E3 ubiquitin ligase isolated by differential display regulates cervical cancer growth *in vitro* and *in vivo* via microRNA-143

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Abstract. Cervical cancer is one of the most common gynecological cancers worldwide. Aberrant expression of E3 ubiquitin ligase isolated by differential display (EDD) has been detected in various types of tumor and has been demonstrated to have an important role in carcinogenesis, tumor growth and drug resistance. However, the role of EDD in cervical cancer and its underlying molecular mechanisms remains unknown. The present study aimed to investigate the role of EDD in the tumorigenicity of cervical cancer. EDD expression levels were measured using reverse transcription-quantitative polymerase chain reaction and western blotting in SiHa, HeLa, CaSki, c-41 and c-33A cervical cancer cell lines and cervical cancer tissue specimens. A functional study was performed using cell proliferation, colony formation, cell apoptosis assays *in vitro* and tumor growth assays *in vivo* with EDD either overexpressed or silenced. In the present study, EDD expression levels were significantly upregulated in cervical cancer cell lines and tissue samples. EDD knockdown significantly inhibited colony formation, cell proliferation and tumor growth and accelerated cell apoptosis in the cervical cancer cell lines and tissue samples. Furthermore, microRNA (miR)-143 expression levels were low in cervical cancer tissue samples and were negatively correlated with EDD expression. miR-143 silencing eliminated the effect of EDD on cell proliferation, colony formation and cell apoptosis in the cervical cancer cells, which suggested that miR-143 is critical for EDD-mediated regulation of cervical cancer cell growth. The results of the present study indicated that EDD may promote cervical cancer growth *in vivo* and *in vitro* by targeting miR-143. In conclusion, EDD may have an oncogenic role in cervical cancer and may serve as a potential therapeutic target for the treatment of patients with cervical cancer.

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Introduction

Cervical cancer is the most common type of gynecological cancer worldwide, accounting for ~8% of all female malignancies, second only to breast cancer (1,2). Every year, cervical cancer affects ~500,000 women worldwide, ~250,000 of which succumb to the disease (3). Although cervical cancer screening has been popularized globally (4,5), large numbers of patients with advanced cervical cancer remain (6). Notably, hh~85% of novel cases and 80% of fatal cases of cervical cancer occur in developing countries (7,8). Human papillomavirus (HPV) is known to be the most common etiological agent of cervical cancer, and 99% of cervical cancer cases are attributed to human HPV infection (9,10). However, HPV infection alone is not sufficient for the malignant transformation of cervical epithelial cells. Various cofactors and molecular events are required in order to promote the pathogenic process of cervical cancer (11,12); therefore, early detection and treatment of precancerous lesions is particularly important in order to prevent the progression of cervical cancer. The molecular pathogenesis of cervical cancer remains poorly understood. Investigating the molecular mechanisms underlying the development of cervical cancer, searching for novel molecular markers for early diagnosis and developing effective therapeutic targets is urgently required.

E3 ubiquitin ligase isolated by differential display (EDD) is a human ortholog of the *Drosophila melanogaster* hyperplastic discs gene (hyd) (13,14), which was initially isolated as a progestin-regulated gene in human T47D breast cancer cells (14,15). Ubiquitin ligase E3 is able to identify degraded proteins and to conduct ubiquitin tagging of the substrate. Ubiquitin-mediated protein degradation is associated with various important protein signaling pathways, including transcription, cell cycle and DNA damage (16-20). Previous studies have demonstrated that EDD participates in the regulation of cyclin levels and cell cycle progression (21-24), regulates ubiquitination and the degradation of protein phosphatase (25), and has a role in transcriptional regulation and the response to DNA damage (15,26-30). Furthermore, it has previously been demonstrated that EDD is ectopically overexpressed in certain types of cancer and has an important role in cancer cell growth, tumorigenesis and drug resistance (31-36). However the effects of EDD on the occurrence and progression of cervical cancer and its associated molecular mechanisms have yet to be fully elucidated.

