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MIR-29B REGULATES CELL PROLIFERATION AND INVASION IN HUMAN OVARIAN CLEAR CELL CARCINOMA BY TARGETING LYSYL OXIDASE (LOX)

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Abstract: Ovarian cancer is the leading cause of death from gynecologic cancer, reflecting its chemoresistance and frequent late diagnosis, and suggesting that a more effective treatment approach is needed. Lysyl oxidase (LOX) is involved in important biological processes such as gene regulation, cell signaling and cell motility, its deregulation contributing to tumor formation and development. Although it is known that LOX is involved in proliferation, migration and invasion in several types of tumors, studies of LOX in ovarian cancers are scarce. To explore the molecular regulation mechanisms in ovarian cancer tumorigenesis, the expression change and the function of LOX was confirmed in ovarian tissues and cells, which suggested that LOX is a tumor suppressor gene. To further understand how LOX expression is regulated in ovarian cancer, microRNAs (miRNAs) were considered because of their role in post-transcriptional regulation of many genes. Recent work has described differential expression of mature miRNAs in human cancers. Bioinformatics prediction which was used to find the appropriate miRNA regulating LOX, revealed that miR-29b regulates LOX protein level via its binding site on the 3'UTR of LOX mRNA in ES-2 cells, a human ovarian clear cell carcinoma cell line. miR-29b knockdown inhibited proliferation and invasion in ES-2 cells. Taken together, these findings suggest that influencing LOX regulation by changing the level of miR-29b expression could provide a novel potential approach for treating human ovarian clear cell carcinoma.

Key words: Ovarian cancer; LOX; miR-29b; proliferation and invasion; human ovarian clear cell carcinoma

INTRODUCTION

Although the clinical outcome post-surgery and chemotherapy for ovarian cancer has improved, ovarian cancer remains the leading cause of death among the gynecological cancers (Torre et al., 2015). Diagnostic results show that more than 75% of ovarian cancer patients are classified according to the International Federation of Gynecology and Obstetrics (FIGO) as being stage III and IV (Hall and Rustin, 2011; Minion et al., 2015), with the 5-year survival rate about 30% in patients with distant metastasis (Jemal et al., 2011; Torre et al., 2015).

LOX is an extracellular copper-containing enzyme which catalyzes the crosslinking of collagen and elastin, and plays a critical role in extracellular matrix organization (da Silva et al., 2015). Increasing evidence suggests that LOX is involved in tumor progression and metastasis, while its role in tumorigenesis is still contradictory (Boufraqech et al., 2015). Both upregulation and downregulation of LOX have been reported in different types of cancer cell lines and primary tumors (Cheng et al., 2015). LOX has been suggested to function as a tumor suppressor in various cancers, including gastric, lung and pancreatic cancers (Bu et al., 2014; Woznick et al., 2005). Inactivation of LOX by

promoter methylation and loss of heterozygosity has been observed in gastric cancer (Kaneda et al., 2004). Our group suggested that LOX G473A polymorphism is a new risk factor for ovarian cancer and that the LOX protein might be a possible therapeutic target in ovarian cancer (Wang et al., 2012). However, the function and regulation of LOX in ovarian cancer has not been elucidated.

MicroRNAs are a group of RNA species about 22 nucleotides length that regulate protein-coding gene expression via their repressive effect on both the stability and translatability of mRNAs in a sequence-specific fashion (Mezzanzanica, 2015). Hundreds of known human microRNAs are aberrantly expressed in cancer and their roles in cancer initiation and progression have been described (Deng et al., 2014). In recent years, knowledge of the function of microRNAs in ovarian cancer has increased, and different microRNAs have been shown to function either as oncogenes or as tumor suppressor genes. Thus, Liu et al. (2015) reported that miR-17 promotes normal ovarian cancer cells to cancer stem-cell development via suppression of the LKB1-p53-p21/WAF1 pathway, Kinose et al. (2015) showed that the hypoxia-related microRNA miR-199a-3p displays tumor suppressor functions in ovarian carcinoma and Chen et al. (2015) reported that microRNA-490-3p targets CDK1 and inhibits ovarian epithelial carcinoma tumorigenesis and progression. In recent years, the observation of microRNA in peripheral blood has attracted attention, and serum microRNA-145 (Liang et al., 2015), microRNA-200c and microRNA-141 (Gao and Wu, 2015) have been identified as novel biomarkers in human ovarian cancer. In order to explore the novel methods in ovarian cancer therapy, we aimed to identify microRNAs that target LOX in ovarian cancer, and regulate LOX protein level.

