

Original Article

MiR-125a suppresses ovarian cancer proliferation and motility by targeting HDAC4

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Received September 6, 2015; Accepted October 21, 2015; Epub February 1, 2016; Published February 15, 2016

Abstract: Increasing evidence has suggested that dysregulation of microRNAs (miRNAs) could contribute to tumor progression. The miR-125a was downregulated in several types of cancer; however, the molecular mechanism of miR-125a in the ovarian cancer remains unclear. The aim of the paper was to study the expression of miR-125a in ovarian cancer and miR-125a's relation to the cell proliferation and invasion in ovarian cancer. In this study, we demonstrated that miR-125a is downregulated in ovarian cancer tissues and cell lines, compared with the corresponding adjacent non-neoplastic tissues and normal human fallopian tube epithelial cell line. Histone deacetylase 4 (HDAC4), which has been reported to be associated with invasive ovarian cancer, was identified as a novel target of miR-125a in OVCAR-3 ovarian cancer cells, and the protein expression of HDAC4 was negatively regulated by miR-125a in OVCAR-3 cells. Additionally, upregulation of miR-125a and downregulation of HDAC4 suppressed ovarian cancer cell proliferation and invasion. These data suggest that miR-125a may suppress ovarian cancer cell proliferation and invasion through direct inhibition of HDAC4 expression. Taken together, our findings show that miR-125a functions as tumor suppressor in ovarian cancer by targeting HDAC4, and miR-125a may therefore serve as a biomarker for diagnosis and therapeutics in ovarian cancer.

Keywords: miR-125a, HDAC4, ovarian cancer, proliferation, invasion

Introduction

Ovarian cancer is the 5th leading cause of cancer-related deaths among women in the world and considered the most lethal gynecologic malignancy [1]. Despite improvements of diagnosis and treatment of ovarian cancer, metastasis leads to a high mortality and low 5-year survival rate of patients with ovarian cancer [2, 3]. It has been demonstrated that dysfunction of oncogenes or tumor suppressors is closely associated with the development and progression of ovarian cancer [4]. Accordingly, developing novel molecular targets may be promising for the development of therapeutic strategies for invasive ovarian cancer.

MicroRNAs (miRNAs), as a class of small (22-nucleotide) non-coding RNAs, have been identified to be aberrantly expressed in several human malignancies [5]. miRNAs regulate gene

expression by binding to the 3'untranslated region (3'-UTR) of their target mRNAs, modulating mRNA stability and/or translation [6]. By negatively regulating the protein expression of their targets, microRNAs exert adverse effects on cell survival, proliferation and motility [4]. Dysfunctions of miRNAs, which act as oncogenes or tumor suppressors, have been demonstrated to be associated with human malignancies [7]. Furthermore, deregulations of numerous miRNAs have been revealed to contribute to the development and progression of invasive ovarian cancer, including miR-186, miR-126, miR-153, miR-129, miR-572 and miR-34a [8-13]. However, the expression and role of miR-125a in ovarian cancer remains unknown.

Histone deacetylases (HDACs) are the key enzymes which regulates the acetylation status of both histone- and non-histone proteins [14]. Recently, HDACs are reported to be frequently

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altered expression in various cancers [15]. It was reported that they involve in cancer initiation and progression by altering cellular epigenetic programming [16]. Moreover, overexpression of HDACs was also reported to promote invasion, migration, angiogenesis, decreased adhesion, and decreased apoptosis in cancer cells [17, 18]. Recently, drug-induced inactivation or gene silencing of class I histone deacetylases suppresses ovarian cancer cell growth, indicating that suppressing HDACs expression is an important direction in anti-cancer drug development in ovarian cancer [19, 20].

In the current study, we confirmed that miR-125a was down-regulated in ovarian cancer. Furthermore, miR-125a targets HDAC4 and inhibited the expression of HDAC4 both at the mRNA and protein levels. Also, overexpression of miR-125a suppressed ovarian cancer cell proliferation and invasion by suppressing HDAC4 expression. In conclusion, we found that miR-125a functions as a tumor suppressor by directly targeting HDAC4. Thus, our findings provide significant clues regarding the role of miR-125a as a tumor suppressor in ovarian cancer.

