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Original Article

MiR-191 inhibits TNF-α induced apoptosis of ovarian endometriosis and endometrioid carcinoma cells by targeting DAPK1

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Abstract: Emerging evidence showed that miRNA dysregulation is involved in the development of endometriosis and may contribute to pathological process of endometriosis associated ovarian cancer (EAOC). miR-191 is one of the most differentially expressed miRNAs in pairwise comparisons among healthy controls, endometriosis, and EAOC patients. However, its regulative network in endometriosis and EAOC are still not clear. This study explored the role of miR-191 in TNF-α induced cell death in ovarian endometriosis and endometrioid carcinoma cells. Based on tissues samples collected from healthy controls, endometriosis, and EAOC patients, this study verified significantly higher expression of miR-191 in endometriosis and endometrioid cancer. Interestingly, we also observed inverse expression trend between miR-191 and DAPK1, a positive mediator of programmed cell death. By conducting luciferase assay, we confirmed miR-191 can directly target DAPK1 and regulate its expression. Functionally, we also found DAPK1 can promote TNF-α induced cell death. DAPK1 knockdown in endometriosis CRL-7566 cells can weaken its response to TNF-α induced cell death, while its overexpression in endometrioid cancer cells CRL-11731 enhanced the response. These functions of DAPK1 can be directly modulated by miR-191. Therefore, the miR-191-DAPK1 axis may play an important role modulating the response of ovarian endometriosis and endometrioid carcinoma cells to death-inducers and might contribute malignant transformation of endometriosis.

Keywords: miR-191, DAPK1, endometriosis, EAOC

Introduction

Endometriosis is one common gynecologic disease characterized as proliferation of endometrial glands and stroma at ectopic sites. The occurrence rate of this disease is around 3% to 15% in premenopausal women [1, 2]. One recent large multinational study showed that patients with endometriosis have about 50% higher risk of some histological subtypes of ovarian cancers, such as clear cell carcinoma, endometrioid carcinoma, and low-grade serous carcinoma [3]. Another recent meta-analysis also demonstrated that endometriosis is strongly associated with increased risk of ovarian endometrioid and clear cell carcinoma [4]. However, although the association is well recognized, the underlying mechanism is still not clearly understood.

MicroRNAs (miRNA) are a group of single-stranded, noncoding, small RNA that regulate gene expression by repressing translation or facilitating degradation of the target mRNA [5]. Emerging evidence showed that miRNA dysregulation is involved in the development of endometriosis and may contribute to pathological progression of endometriosis associated ovarian cancer (EAOC) [6, 7]. For example, miRNA-199a-5p can regulate the expression of VEGFA in endometrial mesenchymal stem cells and thereby affect angiogenesis during endometriosis [8]. The loss of miR-125a/b activity contributes to elevated ERBB2 levels in EAOC, but not in non-EAOC [9, 10]. In addition, multiple dysregulated miRNAs, such as miR-21, miR-26a and miR-214 are involved in the deletion of tumor suppressor gene PTEN and activation of oncogene KRAS, which play critical roles in the