To verify the mechanisms for ovarian cancer tumorigenesis and development, we examined the expression and function of LOX in ovarian tissues and cells, and report that LOX is a tumor suppressor gene. To further explore the regulation of LOX in ovarian cancer, bioinformatics prediction was employed to find the microRNA that might regulate the level of LOX protein. Our results showed that there were three miR-29b binding sites on the 3'UTR of LOX mRNA. Because LOX mRNA and miR-29b showed opposite expression in ES-2 cells, a human ovarian clear cell carcinoma was used. Western blot and luciferase reporter assay indicated that miR-29b could inhibit the level of LOX protein level, whereas miR-29b knockdown inhibited ES-2 cell proliferation and invasion. We suggest that the combined use of miR-29b and LOX might provide a new therapeutic approach for ovarian cancer.

MATERIALS AND METHODS

Ovarian cancer tissue samples

Ovarian cancer tissues were obtained from patients undergoing surgical resection at the Yantai Yuhuangding Hospital of Qingdao University School of Medicine between 2008 and 2014. No patient had undergone radiotherapy, chemotherapy or adjuvant treatment before surgery. The specimens were microscopically confirmed by more than two pathologists. The ovarian carcinoma histological architecture was defined in terms of World Health Organization classification. Informed consent was obtained from all subjects; and the Qingdao University School of Medicine Ethics Committee approved the study.

Immunohistochemistry

Immunohistochemistry was performed according to manufacturer's instruction (Boster Biotech., Wuhan, China). Briefly, samples were embedded in paraffin and cut into deparaffinized slices, slices underwent antigen retrieval in citrate buffer for 15 min, and were then treated with 0.3% H_2O_2 for 10 min at room temperature. The slices were then blocked by PBS containing 0.2% Tween 20 for 1 h, followed by overnight incubation at 4°C with primary anti-LOX antibody (1:500, sc-32409, Santa Cruz). Slices were washed with PBS and incubated with biotin conjugated rabbit anti-mouse IgG for 2 h. After washing in PBS, sections were treated with Streptavidin-HRP at room temperature for 1 h. After another wash step in PBS, visu-

alization was performed using DAB. Each slice was randomly selected and viewed at × 200 magnification.

Cell lines and culturing

Ovarian cancer cell lines ES-2, OV-1063, SKOV3 and OVCAR-3 were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 (HyClone, Logan, UT, USA) supplemented with 10% FBS, 100 U/mL of penicillin and 100 μg/mL of streptomycin. They were maintained in an incubator under a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed every two or three days according to the recommended culture condition. Cell transfection was performed using Lipofectamine 2000 reagent following the manufactures' protocol.

Plasmid construction

The LOX full-length cDNA was amplified and the pcDNA3-LOX overexpression plasmid were all constructed by Genechem Company (Genechem, Shanghai, China). The pmiR-report-luciferase containing LOX mRNA 3'UTR was purchased from Ribo Company (Ribo, Guangzhou, China). The mutation of the miR-29b binding sites on LOX mRNA 3'UTR was performed by the Transgene Company (Transgene, Beijing, China).

Transfection with miR-29b scramble, mimic and inhibitor

miR-29b scramble, mimic and inhibitor were chemically synthesized nucleotide fragments purchased from the Ribo Company (Ribo, Guangzhou, China). The miR-29b mimic shared the same sequence of endogenous miR-29b; the miR-29b inhibitor shared the reverse complementary sequence pairing with endogenous miR-29b; the sequence of scramble was randomly arranged and was used as the control. Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, USA) was used for transfection with miR-29b scramble, mimic or inhibitor into cells according to the manufacturer's instructions.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from tissues or cells using an Rneasy Mini kit (Qiagen, Hilden, Germany). The RNA purity was evaluated by measuring the absorbance at 260 and 280 nm. First-strand cDNA was synthesized from RNA using random or specific primers, SuperScript III reverse transcriptase and RNase inhibitor according to the manufacturer's instructions (Life Technologies, Carlsbad, CA).