Materials and methods

Patients and samples

Human ovarian cancer specimens (n = 25) and paired adjacent specimens (n = 25) were obtained from the patients at Qilu Hospital of Shandong University with documented individual informed consent. Patients undergoing surgery for ovarian cancer provided the written consent to donate tissue for analysis.

Cell culture and transfection

Human ovarian carcinoma cell line OVCAR-3 was maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (HyClone, Logan, Utah, USA) and 1% penicillin/streptomycin and incubated in a humidified (37°C, 5% CO₂) incubator.

Human ovarian carcinoma cells were transfected with miR-125a mimic or miR-Ctrl (50 nM; GenePharma, Suzhou, China) using Lipofectamine 2000 reagent (Invitrogen). HDAC4 siRNA, 5'-CGACTCATCTTGAGCTTATT-3'. Cells were incubated for 48 h after transfection and used for further experiments.

qRT-PCR

The PCR amplification for the quantification of the miR-125a and U6 was performed using TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) and TaqMan Human MiRNA Assay Kit (Applied Biosystems, Foster City, CA, USA). The relative expression of miR-125a was shown as fold difference relative to U6. The PCR amplification for the quantification of the HDAC4 and GAPDH mRNAs was performed using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and a SYBR®Premix Ex Taq™ ii (Perfect Real Time) Kit (Takara Bio, Shiga, Japan).

Western blot

Whole cell extracts were prepared with a cell lysis reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manual, and then, the protein was quantified by a BCA assay (Pierce, Rockford, IL, USA). Then, the protein samples were separated by SDS-PAGE (10%) and detected by Western blot using polyclonal (rabbit) anti-HDAC4 antibody (Santa Cruz Bio-technology, Santa Cruz, CA, USA). Goat anti-rabbit IgG (Pierce, Rockford, IL, USA) secondary antibody conjugated to horseradish peroxidase and ECL detection systems (SuperSignal West Femto, Pierce) were used for detection.

Luciferase assay

After 48 h of transfection, cells were lysed with 1× reporter lysis buffer, and renilla luciferase activities were measured using the Dual-Luciferase Reporter Kit (Promega) according to the manufacturer's instructions. Finally, luciferase activity was standardized to the renilla activity as control.

Cell proliferation and invasion assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to estimate cell viability [21]. Briefly, cells were plated at a density of 1×10⁴ cells per well in 96-well plates. After exposure to specific treatment, the cells were incubated with MTT at a final concentration of 0.5 mg/ml for 4 h at 37°C. After the removal of the medium, 150 mM DMSO solutions were added to dissolve the formazan crystals. The absorbance was read at 570 nm using a multi-well scanning

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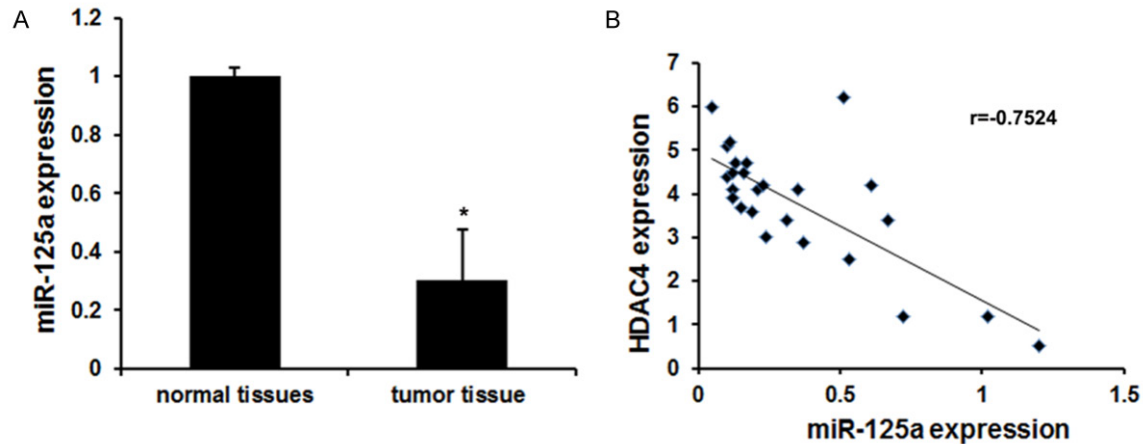


