

# MiR-26a regulates cell cycle and anoikis of human esophageal adenocarcinoma cells through Rb1-E2F1 signaling pathway

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**Abstract** Resistance to anoikis, the subtype of apoptosis induced by lack of matrix adhesion, contributes to malignant transformation and development of metastasis. MicroRNAs play key regulatory roles in tumorigenesis and metastasis. In this study, we described that miR-26a, which is usually downregulated in tumor cells, is involved in the acquisition of anoikis-resistance of human esophageal adenocarcinoma (EA) cells. Results of qRT-PCR in clinical samples showed that downregulated miR-26a expression is related to tumorigenesis and metastasis of EA. In vitro experiments determined that miR-26a directly participates in the regulation of cell cycle and anoikis of human EA OE33 cells. Further, we identified that Rb1 is the direct functional target of miR-26a, and revealed that the reduction of miR-26a expression leads to increased Rb1 protein level and thus inhibits the function of E2F1, by which it influences the phenotypes of cell cycle and anoikis. The findings we reported here presented the evidence that miR-26a may be involved in regulation of anoikis-resistance of EA cells. Targeting miR-26a may provide a novel strategy to inhibit metastasis.

**Keywords** MiR-26a · Cell cycle · Anoikis · Esophageal adenocarcinoma · Rb1-E2F1 signaling pathway

## Introduction

In many areas of the world, especially in the west, the incidence of esophageal adenocarcinoma (EA) has risen dramatically over the past three decades [1]. Surgery is the best curative treatment option but only a small fraction of EA patients benefit because many patients still suffer from metastatic recurrence within 2 years after curative treatment [2]. Therefore, elucidating the mechanisms of how EA cells spread and how metastasis can be blocked has important implications for future treatment strategies.

Anoikis, a Greek word meaning loss of “home” or “homelessness”, was originally defined by Frish and Francis in 1994 as a unique phenomenon reflecting apoptotic cell death consequential to insufficient cell-matrix interactions and was later recognized as a potentially significant player in tumorigenesis and metastasis [3, 4]. As a critical safeguard of nature, anoikis can prevent detached cells from taking up residence and growing in ectopic positions, whereas some tumor cells develop means to evade this process, migrate to new sites and establish secondary tumors [5]. Resistance to anoikis is thus emerging as a hallmark of metastatic cancer cells, especially because anchorage-independent growth of tumor cells is a classic characteristic of different types of human malignancies, including EA [5, 6].

MicroRNAs (miRNAs) are endogenous small noncoding RNAs that regulate cellular gene expression and are functionally linked to tumorigenesis and metastasis [7]. To date, a large number of miRNAs have been implicated in cancer metastasis. For instance, the miR-200 family has been shown to inhibit the first step of metastasis, epithelial

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mesenchymal transition [8], and miR-21, the well known oncogenic miRNA, was reported to promote metastasis in a variety of tumors via different pathways [9]. However, until now, very few miRNAs known to be involved in anoikis resistance and subsequent metastasis have been reported.

In this study, we reported that miR-26a suppression contributed to the acquisition of anoikis resistance of EA cells through regulating Rb1/E2F1 pathway. Our findings not only provide new insights into the metastatic mechanisms in EA, but also have implications for future therapies.

## Methods

### Cell culture and selection for anoikis resistance

The human Barrett's adenocarcinoma derived OE33 cell line used in this study was kindly provided by Dr. Xiaoxin Chen (North Carolina Central University, USA). To obtain a culture condition for suspension, OE33 cells were seeded on plates pre-coated with poly (2-hydroxyethyl methacrylate) (Poly-HEMA, Sigma Aldrich, USA), which is a classical and widely accepted method to obtain detached cells [10]. Via suspension culture, a subpopulation of OE33 cells that acquired resistance to anoikis was designated as OE33/AR (anoikis-resistance) cells.

### Experimental metastasis

An orthotopic mouse model of human EA was used to produce experimental metastasis [11]. Briefly, OE33 cells ( $1 \times 10^7$ ), in a 200  $\mu$ L suspension, were s.c. injected into the flanks of nude mice. When tumor growth reached 5 to 7 mm, tumors were excised and cut into pieces of  $\sim 2\text{--}3 \text{ mm}^3$  size, and then were orthotopically implanted to the abdominal esophagus. After 4 weeks, the mice were killed, and the liver was examined for metastases. The orthotopic tumor and liver metastases were removed under sterile conditions, respectively, and the RNA from each sample was extracted and subjected to miRNA array analysis.

### miRNA array analysis

Total RNA was extracted from cultured cells and tissues by using Takara RNAiso reagent (Takara, Japan) according to the manufacturer's instructions. The purity and quantity of the isolated RNAs were assessed using gel electrophoresis and spectrophotometry. Then the samples were submitted to KangChen-Biotech (Shanghai, China) for array hybridization on a miRCURYTM Array

microarray kit (Exiqon, Denmark). Each microarray chip was hybridized with a single sample labeled with either Cy3 or Cy5. Background subtraction and normalization were performed. MiRNAs whose expression levels differed by more than two folds were selected for further investigation in this study.

### Clinical samples

Twenty-five patients who had undergone esophagectomy with lymph node dissection for EA at Southwest Hospital or Wuhan General Hospital of Guangzhou Command between 2005 and 2010 were included in the study. No patients received neoadjuvant chemotherapy or radiation therapy before surgery. The resected specimens were histologically examined by H&E staining. Total RNA from paraffin-embedded tumor tissues and corresponding non-tumor mucosa and lymph nodes were collected for each patient using the Paraffin-embedded Tissue microRNA Extraction Kit (Biotek, China) according to the manufacturer's instructions. Ethics approval for the trial was obtained from the Ethics Committee of Southwest hospital or Wuhan General Hospital of Guangzhou Command.