MicroRNAs (miRNAs or miRs) are a class of non-coding small RNAs of 20-24 nucleotides in length that regulate gene and protein expression (37). miRNAs participate in a diverse range of biological processes including development, proliferation, differentiation, apoptosis and disease (38-43). Furthermore, certain miRNAs have been demonstrated to function as oncogenes or tumor suppressors, therefore they are directly involved in human cancer, including liver, lung, breast, colon and brain cancer (44-53). The miRNA expression profiles of cervical cancer cells and tissues have previously been analyzed using cDNA cloning (52). The results indicated that aberrant expression of oncogenic and tumor suppressive miRNAs was required for cancer cell growth in cervical cancer, and miR-143 and miR-145 were demonstrated to be the tumor suppressive miRNAs. Furthermore, it was subsequently demonstrated that miR-143 is capable of inhibiting tumor growth and angiogenesis, inducing cancer cell apoptosis and cell cycle arrest, increasing chemosensitivity, and regulating cyclooxygenase stability and expression in colorectal, lung and pancreatic cancer (44,46,50,54). These results suggested that miR-143 had an important role in the carcinogenic process.

The present study aimed to investigate the role of EDD in the tumorigenicity of cervical cancer, and to further elucidate the underlying molecular mechanism, in order to improve the understanding of the pathogenesis of cervical cancer and to aid in the development of novel therapeutics for the disease.

Materials and methods

Tissue samples and cell lines. Human cervical cancer tissue samples (n=39) and normal cervical tissue samples (n=13) were obtained from patients at the Hebei General Hospital (Shijiazhuang, China). The 39 patients with cervical cancer were aged between 27 and 55 years (average age, 46 years) and had an average weight of 54 kg (weight range, 48 to 63 kg). All patients in this group had been diagnosed with stage IA-IVB cervical cancer, according to the FIGO staging system (55). The 13 normal control patients were aged between 30 and 55 years and weighed between 48 and 61 kg. The control patients were undergoing a simple hysterectomy at the Hebei General Hospital due to uterine leiomyomata. All cancer specimens used in the analyses consisted of >90% tumor cells, as examined by a gynecologic pathologist. Cancer specimens from patients with concomitant gynecological problems were excluded from the study. Informed consent was obtained from all patients prior to the surgical procedure, and approval was obtained from the Medical Ethics Committee of the Hebei General Hospital.

Normal cervical epithelial cells and five cervical cancer cell lines, including SiHa, HeLa, CaSki, c-41 and c-33A, were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell culture was conducted according to methods previously described by Liu *et al* (56). Briefly, the cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; both purchased from Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin (all obtained from Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated at 37˚C, in an atmosphere containing 5% CO_2.

Cell proliferation, apoptosis and colony formation assays. SiHa cells ($1x10^4$ cells/ml) were seeded onto 96-well plates, after which cell proliferation and apoptosis were assessed using the MTT Cell Proliferation/Viability Assay kit (cat. no. 11465007001; Sigma-Aldrich Chemie Gmbh, Munich, Germany) and the Annexin-V-FITC Apoptosis Detection kit (cat. no. APOAF-20TST; Sigma-Aldrich Chemie Gmbh) with a flow cytometer (BD FACSCalibur™; BD Biosciences, Franklin Lakes, NJ, USA), respectively, according to the manufacturer's protocols. Subsequently, SiHa cells ($1x10^4$) were suspended in 1.5 ml complete medium [consisting of RPMI 1640 (Invitrogen; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; GE Healthcare Life Sciences, Logan, UT, USA)] containing 0.45% low melting point agarose (Invitrogen; Thermo Fisher Scientific, Inc.) and then seeded into 35 mm tissue culture plates containing 1.5 ml complete medium and 0.75% agarose on the bottom layer, which were then incubated for 10 days at 37˚C. The plates were then stained with 0.005% crystal violet for 30 sec (Sigma-Aldrich Chemie Gmbh) and the SiHa cell colonies (>0.5 mm in diameter) were counted under a microscope (Leica DMI3000; Leica Microsystems GmbH, Wetzlar, Germany), according to a previous study (57).

