

## MDH2 Stimulated by Estrogen-GPR30 Pathway Down-Regulated PTEN Expression Promoting the Proliferation and Invasion of Cells in Endometrial Cancer<sup>1,2</sup>

Yan Zhuang<sup>\*,†</sup>, Jiangdong Xiang<sup>†</sup>, Wei Bao<sup>†</sup>,  
Ya Sun<sup>†</sup>, Lina Wang<sup>†</sup>, Meihua Tan<sup>†</sup>,  
Yinyan He<sup>†</sup> and Xiaowei Xi<sup>\*,†</sup>

<sup>\*</sup>Department of Obstetrics and Gynecology, Shanghai General Hospital of Nanjing Medical University, No. 100, Haining Road, Shanghai 200080, China

<sup>†</sup>Department of Obstetrics and Gynecology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, No. 100, Haining Road, Shanghai 200080, China



### Abstract

**PURPOSE:** The relationship between endometrial carcinoma and cellular metabolism is unknown. In endometrial cancer, mutation rate of PTEN has been reported very high. Malate dehydrogenase 2 (MDH2) is one of the isoforms of malate dehydrogenase, which is involved in citric acid cycle in mitochondria. Our study aimed to investigate the role MDH2 played in PTEN-regulated endometrial carcinoma. **METHODS:** To reveal the expression of MDH2 and the co-localization of PTEN and MDH2, immunohistochemistry and immunofluorescent staining were used. Western blot, Real-time PCR, RNA interference and overexpression plasmid DNA transfection were performed to investigate the relationship between PTEN and MDH2 as well as the impact of E2 on the expression of PTEN and MDH2, while CCK8, transwell and flow cytometric analysis were carried out to evaluate the proliferation, migration and invasion and apoptosis of endometrial carcinoma cell lines. **RESULTS:** Our results demonstrated that as a metabolism related enzyme, MDH2 was overexpressed in endometrial carcinoma tissues and related to the grade of the cancer ( $P = .038$ ). Western blot, Real-time PCR and immunofluorescent staining revealed MDH2 inhibited the expression of PTEN and was co-localized with PTEN in the cytoplasm of endometrial carcinoma. Proliferation, transwell and apoptosis assay suggested that MDH2 enhanced the proliferation, migration and invasion but inhibited the apoptosis of endometrial cancer cell line through suppressing PTEN. Furthermore, E2 inhibited the expression level of PTEN but enhanced MDH2 via GPR30. **CONCLUSIONS:** Our study demonstrated that MDH2, stimulated by estrogen, was involved in the development of PTEN-regulated endometrial carcinoma through GPR30-related pathway.

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### Introduction

Endometrial carcinoma is one of the three gynecologic malignancies, which threaten women's health. In Europe and North America, endometrial carcinoma is the most common gynecologic malignancy [1]. The disease accounts for 6% of all the new cases every year, and 3% of all the cancer-related deaths [2]. Studies in the past focused on the pathogenesis from the angle of molecular mechanism [3]. However, the energy metabolism in endometrial carcinoma remains elusive.

Recent advances demonstrate that activated oncogenes and inactivated tumor suppressors regulate cellular reprogramming. Many oncogenes and tumor suppressors are associated with

Address all correspondence to: Xiaowei Xi, MD, Department of Obstetrics and Gynecology, Shanghai General Hospital of Nanjing Medical University, No. 100, Haining Road, Shanghai 200080, China or Yinyan He, MD, PhD, Department of Obstetrics and Gynecology, Shanghai General Hospital, Shanghai Jiao Tong University School of Medicine, No. 100, Haining Road, Shanghai, 200080, China.  
E-mail: [he\\_yinyan76@sina.cn](mailto:he_yinyan76@sina.cn)

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tumor-suppressive transcription factors [4]. PTEN is a tumor suppressor. As a transcription factor, it modulates cellular activities via PI3K/AKT/mTOR pathway, including proliferation, apoptosis, and energy metabolism [5]. PTEN regulates the energy metabolism of tumor cells by increasing the uptake of glucose and synthesis of lipids via PI3K-AKT-mTOR pathway to modulate the biological behavior of tumor [6]. PTEN mutations or deletions have been reported in almost 80% endometrioid endometrial carcinoma [2].