### Quantitative real-time RT-PCR (qRT-PCR)

TaqMan stem-loop RT-PCR method was used to assess the expression of mature miR-26a (miR-26a-1) with kits from Applied Biosystems (Foster City, USA). The real-time PCR results, recorded as threshold cycle numbers (Ct), were normalized against an internal control (U6). For relative expression levels, the  $2(-\Delta\text{Ct})$  method was used as previously described [12]. Experiments were carried out in triplicate for each data point, and data analysis was done by using Bio-Rad IQ software.

### Cell transfection

Cells were transfected with miR-26a mimics/inhibitor and their respective negative control duplexes (Ambion, USA) using Lipofectamine 2000 (Invitrogen, USA). After 24 h transfection, cells were collected for qRT-PCR analysis or further processing.

### Cell cycle analysis

Cells transfected with miR-26a mimics/inhibitor were harvested 24 h later by trypsinization, washed with ice-cold PBS, fixed in 70 % ethanol, and stored at 4 °C. Following overnight incubation, cells were washed and resuspended in PI staining buffer. DNA content was evaluated by flow cytometry (Coulter Epics XL-MCL, USA).

### Apoptosis analysis

Detection of apoptotic cells by flow cytometry was performed as described previously [13]. Cells transfected with miR-26a mimics/inhibitor were harvested 24 h later then annexin V/PI analysis by flow cytometry (Coulter Epics XL-MCL) was performed. For anoikis analysis, OE33 cells cultured in normal cell plates or OE33/AR cells cultured in plates pre-coated with Poly-HEMA were transfected with miR-26a mimics/inhibitor. After 24 h transfection, both cells were cultured in normal cell plates for 24 h, and then were cultured in plates pre-coated with Poly-HEMA for 48 h before apoptosis analysis. For analysis of chemotherapy-induced apoptosis, OE33 cells after 24 h transfection of miR-26a mimics/inhibitor were incubated with 10  $\mu\text{g}/\text{mL}$  of 5-Fu for 48 h and then apoptosis detection was performed.

### Migration and invasion analysis

Transwell chambers (8- $\mu\text{m}$  pore size, Corning, USA) were used in the migration and invasion analysis. For migration assays,  $1 \times 10^5$  cells were plated in the top chamber lined with a non-coated membrane. For invasion assays, chamber inserts were coated with 200 mg/mL of Matrigel and dried overnight under sterile conditions. Then,  $1 \times 10^5$  cells were plated in the top chamber. In both assays, cells were suspended in medium without serum or growth factors, and medium supplemented with serum was used as a chemo-attractant in the lower chamber. After incubation at 37 °C for 48 h, the top chambers were wiped with cotton wool to remove the non-migratory or noninvasive cells. The invading cells on the underside of the membrane were fixed in 100 % methanol for 10 min, air-dried, stained in 0.1 % crystal violet, and counted under a microscope. The mean of triplicate assays for each experimental condition was used.

### Western blot analysis

Total cell lysates, cytoplasmic and nuclear proteins were prepared as previously described [14]. Equal amounts of protein were analysed by western blotting, using antibodies against Rb1, E2F1, Cyclin E, Bcl-2,  $\beta$ -actin (Santa Cruz Biotechnology, USA), I $\kappa$ B- $\alpha$ , p-I $\kappa$ B- $\alpha$  (Sigma, USA) and NF- $\kappa$ B (Beyotime, China).

### Semi-quantitative RT-PCR

Total cell RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reverse transcription was performed on 1  $\mu\text{g}$  of total RNA from each sample using RevertAid<sup>TM</sup> First Strand cDNA

synthesis kit (Fermentas, Canada). Rb1 cDNA was then amplified using the following primer pairs: forward, 5'-TGACCTGGTAATCTCATTTCAGC-3'; reverse, 5'-GGGTGTTTCGAGGTGAACCAT-3'.  $\beta$ -actin was used as the loading control.

### Luciferase assay

The 3'-UTR segments of Rb1 mRNA containing the miR-26a binding sites were amplified by PCR from human genomic DNA and inserted into the pMIR-REPORT luciferase reporter vector (Ambion) and named pMIR-Rb1-3'UTR. A mutant version with a deletion of 5 bp from the site of perfect complementarity was also generated and named pMIR-mut-Rb1-3'UTR. The recombinant reporter vectors with wild-type or mutant Rb1 3'-UTR were then cotransfected with miR-26a mimics or control into OE33/AR cells, respectively, using Lipofectamine<sup>TM</sup> 2000. The luciferase assay was performed according to the manufacturer's instructions.

### Statistical analysis

The data were expressed as the mean  $\pm$  SEM. Differences were compared by one-way ANOVA analysis followed by LSD-t test. All statistical analyses were performed using SPSS17.0 software (Chicago, IL, USA).  $P < 0.05$  was considered as statistically significant.

