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The mechanism of PDX in regulating cervical cancer HeLa cell proliferation and tumor formation

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Abstract. – OBJECTIVE: As a gynecological malignant tumor, cervical cancer tends to occur in younger patients. Furin is an important member of precursor protein convertase that may affect malignant tumor metastasis and neovascularization. Pancreatic duodenal homeobox (PDX), as a transcription factor, can inhibit Furin. This study intends to explore the impact of PDX on cervical cancer HeLa cell proliferation and mechanism.

PATIENTS AND METHODS: PDX plasmid was transfected to cervical cancer HeLa cells. Cell proliferation, invasion, and migration were tested. HeLa cells were injected to Balb/c nude mice to observe the change of general status, tumor formation rate, tumor weight, and volume.

RESULTS: PDX expression was gradually increased after PDX transfection following time extension. Cell proliferation, invasion, and migration in experimental group were significantly lower than those in normal control ($p < 0.05$). The nude mice injected with PDX transfected HeLa cells showed markedly better general status, with reducing rate of tumor formation, tumor weight and volume compared with control.

CONCLUSIONS: PDX can suppress cervical cancer HeLa cell proliferation, cell invasion and migration, improve general status of tumor-bearing nude mice and reduce tumor weight and volume. Our data highlight the clinical benefits of PDX in inhibiting cervical cancer proliferation, invasion, and metastasis.

Key Words:
PDX, HeLa cell, Proliferation, Invasion, Tumor formation rate.

Introduction

The increasing incidence of cervical cancer has emerged in younger group in recent years. So far, surgery is the main method for cervical cancer therapy in clinic. Adjuvant chemoradiotherapy is also employed and determined according to the staging. However, severe adverse reaction of chemoradiotherapy poses a certain threat to the quality of life and the expensive cost leads to heavy economic burden for patients' family[1,2]. Basically, malignant tumor is characterized as uncontrollable proliferation of cells. Local invasion and distant metastasis are critical standards of judging tumor malignancy in clinic. It has been typically a long development process from precancerous lesions to solid tumor and even multiple metastases. Clinically, it was found that most patients with cervical cancer died of local invasion and distant metastasis[3,4]. Previous finding indicated that cervical cancer cell transformation, migration, invasion, and neovascularization were affected by Furin, which contributes to activating precursor protein Notch-1 and MT1-MMP. Pancreatic duodenal homeobox (PDX) is a selective inhibitor of Furin belonging to Homeobox family[5,6]. We aim to analyze the impact and mechanism of PDX on HeLa cell proliferation and tumor formation, as well as its effect on nude mice with tumor.

Patients and Methods

Experimental Cells, Animals, and Object of Study

Cervical cancer HeLa cells were bought from ATCC (Manassas, VA, USA). Male healthy balb/c nude mice in SPF grade were provided by Laboratory Animal Center in Zhejiang University (Hangzhou, Zhejiang, China). A total of 30 patients with chronic cervicitis with mean age

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at 52.6 ± 4.2 (35-70) years old were enrolled for the collection of cervical epithelial cells. No statistical difference was observed in age and weight in the enrolled patients ($p > 0.05$). The study was reviewed and approved by Ethics Committee of The First People's Hospital of Wenling (Wenling, Zhejiang, China). All patients participated in the study signed the informed consent.

Reagents and Instruments

PDX and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Polymerase Chain Reaction (PCR) and Real-time-PCR amplifier kits were provided by TaKaRa (Otsu, Shiga, Japan). Microscope was bought from Olympus (Tokyo, Japan).

Conventional Cell Culture

HeLa cells were maintained in Roswell Park Memorial Institute (RPMI)-1640 medium and cultured at 37°C and 5% CO_2.

PDX Gene Clone

Total RNA was extracted and reverse transcripted to cDNA. PCR reaction was performed at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 1 min. After digestion and purification, the PCR product was collected to form pEGFP/PDX recombinant. The recombinant was put into the bacterial liquid for ice-bath for 45 min. The bacterial suspension was smeared on agar plate for bacteria propagation at room temperature overnight. The transformed strain containing pEGFP-PDX recombinant was stored at -20°C.

Cell Transfection

Plasmid DNA and lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) were diluted to form DNA-lipofectamine complex for transfection. The HeLa cells were digested for passage.

Real Time-PCR

A total of 200 ng RNA was reverse transcripted to cDNA for PCR. PCR reaction contained 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, with specific primers shown in Table I.

MTT Assay

After transfected with PDX plasmid, the Hela cells were seeded in 96-well plate. The plate was added with 200 µl MTT at 0.5 mg/ml at room temperature for 2 h and read at 570 nm.

