 were mediated by repression of CDK4 and CDK6 in ovarian cancer cells, gain-of-function and loss-of-function studies were performed. First, *CDK4* or *CDK6* were silenced to investigate whether reduced expression of CDK4 or CDK6 could mimic the suppressive effect of miR-506. HeyA8 cells were transfected with siRNAs for *CDK4* and *CDK6* singly or in combination. As shown in Figure 4, knockdown of CDK4, CDK6, or both led to significant cell growth inhibition, similar to that induced by miR-506. Knockdown of CDK6 or both CDK4 and CDK6 also led to cell cycle arrest and increased senescence like those observed with miR-506 overexpression. The levels of FOXM1 were also examined in these cells, and the results showed that knockdown of both CDK4 and CDK6 was required for downregulation of FOXM1.

We then studied whether ectopic expression of *CDK4* or *CDK6* could rescue the suppressive effects of miR-506. HeyA8 cells were transfected with *CDK4* or *CDK6* for 48 hours and then with miR-506 or miR-ctrl altered to encode the full-length sequence without the 3'-UTR region. Overexpression of CDK4 or CDK6 could partially rescue the downregulation of FOXM1 (Figure 4E). Our results showed that ectopic expression of *CDK4* or *CDK6* could partially rescue miR-506-induced cell growth suppression and senescence (Figure 4F,H).

Systemically delivered miR-506 inhibited CDK4 and CDK6 expression and reduced proliferation *in vivo*

In our previous study, miR-506-transfected SKOV3 cells showed epithelial cell features including increased E-cadherin expression. Delivery of miR-506 incorporated in DOPC nanoliposomes (miR-506-DOPC) resulted in significant reductions in numbers of tumor nodules and tumor weights in orthotopic mouse models of SKOV3-IP1 and HeyA8-IP1 ovarian tumors; immunohistochemical analysis of the tumors showed significant suppression of SNAI2 and vimentin and induction of E-cadherin [12]. For the studies reported here, immunohistochemical staining for CDK4, CDK6, and Ki-67 was performed on the tumor tissues collected from these mice. Concordant with our *in vitro* findings, miR-506 treatment led to significantly decreased CDK4 and CDK6 expression ($P < 0.05$ and $P < 0.05$ respectively). Moreover, the number of Ki-67-positive cells was also significantly lower than among controls ($P < 0.01$, $P < 0.01$, respectively, Figure 5).

High miR-506 expression was associated with decreased CDK4/CDK6-FOXM1 signaling and lower proliferation in human serous ovarian carcinoma

To investigate the modulation by miR-506 of the key CDK4/CDK6-FOXM1 signaling proteins in human ovarian cancer tissue, microarrays of tissues from 92 patients with ovarian cancer that we developed for a previous study were subjected to immunohistochemical staining for CDK4, CDK6, FOXM1, and Ki-67 and their correlations

with miR-506 expression were analyzed (determined by RT-PCR and miRNA *in situ* hybridization) [12]. As shown in Figure 6, miR-506 expression was inversely correlated with CDK4, CDK6, and FOXM1 protein expression in the 92 cases of serous ovarian cancer ($P < 0.05$ for all), which was consistent with our *in vitro* and animal model results. Furthermore, high miR-506 expression was significantly associated with low Ki-67 expression ($P < 0.05$). Altogether, our results from *in vitro*, *in vivo*, and human tumor samples demonstrate that miR-506 inhibited the CDK4/CDK6-FOXM1 signaling pathway and consequently decreased cell proliferation in ovarian cancer.

Discussion

We recently reported that miR-506 functions as a tumor suppressor in serous ovarian cancer. Decrease of miR-506 expression by methylation was observed in patients with serous ovarian cancer who had shorter survival, an observation that has been validated in multiple datasets (Bentink dataset and Bagnoli dataset) [12]. Nanoparticle delivery of miR-506 significantly blocked tumor nodule development and growth in an orthotopic mouse model [12, 14], and the antitumor effect of miR-506 was partially attributed to its capacity to inhibit the EMT. In this study, we demonstrate that miR-506 plays a substantial role as a tumor suppressor in ovarian cancer, and that this miRNA has multiple functions, including inhibiting proliferation and promoting senescence, in addition to inhibiting the EMT (Figure 7). During cancer promotion and progression, abrogation of senescence is accompanied by the EMT, illustrating that these two biological processes cross paths in cancer development [15, 16].