A few metabolic enzymes function as transcriptional regulators to modulate the expression of tumor suppressors [7,8]. Malate dehydrogenases are a group of NAD-dependent dehydrogenases. The isoform MDH2 is considered to play an important role in the tricarboxylic acid cycle in mitochondria while MDH1 facilitate the malate/aspartate shuttle across the mitochondrial membrane [9]. Recent studies have shown that specific metabolic enzymes such as malate dehydrogenases function as transcriptional factors to regulate the expression of oncogenes and tumor suppressors [10]. For instance, MDH1 functions as a transcriptional factor in the regulation of p53-dependent metabolism by combining with p53 [11].

Our study investigated the correlation between MDH2 and PTEN, to elucidate the mechanism of PTEN-mediated regulation of endometrial tumorigenesis via suspected modulation of cellular energy metabolism. Endometrial cancer cell lines HEC-1-A and AN3CA were enrolled in our study. Our results suggested that MDH2 overexpressed in endometrial carcinoma tissues and was related to the grade of the tumor. siRNA-MDH2 to knockdown of MDH2 increased the expression of PTEN, and overexpression of MDH2 decreased the expression level of PTEN, vice versa. Immunofluorescent staining revealed that MDH2 and PTEN co-localized in the cytoplasm of endometrial carcinoma. Inhibition of the expression of MDH2 blocked the proliferation, invasion and migration of cells, and increased the apoptosis by suppressing PTEN. Additionally, the stimulation of E2 and G1 increased the expression of MDH2 but decreased the expression of PTEN. In brief, MDH2, stimulated by E2, played a role in PTEN-mediated regulation of endometrial tumorigenesis via altered cellular metabolism through GPR30-related pathway.

## Material and Methods

### *Tissue Chip*

The endometrial carcinoma tissue chip was purchased from Shanghai Outdo Biotech Co. Ltd., with the CGT number HUteA060CS01 and the lot number XT15-033. The chip contained endometrial carcinoma tissues and normal endometrial tissues derived from 34 cases, which were fixed in 60 pores. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and national research committee. Informed consent was obtained from all individual participants included in the study.

### *Cell Culture*

The endometrial carcinoma cell lines HEC-1-A and AN3CA were cultured in DMEM/F12 media (11,030; Gibco, Auckland, NZ) supplemented with 10% FBS (S1810; Biowest, Nuaille, France), 100 units/mL penicillin, and 0.1 µg/mL streptomycin in a humidified atmosphere of 5% CO<sub>2</sub>/95% air at 37 °C. Cells were transfected with siRNAs against PTEN and MDH2, respectively, using Lipo2000 (11668-019, Invitrogen) for 72 h.

### *Western Blot*

The cell culture dish was transferred to ice and the cells were washed with ice-cold PBS. After aspiration of PBS, and addition of ice-cold lysis buffer (1 mL per 10<sup>7</sup> cells/100 mm dish/150 cm<sup>2</sup> flask; 0.5 mL per 5x10<sup>6</sup> cells/60 mm dish/75 cm<sup>2</sup> flask) into cell culture dish, adherent cells were scraped off the dish and the cell suspension was transferred into a pre-cooled microcentrifuge tube under constant agitation for 30 min at 4 °C. Microcentrifuge tubes were centrifuged at 12,000 rpm for 20 min at 4 °C. After SDS-PAGE of 30 µg proteins, the separated protein bands were transferred electrophoretically to polyvinylidene fluoride membranes. The membrane was blocked for 1 h at room temperature or incubated overnight at 4 °C using a blocking buffer (BSA) and incubated with one of the following primary antibodies: anti-PTEN (1:500; Abcam, Cambridge, UK), anti-MDH2 (1:2000, Abcam, Cambridge, UK) and GAPDH (1:2000, Abcam, Cambridge, UK). The membrane was incubated with an appropriate secondary antibody (1:1000) at room temperature for 1 h. The membrane was washed three times in TBST, for 5 mins each. The image was acquired in the dark room according to the manufacturer's recommendations.

### *Real-Time PCR*

Total RNA was extracted from human endometrial carcinoma cells HEC-1-A and AN3CA with TRIzol (Invitrogen, USA). The cDNA was generated with oligo(dT)18 primers using the Revert Aid First Strand cDNA Synthesis Kit (K1622; Fermentas Life Science, Germany). The 25-µL PCR amplification of single-strand cDNA was carried out using 40 cycles of denaturation (95 °C) for 60 s, annealing (60 °C) for 30 s and elongation (72 °C) for 30s using SYBR Green PCR Kit (K0223; Thermo Takara, Dalian, China). The results were analyzed with ABI Prism 7300 SDS Software.