## Results

### MiR-26a is involved in the acquisition of anoikis-resistance and subsequent metastasis of human EA cells

To identify miRNAs potentially involved in the acquisition of anoikis-resistance and subsequent metastasis of human EA cells, human EA OE33 cell line was used in this study. Firstly, we established a mouse model with orthotopic implantation of human EA originated from OE33 cells. Through analyzing the difference in miRNA expression between orthotopic transplantation and corresponding liver metastasis, we identified a group of miRNAs that may be involved in EA metastasis. Further, we generated a subline of OE33 cells with an anoikis-resistant phenotype using suspension culture, named OE33/AR. By microarray analysis, we detected differentially expressed miRNAs between OE33/AR cells and its parental OE33 cells, which is expected to contribute to the regulation of acquisition of anoikis-resistance in human EA cells.

MiR-26a was one of those that exhibited a consistent pattern of expression in both in vivo and in vitro models. Previous studies have shown that miR-26a is consistently

downregulated in a number of cancers, indicating their potential tumor suppressor functions [15–18]. In this work, miR-26a downregulation was found to possibly contribute to the acquisition of anoikis-resistance and subsequent metastasis of human EA cells. To validate the microarray results, we performed qRT-PCR analysis and the data confirmed that miR-26a expression was significantly decreased in liver metastases and OE33/AR cells compared to primary EA tissues and OE33 cells, respectively (Fig. 1a, b, fold changes,  $P < 0.05$ ). Furthermore, we investigated the expression levels of miR-26a in 25 EA tissues (T) and non-tumor mucosa (NT) by qRT-PCR. The term  $-\Delta Ct$  was used to describe the expression level of miR-26a. As presented in Fig. 1c, the miR-26a expression level in EA tissues was significantly lower than that in non-tumor mucosa ( $-2.35 \pm 0.38$  vs.  $-1.88 \pm 0.27$ ,  $P < 0.01$ , paired  $t$  test). Moreover, in the 16 cases of EA with lymph node metastasis, the miR-26a expression level in the primary EA tumors versus the metastatic lymph nodes was compared, and as shown in Fig. 1c, the expression level of miR-26a in metastatic lymph nodes was much lower than that in primary EA tumors ( $-3.04 \pm 0.46$  vs.  $-2.42 \pm 0.38$ ,  $P < 0.001$ , paired  $t$  test), indicating that miR-26a is associated with and may play a role in EA metastasis.

miR-26a downregulation directly causes cell cycle arrest but has little effect on apoptosis, migration and invasion in OE33 cells

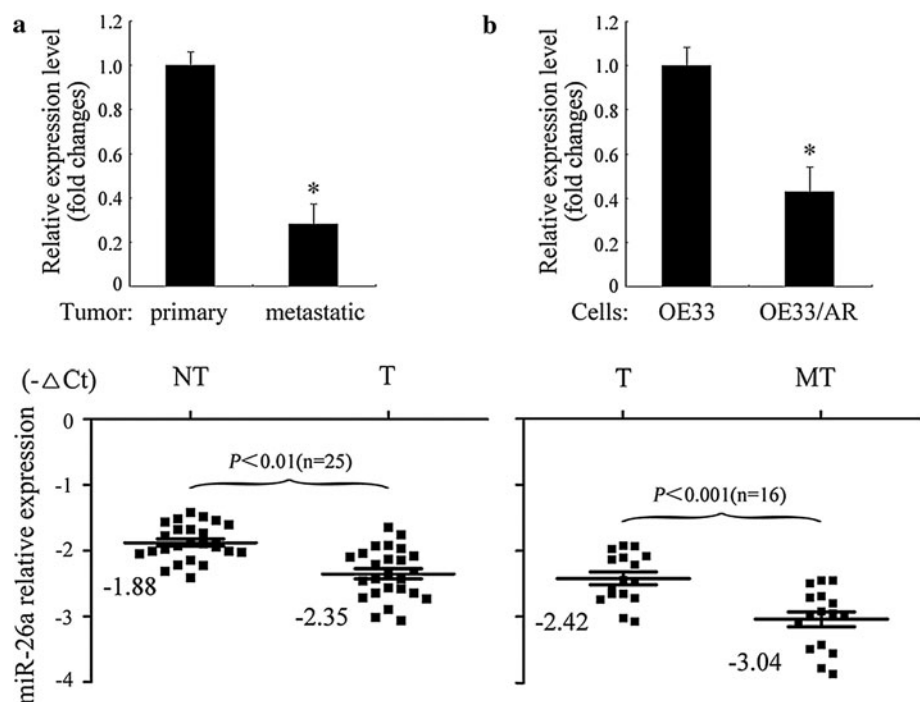
To study the role of miR-26a in the progression of EA, the effect of suppression of miR-26a on cell proliferation,