Quantitative real time PCR (qRT-PCR)

The relative expression levels of different genes were analyzed by RT-qPCR using the SYBR Green approach. SYBR Green I amplification mixtures (12 μl) contained 3 μL of cDNA, 6 μL of 2 X Power SYBR Green I Master Mix (Life Technologies) and primers. The primer sequences were as follows: LOX F: 5'-CGGCGGAGGAAAACTGTCT-3'; R: 5'-TCGGCTGGGTAAGAAATCTGA-3'. GAPDH: F: 5'- ACAACTTTGGTATCGTGGAAGG-3'; R: 5'- GCCATCACGCCACAGTTTC-3' The PCRs were conducted on an ABI Prism 7300 PCR machine (Life Technologies). Quantitative data were normalized relative to the internal housekeeping control genes.

Western blotting

After treatment, cells were lysed in RAPI lysis buffer for 5 min at 4°C. Lysates were then centrifuged at 12000 rpm at 4°C for 15 min, and supernatants were collected for protein quantitation. Cellular proteins were separated by 10% SDS-PAGE, and the proteins were transferred to PVDF membrane electrophoretically. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing 0.1% Tween-20 (PBST) at room temperature for 1 h, and then incubated with primary antibody overnight at 4°C. Primary antibodies included anti-LOX (1:500, sc-32409, Santa Cruz) and anti-GAPDH (1:1000, sc-25778, Santa Cruz). After incubation with horseradish peroxide conjugated secondary antibody, the chemiluminescence detection system ECL (Amersham Biosciences, Buckinghamshire, UK) was employed to visualize the appropriate bands.

Cell proliferation assay

Cell proliferation assay was performed by CCK-8 assay. Cells were transfected with *LOX* overexpression plasmid or vector and miR-29b scramble, mimic and inhibitor, using Lipofectamine LTX Reagent (Invitrogen) and then cultured for 24 h. Cells were plated in a 6-well plate at 1×10^5 per well and cells were counted at indicated time points using a CCK-8 Cell Counting Kit.

Migration and invasion assays

Cells were transfected with *LOX* overexpression plasmid or vector and miR-29b scramble, mimic or inhibitor. Cell invasion were evaluated *in vitro* using a MATRIGEL Invasion Chamber (BD Biosciences, USA). Cells were seeded onto the upper transwell chamber at a density of 1×10^5 cells per chamber and maintained in serum-free medium, whereas the lower chamber contained complete medium. Cells were incubated for 24 h in a 5% CO_2 incubator at 37°C. After incubation, non-migrated cells in the upper chamber were removed with a cotton swab. Cells migrated to the lower chamber were stained with 1% Toluidine Blue O (Sigma, St. Louis, MO), and then detected by microplate reader.

Statistical analysis

The values are presented as means±SD. Statistical analysis was performed using the Student's *t*-test (* $p<0.05$). All experiments were done in triplicate and repeated at least three times.

RESULTS

LOX is downregulated in ovarian cancer

In order to explore the biological functions of LOX in ovarian cancer, we first detected the expression level of LOX in the cancer tissues. There were 45 pairs of ovarian cancer tissues and paraneoplastic tissues were involved to verify the protein level of LOX in ovarian cancer. Immunohistochemistry (Fig. 1A) was employed to detect the expression level of LOX, and the result showed that the LOX expression was greatly decreased in the ovarian cancer tissues compared with the paraneoplastic tissues.

LOX expression level was detected in different ovarian cancer cell lines. Through Western blot assay (Fig. 1B), the results showed that LOX expression maintained a relatively high expression level in normal ovarian epithelial cells, whereas it was greatly downregulated in ovarian cancer cell lines, including ES-2, OV-1063, SKOV3 and OVCAR-3.