Cell transfection. In order to analyze the effects of EDD overexpression or knockdown, SiHa cells ($1x10^4$) were transfected with adenovirus-expressing Ad-EDD or retrovirus expressing short hairpin (Sh)-EDD, respectively. Ad-EDD/Ad-negative control (NC) and Sh-EDD/Sh-NC were designed and synthesized by Invitrogen (Thermo Fisher Scientific, Inc.). For miR-143 silencing, SiHa cells ($1x10^4$) were transfected with the pLL3.7 lentivirus vector (Addgene, Cambridge, MA, USA) encoding a miR-143 inhibitor sponge. The miR-143 inhibitor sponge was synthesized by GenePharma (Shanghai, China). The cells were cultured in six-well plates to 70-80% confluency and transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The cells were harvested 48 h post-transfection for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in order to determine the transfection efficiency.

Tumor xenograft experiments. Xenograft mice experiments were performed as previously described (56). Briefly, SiHa cells ($5x10^6$) transfected with Sh-NC, Sh-EDD, Ad-NC or Ad-EDD were injected subcutaneously into the flanks of female athymic nude mice (age, 3-4 weeks; n=6/group), purchased from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China). The mice were maintained under at 21-22˚C under a 12-h light/dark cycle. To obtain the tumors, the mice were sacrificed by an overdose of pentobarbital (50 mg/kg; Sigma-Aldrich Chemie Gmbh) 4 weeks following inoculation.

RT-qPCR analysis. Total RNA (100 ng) was extracted from the harvested tissue samples and cell lines using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was purified using RNase-Free DNase Set (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocols. Subsequently, 5 ng RNA was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR was performed using TaqMan MicroRNA Assays

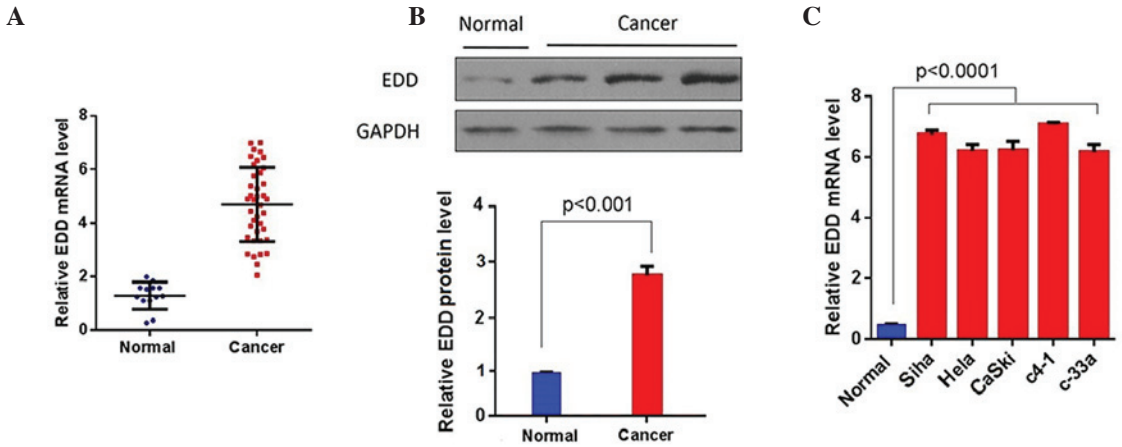

Figure 1. EDD expression levels are upregulated in cervical cancer tissue samples and cell lines. (A) The mRNA expression levels of EDD are upregulated in cervical cancer tissue samples (n=13 in normal group, n=39 in cancer group). (B) The protein expression levels of EDD are upregulated in cervical cancer tissue samples (n=2 in normal group, n=5 in cancer group). (C) The mRNA expression levels of EDD are upregulated in the cervical cancer cell lines (n=3 in each group). EDD, E3 ubiquitin ligase isolated by differential display.