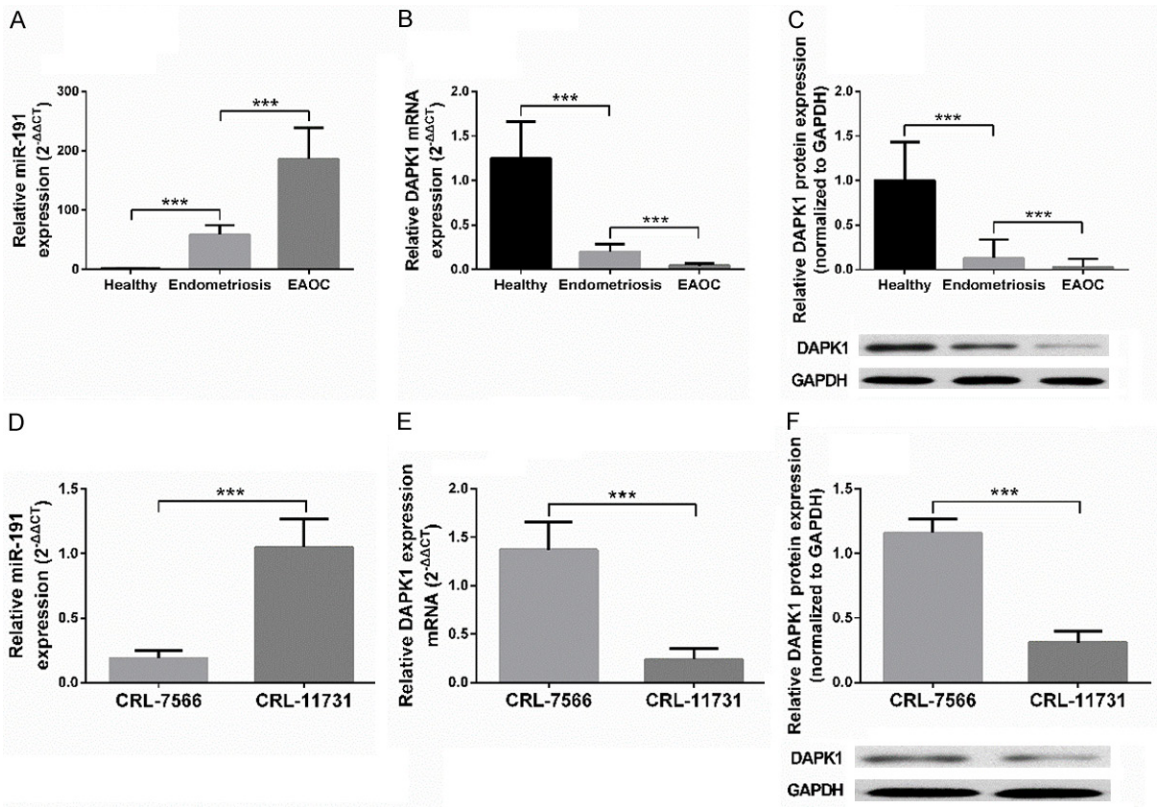


Figure 1. miR-191 expression is significantly increased in ovarian endometriosis and endometrioid carcinoma. (A) qRT-PCR analysis of miR-191 expression in tissue samples from healthy (n=10), ovarian endometriosis (n=10) and ovarian endometrioid carcinoma (n=10) participants. (B and C) qRT-PCR analysis of DAPK1 mRNA expression (B) and western blot analysis of DAPK1 protein expression (C) in the tissue samples from healthy (n=10), ovarian endometriosis (n=10) and ovarian endometrioid carcinoma (n=10) participants. (D) qRT-PCR analysis of miR-191 expression in endometriosis cell line CRL-7566 and ovarian endometrioid carcinoma cell line CRL-11731. E and F. qRT-PCR analysis of DAPK1 mRNA expression (E) and Western blot analysis of DAPK1 protein expression (F) in CRL-7566 and CRL-11731 cells.

progression from endometriosis to endometrioid cancer [11]. MiR-191 is one of the miRNAs significantly upregulated in endometriosis and EAOC [7]. In fact, miR-191 is viewed as an oncomiR in several types of cancer, including ovarian cancer [12, 13]. However, its role and regulative network in ovarian endometriosis and EAOC are still not clear.

In progression from endometriosis to endometrioid cancer, enhanced cell survival and reduced sensitivity to apoptotic signal are two of the features of malignant transformation. In this study, we explore the role of miR-191 in TNF-α induced cell death in ovarian endometriosis and endometrioid carcinoma cells. We firstly demonstrated that miR-191 can regulate survival of ovarian endometriosis and endometrioid carcinoma cells by directly targeting and regulating the expression of death-associated protein kinase 1 (DAPK1).

Methods

Human tissue samples

All patients were recruited from the Scientific research cooperation hospital. Informed consent was obtained from each participant before any physical procedures. Histologically confirmed ectopic endometrial tissues were collected from 10 patients (mean age: 36.0 years, range: 27-42) who have ovarian endometriomas and received laparoscopic surgical examination. Cancer tissues were obtained from 10 patients (mean age: 41.0 years, range: 32-45) with endometrioid ovarian cancer (n=10) and received laparoscopic surgical resection. Healthy ovarian tissues were collected from 10 women (mean age: 35.0 years, range: 28-44) who had laparoscopic biopsy due to suspicious ovarian cyst but found no evidence of ovarian pathology.