Figure 1. miR-125a is negatively associated with HDAC4 in clinical ovarian tissues. A. miR-125a expression levels were examined by qRT-PCR in 25 cases of clinical ovarian tissues and paired non-tumorous tissues. B. Correlation of miR-125a levels with HDAC4 mRNA levels was examined by qRT-PCR in 25 cases of clinical ovarian tissues. Statistically significant differences are indicated: * $P < 0.01$ versus paired non-tumorous tissues.

spectrophotometer reader. Cells in the control group were considered 100% viable at 48 h.

The capability of cell invasion was examined by transwell invasion assay. Cells were cultivated to 80% confluence on the 12-well plates. Then, we observed the procedures of cellular growth at 48 h. All the experiments were repeated in triplicate. The transwell migration chambers were used to evaluate cell invasion. Then cells invading cells across the membrane were counted under a light microscope.

Statistical analysis

Each experiment was repeated at least three times. Data were shown as mean \pm s.d. and analyzed using SPSS 18.0. Statistical comparisons between groups were analyzed using Student's t-test and a two-tailed $P < 0.05$ was considered to indicate statistical significance.

Results

miR-125a is negatively associated with HDAC4 in clinical ovarian cancer tissues

HDACs was overexpression in various cancer [17, 18], however, the role and mechanism of HDAC4 in ovarian cancer progression and metastasis remains understood. Here, we analyzed the miR-125a expression in 25 paired clinical ovarian cancer and adjacent noncancerous liver tissues using qRT-PCR. When compared with their noncancerous counterparts, significant downregulation of miR-125a was

observed in all the 25 ovarian cancer samples (Figure 1A). Then we assessed the correlation between miR-125a and HDAC4. As expected, we found that the levels of miR-125a exhibited a significant negative correlation with the levels of HDAC4 mRNA (Pearson's correlation coefficient of -0.7524 , $P < 0.01$) (Figure 1B). Overall, our finding indicates that the levels of miR-125a are negatively associated with those of HDAC4 mRNA in clinical ovarian cancer tissues.

miR-125a directly targets HDAC4 in ovarian cancer cells

In this study, the miRNA target prediction websites www.microRNA.org and TargetScan were used and identified a conserved miR-125a-binding site in the 3'-UTR of HDAC4 mRNA. We then cloned WT or Mut target region sequence of the HDAC4 3'-UTR, which was inserted into a luciferase reporter vector (Figure 2A). Subsequently, these reporter vectors were cotransfected with miR-125a mimics and mimics control (mimics_con) into the HEK293T cell line. As shown in Figure 2A, co-transfection of miR-125a mimics suppressed the luciferase activity of the reporter containing wild-type HDAC4 3' UTR sequence, but failed to inhibit that of mutated HDAC4 by dual-luciferase reporter assay. These data indicate that HDAC4 is one of the direct targets of miR-125a in ovarian cancer.

To further investigate the effect of miR-125a on HDAC4, we transfected miR-125a mimics (miR-

