

## Original Article

# miR-20a promotes proliferation and invasion by targeting APP in human ovarian cancer cells

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**MicroRNAs (miRNAs) are emerging as a class of small regulated RNAs, and the alterations of miRNAs are implicated in the initiation and progression of human cancers. Our study shows that inhibition of miR-20a in OVCAR3 ovarian cancer cell line could suppress, whereas overexpression of miR-20a could enhance cell long-term proliferation and invasion. We also confirmed amyloid precursor protein (APP) as a direct target gene of miR-20a. Furthermore, suppression of APP expression could also promote ovarian cancer cell proliferation and invasion, which is consistent with the results of miR-20a overexpression. Therefore, we concluded that the regulation of APP is an important mechanism for miR-20a to promote proliferation and invasion in ovarian cancer cells.**

**Keywords** miR-20a; ovarian carcinoma; colony formation; invasion; amyloid precursor protein (APP)

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## Introduction

MicroRNAs (miRNAs) are a group of non-coding single-strand RNAs ~22 nucleotides in length, which modulate generous protein-coding gene expression post-transcriptionally by interacting with complementary sites within 3' UTR of target mRNA [1]. They are implicated in a multitude of cellular processes including cell differentiation, proliferation, migration, metabolism, and apoptosis [2–4]. Previous studies have pointed out the potential roles of miRNAs in cancers and suggested that abnormal expression of miRNAs may be associated with tumor initiation and progression by regulating cancer-related genes and pathways involved in cancer pathogenesis. Many cancer-specific miRNAs have been identified in some types of cancers, containing B-cell chronic lymphocytic leukemia [5], lung cancer [6], colorectal cancer [7,8], breast cancer [9], papillary thyroid cancer [10], hepatocellular carcinoma [11], and gastric

adenocarcinoma [12]. Recently, using miRNA microarray analysis, we found that miR-20a was up-regulated in high-metastatic colon cancer cells and may contribute to the metastatic activity of colon cancer cells (data not shown). Furthermore, previous reports have demonstrated that miR-20a was involved in the regulation of cellular proliferation in human lung cancer [13] and chronic myeloid leukemia [14]. These facts led us to ask whether miR-20a also contributed to the invasive activity of ovarian carcinoma cells.

In this research, we found that miR-20a contributed to the long-term proliferative activity and invasive activity of human ovarian cancer cell line OVCAR3. Moreover, the amyloid precursor protein (APP) was confirmed to be a direct and important functional target for miR-20a in ovarian carcinoma. Given that *APP* gene plays a major role in Alzheimer's disease (AD) [15–17] and may also be involved in some other cellular pathways, we demonstrated that APP also affects cell proliferation in OVCAR3 ovarian cancer cells. Understanding the modulation pathway of miR-20a in ovarian cancer cells might be helpful to characterize the molecular mechanisms of ovarian cancer progression.

## Materials and Methods

### Cell lines, transfection, and RNA extraction

The ovarian cancer cell line OVCAR3 was cultured at 37°C, with 5% CO<sub>2</sub> in RPMI 1640 media (Gibco, Gaithersburg, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Transient transfection was performed using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. All the transfections were carried out in three independent experiments. Enriched miRNAs were obtained from OVCAR3 cells using the *mir*Vana miRNA isolation kit (Ambion, Austin, USA) according to the manufacturer's recommendations. Total RNA was extracted by using the Trizol reagent (Invitrogen).

**Table 1** The oligonucleotides used in this work

Name	Sequence (5' → 3')
APP-3' UTR-sense	GACAAGATCTAAGAGGATACACACGTTTG
APP-3' UTR-antisense	CACGCTCGAGTCTGTAGTCATCCTTC
APP-3' UTRmut-sense	CATTGTAAGGACATATACGGGGCGG
APP-3' UTRmut-antisense	CCGCCCCGTATATGTCCTTACAATG
APP-siR-Top	GATCCCGGCTGAAGAAAGTGACAATGTTCAAGAGACATTGTCACCTT- TCTTCAGCCTTTTTTGGAAA
APP-siR-Bottom	AGCTTTTCCAAAAAAGGCTGAAGAAAGTGACAATGTCTCTTGAACA- TTGTCACTTTCTTCAGCCGG

The underlined sequences indicate the restriction enzyme sites.