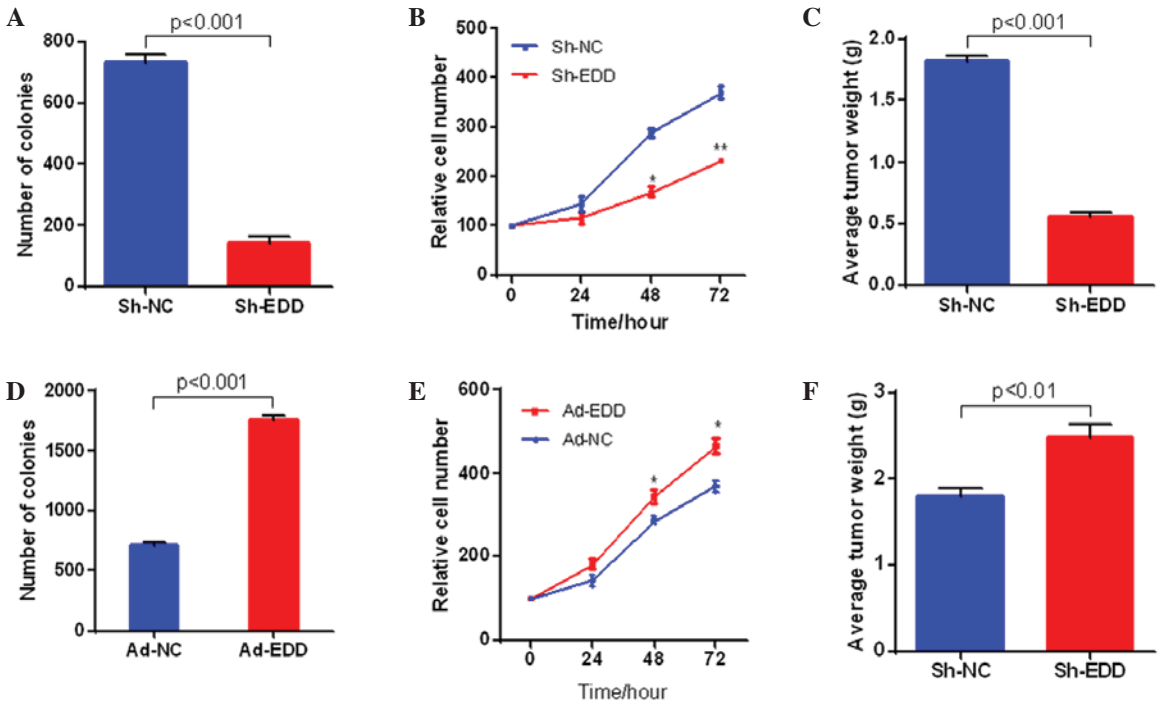

Figure 2. EDD promoted cervical cancer growth *in vitro* and *in vivo*. SiHa cervical cancer cells were infected with retrovirus expressing Sh-NC or Sh-EDD and adenovirus expressing control green fluorescent protein (Ad-NC) and Ad-EDD. (A) EDD knockdown inhibited the colony formation of SiHa cells (n=3 in each group). (B) EDD knockdown inhibited cell proliferation of SiHa cells. *P<0.05 and **P<0.01, vs. sh-NC. (C) EDD knockdown inhibited tumor growth (n=3 in each group). (D) EDD overexpression promoted the colony formation of SiHa cells (n=3 in each group). (E) EDD overexpression promoted the cell proliferation of SiHa cells. *P<0.05, vs. ad-NC. (F) EDD overexpression promoted tumor growth (n=3 in each group). EDD, E3 ubiquitin ligase isolated by differential display; Sh-NC, short hairpin negative control; Sh-EDD, short hairpin EDD.