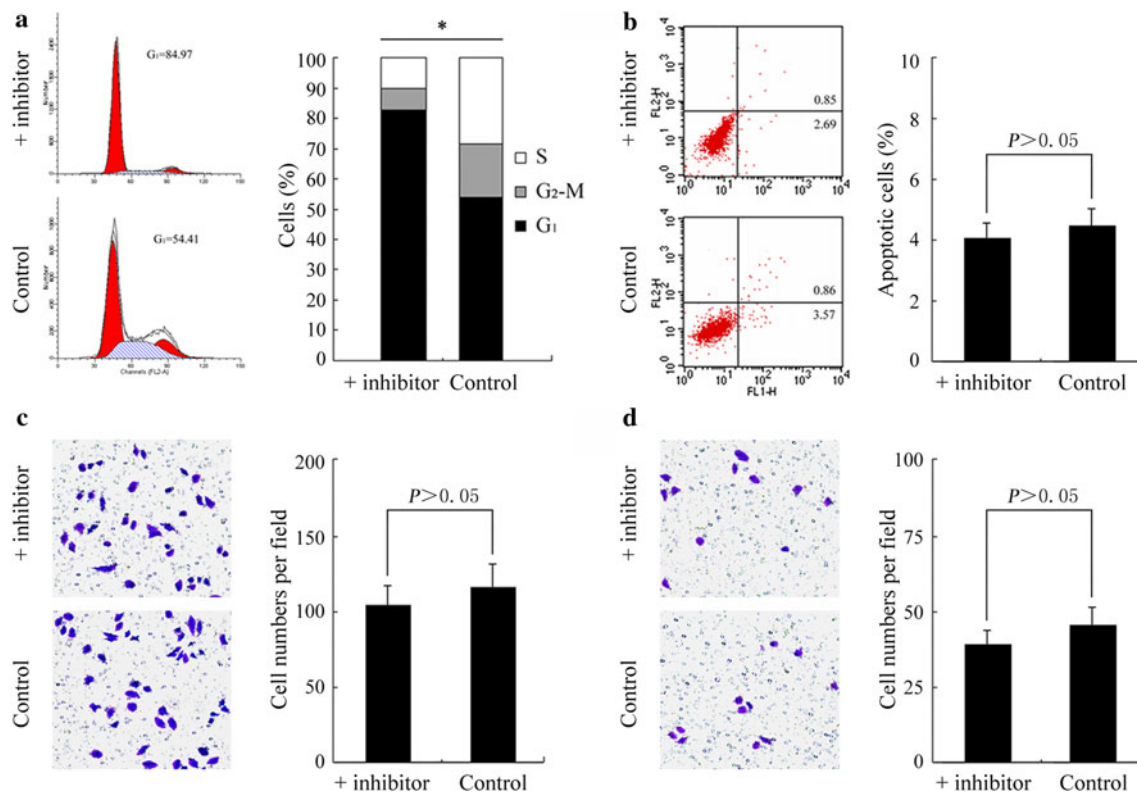
apoptosis, migration and invasion was examined in vitro using OE33 cells. Taqman real-time PCR revealed that transfection of miR-26a specific antisense oligonucleotides (inhibitor) caused a 54 % decrease of miR-26a expression in OE33 cells, compared to control oligonucleotides (fold changes,  $0.36 \pm 0.094$  vs.  $1 \pm 0.131$ ,  $P < 0.05$ ). As shown in Fig. 2a, miR-26a suppression resulted in decreased cellular proliferation by cell cycle arrest of the G1 phase ( $83.13 \pm 1.83$  % in miR-26a inhibited cells vs.  $54.01 \pm 0.73$  % in control cells,  $P < 0.05$ ), while no significant changes in cell apoptosis, migration and invasion were observed in OE33 cells after miR-26a downregulation (Fig. 2b, c, d,  $P > 0.05$ ). In addition, we also investigated the effect of miR-26a upregulation on proliferation, apoptosis, migration and invasion of OE33 cells, and however no significant changes were observed (data not shown). Kota et al. [19] reported that miR-26a could inhibit cell proliferation and induce tumor-specific apoptosis in human hepatoma HepG2 cells. In this study, we observed that in OE33 cells miR-26a downregulation, but not upregulation, could cause a significant inhibition of proliferation, and miR-26a upregulation also couldn't induce significant apoptosis, which are contrary to the findings of Kota et al. [19], indicating that in different cell types miR-26a may play different, and even antagonistic functional roles.

MiR-26a downregulation increases survival of OE33 cells and resistance to anoikis and apoptosis

Because miR-26a downregulation is likely to contribute to the acquisition of anoikis-resistance, we next examined the

**Fig. 1** MiR-26a suppression correlates with resistance to anoikis and subsequent metastasis in EA. The expression of miR-26a was investigated by qRT-PCR. **a** Data are shown as fold changes of miR-26a expression in liver metastases relative to primary EA tissues, which is set as 1. **b** The relative expression level of miR-26a in OE33/AR cells compared to OE33 cells. Experiments were carried out in triplicate for each data point.  $*P < 0.05$ . **c** MiR-26a expression in clinical EA specimens. The term  $-\Delta Ct$  was used to describe the expression level of miR-26a ( $-\Delta Ct = Ct_{U6} - Ct_{miR-26a}$ )





**Fig. 2** The effects of miR-26a suppression on the malignant phenotypes of EA OE33 cells. **a** MiR-26a suppression induced significant cell cycle arrest at G1 phase. **b, c, d** MiR-26a suppression has no

obvious effects on apoptosis, cell migration and invasion. All data are representative of five independent experiments. \* $P < 0.05$

effect of miR-26a on anoikis sensitivity in OE33 cells and its subline OE33/AR cells. As shown in Fig. 3a, antisense-mediated suppression of miR-26a expression in OE33 cells resulted in significant inhibition of anoikis ( $40.26 \pm 2.63$  % in miR-26a inhibited cells vs.  $63.84 \pm 2.91$  % in control cells,  $P < 0.05$ ), whereas elevating the levels of miR-26a by transfection of miR-26a mimics increased the sensitivity of OE33/AR cells to anoikis ( $31.08 \pm 2.57$  % in miR-26a elevated cells vs.  $17.40 \pm 1.66$  % in control cells,  $P < 0.05$ ). Furthermore, we evaluated the effect of miR-26a on the sensitivity of OE33 cells to 5-Fu-induced apoptosis. As shown in Fig. 3b, miR-26a downregulation was able to inhibit the apoptosis induced by 5-FU, and however, miR-26a upregulation appeared to have no significant effect on 5-Fu-induced apoptosis. These results indicated that miR-26a suppression could increase the survival of OE33 cells and decrease its sensitivity to anoikis or chemotherapy-induced apoptosis.