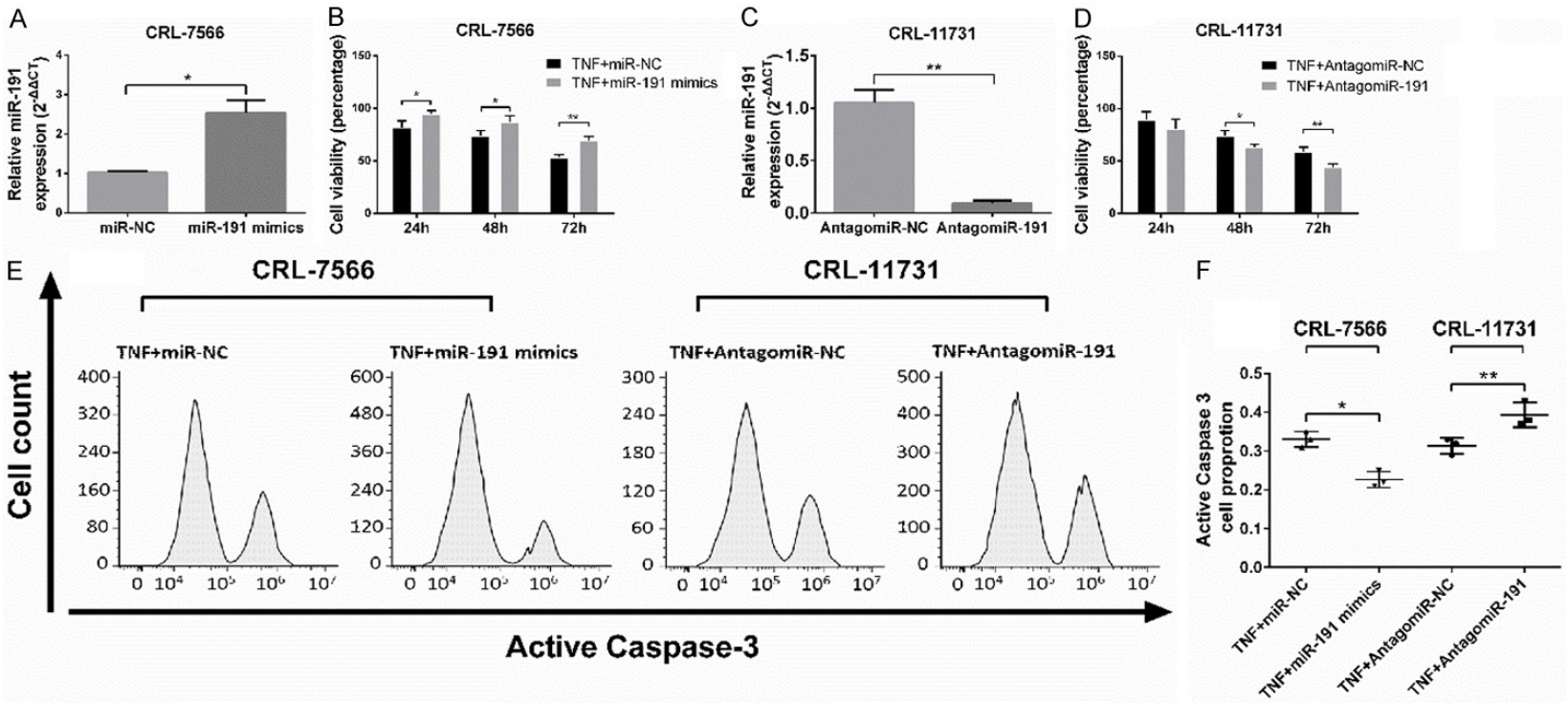


Figure 2. miR-191 negatively regulates TNF-α induced apoptosis. (A) qRT-PCR analysis of CRL-7566 cells transfected with miR-191 mimics (50 nM) for overexpression. (B) MTT assay of cell viability of CRL-7566 cells with miR-191 overexpression under the treatment with TNF-α (30 ng/ml) at 24, 48 and 72 h. (C) qRT-PCR analysis of CRL-11731 cells transfected with antagomiRn-191 (200 nM) for knockdown. (D) MTT assay of cell viability of CRL-11731 cells with miR-191 knockdown under TNF-α (30 ng/ml) treatment at 24, 48 and 72 h. (E) CRL-7566 and CRL-11731 cells with miR-191 overexpression or knockdown were treated with TNF-α (30 ng/ml) for 48 h. Then, the proportion of cells with active caspase 3 was measured by using flow cytometry analysis. (F) Quantification of active caspase 3 of cells in (E).

Cell culture

HEK293T cells, ovarian endometriosis cell line CRL7566 and ovarian endometrioid carcinoma cell line CRL-11731 were obtained from ATCC. HEK293T cells were grown in DMEM medium (Sigma-Aldrich Co.) supplemented with 10% heat-inactivated FBS, while CRL-7566 and CRL-11731 cells were cultured in RPMI-1640 medium (Sigma-Aldrich Co.) supplemented with 10% heat-inactivated FBS. All cells were maintained in an incubator with a humidified atmosphere and 5% CO_2 at 37°C.

Regents and cell transfection

TNF-α was purchased from Sigma Aldrich (H8916, USA). Chemically synthesized miR-191mimics, antagomiR-191, DAPK1 siRNA and the corresponding negative controls were purchased from Ribo Life Science (China). To knockdown or overexpress miR-191, 200 nM antagomiR-191 or 50 nM miR-191 mimics were transfected to the cells by using lipofectamine 2000 (Invitrogen). To knockdown the expression of DAPK1, DAPK1 siRNA (30 nM) was transfected to the cells by using Oligofectamine (Invitrogen). To induce apoptosis, cells were treated with 30 ng/ml TNF-α.