Our mechanistic investigations revealed that miR-506 regulates cell proliferation and senescence by directly targeting its binding sites on the 3'-UTRs of *CDK4* and *CDK6*. CDK4/6 plays an important role in G₀/S phase transition by associating with the D-type cyclins and phosphorylating the retinoblastoma protein (Rb) [17]. Deregulation of the CDK4/6–cyclinD signaling pathway is among the most common aberrations found in human cancer, including ovarian cancer [18–20]. Alteration of the p16–cyclinD1/CDK4–pRb pathway (i.e., the Rb pathway) has been reported to be a significant and independent indicator of poor overall survival in ovarian cancer [21]. CDK4/6 has been considered a desirable target for cancer therapies [22]. The CDK4/6 inhibitor PD-0332991 is currently undergoing clinical testing in several cancer types [23–25].

CDK4/6 has been reported to stabilize and activate the transcription factor FOXM1, whose network is activated in 84% of serous ovarian cancer cases (TCGA data) [13]. CDK4/6–FOXM1 signaling may suppress levels of reactive oxygen species and protect cancer cells from senescence [26, 27]. Senescence is a tumor-suppressive mechanism and must be overcome during immortalization and transformation [28, 29]. Our results demonstrate that miR-506 caused little to no increase in ovarian cancer cell apoptosis. However, miR-506 induced a dramatic increase in cellular senescence, and FOXM1 protein levels were decreased following miR-506 overexpression.

Emerging evidence from *in vitro* and *in vivo* studies has implicated the oncogenic transcription factor FOXM1 in cell migration, invasion, angiogenesis, and

metastasis[30-32], which affirms its significance for therapeutic intervention [33]. FOXM1 has been shown to be an important regulator of EMT. Specifically, FOXM1 promoted EMT in breast cancer cells by stimulating SNAI2 [34]. FOXM1 was involved in EMT induced by TGF β 1 [35, 36]. H292-FoxM1 cells expressed lower levels of E-cadherin and higher levels of vimentin and N-cadherin [37]. This finding that miR-506 can repress CDK4/6-FOXM1 signaling provides a rationale for treatment of ovarian cancer with miR-506.

Taken together, our findings shed light, for the first time, on the mechanism of miR-506 as a tumor-suppressive miRNA in human ovarian cancer, revealing that this suppression is mediated, at least partly, through repression of CDK4/6-FOXM1 signaling. Our data provide evidence that re-introduction of miR-506 into tumor cells may be an effective therapeutic strategy, reducing the expression of oncogenic target genes. Although miRNA-based therapeutics are still in their infancy [14], our findings on miR-506 are encouraging and suggest that this miRNA is a potential target for the treatment of ovarian cancer. This finding is consistent with those reported in malignant transformed human bronchial epithelial cells [9]. In another study, however, the miR-506-514 cluster was shown to promote cell growth in melanoma [10]. These controversial results suggest that the role of miR-506 may be tumor specific and highly dependent on its targets in different cancer cells. Nevertheless, miR-506 emerges as a strong ovarian cancer-suppressing agent that blocks two clinically important cancer hallmarks, EMT and cell proliferation, while enhancing cell senescence.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin.* 2013; 63:11–30. [PubMed: 23335087]
2. Heintz AP, Odicino F, Maisonneuve P, et al. Carcinoma of the ovary. FIGO 26th Annual Report on the Results of Treatment in Gynecological Cancer. *Int J Gynaecol Obstet.* 2006; 95(Suppl 1):S161–192. [PubMed: 17161157]
3. Clarke-Pearson DL. Clinical practice. Screening for ovarian cancer. *N Engl J Med.* 2009; 361:170–177. [PubMed: 19587342]
4. Coleman MP, Forman D, Bryant H, et al. Cancer survival in Australia, Canada, Denmark, Norway, Sweden, and the UK, 1995–2007 (the International Cancer Benchmarking Partnership): an analysis of population-based cancer registry data. *Lancet.* 2011; 377:127–138. [PubMed: 21183212]