### *RNA Interference*

The siRNAs against MDH2 were designed and ordered from Shanghai JRDUN Biotechnology co. Ltd. HEC-1-A and AN3CA cells were transfected with the siRNAs using Lipo2000 (11668-019, Invitrogen) according to the manufacturer's protocol. The cells were incubated for 72 h, and the knockdown efficiency was detected by qRT-PCR and western blot respectively. The siRNA sequence with the most efficient knockdown of MDH2 was 5'- CCCA GAACAAUGC UAAAGU -3. The sequence against negative control group was UUCUCCGAACGUGUCACGU.

### *Overexpression Plasmid DNA Transfection*

The PTEN sequence, MDH2 sequence and negative control were synthesized and subcloned into GV219 vector purchased from Shanghai Genechem Company. Then the plasmid GV219-PTEN, GV219-MDH2 or negative control was transfected into HEC-1-A and AN3CA cell lines and cultured using Lipo2000 (11668-019, Invitrogen) according to manufacturer's protocol for 72 h.

### *Proliferation Assay*

Cells were seeded in 96-well plates at 1-5 × 10<sup>4</sup> cells/mL and cultured for 24 h. Every plate was divided into 3 groups: normal, negative control and RNAi groups. At 0 h, 24 h, 48 h and 60 h after transfer, 100 µL of the liquid was mixed with Cell Counting Kit -8 (CP002, SAB) to which 10 times serum-free essential medium was added to each well in an atmosphere of 5% CO<sub>2</sub>, and 95% air at 37 °C for 1 h. Determination of absorbance at 450 nm was measured

with enzyme-labeled instrument (DNM-9602, Perlong New Technology Co., Ltd., Beijing).

### Transwell Assay

Human endometrial cells HEC-1-A and AN3CA were transferred and divided into three groups: normal, negative control and transfected groups. One day before the experiment, cells were changed into serum-free culture medium. The 24-well plate and Transwell chambers were soaked in 1 × PBS for 5 min (During the invasion test, the chambers were covered with 80 μL matrigel and transferred to an incubator at 37 °C for 30 min). Cells were seeded in Transwell chambers at 1 × 10<sup>5</sup> cells/ml. The lower chamber was filled with 0.75 mL DMEM with 10% FBS at 37 °C for 48 h. Each well was fixed with 1 mL of 4% formaldehyde for 10 min. After staining with crystal violet, non-migrating (or non-invasion) cells in the chambers were wiped and counted microscopically under 200 times magnification. The assay was repeated three times, and the results were expressed as an average percentage of the three controls. Each experiment was carried out in triplicate and repeated three times.

### Apoptosis Assay

Cell culture medium treated with PBS and trypsin-EDTA solution was transferred to the centrifuge tubes and centrifuged at 1000 g for 5 min. Cells were collected and re-suspended with PBS before counting, and 5000 to 10,000 re-suspended cells were centrifuged at 1000 g for 5 min, and mixed with 195 μL Annexin V-FITC before resuspension. Next, 5 μL Annexin V-FITC was added to the re-suspended cells and incubated in the dark at 4 °C for 15 min. Finally, 5 μL PI was added to the mixture and incubated in the dark at 4 °C for 15 min. The results were determined with flow cytometry.

### Immunohistochemistry and Immunofluorescent Staining

For immunohistochemistry, the paraffin tissue chip was dehydrated in PBS and underwent antigen retrieval in sodium citrate buffer (10 mM, 0.05% Tween 20, pH 6.0). Then the tissue chip was washed 2 × 5 min in TBS plus 0.025% Triton X-100 with gentle agitation followed by being blocked in 10% normal serum with 1% BSA in TBS for 2 hr. at room temperature. The chip was incubated with rabbit anti-MDH2 antibody (ab181857, abcam) and peroxidase-labeled anti-rabbit IgG (H + L) antibody (03-15-06, KPL). Next, the chip was counterstained with hematoxylin.

The endometrial carcinoma cell line HEC-1-A was incubated in 100% methanol (chilled at -20 °C) at room temperature for 5 min and fixed with 4% paraformaldehyde in PBS at pH 7.4 for 10 min at room temperature. To permeabilize the cells, the sample was incubated for 10 min with PBS containing either 0.25% Triton X-100. Next, the cells were blocked with 1% BSA, 22.52 mg/mL glycine in PBST (PBS+ 0.1% Tween 20) for 30 min. The cells were stained with rabbit anti-MDH2 antibody (ab181857, abcam) and TRITC-labeled anti-rabbit IgG (H + L) Antibody (03-15-06, KPL) and with mouse anti-PTEN antibody (ab79156, abcam) and FITC-conjugated anti-mouse IgG (H + L) antibody (172-1806, KPL). The results were detected using a fluorescence microscope.