DAPK1 lentiviral expression vectors (DAPK1-wt: with 3'-UTR region; DAPK1-mut: without 3'-UTR region) and the packaging mix were purchased from GENECHEM (China). The lentiviral vectors were co-transfected with packaging mix to HEK293T cells to produce lentiviral particles. 48 h post transfection, the viral titer was measured and the viral supernatant was harvested. To overexpress DAPK1, the host cells were treated with viral supernatants with the presence of 8 μg/ml polybrene (Sigma-Aldrich).

qRT-PCR analysis of miR-191 and DAPK1 expression

Total miRNA from tissue was extracted by using the mirVana miRNA isolation kit (Ambion). Then, TaqMan MicroRNA Reverse Transcription Kit was used to reversely transcript miRNA to specific cDNA. Taqman miRNA Assays (Applied Biosystems) was further used to quantify mature miR-191 expression according to recommended protocol. RNU6B was used for normalization.

To measure the expression of DAPK1 mRNA, total RNAs in tissue were firstly extracted using Trizol Reagent. And then the first strand cDNA was generated using RevertAid first strand cDNA synthesis kit (Fermentas) according to the manufacturer's instructions. Based on the template, DAPK1 mRNA level was detected by using DAPK1 specific primers: forward: 5'-CAAGACAGGCACGGCAATAC-3', reverse: 5'-GGCTCCCATCAGACAGAGATAC-3'. qRT-PCR was performed using Syber Green PCR mastermix (Applied Biosystems). GAPDH served as internal control.

MTT assay of cell viability

Cells after transfection were plated at 5×10^3 cells/well in 96-well plates and treated with TNF-α (30 ng/ml). Cells were cultured for 24 h, 48 h and 72 h and the cell viability at each time point was measured by MTT (Sigma-Aldrich) assay according to recommended protocol. Absorbance at 490 nm of the solution was read by using a spectrophotometric plate reader. Each test was performed with three repeats.

Western blot analysis

Total proteins from tissue or cell samples were extracted using RIPA buffer and the concentration was quantified using BCA protein assay (Pierce, Thermo Scientific). The proteins were separated on 10% SDS PAGE gel and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in TBST and probed with primary antibodies (anti-DAPK1, 1:3000, ab109382, Abcam; anti-GAPDH, 1:2000, ab125247, Abcam) overnight at 4°C and then probed with secondary antibody (Anti-Rabbit IgG (HRP), 1:10000, ab191866, Abcam) for 2 h at room temperature. The signals were visualized by using ECL Western Blotting Substrate (Thermo Scientific Pierce). The band intensity was detected using Image-J software. Experiments were performed in triplicate.

Flow cytometry analysis of apoptosis

48 h after treatment, CRL-7566 and CRL-11731 cells were plated in six-well plates at 4×10^5 cells/well. The proportion of cells with active caspase 3 expression was measured by using Fluorescein Active Caspase 3 Staining Kit (88-7004, eBioscience) and FACSCalibur (BD Biosciences). The results were analyzed using ModFit (BD Biosciences).