5. Dinh P, Harnett P, Piccart-Gebhart MJ, et al. New therapies for ovarian cancer: cytotoxics and molecularly targeted agents. *Crit Rev Oncol Hematol*. 2008; 67:103–112. [PubMed: 18342536]
6. Wan SM, Lv F, Guan T. Identification of genes and microRNAs involved in ovarian carcinogenesis. *Asian Pac J Cancer Prev*. 2012; 13:3997–4000. [PubMed: 23098506]
7. Vaksman O, Stavnes HT, Kaern J, et al. miRNA profiling along tumour progression in ovarian carcinoma. *J Cell Mol Med*. 2011; 15:1593–1602. [PubMed: 20716115]
8. Mezzanzanica D, Bagnoli M, De Cecco L, et al. Role of microRNAs in ovarian cancer pathogenesis and potential clinical implications. *Int J Biochem Cell Biol*. 2010; 42:1262–1272. [PubMed: 20035894]
9. Zhao Y, Liu H, Li Y, et al. The role of miR-506 in transformed 16HBE cells induced by anti-benzo[a]pyrene-trans-7,8-dihydrodiol-9,10-epoxide. *Toxicol Lett*. 2011; 205:320–326. [PubMed: 21726609]
10. Streicher KL, Zhu W, Lehmann KP, et al. A novel oncogenic role for the miRNA-506-514 cluster in initiating melanocyte transformation and promoting melanoma growth. *Oncogene*. 2012; 31:1558–1570. [PubMed: 21860416]
11. Tong JL, Zhang CP, Nie F, et al. MicroRNA 506 regulates expression of PPAR alpha in hydroxycamptothecin-resistant human colon cancer cells. *FEBS Lett*. 2011; 585:3560–3568. [PubMed: 22036718]
12. Yang D, Sun Y, Hu L, et al. Integrated analyses identify a master microRNA regulatory network for the mesenchymal subtype in serous ovarian cancer. *Cancer Cell*. 2013; 23:186–199. [PubMed: 23410973]
13. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011; 474:609–615. [PubMed: 21720365]
14. Rupaimoole R, Han HD, Lopez-Berestein G, et al. MicroRNA therapeutics: principles, expectations, and challenges. *Chin J Cancer*. 2011; 30:368–370. [PubMed: 21627858]
15. Ansieau S, Morel AP, Hinkal G, et al. TWISTing an embryonic transcription factor into an oncoprotein. *Oncogene*. 2010; 29:3173–3184. [PubMed: 20383196]
16. Smit MA, Peeper DS. Epithelial-mesenchymal transition and senescence: two cancer-related processes are crossing paths. *Aging (Albany NY)*. 2010; 2:735–741. [PubMed: 20975209]
17. Blain SW. Switching cyclin D-Cdk4 kinase activity on and off. *Cell Cycle*. 2008; 7:892–898. [PubMed: 18414028]
18. Nam EJ, Kim YT. Alteration of cell-cycle regulation in epithelial ovarian cancer. *Int J Gynecol Cancer*. 2008; 18:1169–1182. [PubMed: 18298566]
19. Shapiro GI. Cyclin-dependent kinase pathways as targets for cancer treatment. *J Clin Oncol*. 2006; 24:1770–1783. [PubMed: 16603719]
20. Yu Q, Sicinska E, Geng Y, et al. Requirement for CDK4 kinase function in breast cancer. *Cancer Cell*. 2006; 9:23–32. [PubMed: 16413469]
21. Hashiguchi Y, Tsuda H, Inoue T, et al. Alteration of cell cycle regulators correlates with survival in epithelial ovarian cancer patients. *Hum Pathol*. 2004; 35:165–175. [PubMed: 14991533]
22. Graf F, Mosch B, Koehler L, et al. Cyclin-dependent kinase 4/6 (cdk4/6) inhibitors: perspectives in cancer therapy and imaging. *Mini Rev Med Chem*. 2010; 10:527–539. [PubMed: 20370706]
23. Leonard JP, LaCasce AS, Smith MR, et al. Selective CDK4/6 inhibition with tumor responses by PD0332991 in patients with mantle cell lymphoma. *Blood*. 2012; 119:4597–4607. [PubMed: 22383795]
24. Dickson MA, Tap WD, Keohan ML, et al. Phase II trial of the CDK4 inhibitor PD0332991 in patients with advanced CDK4-amplified well-differentiated or dedifferentiated liposarcoma. *J Clin Oncol*. 2013; 31:2024–2028. [PubMed: 23569312]
25. Flaherty KT, Lorusso PM, Demichele A, et al. Phase I, dose-escalation trial of the oral cyclin-dependent kinase 4/6 inhibitor PD 0332991, administered using a 21-day schedule in patients with advanced cancer. *Clin Cancer Res*. 2012; 18:568–576. [PubMed: 22090362]
26. Anders L, Ke N, Hydbring P, et al. A systematic screen for CDK4/6 substrates links FOXM1 phosphorylation to senescence suppression in cancer cells. *Cancer Cell*. 2011; 20:620–634. [PubMed: 22094256]