### Vector constructions

To construct the enhanced green fluorescent protein (EGFP) reporter plasmid, the EGFP coding region from the pEGFP-N2 vector was subcloned into pcDNA3.1 poly-clone sites with *Hind*III and *Bam*HI sites. And then, the wild type or mutant of APP mRNA 3' UTR was amplified by polymerase chain reaction (PCR) from OVCAR3 cDNA library, digested by *Bgl*II and *Xho*I, and inserted into the downstream of *EGFP* gene between *Bam*HI and *Xho*I sites. pSilencer/sh-APP vector was constructed by annealing top and bottom strands of hairpin RNA and inserting it into pSilencer2.1 neo vector (Ambion) by *Bam*HI and *Hind*III sites. All the oligonucleotides used were shown in **Table 1**.

### Real-time reverse transcriptase PCR

mRNAs or miRNAs were reverse transcribed to generate cDNA using oligo-dT primers or stem-loop reverse transcriptase (RT) primers [18], respectively. Then, U6 snRNA (for miRNA) or the home-keeping gene  $\beta$ -actin (for mRNA) was considered as the endogenous control. Target genes and controls were treated with the same condition and analyzed by real-time RT-PCR using SYBR Premix Ex Taq™ (TaKaRa, Dalian, China) according to the manufacturer's protocol.

### EGFP reporter assay

To confirm the direct interaction between miR-20a and APP mRNA, OVCAR3 cells were transiently co-transfected with the fluorescent reporter plasmid and miR-20a antisense oligonucleotides (ASO) or control oligonucleotides in 48-well plates. The RFP expression vector pDsRed2-N1 plasmid was used as the loading control. After transfection for 48 h, cells were lysed using radio-immunoprecipitation assay (RIPA) lysis buffer [150 mM NaCl, 50 mM Tris-HCl, pH 7.2, 1% Triton X-100, and 0.1% sodium dodecylsulfate (SDS)], and the EGFP and RFP intensities were measured with the fluorescence spectrophotometer F4500 (Hitachi, Tokyo, Japan).

### Western blot analysis

Total protein of OVCAR3 cells transiently transfected with miR-20a ASO, control oligonucleotides, pSilencer/sh-20a, or pSilencer/NC was extracted by using RIPA lysis buffer. Protein expression was analyzed by western blot analysis. Briefly, total protein extract was separated on 10% SDS denatured polyacrylamide gel electrophoresis (PAGE) gels and transferred to the nitrocellulose membrane. The expression level of APP was evaluated out by using the rabbit polyclonal APP antibody. As a loading control, glyceraldehyde phosphate dehydrogenase (GAPDH) expression levels were measured by rabbit polyclonal GAPDH antibody. The membrane was incubated with the first antibodies overnight at 4°C, and then washed and incubated with horseradish peroxidase (HRP) conjugated secondary antibody on the next day. Protein expression was assessed by enhanced chemiluminescence and exposure to the chemiluminescent film (Fujifilm, Tokyo, Japan). LabWorks image acquisition and analysis software (UVP) was used to quantify the band intensities.

### MTT assay

The OVCAR3 cells were seeded in 96-well plates at 7000 cells/well. Forty-eight hours after transient transfection, the cells were incubated with 15  $\mu$ l of 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazoliumbromide (MTT, at a final concentration of 0.5 mg/ml) at 37°C for another 4 h. Then the medium was removed and the precipitated formazan was dissolved in 100  $\mu$ l DMSO. After shaking for 20 min, the absorbance at 570 nm ( $A_{570}$ ) was detected using  $\mu$ Quant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, USA).

### Colony formation test in soft agar

At 48 h after transient transfection, OVCAR3 cells were trypsinized, and  $10^3$  cells/well were suspended in 1 ml of 0.3% agarose gel supplemented with a complete culture medium. This suspension was layered over 1 ml of 0.5%

agar-medium base layer in a 12-well plate, and 1 ml of complete culture medium was added over the agarose to keep the cells from drying and to provide more nutrition. The cells were cultured for 21 days, with the culture medium replaced every 3 days. We calculated the numbers of cell clones that were considered as a colony when it contained beyond 30 cells. Experiments were performed in triplicates.