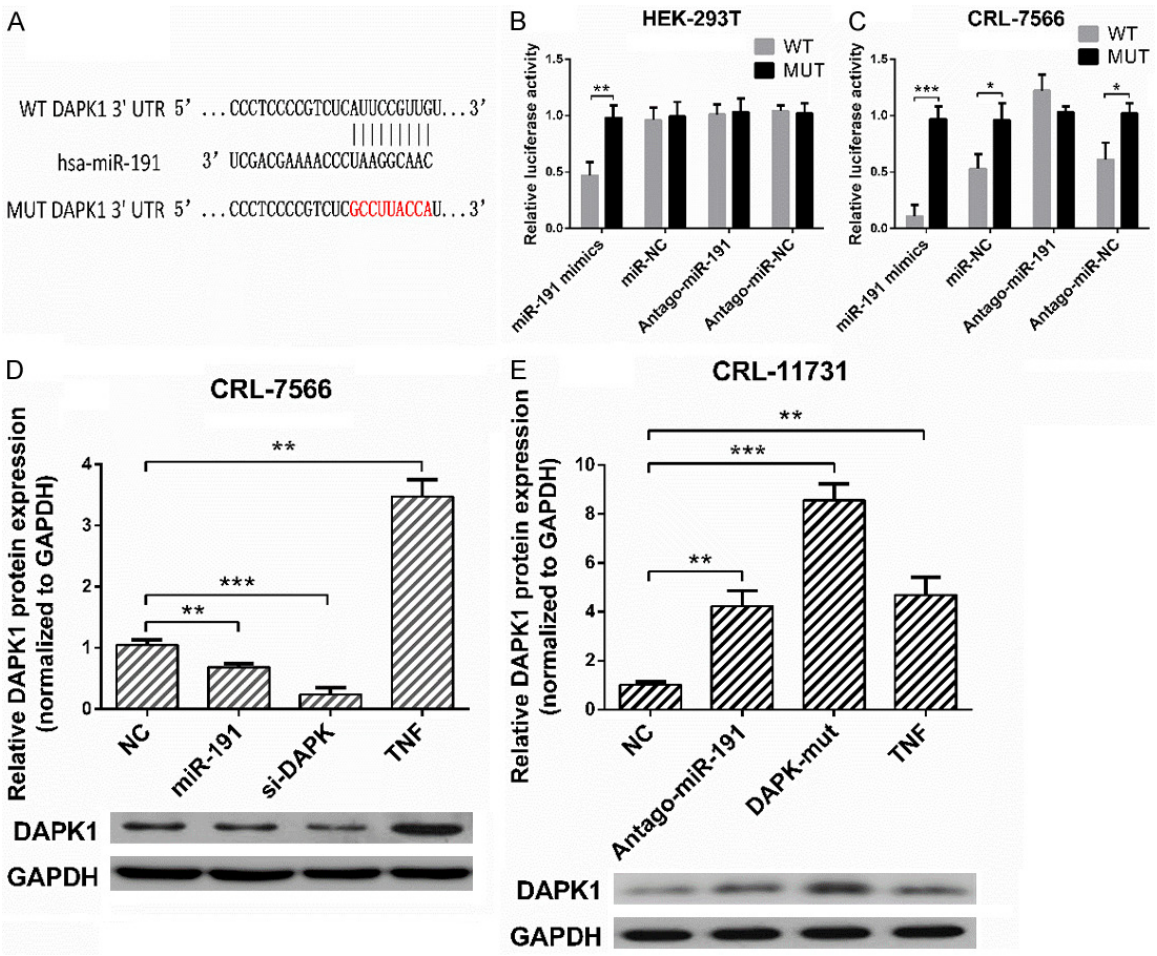


Figure 3. miR-191 directly targets DAPK1 mRNA and regulates its expression. (A) The putative pairing and designed mutant sequence between miR-191 and DAPK1 mRNA. (B and C) Dual luciferase assay was performed to verify the pairing in HEK293T (B) and CRL-7566 (C) cells. HEK293T and CRL-7566 cells were co-transfected with 200 ng reporter plasmids and 50 nM miR-191 mimics or 200 nM antagomiR-191 respectively. 24 h post transfection, fluorescence activity was measured. The relative firefly luciferase activity was normalized with renilla luciferase. (D) qRT-PCR and western blot analysis of DAPK1 expression at mRNA and protein level in CRL-7566 cells transfected with miR-191 mimics (50 nM), siDAPK1 (30 nM) and treated with TNF-α (30 ng/ml) for 48 h. (E) qRT-PCR and western blot analysis of DAPK1 expression at mRNA and protein level in CRL-11731 cells transfected with antagomiR-191 (200 nM), DAPK1-mut and treated with TNF-α (30 ng/ml) for 48 h.

Luciferase reporter assay

The putative pairing between miR-191 and DAPK1 mRNA were predicted by using miRWalk (2.0). DNA oligonucleotides representing 3'-UTR of DAPK1 with wide type or mutant pairing with miR-191 and with flanking SacI and SalI sites were chemically synthesized. The sequence details were: WT, forward: 5'-cCCCTCCCCGTCTCATTCCGTTGTCTGTGGATGGTCATTGCg-3'; reverse: 5'-tcgacGCAATGACCATCCACAGACAACGGAATGAGACGGGGAGGGgagct-3'. MUT, forward: 5'-cCCCTCCCCGTCTCGCCTTACCATCTGTGGATGGTCATTGCg-3'; reverse: 5'-tcgacGCAATGACCATCCACAGATGGTAAGGCGAGACGGGGAGGGgagct-3'. These two pairs of sequences were inserted into the SacI and SalI sites in pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega). The reconstructed luciferase report vectors were designated as Luc-DAPK1-WT and Luc-DAPK1-MUT respectively. The insertion was confirmed by sequencing. HEK293T and CRL7566 cells were co-transfected with 200 ng reporter plasmids and 50 nM miR-191 mimics or 200 nM antagomiR-191 respectively. 24 h post transfection, the cells were lysed and the fluorescence activity was detected by GloMax 20/20 Luminometer. The firefly luciferase activ-

ity was normalized to the renilla luciferase activity.