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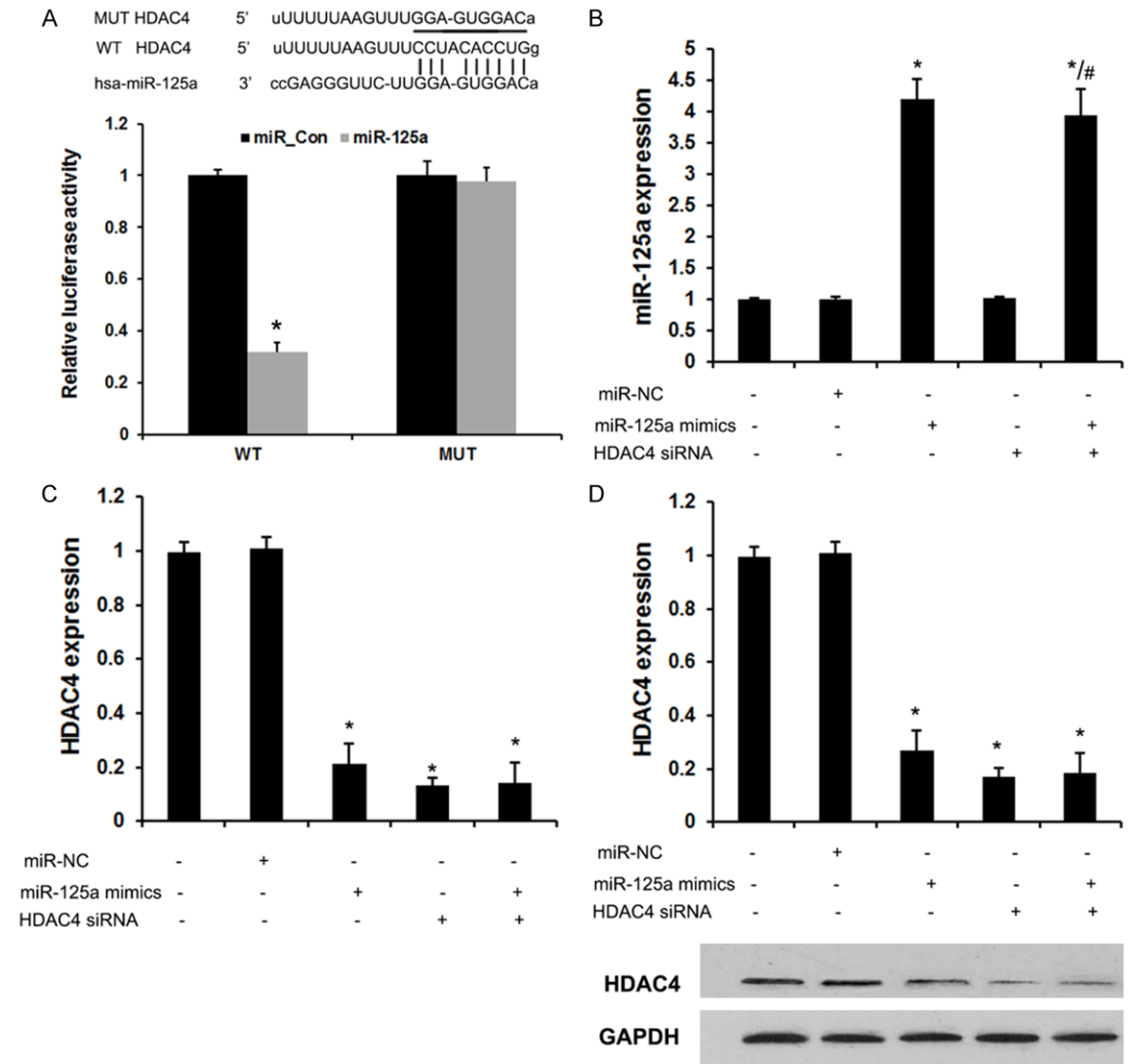


Figure 2. miR-125a directly targeted HDAC4. A. Sequence alignment of miR-125a and 3' UTR of HDAC4 using mircoRNA.org. Luciferase reporter assay with co-transfection of wild-type or mutant HDAC4 and miR-125a mimics or miR-control in HEK293T cells. Error bars represent \pm S.E. and *, $P < 0.01$ versus negative control (NC). B. qRT-PCR analysis revealed the effects of miR-125a mimics (miR-125a mimics group), HDAC4 siRNA (HDAC4 siRNA group), or cotransfected miR-125a mimics and HDAC4 siRNA on the expression level of miR-125a. C. qRT-PCR analysis. D. Western blot analysis revealed the effects of miR-125a mimics, HDAC4 siRNA, or cotransfected miR-125a mimics and HDAC4 siRNA on the expression level of HDAC4. Error bars represent \pm S.E. and *, $P < 0.01$ versus negative control (NC). #, $P < 0.01$ versus HDAC4 siRNA group.