### Invasion assay

The invasion assay was performed using Transwell (Corning, Cambridge, USA). The well was filled with Matrigel (BD Biosciences, Franklin Lakes, USA), which was diluted to 1  $\mu\text{g}/\mu\text{l}$  with the RPMI 1640 medium without FBS. The lower chamber was filled with 600  $\mu\text{l}$  of the RPMI 1640 medium with 10% FBS to act as the nutritional attraction. The transfected cells were harvested with trypsin ethylene diamine tetraacetic acid (EDTA), washed once using the serum-free RPMI 1640 medium and resuspended to  $8 \times 10^5$  cells/ml. The suspension (100  $\mu\text{l}$ ) was added to the well and the cells were allowed to migrate at 37°C, with 5% CO<sub>2</sub> for 36 h. The upper surface of the membrane was wiped with a cotton tip to mechanically remove non-migratory cells and the migrant cells attached to the lower surface were stained for 20 min with crystal violet. Then, the membranes were carved and embedded under cover slip with cells on the top side. Cells in nine random fields of view at 100 $\times$  magnification were counted and expressed as the average number of cells per field of view. All assays were performed in triplicates.

### cDNA microarray assay

The differentially expressed genes in miR-20a ASO-treated OVCAR3 cells were detected on a cDNA microarray using MICROMAX TSA labeling and detection kit

(PerkinElmer, Waltham, USA) according to the manufacturer's instructions. Briefly, 10  $\mu\text{g}$  of large RNA was converted into fluorescein (FL, for control group) or biotin (for experimental group) labeled cDNA using RT. The labeled cDNA was then hybridized overnight to the microarray, which contained 7267 human gene probes with each probe in duplex. The microarray was sequentially incubated with anti-FL-HRP, which catalyzed deposition of Cy3, and streptavidin-HRP, which catalyzed deposition of Cy5. The slide was scanned with the Packard Biochip Technologies' ScanArray Express microarray acquisition system.

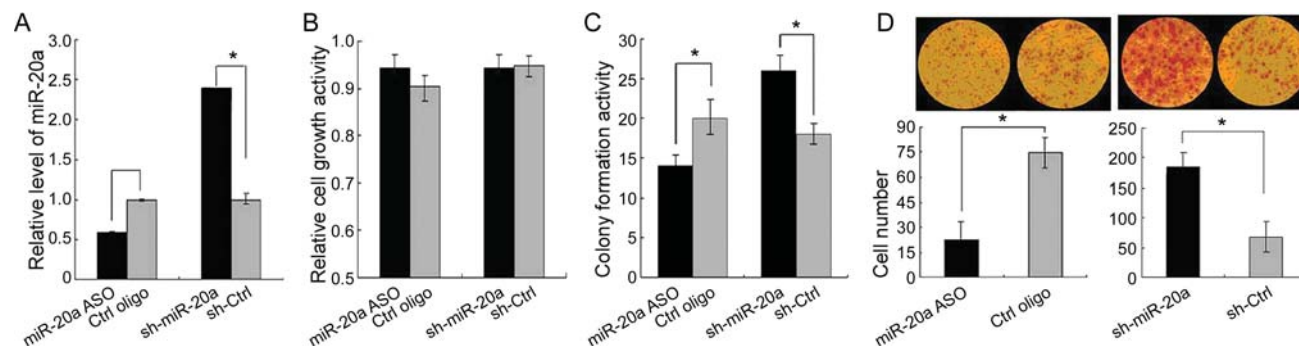
### Statistical analysis

All experiments were performed three times and the results were expressed as the mean  $\pm$  standard deviation (SD) and statistically analyzed using the Student's *t*-test for comparison of two groups, with  $P < 0.05$  from two-sided tests considered to be significant.

## Results

### Expression level of miR-20a was effectively altered in OVCAR3 cells

In order to investigate the effect of miR-20a on cell phenotypes, we used miR-20a blockage (miR-20a antisense oligonucleotides, miR-20a ASO) or precursor expression vector (small hairpin miR-20a, sh-miR-20a) to inhibit or enhance the mature miR-20a activity in OVCAR3 cells, respectively. Real-time PCR assay was used to validate the alteration of miR-20a expression level. As a result, the miR-20a level in miR-20a ASO-treated group was decreased by  $\sim 41.7\%$  compared with the control group, whereas the miR-20a level in pSilencer/sh-miR-20a-treated group was 2.38 folds compared with the control group



**Figure 1** miR-20a affected the colony formation activity and invasive activity of the OVCAR3 cells (A) Real-time PCR was performed to detect the miR-20a level in OVCAR3 cells treated with miR-20a ASO or pSilencer/sh-miR-20a. (B) MTT array was used to determine the effect of miR-20a on OVCAR3 cell proliferation activity. (C) Soft-agar colony formation assay was performed to determine the effect of miR-20a on the long-term proliferative activity of OVCAR3 cells. (D) Invasive activity of OVCAR3 cells treated with miR-20a ASO or pSilencer/sh-miR-20a was detected using Transwell assay. Cell numbers traversing the membrane were determined by averaging nine random fields. The bars represent the mean  $\pm$  SD of three independent experiments. Ctrl oligo, control oligonucleotides; sh-miR-20a, pSilencer/sh-miR-20a; sh-Ctrl, pSilencer/sh-control. \* $P < 0.05$ .