27. Ruas M, Gregory F, Jones R, et al. CDK4 and CDK6 delay senescence by kinase-dependent and p16INK4a-independent mechanisms. *Mol Cell Biol.* 2007; 27:4273–4282. [PubMed: 17420273]
28. Chandler H, Peters G. Stressing the cell cycle in senescence and aging. *Curr Opin Cell Biol.* 2013
29. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol.* 2007; 8:729–740. [PubMed: 17667954]
30. Halasi M, Gartel AL. Targeting FOXM1 in cancer. *Biochem Pharmacol.* 2013; 85:644–652. [PubMed: 23103567]
31. Raychaudhuri P, Park HJ. FoxM1: a master regulator of tumor metastasis. *Cancer Res.* 2011; 71:4329–4333. [PubMed: 21712406]
32. Gomes AR, Zhao F, Lam EW. Role and regulation of the forkhead transcription factors FOXO3a and FOXM1 in carcinogenesis and drug resistance. *Chin J Cancer.* 2013; 32:365–370. [PubMed: 23706767]
33. Koo CY, Muir KW, Lam EW. FOXM1: From cancer initiation to progression and treatment. *Biochim Biophys Acta.* 2012; 1819:28–37. [PubMed: 21978825]
34. Yang C, Chen H, Tan G, et al. FOXM1 promotes the epithelial to mesenchymal transition by stimulating the transcription of Slug in human breast cancer. *Cancer Lett.* 2013; 340:104–112. [PubMed: 23856032]
35. Ke Y, Zhao W, Xiong J, et al. miR-149 inhibits non-small-cell lung cancer Cells EMT by targeting FOXM1. *Biochem Res Int.* 2013; 2013:506731. [PubMed: 23762558]
36. Balli D, Ustiyani V, Zhang Y, et al. Foxm1 transcription factor is required for lung fibrosis and epithelial-to-mesenchymal transition. *EMBO J.* 2013; 32:231–244. [PubMed: 23288041]
37. Xu N, Jia D, Chen W, et al. FoxM1 is associated with poor prognosis of non-small cell lung cancer patients through promoting tumor metastasis. *PLoS One.* 2013; 8:e59412. [PubMed: 23536876]

MiR-506 suppresses proliferation and induces senescence by directly targeting the CDK4/6–FOXM1 axis in ovarian cancer

Figure 1. CDK4 and CDK6 were directly targeted by miR-506

A. Microarray analysis demonstrated that *CDK4* and *CDK6* mRNA levels decreased after transfection of miR-506 mimic in HeyA8 cells.

B,C. Luciferase reporter assay showed that CDK4 and CDK6 were direct targets of miR-506. HeLa cells were co-transfected with CDK4-(B) or CDK6-3'-UTR- (C) luciferase reporter, wild-type (wt) or mutant (mt), and miR-ctrl or miR-506 mimic for 48 hours before analysis. Firefly luciferase activity of the reporter was normalized to the internal Renilla luciferase activity. The means $\pm$ SD of three independent experiments are shown; * $P < 0.05$.

D,E. Western blot analysis showed that miR-506 downregulated the levels of CDK4 and CDK6 proteins in three ovarian cancer cell lines, while anti-miR-506 upregulated CDK4/6.

F. MiR-506 binding sites were predicted in the 3'-UTRs of CDK4 and CDK6 by TargetScan.

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Figure 2. Forced expression of miR-506 induced growth inhibition in ovarian cancer cells

A. MTT assay results showed that miR-506 suppressed viability of HeyA8 and SKOV3 cells after transfection of miR-506 or miR-ctrl. The means $\pm$ SD of three independent experiments are shown.