Overexpressing LOX can suppress ovarian cancer cell proliferation and invasiveness

Ovarian cancer cells exhibited high proliferation and invasiveness. To further study whether LOX participates in cell growth and invasion, the LOX was overexpressed in ES-2 cells via transfecting the pcDNA3-LOX plasmid, which contains the coding sequence (CDS) region of the LOX gene. Western blot showed that the protein level of LOX was significantly in-

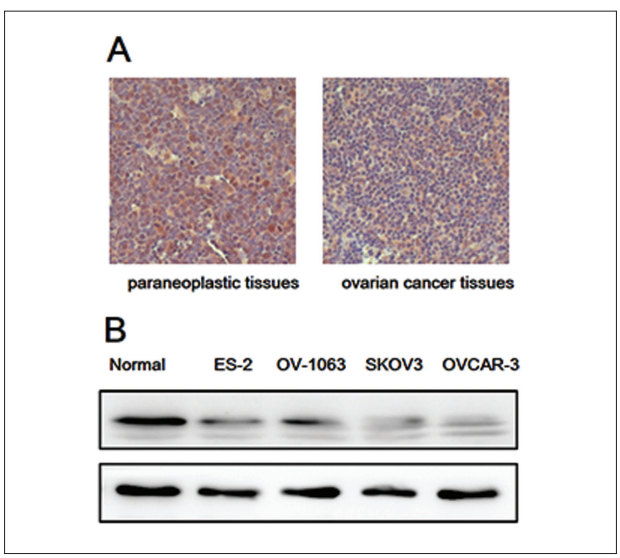

Fig. 1. LOX downregulation in ovarian cancer. **A** – the expression level of LOX detected by immunohistochemistry in the paraneoplastic tissues and ovarian cancer tissues. **B** – detection of LOX protein by Western immunoblotting in normal ovarian epithelial cells and ovarian cancer cell lines, ES-2, OV-1063, SKOV3 and OVCAR-3.

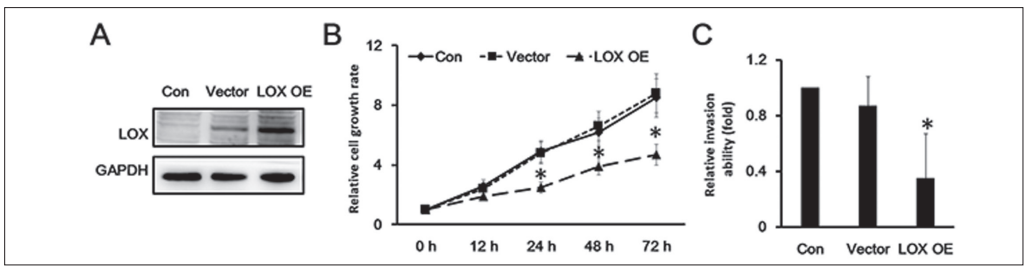

Fig. 2. LOX overexpression suppresses ovarian cancer cells proliferation and invasion. **A** – the expression level of LOX in ES-2 cells transfected with pcDNA3-LOX overexpression plasmid (LOX OE) or with an empty vector. Nontransfected cells served as the negative control. **B** – cell viability assay performed with CCK-8 on ES-2 cells overexpressing LOX and in cells possessing an empty vector. Nontransfected cells served as the negative control. The experiment was repeated at least three times. **C** – transwell analysis performed on ES-2 cells overexpressing LOX or in cells possessing an empty vector; the luciferase unit value for the different cells was compared. Nontransfected cells served as the negative control. The experiment was repeated at least three times. *$p < 0.05$ compared with the negative control.