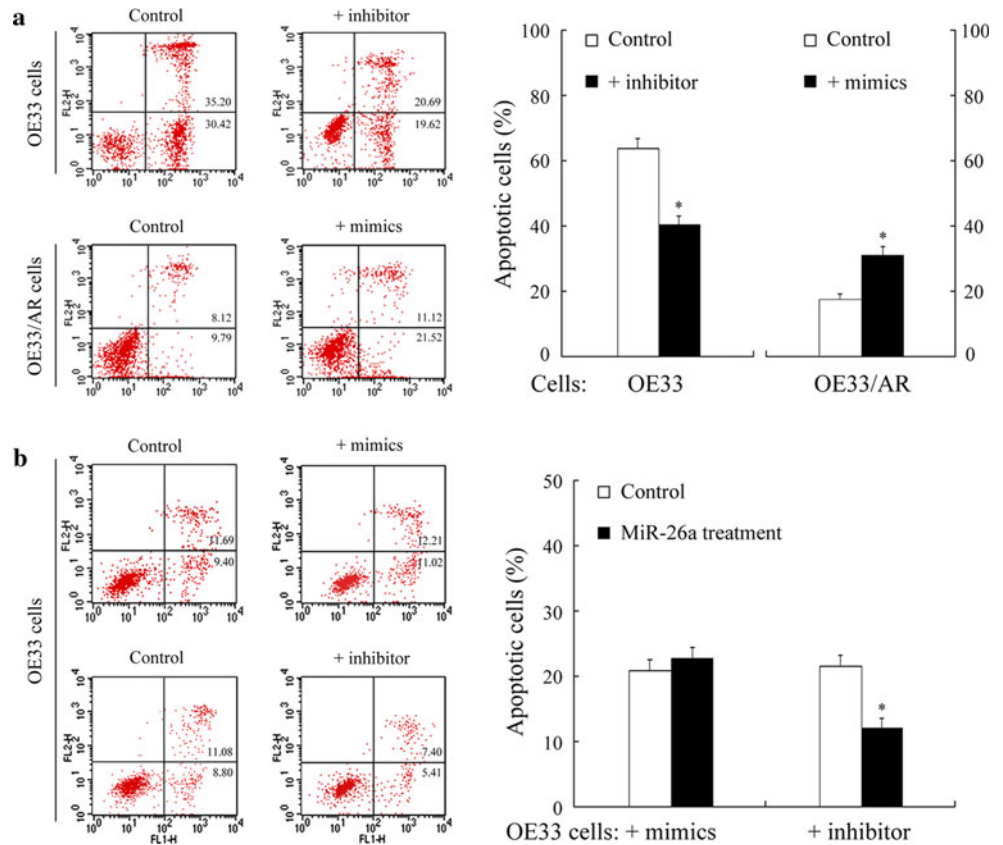
Rb1 is a direct functional target of miR-26a in EA cells

To assess how suppression of miR-26a expression leads to decreased proliferation and resistance to anoikis of EA cells, we searched for the potential regulatory targets of miR-26a using prediction tools, including miRanda,

miRBase, PicTar, and TargetScan. Although hundreds of different targets were predicted, those genes involved in cell proliferation and apoptosis may be the relevant targets with respect to the biological functions of miR-26a in EA cells. We then performed a functional classification of the predicted targets using the miRGEN database (<http://www.diana.pcbi.upenn.edu/miRGen.html>). Of these selected genes, Rb1 has been demonstrated to be a direct functional target of miR-26a in some cell types and has the functions of regulating cell proliferation and apoptosis mainly through direct interaction with E2F1 [20, 21]. E2F1 plays a critical role in regulating cell proliferation, and its function is mostly controlled by Rb1 protein [22]. In addition, E2F1 was found to be able to repress NF- $\kappa$ B activation through inhibiting the degradation of I $\kappa$ B- $\alpha$  [23–25]. And for that, increased Rb1 protein expression resulted from miR-26a suppression could enhance NF- $\kappa$ B activation, by which cells counteract apoptosis. These findings suggest that in EA cells miR-26a suppression may cause increased expression of Rb1 protein, and thus result in inhibition of E2F1 activity, which leads to arrest of cell cycle progression and decreased sensitivity to apoptosis.

To test our hypothesis, we analyzed the expression of Rb1 in OE33 and OE33/AR cells. The results showed that the Rb1 protein level in OE33/AR cells or OE33 cells transfected with

**Fig. 3** MiR-26a suppression increases the resistance of OE33 cells to anoikis and apoptosis. The sensitivity of cells to anoikis or 5-Fu-induced apoptosis was evaluated by flow cytometry. **a** The effects of miR-26a expression on the sensitivity to anoikis in OE33/AR or OE33 cells. **b** Effects of miR-26a expression on the sensitivity of OE33 cells to 5-Fu-induced apoptosis. All data are representative of five independent experiments. \* $P < 0.05$



miR-26a inhibitor was significantly greater than that in their corresponding control cells, respectively. In contrast, RT-PCR did not show any difference in the mRNA level (Fig. 4a). Further, to ascertain whether miR-26a directly regulate Rb1 expression through the target site in the 3'UTR of Rb1 mRNA, we constructed a luciferase reporter vector with the putative Rb1 3'UTR target site for miR-26a downstream of the luciferase gene (pMIR-Rb1-3'UTR) and mutant version thereof with a deletion of 5 bp from the site of perfect complementarity (pMIR-mut-Rb1-3'UTR) (Fig. 4b). Luciferase reporter vector together with miR-26a mimics or mimics control were transfected into OE33/AR cells that weakly expressed miR-26a. A significant decrease in relative luciferase activity was noted when pMIR-Rb1-3'UTR was cotransfected with miR-26a mimics but not with scrambled oligonucleotides ( $P < 0.05$ ). As expected, this suppression was abolished by deleting part of the perfectly complementary sequences in the Rb1 3'UTR (pMIR-mut-Rb1-3'UTR) which disrupts the interaction between miR-26a and Rb1 (Fig. 4c).