Statistical analysis

Data were presented as mean ± SD. Group comparison was based on unpaired T test. *P* value <0.05 is considered as significant difference. *, **, and *** donate significance at 0.05, 0.01 and 0.001 level respectively.

Results

miR-191 expression is significantly increased in ovarian endometriosis and ovarian endometrioid carcinoma

qRT-PCR analysis was performed to quantify miR-191 expression in tissue samples from healthy, endometriosis and ovarian endometrioid carcinoma participants. Compared with healthy controls, miR-191 expression was dramatically increased in endometriosis and was further increased in ovarian endometrioid carcinoma patients (**Figure 1A**). On the contrary, the expression of DAPK1 mRNA (**Figure 1B**) and protein (**Figure 1C**) in the tissues showed an inverse trend. The expression was highest in healthy tissues, but was significantly decreased in endometriosis tissues and was further decreased in ovarian endometrioid carcinoma (**Figure 1B** and **1C**). These expression trends were verified in ovarian endometriosis cell line CRL7566 and ovarian endometrioid carcinoma cell line CRL-11731. MiR-191 expression was significantly higher in CRL-11731 than in CRL-7566 cells (**Figure 1D**). However, DAPK1 expression in CRL-11731 cells was significantly lower than in CRL-7566 cells at both mRNA (**Figure 1E**) and protein level (**Figure 1F**). These results suggest that miR-191 expression is positively, while DAPK1 is negatively related to ovarian endometriosis and endometrioid carcinoma.

miR-191 negatively regulate TNF-α induced apoptosis

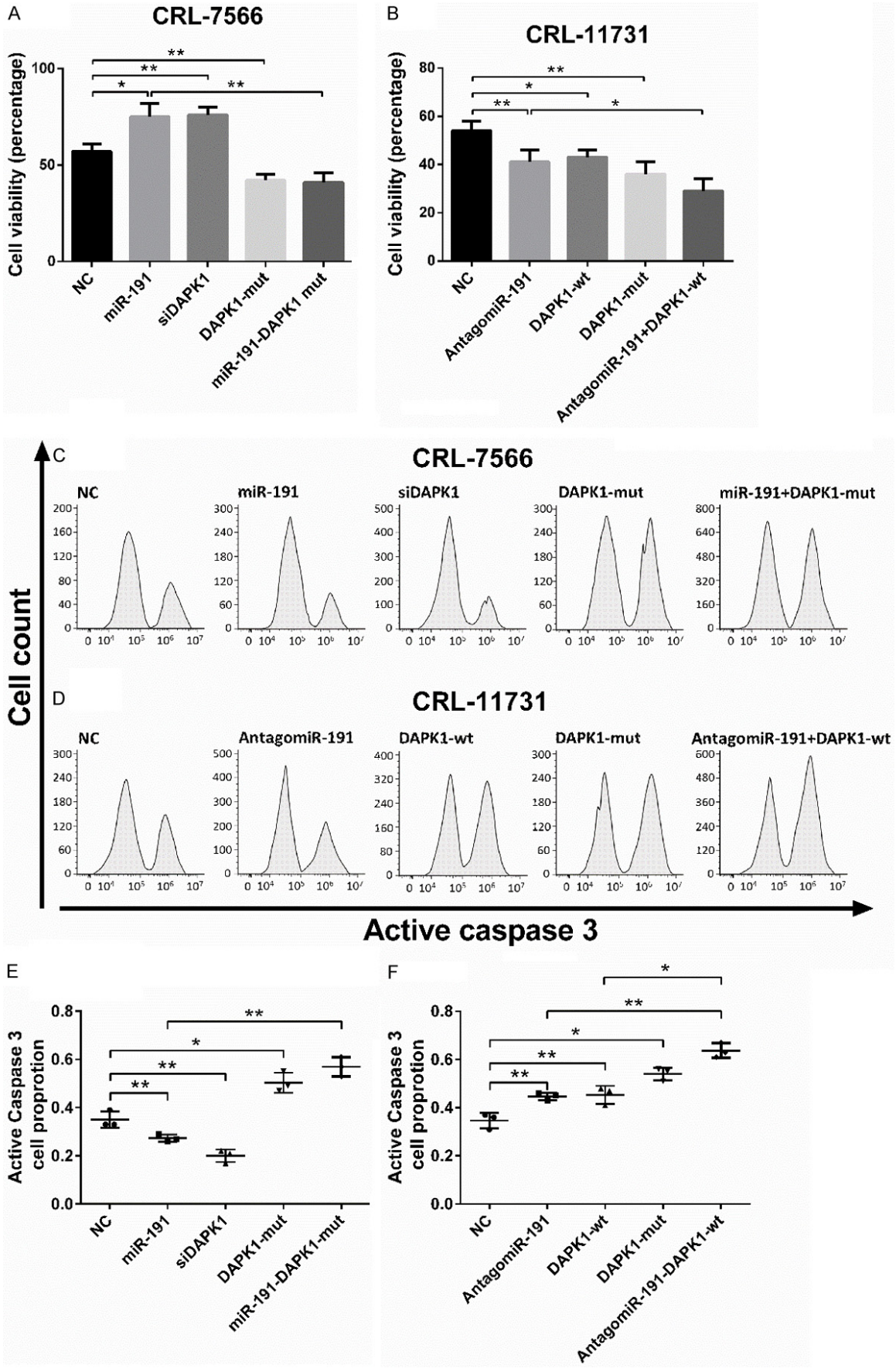
To explore the effect of different miR-191 expression on survival of endometriosis and endometrioid carcinoma cells under apoptotic signals, CRL-11731 and CRL-7566 cells were firstly transfected with antagomiR-191 or miR-191 mimics respectively (**Figure 2A** and **2C**). In CRL-11731 cells, miR-191 knockdown could significantly increase TNF-α induced growth inhibition (**Figure 2B**). In contrast, its overexpression in CRL-7566 cells alleviated the survival inhibition induced by TNF-α (**Figure 2D**). Preapoptotic marker, Active caspase 3 was also measured under these treatments. AntagomiR-191 transfection enhanced apoptosis induced by TNF-α in CRL-11731 cells, while miR-191 mimics transfection weakened the apoptosis (**Figure 2E** and **2F**). These results suggest that miR-191 can modulate TNF-α induced apoptosis in ovarian endometriosis and endometrioid carcinoma cells.