[Fig. 1(A)]. These results suggested that the expression of miR-20a was successfully altered in our experiments.

### miR-20a promotes colony formation and invasion of OVCAR3 cells without affecting cell growth activity

Given that miR-20a acts as an oncogene in many cancers, we asked here whether miR-20a has an impact on cell malignant phenotypes, i.e. growth and invasion activity, in ovarian cancer cells. Our data suggested that when miR-20a was blocked or overexpressed, the cell growth activity showed no differences detected by MTT assay [Fig. 1(B)]. However, the soft-agar colony formation activity of OVCAR3 cells was affected by miR-20a [Fig. 1(C)]. Furthermore, in Transwell assay, cells treated with miR-20a ASO displayed significant inhibition of invasive activity vs. control cells, whereas the pSilencer/sh-miR-20a group exhibited intense capability of invasion than the control group [Fig. 1(D)]. From these results, we knew that miR-20a was involved in the regulation of long-term proliferation and invasion activities in OVCAR3 cells.

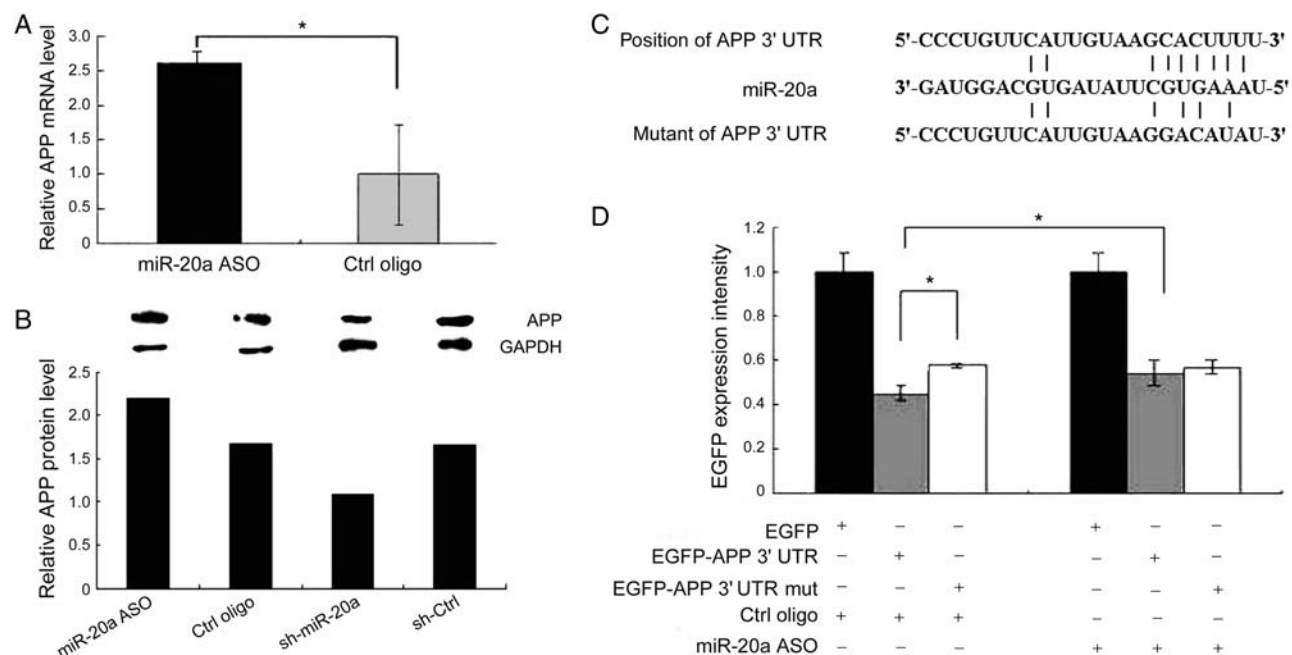
### miR-20a regulates APP at both the mRNA and protein levels

To illuminate the mechanism of miR-20a function in ovarian cancer, we predicted the candidate target genes of miR-20a. First, the algorithm programs (PicTar, TargetScan, and

miRBase Targets) were used to pick out the potential target genes. Second, we used cDNA microarray to detect the up-regulated genes in miR-20a-blocked OVCAR3 cells (Supplementary Table S1), some of which may be negatively regulated by miR-20a. As a result, *APP* (GenBank accession no. NM\_000484) was predicted as a potential target of miR-20a combining cDNA microarray analysis and bioinformatics. Next, to determine the effect of miR-20a on the APP expression level, total RNA was isolated from OVCAR3 cells transfected with miR-20a ASO, and then the APP mRNA level was examined by real-time PCR. We found that when miR-20a was inhibited, the mRNA level of APP was increased [Fig. 2(A)]. Furthermore, the data of western blot assay also showed a reverse correlation of miR-20a and APP protein level [Fig. 2(B)].