Transwell Assay

For invasion experiment, the matrigel was mixed with serum-free medium at 1:8 for 24h. A total of 60 µl diluted Matrigel was put onto the upper chamber. HeLa cells were seeded to the upper chamber in 200 µl serum-free medium (Thermo Fisher Scientific, Waltham, MA, USA) at 10^6/ml. Another 1300 µl complete medium was added to the lower chamber. After cultured at 37°C and 5% CO_2 for 24 h, the membrane was fixed by ethanol and stained for observation.

For migration experiment, HeLa cells in 1300 µl complete medium were added to the upper chamber at 10^6/ml. Another 1300 µl complete medium was added to the lower chamber. After cultured at 37°C and 5% CO_2 for 24 h, the membrane was fixed by ethanol and stained for observation.

Establishment of Nude Mice Subcutaneous Transplantation Model

The nude mice were randomly divided into two groups with 10 in each group. HeLa cells transfected with PDX were prepared as single cell suspension and seeded to the subcutaneous axilla of right forelimb at 0.2 ml/time for 30 days. Equal amount of wild-type HeLa cells were seeded to the mice as control. General status, tumor weight, and volume were observed.

Table I. Primer sequences.

Gene		Sequence (5'-3')
PDX	Forward	5'-ACGAAGCTTGCTGCCACCATGAACAGTG-3'
	Reverse	5'-GCTGGATCCCGGGGTTCCTGCGGTC-3'
GAPDH	Forward	5'-CTCGCTTCGGCAGCACA-3'
	Reverse	5'-AACGCTTCACGAATTTGCGT-3'

Statistical Analysis

SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) was applied for data analysis. Measurement data was presented as mean ± standard deviation and tested by x^2-test or ANOVA with the Tukey's post-hoc test. Enumeration data were analyzed by the *t*-test. $p < 0.05$ was depicted as statistical significance.

Results

PDX Expression in HeLa Cells and Normal Cervical Epithelial Cells

After PDX transfection, PDX level in HeLa cells was significantly increased following time extension ($p < 0.05$). Generally, PDX also expressed in HeLa cells control without PDX transfection and normal cervical epithelial cells, while its level in HeLa cells was significantly higher than that in normal epithelial cells ($p < 0.05$) (Figure 1).

HeLa Cell Proliferation After PDX Transfection

HeLa cell proliferation was detected after PDX transfection. It was found that HeLa cell proliferation activity at 24 h and 48 h were markedly lower than that of control, and continued to decrease in a time-dependent manner ($p < 0.05$) (Figure 2).

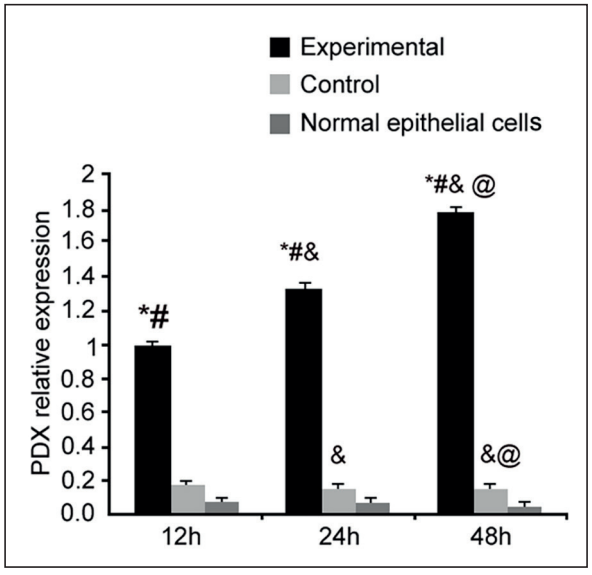

Figure 1. PDX expression in HeLa cells and normal cervical epithelial cells. $^{*}p < 0.05$, *vs.* control. $^{\#}p < 0.05$, *vs.* normal epithelial cells. $^{\&}p < 0.05$, *vs.* 12 h. $^{@}p < 0.05$, *vs.* 24 h.

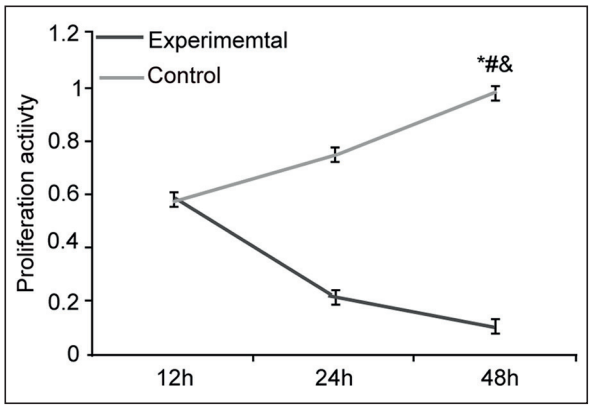

Figure 2. HeLa cell proliferation after PDX transfection. $^{*}p < 0.05$, *vs.* control. $^{\#}p < 0.05$, *vs.* 12 h. $^{\&}p < 0.05$, *vs.* 24 h

HeLa Cell Invasion and Migration After PDX Transfection

HeLa cell invasion and migration were significantly decreased after PDX transfection compared to that in the control without the transfection ($p < 0.05$) (Figure 3).