(Thermo Fisher Scientific, Inc.) on an ABI 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.), according to a previous study (44). The primer sequences were as follows: EDD forward, 5'-TTAGGCTTTTGGTAAATGGCTGCG-3' and reverse, 5'-TGAGGGCATAGGCTGGAATCCTTC-3'; miR-143 forward, 5'-AGTGCGTGTCGTGGAGTC-3' and reverse, 5'-GCCTGAGATGAAGCACTGT-3'; and β-actin forward, 5'-CATCCTGCGTCTGGACCT-3' and reverse, 5'-CAGGAGGAGCAATGATCTTG-3'. β-actin was used as the internal control. EDD, miR-143 and β-actin primers were designed as previously described by Liu *et al* (54) and Clancy *et al* (30). The PCR cycling conditions were as follows: Pre-heating at 95˚C for 5 min, followed by 35 cycles of denaturation for 30 sec at 95˚C, annealing for 1 min at 55˚C and extension for 1 min at 72˚C, with a final extension for 5 min at 72˚C. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Cq}$ method (58).

Western blotting. Western blotting was performed as previously described by Zhang *et al* (44). Briefly, total protein (50 μg)

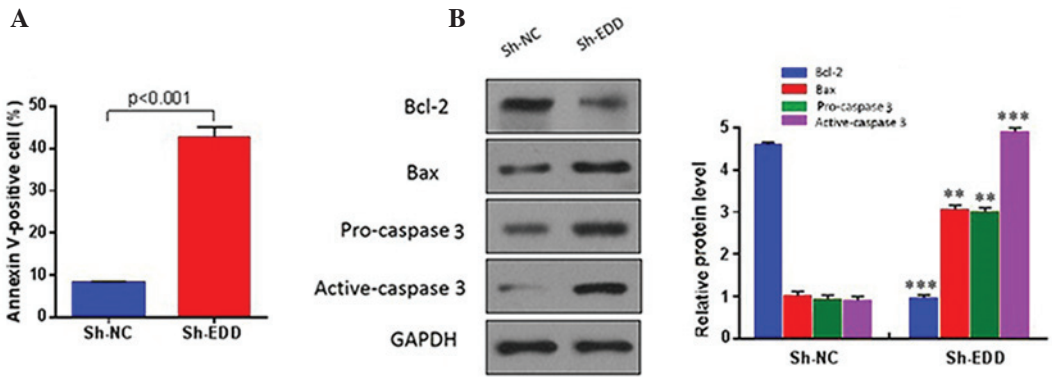

Figure 3. EDD knockdown induces cervical cancer cell apoptosis. SiHa cells were infected with retrovirus expressing Sh-NC or Sh-EDD for 48 h, and fluorescence-activated cell sorting analysis was performed on the cells. The proteins of the SiHa cells were extracted for western blot analysis. (A) EDD knockdown induced the apoptosis of cervical cancer cells (n=3 in each group). (B) EDD knockdown induced the downregulation of anti-apototic protein Bcl-2 expression, and the upregulation of pro-apoptotic Bax and active-caspase 3 expression. **P<0.01 and ***P<0.001 , vs. sh-NC. EDD, E3 ubiquitin ligase isolated by differential display; Bcl-2, B cell lymphoma 2; Bax, Bcl-2-associated X protein; Sh-NC, short hairpin negative control; Sh-EDD, short hairpin EDD.