### Statistics

The expression difference between normal endometrial tissues and endometrial carcinoma tissues was calculated using Chi-squared test. The correlation of MDH2 and the clinic-pathological factors was analyzed using Ridit analysis. The analysis of Real-time PCR, cell

proliferation, migration and invasion and apoptosis was performed using the Student's t-test or one-way ANOVA analysis. All the statistics was performed with SPSS 20.0. A two-sided test with  $P < .05$  was considered statistically significant.

## Results

### *PTEN Inhibited the Proliferation, Invasion and Migration but Promoted the Apoptosis of Endometrial Cancer Cell Line*

As PTEN downregulations and mutations have been found in nearly 80% of all endometrial carcinoma cases [2], it has been regarded as a tumor suppressor in endometrial carcinoma. To confirm the role of PTEN in endometrial cancer, we respectively transfected siRNA-PTEN (siPTEN), overexpression plasmid of PTEN GV219-PTEN (OE-PTEN) to endometrial cancer cell line HEC-1-A. Here we showed siRNA-PTEN promoted the proliferation, migration and invasion but inhibited the apoptosis of cancer HEC-1-A (Figure 1a, b and c). Accordingly, the overexpression of PTEN inhibited the proliferation, migration and invasion but promoted the apoptosis of cancer cell line (Figure 1d, e and f). What's more, siRNA-PTEN to knockdown PTEN upregulated the expression of MDH2 (Figure 1g and h) and accordingly the overexpression of PTEN downregulated the expression of MDH2 (Figure 1i and j).

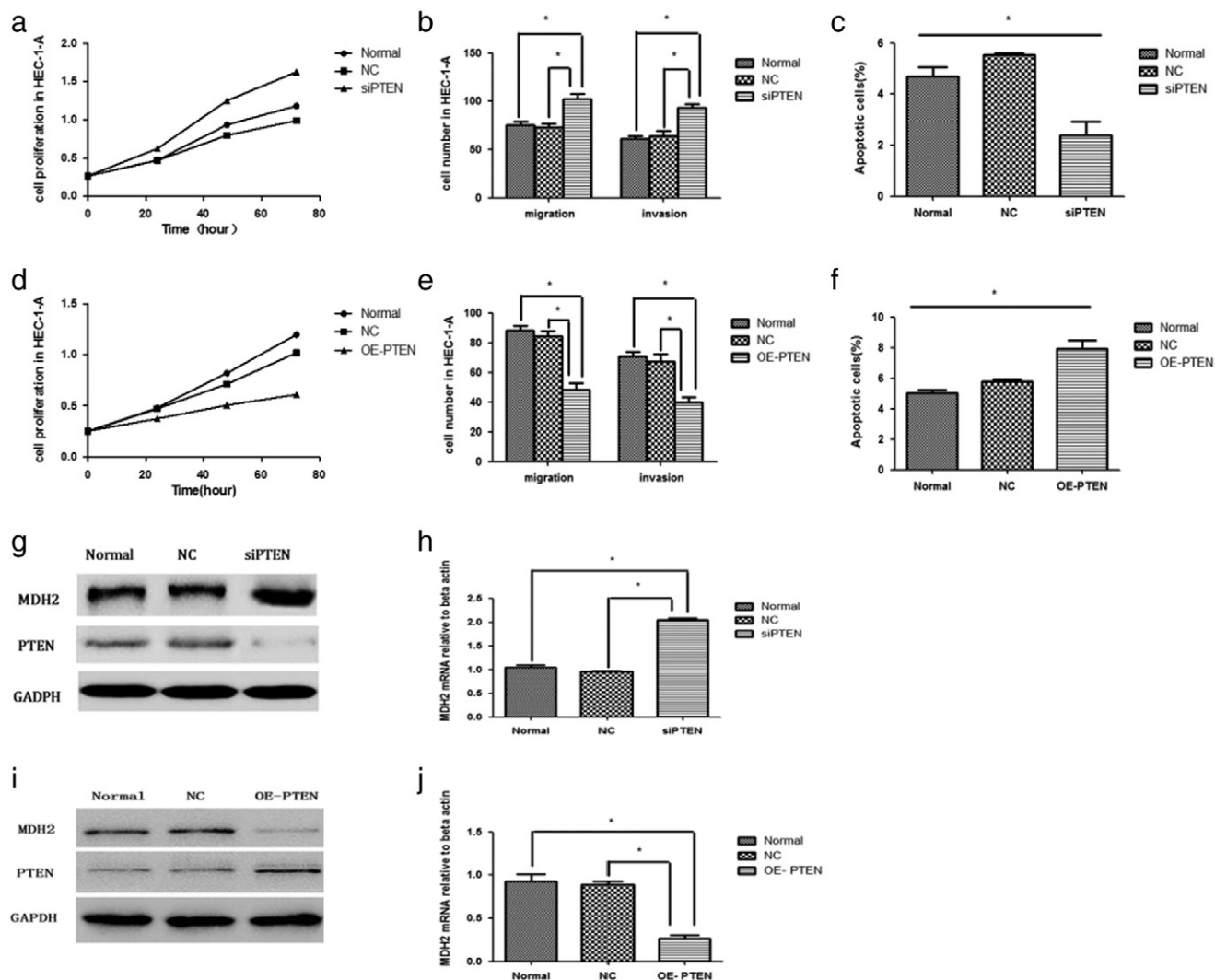
### *Overexpression of MDH2 in Endometrial Cancer*