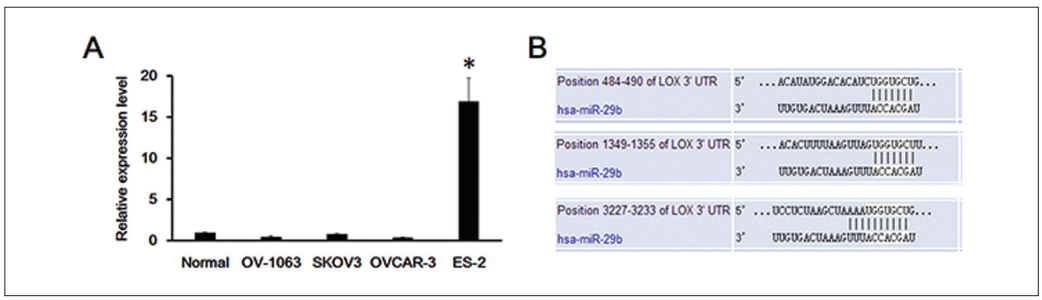

Fig. 3. Expression of miR-29b displayed an aberrant profile in different ovarian cancer cells. **A** – the expression level of miR-29b detected in normal ovarian epithelial cells and ovarian cancer cell lines, including ES-2, OV-1063, SKOV3 and OVCAR-3. The experiment was repeated at least three times. **B** – putative miR-29b-binding sites on LOX mRNA 3'UTR are presented; the potential complementary residues are shown on the white background. *$p < 0.05$ compared with the negative control.

creased in the LOX overexpression (LOX OE) group (Fig. 2A). We then performed CCK-8 and transwell assays, using ES-2 cells to verify their proliferation and invasiveness. We found that LOX overexpression in both cells resulted in a decreased cell survival rate by CCK-8 assay (Fig. 2B), and the invasion rate was also reduced in the transwell assay (Fig. 2C) compared to the control.

Expression of miR-29b showed an aberrant profile in different ovarian cancer cells

To elucidate the detailed molecular mechanisms by which microRNA might target LOX, we screened for potential microRNAs using TargetScan, whereby we found that there were three miR-29b binding sites on the 3'UTR of LOX mRNA (Fig. 3A). To further confirm whether LOX was regulated by miR-29b in ovarian cancer cells, the expression profile of miR-29b was examined via qRT-PCR in different cancer cell lines. The results showed that the miR-29b was down-regulated in OV-1063, SKOV3 and OVCAR-3 cells, whereas the expression level of miR-29b was greatly increased in ES-2 cells (Fig. 3B). Because these cell lines were derived from different positions, for example, OV-1063 was human epithelium ovarian cancer cells, SKOV3 and OVCAR-3 were human ovarian adenocarcinoma cells, whereas ES-2 was human ovarian clear cell carcinoma, it was possible that miR-29b showed different expression levels in different types ovarian cancers. Because LOX mRNA and miR-29b showed opposite expressing trends in ES-2 cells, human ovarian clear cell carcinoma was the focus for the further investigation.

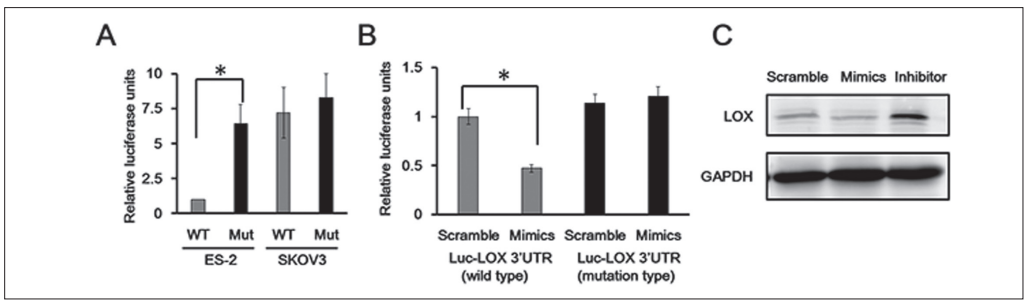

Fig. 4. LOX mRNA is a direct target for miR-29b in ES-2 cells. **A** – luciferase activity was measured in ES-2 cells and SKOV3 cells transfected with either Luc-LOX UTR WT or Luc-LOX UTR Mut. The luciferase unit of Renilla was used as an internal control. The experiment was repeated at least three times. **B** – luciferase activity was measured in HeLa cells co-transfected with miR-29b and either Luc-LOX UTR WT or Luc-LOX UTR Mut. The luciferase unit of Renilla was used as an internal control. The experiment was repeated at least three times. **C** – LOX protein expression level detected in ES-2 cells treated with scramble, mimic or inhibitor miR-29b. GAPDH was used as a loading control. The experiment was repeated at least three times. *$p < 0.05$ compared with the negative control.