125a mimics group) or HDAC4 siRNA (siRNA group) into ovarian cancer cells. We found that miR-125a mimics markedly increased the expression of miR-125a (Figure 2B) and decreased the expression of HDAC4 both at mRNA and protein levels in ovarian cancers (Figure 2C and 2D). In addition, HDAC4 did not affect miR-125a expression (Figure 2B). In conclusion, these results demonstrated that HDAC4 is a direct target of miR-125a in ovarian cancer.

miR-125a suppresses proliferation and invasion of ovarian cancer cells via HDAC4

It has been reported that miR-125a is implicated in various cancer progression [22, 23]. Here, we detected that effect of miR-125a on cell proliferation and invasion of ovarian cancer cells (Figure 3). MTT assay revealed that cell proliferation was greatly decreased by miR-125a mimics and HDAC4 siRNA (Figure 3A). Consistent with these results, the transwell inva-

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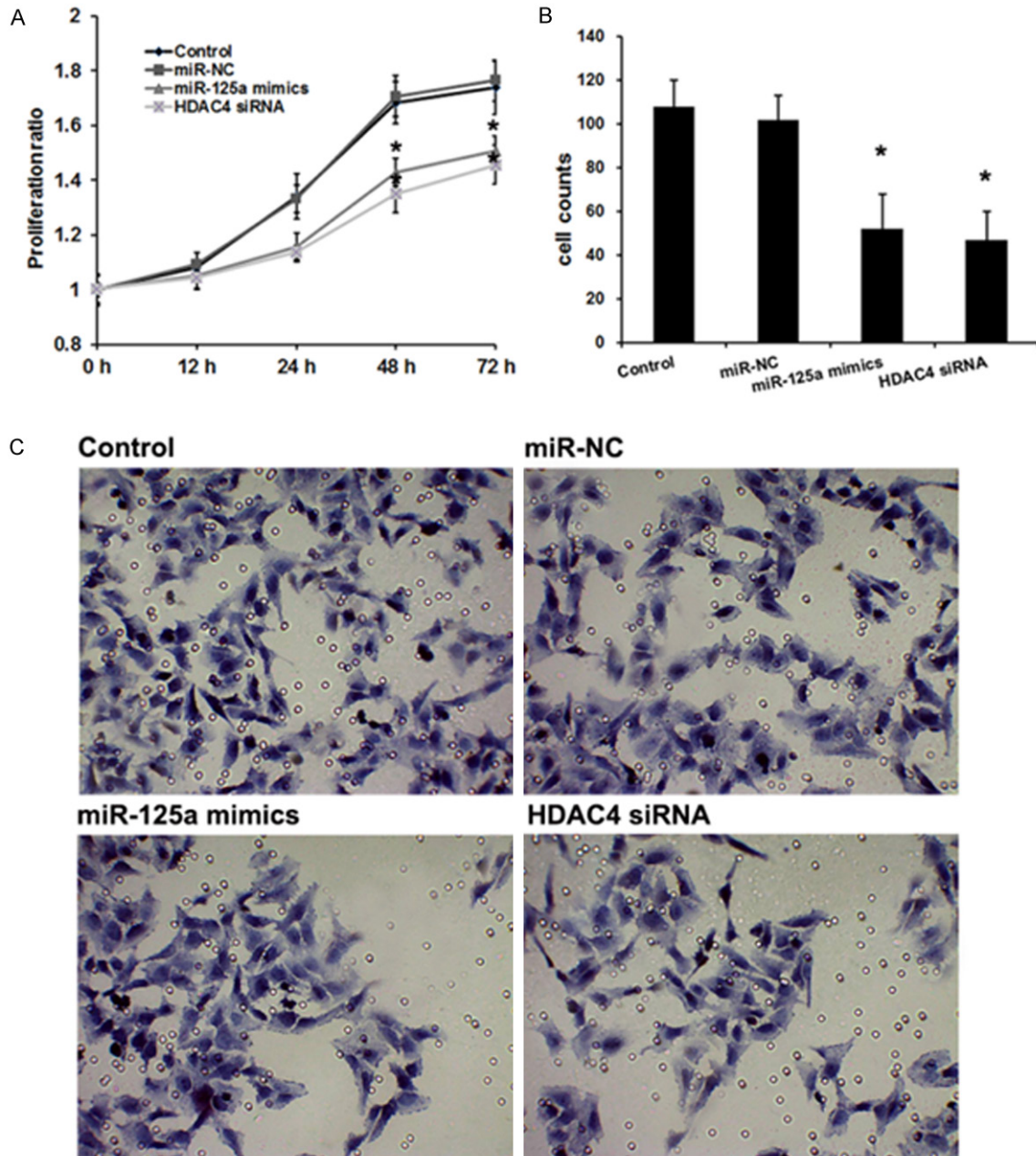