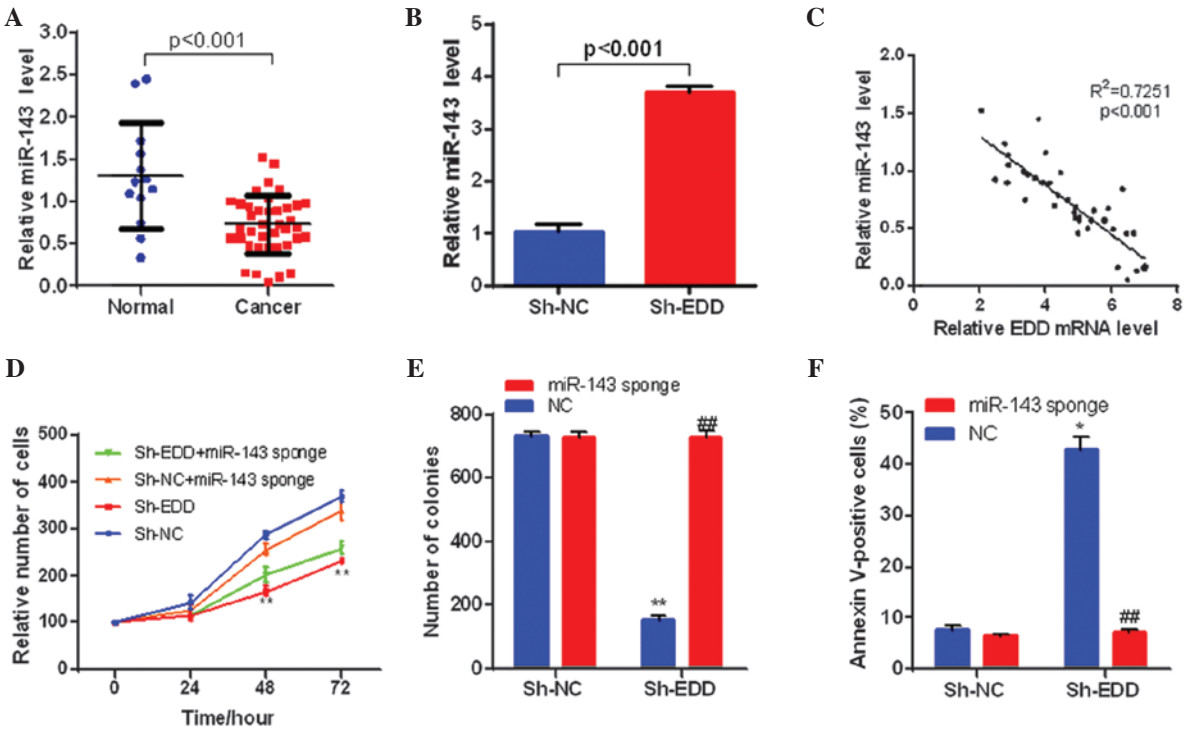

Figure 4. EDD regulates cervical cancer cell growth through miR-143. (A) The expression levels of miR-143 are downregulated in cervical cancer tissue samples (n=13 in normal group, n=39 in cancer group). (B) EDD knockdown increases miR-143 expression levels in SiHa cells (n=3 in each group). (C) The expression of miR-143 was negatively correlated with the expression of EDD in cervical cancer tissue samples (n=39). (D) miR-143 silencing partly reversed the inhibition effect on cell proliferation caused by EDD knockdown in SiHa cells. **P<0.01, vs. sh-NC. (E) miR-143 silencing eliminated the effect on colony formation caused by EDD knockdown in SiHa cells (n=3 in each group). **P<0.01, vs. sh-NC + NC; $^{\#\#}$P<0.01, vs. sh-EDD + NC. (F) miR-143 silencing prevented the apoptosis caused by EDD knockdown in SiHa cells (n=3 in each group). *P<0.05, vs. sh-NC + NC; $^{\#\#}$P<0.01, vs. sh-EDD + NC. EDD, E3 ubiquitin ligase isolated by differential display; Sh-NC, short hairpin negative control; Sh-EDD, short hairpin EDD; miR, microRNA.

was extracted from the tissue samples and SiHa cell line using lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 25 mM 2-mercaptoethanol, 0.5 mM AEBSF, 0.5% Triton X-100, 2 μg/ml leupeptin and 3 μg/ml aprotinin) and the protein concentrations were measured using a Bio-Rad Protein Assay kit (cat. no. 5000002; Bio-Rad Laboratories, Inc., Hercules, CA, USA). Proteins were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Merck Millipore, Darmstadt, Germany). Subsequently, the membranes were blocked with 10% skimmed milk solution and then incubated overnight at 4˚C with goat anti-EDD (cat. no. sc-9562), rabbit anti-B-cell lymphoma (Bcl)-2 (cat. no. sc-492), rabbit anti-Bcl-2-associated X protein (Bax; cat. no. sc-493), goat anti-caspase 3 p11 (cat. no. sc-1224) and goat anti-GAPDH (cat. no. sc-48166) polyclonal antibodies (1:2,000; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Following washing with phosphate-buffered saline, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG, $F(ab')_2$ (1:3,000; cat. no. sc-3836) and chicken anti-goat IgG (1:3,000; cat. no. sc-516086; both Santa