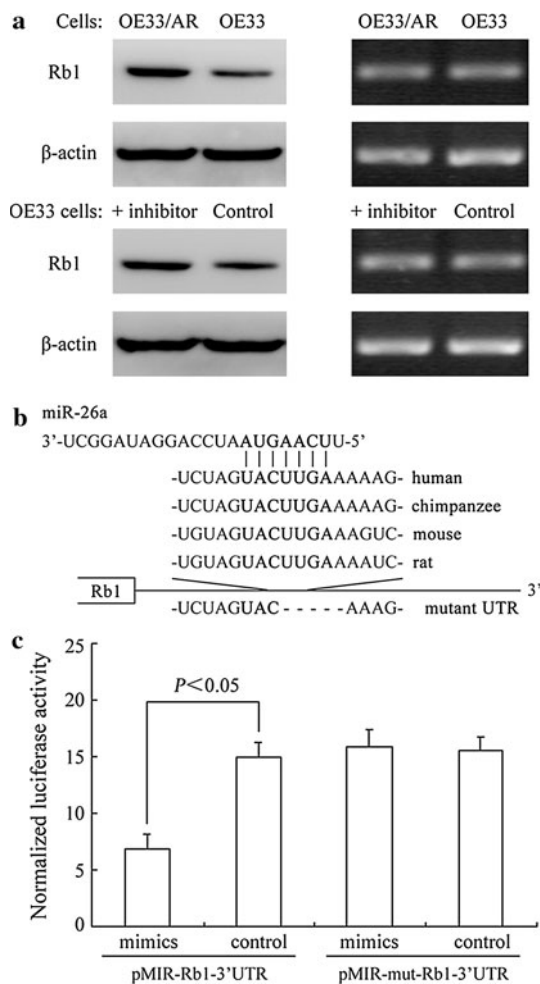
RB1 interacts with E2F1 and represses its transcription activity, leading to cell cycle arrest

As Rb1 interacts with E2F1 and suppresses E2F1-driven transcription, we tested the possibility that Rb1 suppresses

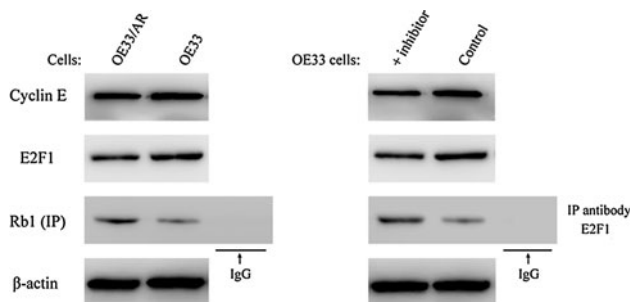
the expression of Cyclin E induced by E2F1. Cyclin E is one of the key regulators of the G1/S transition in the cell cycle, and is normally induced by E2F1 at the transition from G1 into S phase [26, 27]. By using co-immunoprecipitation, we identified that in OE33/AR cells or miR-26 downregulated OE33 cells the level of Rb1 complexed with E2F1 increased significantly compared with their corresponding controls. And as a consequence, the free E2F1 protein levels decreased, leading to the inhibition of Cyclin E activity. (Fig. 5).

Increased interaction of Rb1 with E2F1 promotes resistance to anoikis and apoptosis through activation of NF- $\kappa$ B pathway.

Activation of NF- $\kappa$ B is a common event in response to stress and seems to be an important mediator of cell survival in many contexts [28]. E2F1 has been shown to inhibit the phosphorylation of I $\kappa$ B- $\alpha$  and thereby block NF- $\kappa$ B activation [23–25], by which it can inhibit protective pathways in response to stress signals. In this study, we analyzed whether NF- $\kappa$ B activation was involved in the acquisition of anoikis-resistance. As revealed in Fig. 6a and 6b, after detachment, the phosphorylation level of I $\kappa$ B- $\alpha$  in miR-26a-downregulated OE33 cells was significantly greater than that in control cells, while the NF- $\kappa$ B p65 protein level in the nucleus was also increased markedly. We also compared the expression levels of NF- $\kappa$ B p65 in



**Fig. 4** Rb1 is a direct target of miR-26a. **a** The protein and mRNA levels of Rb1 in OE33/AR cells or OE33 cells transfected with miR-26a inhibitor are respectively detected by Western blotting (*left panel*) and RT-PCR (*right panel*). **b** The Rb1 3'UTR was a potential target of miR-26a. **c** Assay for luciferase activity was performed in OE33/AR cells as described in “Methods” Section. The values are the means from three independent experiments  $\pm$  SEM



**Fig. 5** MiR-26a suppression leads to increased interaction of Rb1 with E2F1 and thus decreased the transcriptional activity of E2F1. Whole-cell lysates were prepared and subjected to co-immunoprecipitation with anti-E2F1 antibody or a control antibody (IgG), followed by Western blot analysis