MDH is a key enzyme in the tricarboxylic acid cycle that catalyzes the interconversion of malate and oxaloacetate by utilizing the NAD/NADH coenzyme system. In addition, MDH has been recognized as an attractive target for cancer treatment, which means an important role of MDH in the relationship between cell metabolism and cancer regulation. Therefore we carried out IHC to determine the MDH2 expression in endometrial carcinoma.

As showed in Figure 2, in total, 34 cases including 26 cases of carcinoma and para-carcinoma carcinoma tissues were collected to perform a tissue microarray. Hematoxylin staining showed that compared with normal tissues, MDH2 were overexpressed in the cytoplasm of carcinoma tissues (Figure 2). In addition, the frequency analysis of IHC staining indicated that 74% (25/34) of carcinoma tissue expressed MDH2 whereas only 31% (8/26) normal tissues were MDH2 positive ( $P = .001$ , Table 1). Further, the expression of MDH2 was related to the endometrial carcinoma grade. However, no correlation was found between MDH2 expression and other clinical factors, such as age, stage and lymph node metastasis (Table 2).

### *MDH2 Inhibited the Expression of PTEN and was Co-Localized with PTEN*

Next, we used RNA interference and overexpression plasmid DNA transfection to determine the relationship between MDH2 and PTEN. Real-time PCR and Western blot were performed to evaluate the expression of MDH2 and PTEN in endometrial cell lines. The result indicated that the suppression of MDH2 significantly upregulated the expression of PTEN (Figure 3a and b). The overexpression of MDH2 downregulated the level of PTEN in AN3CA cell line (Figure 3c and d). Furthermore, the overexpression of PTEN by transfection of overexpression plasmid GV219-PTEN was inhibited by addition of overexpressed MDH2 (Figure 3e and f). Thus, the results showed that MDH2 inhibited the expression of PTEN, which suggested MDH2 might contribute to the decreased function of PTEN in endometrial carcinoma cells, which may be linked to



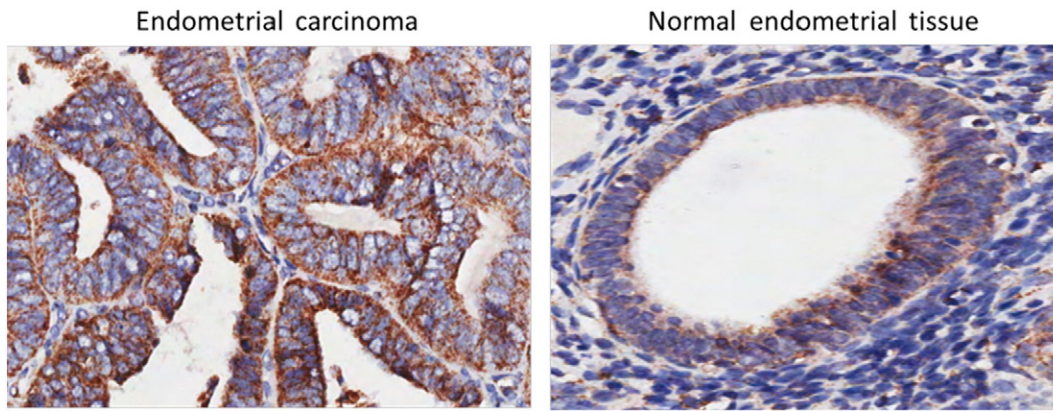
**Figure 1.** PTEN inhibited the proliferation, invasion and migration but promoted the apoptosis of endometrial cancer cell line. It suppressed the expression of MDH2. To investigate the impact of PTEN on the biological behavior of endometrial cancer cell line, we respectively transfected endometrial cancer cell line HEC-1-A with siRNA-PTEN (siPTEN) and overexpression plasmid GV219-PTEN, with which PTEN was overexpressed (OE-PTEN). Proliferation, invasion, migration and apoptosis were analyzed to determine the difference among normal group(Normal), negative control(NC) group and siPTEN group or OE-PTEN group. (a-c): siRNA-PTEN to knockdown of PTEN promoted the proliferation, invasion and migration of HEC-1-A but inhibited the apoptosis. (d-f): the overexpression of PTEN inhibited the proliferation, invasion and migration of HEC-1-A but promoted the apoptosis. To detect the impact of PTEN on the expression of MDH2, western blot and real-time PCR were carried out in the endometrial carcinoma cell line HEC-1-A transfected with siPTEN and overexpression plasmid GV219-PTEN. (g and h): siRNA-PTEN upregulated the expression of MDH2. (i and j): The overexpression of PTEN downregulated the expression of MDH2.\* $P < .05$ , One-way ANOVA.