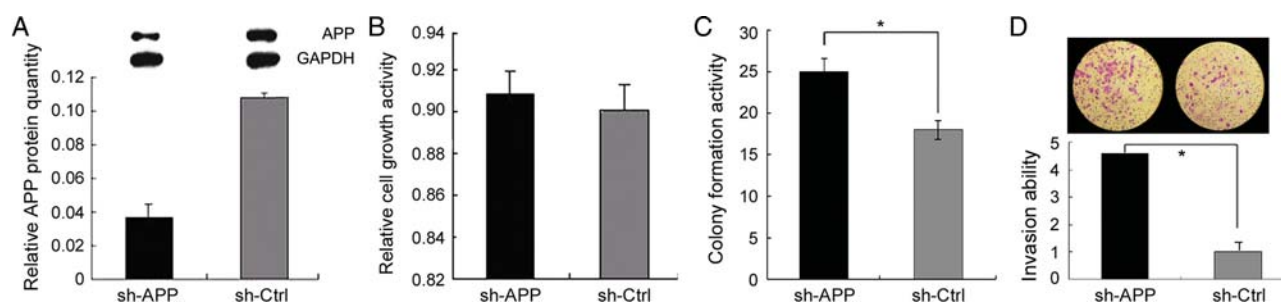
### miR-20a targets directly at the APP 3' UTR

Next, we adopt an EGFP reporter system to confirm the direct regulation of miR-20a on APP mRNA. The alignment of miR-20a with the APP 3' UTR sequence is illustrated [Fig. 2(C)]. First, the APP 3' UTR fragment was cloned into the downstream of EGFP codon region. Meanwhile, we constructed another vector bearing three bases mutation in the seed sequence of APP 3' UTR complementary to miR-20a [Fig. 2(C)]. The EGFP reporter analysis showed that with wild-type 3' UTR inserting,



**Figure 2 APP was a direct target gene of miR-20a** (A) Real-time RT-PCR assay was performed to detect the APP mRNA level in OVCAR3 cells treated with miR-20a ASO. (B) Western blot analysis was used to detect the protein expression level of APP in OVCAR3 cells transfected with miR-20a ASO or pSilencer/sh-miR-20a. (C) Complementary sequences of miR-20a on APP mRNA 3' UTR were obtained from publicly available algorithms. The mutant of APP 3' UTR bearing three mutated bases was also shown. (D) The EGFP reporter assay was performed to confirm the direct regulation of miR-20a on APP mRNA. Histogram showed the normalized mean  $\pm$  SD of the normalized fluorescent intensity from three independent experiments. Ctrl oligo, control oligonucleotides; sh-miR-20a, pSilencer/sh-miR-20a; sh-Ctrl, pSilencer/sh-control. \* $P < 0.05$ .





**Figure 3** APP affected the colony formation activity and invasive activity of the OVCAR3 cells (A) APP protein level in OVCAR3 cells transfected with pSilencer/sh-APP was detected using western blot assay. (B) MTT assay was used to determine the effect of APP on OVCAR3 cell short-term proliferation. (C) Soft-agar colony formation assay was performed to determine the effect of APP on the long-term proliferation activity of OVCAR3 cells. (D) Invasive activity of OVCAR3 cells treated with pSilencer/sh-APP was detected using Transwell assay. Cell numbers traversing the membrane were determined by averaging nine random fields. All data were analyzed by three independent experiments. sh-APP, pSilencer/sh-APP; sh-Ctrl, pSilencer/sh-control. \* $P < 0.05$ .

the EGFP value was significantly lower than mutants when they were all treated with irrespective sequences [Fig. 2(D)], demonstrating the effects of endogenous miR-20a on APP. When miR-20a activity was inhibited, the EGFP expression level in the vector bearing the wild-type APP 3' UTR fragment was significantly higher. However, the EGFP expression in the reporter vector bearing the mutated APP 3' UTR was not affected by miR-20a [Fig. 2(D)]. From these results, we concluded that miR-20a could negatively regulate APP expression through directly binding to the special sequence of APP mRNA 3' UTR.