General Status of Nude Mice

The nude mice were employed as an animal model. The subcutaneously inoculated cells grew slowly, as the tumor in sphere without tenderness. The tumor gradually grew after the inoculation, which became hard, fixation, and tenderness. Some nude mice were found with rubefaction, ulceration, and exudation. Poor general status was observed with reduced eating, drinking, and activity. No mice died within 30 days after inoculation.

Tumor Formation Rate Comparison

After receiving inoculation with HeLa cells, one mouse appeared subcutaneous tumor on the fifth day, while all of ten mice formed tumor on the 25th day in control group. The tumor formation rate was 100%. At the same time, in experimental group, mice were inoculated with HeLa cells with overexpression of PDX. Of note, a mouse appeared tumor on the 10th day, whereas in another 5 mice, tumor was formed on the 30th day. The tumor formation rate was 50%.

Tumor Weight and Volume Changes After HeLa Cell Inoculation

Tumor weight and volume in experimental group were significantly lower than that of control ($p < 0.05$) (Figures 4,5).

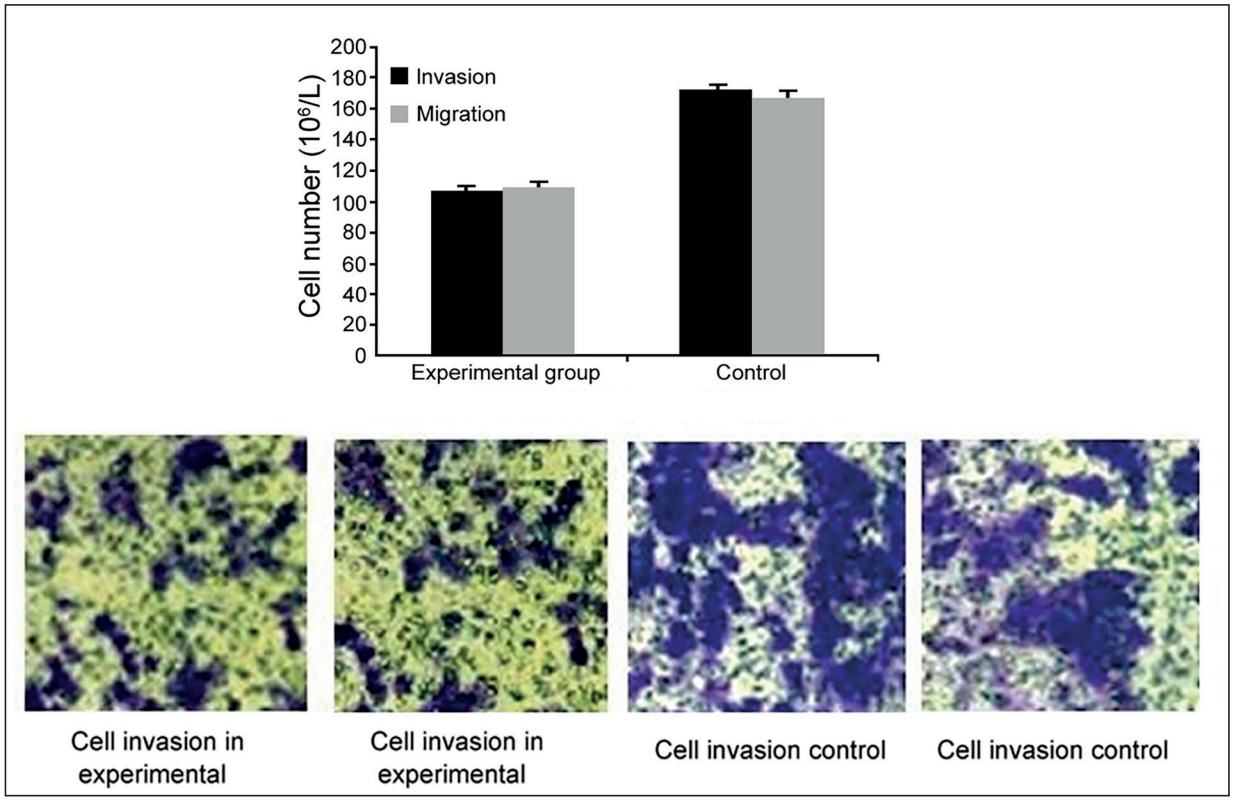

Figure 3. HeLa cell invasion and migration after PDX transfection (×200). *$p < 0.05$, *vs.* control.