Cruz Biotechnology, Inc.) for 45 min at room temperature, prior to incubation with an enhanced chemiluminescence substrate (Thermo Fisher Scientific, Inc.). Subsequently, the membranes were visualized by exposure to ECL film and the band intensities were quantified using UN-SCAN-IT gel analysis software, version 5.1 (Silk Scientific, Inc., Orem, UT, USA). GAPDH was used as a reference gene.

Statistical analysis. All experiments were performed ≥3 times. Data were presented as the mean ± standard deviation, and were analyzed using SPSS 16.0 software (SPSS, Inc., Chicago, IL, USA). Statistical differences between two independent groups were determined using the unpaired Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

EDD is overexpressed in cervical cancer tissue samples and cell lines. To investigate the role of EDD in cervical cancer, the expression levels of EDD were measured in cervical cancer tissue samples and cell lines using RT-qPCR and western blotting. EDD was significantly upregulated in the cervical cancer tissue samples at both the mRNA and protein levels ($P<0.01$; Fig. 1A and B). Furthermore, the mRNA expression levels of EDD were significantly increased in the cervical cancer cell lines, as compared with the normal cervical epithelial cells ($P<0.0001$; Fig. 1C). These results suggest that EDD is overexpressed in cervical cancer tissue samples and cell lines.

EDD promotes cervical cancer growth in vitro and in vivo. To further explore the role of abnormal EDD expression in cervical cancer, EDD expression was knocked down or upregulated in SiHa cells. EDD knockdown in SiHa cervical cancer cells significantly inhibited the growth of the cancer cells *in vitro* and *in vivo*. As shown in Fig. 2A and B, colony formation and cell proliferation were significantly inhibited in the SiHa cells when EDD was knocked-down *in vitro*. Furthermore, EDD silencing significantly suppressed the growth of cervical cancer tumors *in vivo* ($P<0.001$; Fig. 2C). The opposite results were observed following EDD overexpression. EDD overexpression significantly increased colony formation, cell proliferation and tumor growth in cervical cancer ($P<0.05$; Fig. 2D-F).

To investigate whether EDD regulates cervical cancer growth via an apoptotic mechanism, quantitative analysis of apoptotic cells was performed using fluorescence-activated cell sorting. The results demonstrated that EDD knockdown significantly increased the apoptosis of SiHa cells ($P<0.001$; Fig. 3A), and reduced the protein expression levels of anti-apoptotic proteins, including Bcl-2, and increased the protein expression levels of pro-apoptotic proteins, including Bax and caspase 3 (Fig. 3B). These results suggest that EDD may have an oncogenic role in cervical cancer.

EDD regulates the proliferation of cervical cancer cells via miR-143. As previously demonstrated by Liu *et al* (56), miR-143 promotes apoptosis and inhibits tumor formation in cervical cancer. Therefore, it was hypothesized that EDD may regulate cervical cancer growth via miR-143. The expression levels of miR-143 were analyzed in human cervical cancer tissue samples. The results demonstrated that miR-143 expression levels were significantly downregulated in cervical cancer tissue samples, as compared with the normal tissue samples ($P<0.001$; Fig. 4A).

Following this, the present study investigated whether the expression levels of miRNA-143 were correlated with EDD. EDD knockdown significantly increased miR-143 expression levels in SiHa cells ($P<0.001$; Fig. 4B), and miR-143 expression was negatively correlated with the expression levels of EDD in cervical cancer tissue samples ($P<0.001$; Fig. 4C), which suggests that EDD represses miR-143 expression in cervical cancer.