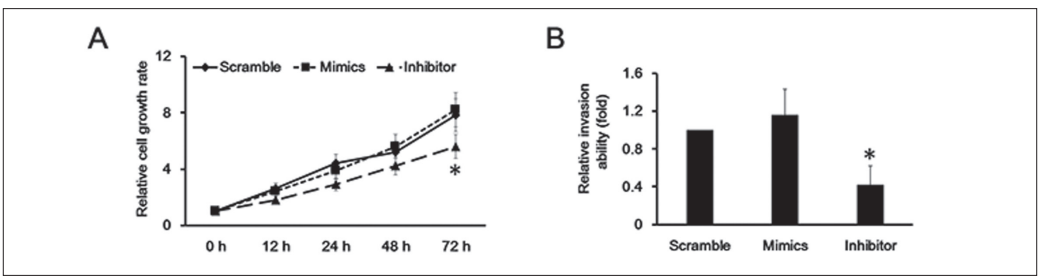

Fig. 5. Knockdown miR-29b could suppress ovarian cancer cells proliferation and invasion. **A** – cell viability assay was performed with CCK-8 on ES-2 cells transfected with scramble, mimic or inhibitor miR-29b; cells treated with scramble served as the negative control. The experiment was repeated at least three times. **B** – transwell analysis performed on ES-2 cells transfected with scramble, mimic or inhibitor miR-29b; the luciferase unit value for the different cells was compared. Cells treated with scramble miR-29b served as the negative control. The experiment was repeated at least three times. *$p < 0.05$ compared with the negative control.

LOX mRNA is a direct target for miR-29b in ES-2 cells

To establish where the LOX gene is a direct target of miR-29b, we placed the wild type or mutant 3'-UTR region downstream of the pmiR-report-luciferase to create Luc-LOX UTR WT and the Luc-LOX UTR Mut. Both constructs were transfected into ES-2 cells and SKOV3 cells, respectively, to determine whether the expression of miR-29b in ovarian cancer cells is indeed functional. The results showed that Luc-LOX UTR WT but not Luc-LOX UTR Mut gave a significantly lower luciferase activity in ES-2 cells than SKOV3 cells (Fig. 4A). Furthermore, the luciferase activity of Luc-LOX UTR WT but not Luc-LOX UTR Mut was brought down by the transfection of miR-29b mimic in HeLa cells (Fig. 4B). In addition, Western blot analysis showed that overexpression of miR-29b did not attenuate LOX expression greatly, whereas knocking down miR-205 elevated the expression of LOX in ES-2 cells significantly (Fig. 4C). Taken together, these results indicate that LOX is a direct target for miR-29b.

Knockdown miR-29b can suppress ovarian cancer cell proliferation and invasion

Next, the functional effects of miR-29b in ES-2 cells were investigated. The growth assay results showed that miR-29b overexpression could slightly increase cell growth, whereas miR-29b knockdown could markedly reduce the cell proliferation rate (Fig. 5A). In addition, results from the transwell assay suggested that overexpression of miR-29b in ES-2 cells significantly increased the invasiveness; on the other hand, down-

regulation of miR-29b by inhibitor could reduce the capability of invasion in ES-2 cells (Fig. 5B). It is likely that miR-29b may exert its oncogene effects via LOX in ES-2 cells through promotion of growth and invasion.