Discussion

Cervical cancer is a type of common gynecologic malignant tumor in developing countries. The main cause of poor prognosis even death is tumor local invasion and distant metastasis[7]. During local invasion and distant metastasis, cancer cells first break through the basement membrane and then invade to distant organ through blood vessel s or lymphatic vessels[8]. Furin is an important member of precursor protein convertase that may affect malignant tumor metastasis and neovascularization[9]. Furin highly expresses in a variety of malignant tumors and regulates humoral immunity, cellular immunity, and malignant tumor cell invasion and migration[10]. PDX is an important selective inhibitor of Furin[11,12].

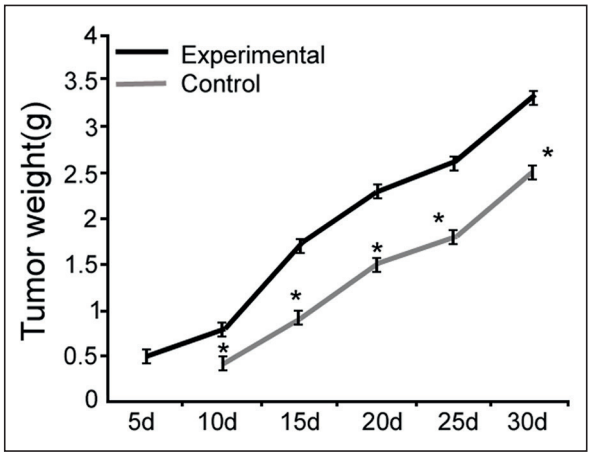

Figure 4. Tumor weight changes. *$p < 0.05$, *vs.* control

In this study, we tested PDX expression in transfected HeLa cells, normal HeLa cells, and cervical epithelial cells. The expression of PDX was higher in HeLa cells than that in normal cervical epithelial cells, which was in agreement with previous study. However, it was reported that the occurrence and development of breast cancer, stomach cancer, and colon cancer were related to PDX downregulation or deletion[13,14]. It was observed that PDX expression in gastric cancer cells was obviously lower than that in normal gastric mucosal cells, indicating PDX may participating in gastric cancer occurrence[15]. The result above suggests that the level of PDX in cancer cells was abnormally expressed.

Nishimura et al[16] and Coppola et al[17] considered that recombinant PDX exhibited effective role on cancer cells proliferation and invasion. As the inhibitor of Furin, PDX can reduce MT1-MMP expression, resulting in losing the ability to degrade collagen and fibronectin. We proposed that PDX may suppress cervical cancer HeLa cells proliferation and metastasis through regulating the abovementioned cytokines and signaling pathway. Of note, proliferation detection revealed that HeLa cell proliferation activity in experimental group was lower than that in control and the data suggested that PDX positively expressed in HeLa cells and can decline the proliferation activity.

We then evaluate the therapeutic effect of PDX on cancer by using *in vivo* test. HeLa cells transfected with PDX were inoculated to nude mice. Interestingly, the tumor formation rate from mice with PDX-treated HeLa cells was obviously decreased compared with that in mice with inoculation of HeLa cells. Also, we found that PDX transfection may improve general status of nude mice and reduce tumor weight and volume. As a molecular marker of tumor progression, the activity of Furin was positively correlated with the malignant phenotype[18]. Furin may be involved in malignant tumor invasion and migration by producing various small molecule proteins. PDX can reduce MT1-MMP expression[19]. PDX may promote malignant tumor proliferation and formation through regulating furin, MT1-MMP, collagen and fibronectin[20]. However, it was considered that invasion marker MT1-MMP may be activated in human colon cancer LoVo cells independent of Furin pathway, and the limitation in our study still exists that underlying mechanisms of PDX on the regulation of various signaling pathways ought to be further investigated. The role of PDX in this study offers alternative leads for the treatment against cervical cancer[21].

Conclusions

Our data demonstrate that the level of PDX was elevated in cervical cancer HeLa cells, compared to normal cervical epithelial cells. However, the overexpression of PDX limits HeLa cells proliferation, invasion and migration *in vitro*, while it also improves general status of tumor-bearing nude mice, and reduces tumor weight and volume. Our research provides a future basis for the therapy of cervical cancer.

Acknowledgements

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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