To further investigate whether miR-143 affected the function of EDD during the growth of cervical cancer, EDD and miR-143 were knocked-down and silenced respectively or simultaneously in SiHa cells. miR-143 sponge was used to eliminate miR-143 function. miR-143 silencing significantly reversed the inhibitory growth effect of EDD knockdown in SiHa cells ($P<0.01$; Fig. 4D). Furthermore, miR-143 silencing eliminated the effect of EDD knockdown on colony formation and prevented the apoptosis induced by EDD knockdown in SiHa cells ($P<0.01$; Fig. 4E and F). Therefore, these results demonstrate that EDD may regulate the proliferation of cervical cancer cells via miR-143.

Discussion

Previous studies have demonstrated that malignancies are frequently accompanied by the abnormal expression of oncogenes or tumor suppressor genes (59-65). EDD, as a human ortholog of the *Drosophila melanogaster* hyd gene (14), was shown to be frequently overexpressed in breast and ovarian cancer, which suggests that it may have a role in the progression of gynecological cancer (31,33,36). Furthermore, Bradley *et al* (36) demonstrated that EDD downregulation decreased ovarian cancer cell viability, increased cell apoptosis, inhibited tumor growth and promoted platinum sensitivity through mediation of ubiquitin ligase activity. However, the function and molecular mechanisms of EDD in human cervical cancer have yet to be elucidated. The results of the present study demonstrated that EDD expression levels were significantly upregulated in cervical cancer cell lines and tissues. Functional studies showed that abnormal expression of EDD impacted cell proliferation and tumor growth in cervical cancer. Furthermore, EDD knockdown significantly inhibited colony formation, cell proliferation and tumor growth *in vitro* and *in vivo* via the activation of the apoptosis signal pathway. These results suggested that EDD may have an oncogenic role in human cervical cancer. Abnormal expression of EDD may result in the disorder of ubiquitination and deubiquitination via ubiquitin ligase E3 and mediate the aberrant expression of oncogenes or tumor suppressor genes, thus inducing tumor occurrence and development.

miRNAs are involved in a diverse range of biological processes (38-43) and previous studies have demonstrated that certain miRNAs may function as oncogenes or tumor suppressors, which are directly involved in cancer occurrence and development (44-53). cDNA cloning demonstrated that miR-143 is a tumor-suppressive miRNA in cervical cancer (52), and Liu *et al* (56) reported that miR-143 expression was

downregulated in cervical cancer. The results of the present study demonstrated that miR-143 expression was downregulated in cervical cancer tissue samples. Furthermore, miR-143 expression levels were significantly increased following EDD knockdown and were negatively correlated with the expression of EDD, which suggested that miR-143 may be a functional target of EDD in cervical cancer. Subsequent functional investigation revealed that miR-143 silencing eliminated the effect of EDD knockdown on cell proliferation, colony formation and cell apoptosis in SiHa cells, indicating that miR-143 may be crucial for the function of EDD in regulating the growth of cervical cancer cells. These results are concordant with those of a previous study, which demonstrated that EDD regulates miRNA-mediated gene silencing and impacts the proliferation of cancer cells (27). Su *et al* (27) identified EDD as a key mediator for miRNA silencing via genetic screening in mouse embryonic stem cells. It was demonstrated that E3 ubiquitin ligase activity was dispensable for EDD function in miRNA silencing (27). However, the C-terminal domain of polyadenylate binding protein 1 (PABC) of EDD was demonstrated to be essential for its silencing function, as EDD regulated miRNA silencing via its PABC domain and PABC interactors (27). Furthermore, it has previously been demonstrated that miR-143 is able to promote cervical cancer cell apoptosis and inhibit tumor formation by targeting Bcl-2 (56). These findings, and the results of the present study, suggest that EDD may regulate miRNA-143 expression via its PABC domain which, in turn, impacts carcinogenesis and tumor growth.

In conclusion, the results of the present study demonstrated that EDD regulates cervical cancer growth *in vivo* and *in vitro* partly via miR-143. Furthermore, EDD may have an oncogenic role in cervical cancer and may be a potential target for cervical cancer therapy.

Acknowledgements

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