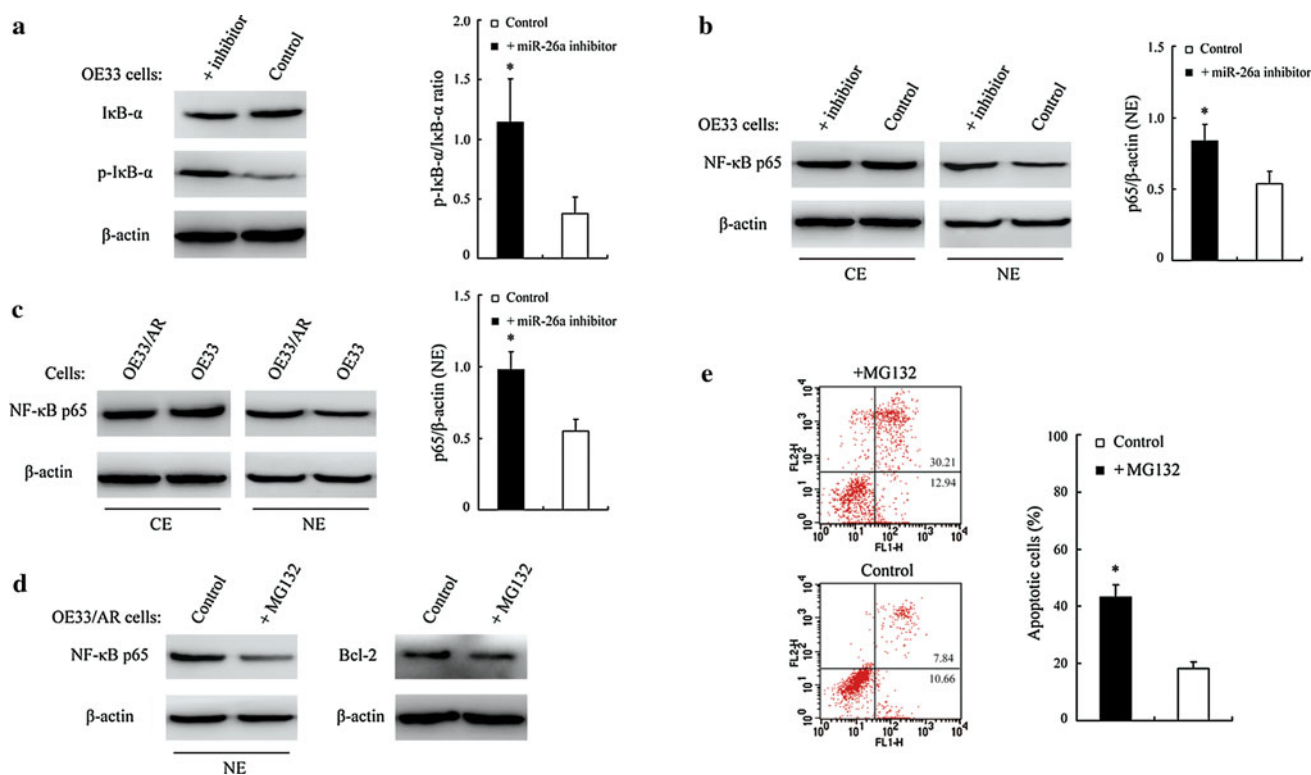
OE33/AR and OE33 cells. As revealed in Fig. 6c, the protein level of NF- $\kappa$ B p65 in the cytoplasm was not significantly different between OE33/AR and OE33 cells, while for the NF- $\kappa$ B p65 protein level in the nucleus it was significantly higher in OE33/AR cells than in OE33 cells. To further ensure that the enhanced activation of NF- $\kappa$ B plays a key role in the acquisition of anoikis-resistance of OE33/AR cells, we detected the effect of MG132, an inhibitor of NF- $\kappa$ B activation, on the anoikis-resistant phenotype of OE33/AR cells. As shown in Fig. 6d, e, MG132 treatment markedly decreased the levels of nuclear NF- $\kappa$ B p65 protein and its downstream target protein Bcl-2, resulting in increased anoikis of OE33/AR cells after detachment ( $43.25 \pm 4.38$  % in MG132 treated cells vs.  $18.13 \pm 2.42$  % in control cells,  $P < 0.05$ ).

## Discussion

The miR-26a (hsa-miR-26a-1) gene is now generally considered as a tumor suppressor gene. Several studies indicated that ectopic miR-26a expression in tumor cells could induce cell cycle arrest at G1 phase through inhibiting the expression of EZH2 at post-transcriptional level [17, 29, 30]. Kota et al. [19] also reported that in hepatocellular carcinoma HepG2 cells miR-26a directly down-regulates cyclins D2 and E2 and induces a G1 arrest. In addition, Kota et al. [19] found that delivery of miR-26a by adeno-associated virus could not only reduce cell progression, but also induce tumor-specific apoptosis in vivo, indicating that miR-26a is involved in the regulation of both cell proliferation and apoptosis.

In this study, we investigated the effects of miR-26a on the malignant phenotypes of human EA OE33 cells. As shown in the results, miR-26a expression was found to directly participate in the regulation of cell proliferation and apoptosis. However, differently, it was observed that downregulation but not upregulation of miR-26a can induce a significant cell-cycle arrest in OE33 cells. Next, we found that miR-26a upregulation can not directly induce obvious apoptosis of OE33 cells, whereas miR-26a downregulation can increase the resistance of OE33 cells to anoikis or 5-Fu-induced apoptosis. Our observation differs from previous reports, suggesting that in different cell contexts, miR-26a may exhibit markedly different characteristics, many of which are unique or even opposite from the pattern found in other cells.