Figure 3. miR-125a affects the invasion and migration of ovarian cancer cells via HDAC4. A. MTT assays revealed the proliferation ability of OVCAR-3 cell transfected with miR-NC, miR-125a or HDAC4 siRNA. B and C. Transwell assays revealed the invasion ability of OVCAR-3 cell transfected with miR-NC, miR-125a or HDAC4 siRNA. Data are the mean \pm SD of duplicates from a representative experiment of three independent experiments. * $p < 0.01$ vs. NC group and control group.

sion assay manifested that cell invasion was decreased when the cells were treated with miR-125a and HDAC4 siRNA (Figure 3B and 3C). Collectively, we concluded that miR-125a inhibits the proliferation and invasion of ovarian cancer cells relying on HDAC4 in part.

Discussion

Accumulated studies revealed deregulated miRNAs in various human cancers including ovarian cancer. Identifying the miRNAs and their targets that are essential for ovarian can-

cer progression may provide promising therapeutic opportunities. In this study, we demonstrated HDAC4 as a direct target of miR-125a and revealed the mechanism of miR-125a-mediated HDAC4 in metastasis of ovarian cancer.

It has been well established that miRNAs were involved in the regulation of diverse physiological and pathological processes, especially tumorigenesis [24, 25]. It was reported that miR-125a was deregulated in various cancers and function as diagnostic and prognostic markers [26, 27]. For example, the reduced expression of miR-125a has shown diagnostic and prognostic significance in non-small cell lung cancer (NSCLC) [28]. miR-125a was also reported as a tumor suppressor by targeting genes associated with cell proliferation and tumor progression [22]. Xu et al. showed that ectopic expression of miR-125a substantially inhibited the proliferation, migration and invasion activities of gastric cancer cells via targeting E2F3 [29]. However, the role and mechanism of miR-125a in ovarian cancer remains unknown. In this study, our data demonstrated that miR-125a expression was greatly decreased in ovarian cancer tissues, and low levels of miR-125a were correlated with high levels of HDAC4 in clinical ovarian cancer samples. Next, we first time to identify the HDAC4 as the direct target of miR-125a and the protein expression of HDAC4 were negatively regulated by miR-125a in ovarian cancer. It has been reported that HDACs and miR-125a involves in proliferation and metastasis in various cancers [17, 18]. Here, by the gain of function assay, it was found that miR-125a overexpression led to a significant inhibition of ovarian cancer cell proliferation and invasion, similar to the effect of HDAC4 inhibition. These findings confirmed that miR-125a plays a role in the regulation of ovarian cancer cell proliferation and metastasis by direct targeting of HDAC4.

In conclusion, we first time demonstrated that miR-125a is downregulated in ovarian cancer tissues and HDAC4 is a novel target genes of miR-125a. Furthermore, we demonstrated the important role of miR-125a-suppressed HDAC4 on ovarian cancer proliferation and metastasis, providing potential therapeutic pathway in ovarian cancer therapy.

Acknowledgements

This work was supported by a grant of Roadmap for Science and Technology Development of Shandong Province (2011GSF11805).

Disclosure of conflict of interest

None.

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