miR-191 directly targets DAPK1 mRNA and regulates its expression

Considering the inverse expression between miR-191 and DAPK1, we further explored their association. By searching in online databases, we identified a putative pairing between miR-191 and DAPK1 mRNA. Therefore, we designed a mutant DNA oligonucleotides sequence without the matching sites (**Figure 3A**) and construct two luciferase reporters. In both HEK293T and CRL-7566 cells, transfection of miR-191 mimics could significantly inhibited luciferase activity of Luc-DAPK1-WT (**Figure 3B** and **3C**). In addition, in CRL-7566 cells, knockdown of endogenous miR-191 by antagomiR-191 abrogated its inhibition on luciferase activity of Luc-DAPK1-WT (**Figure 3C**). TNF-α induced significantly higher DAPK1 expression in both CRL-7566 and CRL-11731 cells (**Figure 3D** and **3E**). In CRL-7566 cells, transfection of miR-191 mimics, similar as siDAPK1, could significantly reduce DAPK1 expression (**Figure 3D**). In CRL-11731 cells, transfection of antagomiR-191, similar as infection with DAPK1-mut lentiviral particles, could significantly increase DAPK1 expression (**Figure 3E**). These results suggest that miR-191 can directly target DAPK1 mRNA and regulate its expression.

MiR-191 inhibits apoptosis of ovarian endometriosis and endometrioid carcinoma cells by targeting DAPK1

In CRL-7566 cells, miR-191 overexpression, similar as DAPK1 knockdown, could significantly increase cell survival under TNF treatment. DAPK1-mut lentiviral vector had synchronic effect with TNF in inhibiting cell survival. However, miR-191 overexpression had no effect on DAPK1-mut induced cell survival inhi-


A
CRL-7566
Cell viability (percentage)
NC
miR-191
siDAPK1
DAPK1-mut
miR-191-DAPK1 mut
B
CRL-11731
Cell viability (percentage)
NC
AntagomiR-191
DAPK1-wt
DAPK1-mut
AntagomiR-191+DAPK1-wt
C
CRL-7566
NC
miR-191
siDAPK1
DAPK1-mut
miR-191+DAPK1-mut
D
CRL-11731
NC
AntagomiR-191
DAPK1-wt
DAPK1-mut
AntagomiR-191+DAPK1-wt
Cell count
Active caspase 3
E
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NC
miR-191
siDAPK1
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miR-191-DAPK1-mut
F
Active Caspase 3 cell proprotion
NC
AntagomiR-191
DAPK1-wt
DAPK1-mut
AntagomiR-191-DAPK1-wt

Figure 4. miR-191 inhibits apoptosis of ovarian endometriosis and endometrioid carcinoma cells by targeting DAPK1. (A) MTT assay of cell viability of CRL-7566 cells transfected with miR-191 mimics, si-DAPK1, DAPK1-mut lentiviral vector or co-transfection of miR-91 mimics and DAPK1-mut lentiviral vector 48 h after TNF-α (30 ng/ml) treatment. (B) MTT assay of cell viability of CRL-11731 cells transfected with antagomiR-191, DAPK1-wt lentiviral vector, DAPK1-mut lentiviral vector or co-transfection of antaogomiR-191 and DAPK1-wt lentiviral vector 48 h after TNF (30 ng/ml) treatment. (C and D) The proportion of cells with active caspase 3 under treatments in (A, C) and (B, D) were measured by using flow cytometry analysis. (E and F) Quantification of active caspase 3 of cells in (C, E) and (D, F).