As part of our research on how miR-26a affects proliferation and apoptosis, we demonstrated that Rb1 was a critical downstream target of miR-26a in human EA OE33 cells. It is known that Rb1 has a crucial role in cell cycle control, mainly acting as the gatekeeper of the G1-S transition through its interaction with E2F proteins, especially



**Fig. 6** Promoted NF- $\kappa$ B activation plays a critical role in the acquisition of anoikis-resistance upon miR-26a suppression. **a**, **b**, **c** I $\kappa$ B- $\alpha$  phosphorylation and NF- $\kappa$ B p65 nuclear translocation were analyzed by Western blotting (CE cytoplasmic extracts; NE nuclear extracts). These results represent averages  $\pm$  SEM from three

separate experiments. **d** MG132 inhibited the nuclear translocation of NF- $\kappa$ B p65 and its promoter activity. **e** MG132 partially reversed the phenotype of resistance to anoikis of OE33/AR cells. The data for apoptosis detection are representative of five independent experiments. \* $P < 0.05$

E2F1 [21]. In addition to the role of Rb1/E2F1 pathway in the control of cell proliferation, it is also clear that this pathway is linked to events that determine cell fate through induction or regulation of apoptosis [21, 22]. Great observations have established that the release of free E2F1 resulting from loss of Rb1 function activates the ARF/MDM2/p53 pathway, leading to induction of apoptosis [31]. However, since OE33 cells harbor mutant p53 [32], the pathway of p53-dependent apoptosis was excluded. E2F1 can also sensitize cells to apoptosis by inhibiting survival signals, in particular those mediated by the transcription factor NF- $\kappa$ B [23–25, 33]. Because Rb1 has already been proven to be a direct target of miR-26a, we propose that downregulation of miR-26a in OE33 cells enhances the expression of Rb1, and thus decreases the release of transcription factor E2F1 from binding to Rb1 protein, which leads to cell cycle arrest on one hand, and on the other hand, for the increased activation of NF- $\kappa$ B, promotes the cell survival against apoptotic stress. To test our hypothesis, we performed luciferase reporter assays with 3'UTR of the Rb1 gene with putative miR-26a binding site, and confirmed Rb1 to be a direct target of miR-26a in EA cells. We also compared the protein and mRNA expression levels of Rb1 in OE33 cells after transfection of

miR-26a inhibitor, or in OE33/AR cells, the results of which further confirmed the posttranscriptional regulation of Rb1 by miR-26a.

Worth of note, in this study, we observed that downregulation of miR-26a in EA cells causes cell cycle arrest but prevents apoptosis. Several lines of evidence have suggested that arrest in G1 stage of the cell cycle can provide resistance to different forms of apoptotic stress [34–36]. In fact, Collins et al. [37] has reported that G1 cell cycle arrest by overexpression of the cyclin-dependent kinase inhibitors p16INK4a, p21Cip1, or p27Kip1 or by treatment with mimosine or aphidicolin all confers anoikis resistance in MCF-10A mammary epithelial cells, suggesting that anoikis resistance, at least in part, is the epiphenomenon of cell cycle arrest. However, because Rb1/E2F1 pathway is of central significance for the control of cell proliferation and also the regulation of apoptosis, we further explored the other possible mechanisms and revealed that promoted NF- $\kappa$ B activation is an important factor in the acquisition of anoikis-resistance upon miR-26a suppression.

As a multifunctional transcription factor, NF- $\kappa$ B plays a pivotal role in a variety of physiological and pathological processes including development, tissue homeostasis and



carcinogenesis [38]. In tumor cells, NF- $\kappa$ B is generally seen as a survival factor because numerous NF- $\kappa$ B targets (such as Bcl-2, Bcl-xL, cIAP-1, cIAP-2, etc.) have anti-apoptotic properties [39]. The adhesion of cells to the extracellular matrix (ECM) by interacting with integrins is essential for cell survival and proliferation [40]. Integrins are a family of transmembrane proteins, contributing for survival signal transduction from the extracellular environment to the intracellular network. If a cell is detached from ECM, it loses integrin-mediated survival signals, and will undergo anoikis [40]. Detachment and subsequent anoikis is a kind of stress to cells. For NF- $\kappa$ B is a major signaling molecule activated in response to various types of stress (including detachment), its activation level is unequivocally considered as the key factor that determines survival or death of the cells [28]. In this study, we observed that the translocation of NF- $\kappa$ B p65 subunit into the nucleus in OE33/AR cells is obvious greater than in OE33 cells upon detachment. Further, we provided evidence supporting that miR-26a suppression can lead to increased phosphorylation of I $\kappa$ B- $\alpha$  and thus contribute to enhanced nuclear translocation of NF- $\kappa$ B p65 subunit. These results clearly indicated that the hypothesized mediating pathway from miR-26a suppression to promoted NF- $\kappa$ B activation actually exists in human EA cells.

In closing, we presented the evidence that miR-26a may be involved in regulation of anoikis-resistance of EA cells. This result provides a novel insight into the mechanisms of anoikis-resistance and maybe useful for the future development of metastasis prevention strategy.

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**Conflict of interest** The authors state no conflicts of interest

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