### APP could suppress OVCAR3 cell long-time proliferation and invasion

Now that APP is a target of miR-20a, we conjectured that miR-20a affected proliferation and invasion through regulating APP. Therefore, we knocked down APP expression with the small interfering RNA method [Fig. 3(A)] and detected the capability of soft-agar colony formation and invasion in OVCAR3 cells. We got similar results with miR-20a overexpression [Fig. 3(B–D)]. Thus, we could conclude that miR-20a facilitates proliferation and invasion by down-regulating APP gene in ovarian carcinoma.

### Discussion

miRNAs are discovered as important regulators of gene expression, which could suppress the expression of target genes through translational repression or degradation of a target's transcript. Previous studies have indicated that alterations of special miRNA expression may play a critical role in cancer initiation and progression [13]. In this study, computational algorithms were used to identify the target gene of miR-20a. Then, based on the cDNA microarray, we detected the up-regulated genes in OVCAR3 cells transfected with miR-20a ASO compared with those treated with control oligonucleotides. We integrated the bioinformatics-based

predicting results with cDNA microarray results and selected APP gene from the list that had the highest recurrence rate as a would-be target gene of miR-20a.

It was suggested that inhibition of an endogenous miRNA can allay the mRNA targets from the increased degradation [19]. Accordingly, we utilized real-time PCR and western blot assays to confirm the presumption that APP was regulated by miR-20a. We found that when miR-20a was blocked, APP expression was enhanced at both the mRNA and protein levels, and when miR-20a was overexpressed, APP mRNA and protein expression levels became lower. Thus, we concluded that miR-20a negatively regulated the expression of APP.

Naturally, we faced another question whether the regulation of miR-20a on APP expression was direct. Generally speaking, miRNAs are believed to bind partially to the homologous sequence of target gene at 3' UTR. So we constructed EGFP reporter plasmids bearing wild-type 3' UTR fragment of APP mRNA following the coding sequence of EGFP *in vitro*. As a result, we found that inhibition of miR-20a by its antisense oligonucleotide could significantly enhance EGFP expression. Furthermore, we synthesized another EGFP reporter vector containing a mutational miR-20a 'seed region' binding site, yet we no longer detected the increased fluorescence mediated by miR-20a ASO. These results suggested that miR-20a can directly and negatively regulate APP gene expression by binding to the 3' UTR of APP mRNA. This target gene of miR-20a is also testified in brain cells (data not shown).

Previous studies have reported that miR-20a is associated with cellular proliferation [20] and cell cycle [21]. In our work, we observed that miR-20a is not only related to long-term cell growth, but also associated with invasion activity in ovarian cancer cells. Soft-agar colony formation assay showed that miR-20a brings a great difference to cell growth in OVCAR3. We conjecture that MTT might not work long enough to show any difference. The most

attracting results are the impact of miR-20a on cell invasion through APP. We found that the expression of APP was closely related to miR-20a, and either when miR-20a was in a high level or when APP was lower, the ability of invasion is far stronger. So we suggested that miR-20a plays a critical role in ovarian cancer cellular invasion by targeting APP.

APP, known as amyloid precursor protein, is closely related to the AD [15–17]. APP was considered as one of the most important factor contributing to AD. Recent studies propose that APP was involved in cell adhesion, motility, proliferation [22,23], as well as in neuroprotection and neurite outgrowth [24,25]. APP also functions as an autocrine growth factor [23,26]. Additionally, because it structurally resembles a receptor and could target to the cell membrane, APP has been suggested to play a role in signal transduction and gene transcription [27,28], suggesting that APP may be relative to more complex cellular progress by regulating downstream genes. In our research, function of APP in the regulation of proliferation and invasion is completely a new found, yet the extensional mechanism is needed to be illuminated in the future.

In summary, our study identified a new mechanism that can fully explain the reverse correlation between the expression of APP and miR-20a in OVCAR3 cells. Reduction in miR-20a expression could be related to tumor progression by affecting cell invasion and long-term proliferation. Albeit this study shows the mechanism of miR-20a in OVCAR3 cells, it remains to be determined if the target gene of miR-20a is variant in different cell lines through diverse pathways. Thus, further studies will be surely required to illuminate the target molecules and its mechanisms.

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## Supplementary Data

Supplementary data are available at *ABBS* online.

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