B. BrdU assay confirmed that miR-506 suppressed proliferation of HeyA8 and SKOV3 cells. BrdU-positive rates were evaluated after transfection of miR-506 or miR-ctrl. The means $\pm$ SD of three independent experiments are shown. ** $P < 0.01$.

C. Colony-forming capacity of various ovarian cancer cell lines was evaluated; values are reported as the ratios between cells transfected with miR-506 or miR-ctrl or with anti-miR-506 or anti-miR-ctrl. * $P < 0.05$, ** $P < 0.01$.

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Figure 3. Forced expression of miR-506 induced G₀/1 phase arrest and senescence and inhibited CDK4/6-FOXM1 signaling in ovarian cancer cells

A. miR-506 induced G₀/1 phase arrest in ovarian cancer cells. Cell cycle distributions of HeyA8 and SKOV3 cells were measured after transfection with miR-506 or anti-miR-506 for 48 hours. * P<0.05.

B, C. miR-506 did not significantly increase apoptosis rate in ovarian cancer cells. Shown are Apo-BrdU assay and annexin V assay results for apoptosis in HeyA8 cells 48 hours after transfection with miR-506 or miR-ctrl. Three independent experiments were performed.

D. Western blot result showed that miR-506 inhibited the CDK4/CDK6-FOXM1 signaling pathway in HeyA8 and SKOV3 cells.

E. The SA-β-Gal staining results demonstrated that miR-506 promoted senescence in HeyA8 and SKOV3 cells. Scale bar represents 50μm. **P<0.01.

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Figure 4. CDK4 and CDK6 were involved in miR-506-induced inhibition of proliferation, senescence, and FOXM1 signaling

A-D. HeyA8 cells were transfected with si-ctrl or with si-CDK4, si-CDK6, or both (siRNAs for CDK4 (SASI_Hs01_00122488, 00122490) and CDK6 (SASI_Hs01_00048792, 00048790) were obtained from Sigma). Forty-eight hours later, protein level (A), cell growth rate (B), percentage of cells in G₀/1 (C), and senescence (D) were measured by western blot, MTT assay, flow cytometry, or senescence assay, respectively. *P<0.05, **P<0.01.

E-H. HeyA8 cells were co-transfected with CDK4, CDK6, or empty vector (EV) together with miR-506 or miR-ctrl. Forty-eight hours later, protein level (E), cell growth rate (F), percentage of cells in G₀/1 phase (G), and senescence (H) were measured by western blot, MTT assay, flow cytometry, or senescence assay, respectively. *P<0.05, **P<0.01.

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Figure 5. miR-506 inhibited proliferation and was inversely related to CDK4,CDK6, and Ki-67 expression in an orthotopic mouse models of ovarian cancer

A. Samples of HeyA8-ip1 and SKOV3-ip1 tumors from control and miR-506–treated mice were subjected to hematoxylin and eosin and immunohistochemical staining for Ki-67, CDK4, and CDK6. Scale bar represents 100 μ m.

B. Expression of Ki-67, CDK4, and CDK6 proteins was calculated as immunohistochemical staining scores. Error bars represent standard error; ** $P < 0.01$, * $P < 0.05$.

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Figure 6. miR-506 expression was inversely associated with CDK4/CDK6-FOXM1 signaling and with proliferation in serous ovarian cancers from human patients

A. Representative images of immunohistochemical staining for Ki-67, CDK4, CDK6, and FOXM1 in cases expressing low or high levels of miR-506 are shown. Scale bar represents 100 μ m.

B. Bar charts show the association between miR-506 expression and expression of Ki-67, CDK4, CDK6, and FOXM1. The x-axes represent relative expression of Ki-67, CDK4, CDK6, and FOXM1 as indicated by immunohistochemical staining (IHC); *P<0.05.

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Figure 7. miR-506 has dual roles in cellular senescence and epithelial differentiation

Results from this study and the literature support a central role for miR-506 in two cross-talking processes important for cancer. miR-506 directly targets the CDK4/6-FOXM1 axis in promoting cellular senescence, as represented by SA-β-Gal positivity (blue cells). miR-506 inhibits EMT by directly targeting SNAI2, resulting in augmented expression of E-cadherin protein as represented by the cobblestone morphology of epithelial cells (green color) and blockage of invasive mesenchymal cells (red color). Decreased expression of miR-506 can be a result of promoter methylation[12].