bition (**Figure 4A**). In CRL-11731 cells, miR-191 knockdown, similar as DAPK1 overexpression, could significantly decrease cell survival under TNF treatment. However, DAPK1-mut had stronger inhibiting effect than DAPK1-wt, which could be explained by the effect of endogenous miR-191. miR-191 knockdown enhanced DAPK1-wt induced survival inhibition (**Figure 4B**). The proportion of cells with active caspase 3 also presented the same trends as cell viability (**Figure 4C-F**). These results suggest that miR-191 modulates responses of ovarian endometriosis and endometrioid carcinoma cells to TNF-α by targeting DAPK1.

Discussion

During the past years, there was emerging evidence about dysregualted expression of miRNAs in the progression from endometriosis to endometrioid cancer. For example, PTEN inactivation considered as an early marker of malignant transformation of endometriosis [14]. In endometrioid cancers, elevated expression of miR-21 [7] and miR-214 [15] were observed. In fact, these two miRNAs can directly target and modulate the expression of PTEN [11]. Conditional deletion of endometrial PTEN, particularly when combined with loss of p53, can lead to aggressive development of endometriotic cancer lesion in mouse cancer model [14]. MiR-200C expression was also increase in endometrial tumors as compared to endometrial tissues from peri- and postmenopausal period [16]. This miRNA can simultaneously modulate the expression of ZEBs, VEGFA, FLT1, IKKβ, KLF9, and FBLN5, which are involved in the regulation of tumor transformation, angiogenesis inflammation response and cell proliferation [16]. Several other miRNAs were also found in the progression from endometriosis to endometrioid cancer, such as miR-15b, miR-16, miR-191 and miR-195 [7]. Typically, miR-191 is one of the most differentially expressed miRNAs in pairwise comparisons among healthy controls, endometriosis, and EAOC patients [7]. Its oncogenic role is also observed in several types of cancer, including ovarian cancer [12, 13]. However, its targets in different types of cancer might be different. Experimentally validated targets of miR-191 include NDST1 in gastric cancer, TIMP3 in colorectal cancer, CDK6 in thyroid follicular tumors and MDM4-C in ovarian cancer [13]. Therefore, its regulation might be cancer specific and its regulative network in endometriosis and EAOC are still not clear.

In this study, we verified significantly higher expression of miR-191 in endometriosis and endometrioid cancer and further found its expression was related to cell apoptosis. Interestingly, we also observed inverse expression trend between miR-191 and DAPK1. DAPK1 is a positive mediator of programmed cell death and is generally viewed as a tumor suppressor [17, 18]. In several cancers, including head and neck cancer, non-small cell lung cancer, and cervical cancer, DAPK1 is downregulated and thereby contributes to increased tumor cell survival and proliferation [19-21]. The DAPK family members can modulate apoptosis initiated by several death-inducers such as oncogenes, INF-γ and TNF-α [22]. TNF-α can enhance cell apoptosis by interacting FADD, TRADD, and caspase-8 and also regulate caspase-3 and caspase-9 [22].

Enhanced cell survival and reduced sensitivity to apoptotic signal is associated with the malignant transformation from endometriosis to endometrioid cancer. Considering the important role of DAPK1 in cell survival and apoptosis and the possible regulative role of miR-191 on DAPK1, we decided to explore their association in ovarian endometriosis and endometrioid cancer cells. By using online bioinformatics databases, we identified a putative binding site between them. By conducting luciferase assay, we verified this binging in both HEK293 and CRL-7566 cells. Due to this direct targeting, miR-191 mimics can reduce DAPK1 expression in CRL-7566 cells, while antagomiR-191 can

increase its expression in CRL-11731 cells. Functionally, we also found DAPK1 can promote TNF-α induced cell death. DAPK1 knockdown in 7566 cells can weaken its response to TNF-α induced cell death. These functions of DAPK1 can be directly modulated by miR-191. Therefore, miR-191 might be an important miRNA modulating the responses to apoptotic signals in endometriosis and endometrioid cancer cells. Its decline in endometriosis and further decline in EAOC might be closely related to malignant transformation of endometriosis.

In summary, the miR-191-DAPK1 axis may play an important role modulating the response of ovarian endometriosis and endometrioid carcinoma cells to death-inducers and might contribute malignant transformation of endometriosis.

Disclosure of conflict of interest

None.

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