DISCUSSION

LOX-family members are reported to exert both intracellular and extracellular effects, and to share or display specific activities (Baker et al., 2013); LOX affects cell behavior by oxidizing lysine residues and influencing H_2O_2 production (Lucero and Kagan, 2006). How LOX activity affects cancer cell growth is still a matter of debate, as in some cases it was reported to have no effect and in others to favor cancer cell growth *in vitro* or *in vivo* (Baker et al., 2011). For example, Agra et al. (2013) showed that EWS/FLI1 downregulates LOX expression and that, remarkably, LOX propeptide exhibits tumor suppressor activities in Ewing tumor cells. Da Silva et al. (2015) confirmed that LOX plays an important role in migration and angiogenesis in diffusively infiltrative astrocytomas, and LOX expression is influenced by IDH1 mutational status. Furthermore, cancer progression is closely related to its microenvironment, such as the extracellular matrix around the tumor (Allen and Louise Jones, 2011). The hypoxic environment of the tumor increases LOX signaling, which stimulates extracellular matrix remodeling and the activation of focal adhesion kinase (Csiszar, 2001). In the ovary, LOX protein is known to regulate collagen degradation during the ovulatory process: increased levels of LOX mRNA have been observed in the ovaries of rats with polycystic ovary syndrome (Papachroni et al., 2010). The excess synthesis of collagen in polycystic ovarian tissue is partly due to the interaction of angiotensin-converting enzyme signaling and LOX, which stimulates LOX activity (Papacleovoulou et al., 2011). Disruption of the normal activities of LOX in the ovaries can lead to abnormalities that may eventually develop into cancer (Nishioka et al., 2012). A study carried out by our group showed that *LOX* G473A polymorphism is associated with increased susceptibility to ovarian cancer (Wang et al., 2012). Therefore, exploring the function of LOX in ovarian cancer still needs to be done.

In recent years, the field of microRNAs has become the hot area for basic medical research. The function of miR-29b in ovarian cancer cells was reported by several groups. Sugio et al. (2014) reported that BAG3 knockdown appears to downregulate the expression of Mcl-1 through upregulation of miR-29b, thereby increasing the chemosensitivity of ovarian clear cell carcinoma cells. Dai et al. (2014) investigated the expression of miR-29b mRNA and its targeted genes, myeloid cell leukemia sequence 1 (Mcl-1), mitogen-activated protein kinase 10 (MAPK10), and autophagy-related protein 9A (ATG9A) in ovarian carcinomas, as well as their association with clinicopathological characteristics and survival of patients with ovarian cancer; it was established that they are closely related to the chemosensitivity of ovarian carcinoma. Teng et al. (2014) showed that Id-1 expression was increased and miR-29b expression was repressed in TGFβ1-responsive ovarian cancer cells; in addition, Id-1, a protein repressed by miR-29b, facilitates TGFβ1-induced EMT in human ovarian cancer cells. Dai et al. (2013) found that intratumoral injection of a miR-29b chimera significantly inhibited the growth of xenograft OVCAR-3 tumors through downregulation of PTEN methylation and subsequent PTEN expression, as well as via downregulation of MAPK4 and IGF1 expressions. Flavin et al. (2009) showed that miR-29b is downregulated in a significant proportion of ovarian serous carcinomas and is associated with specific clinicopathological features, most notably high miR-29b expression being associated with reduced disease-free survival. However, the relationship between miR-29b and LOX was poorly understood. In the present paper, we have indicated that LOX might play important roles in ovarian cancer tumorigenesis and development.

CONCLUSION

In our study, we studied LOX expression and function in ovarian tissues and cells and suggest that overexpression of LOX could inhibit cell proliferation and invasion. By bioinformatics prediction we established that there were three miR-29b binding sites on the 3'UTR of LOX mRNA. Because of the high expression level of miR-29b in human ovarian clear cell carci-

noma, ES-2 cell line was used for further investigation. Next, miR-29b targeting of LOX was confirmed by Western blot and luciferase reporter assay. Knockdown of miR-29b revealed its antiproliferative and anti-invasive ability in ES-2 cells. LOX targeting by miR-29b in ES-2 cells provides new insight for the design of targeted therapies to control ovarian cancer.

Authors' contributions: Xuan Wang and Peishu Liu conceived and designed the study. Xuan Wang and Yan Wang performed the experiments. Xuan Wang wrote the paper. Xuan Wang, Yan Wang, Guichan Wang and Peishu Liu reviewed and edited the manuscript. All authors read and approved the manuscript.

Conflict of interest disclosure: All authors declare no interest of conflicts.

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