mutations and deletion of PTEN. To further explore whether a direct relationship between MDH2 and PTEN existed, immunofluorescent staining was performed in endometrial carcinoma cell line HEC-1-A. The results demonstrated that PTEN co-localized with MDH2 in the cytoplasm (Figure 3g) providing further evidence implying that MDH2 was related to PTEN, and combined with PTEN regulating the activity of endometrial carcinoma cells.

**MDH2 Promoted the Proliferation and Invasion, Inhibited the Apoptosis of Endometrial Carcinoma Cells Through Suppressing the Expression of PTEN**

To investigate the effect of MDH2 on the behavior of endometrial carcinoma cell lines, siRNA-MDH2 and overexpression plasmid

GV219-MDH2 were transfected to HEC-1-A and AN3CA respectively. Then we compared the proliferation, invasion, migration and apoptosis among three groups, normal group(Normal), negative control(NC) group and the group transfected with MDH2 RNAi(siMDH2) or the group transfected with GV219-MDH2(OE-MDH2). Interestingly, siRNA-MDH2 to knock-down of MDH2 inhibited the proliferation, migration and invasion but promoted the apoptosis of HEC-1-A (Figure 4a-c). Accordingly, the overexpression of MDH2 promoted the proliferation, migration and invasion but suppressed the apoptosis of AN3CA (Figure 4d-f). In addition, the suppression of cell proliferation, migration, invasion and the enhancement of apoptosis resulted from the overexpression of PTEN were reversed by addition of overexpressed MDH2 (Figure 4g-i).



**Figure 2.** MDH2 protein was overexpressed in endometrial carcinoma tissue. The expression of MDH2 protein was detected in normal endometrial tissue and endometrial carcinoma tissue with immunohistochemical staining. MDH2 was expressed in cytoplasm. Magnification,  $\times 200$ .

In short, we confirmed that MDH2 played an important role in promoting the proliferation, invasion and migration of endometrial cell lines through suppressing the expression of PTEN.

***E2 Might Inhibit the Expression Level of PTEN but Enhanced MDH2 Through GPR30-Related Pathway***

Traditionally, estrogen played an important role in the initiation and development of endometrial carcinoma via binding to ER in nuclear. However, in recent years, GPR30, a member of GPCR family, has been found as a new estrogen receptor which mediates a non-genomic, rapid signaling of  $17\beta$ -estradiol (E2), which is independent on ER. Our previous study had proved that GPR30, a transmembrane receptor of estrogen, promoted the initiation and development of endometrial carcinoma [12,13]. In present study, it demonstrated that in endometrial carcinoma cells HEC-1-A (ER positive and GPR30 positive) and AN3CA (ER negative and GPR30 positive), estrogen and G1, a non-steroidal GPR30-specific agonist in both ER-negative and ER-positive endometrial carcinoma cell lines, upregulated the expression of MDH2 but downregulated the expression of PTEN. On the contrary, G15, an antagonist down-regulated the expression level of MDH2 (Figure 5a-b). Therefore, it implied that estrogen stimulated the expression of MDH2 through the activation of GPR30.

**Discussion**

PTEN is one of the tumor suppressor genes with a high mutation rate in endometrial carcinoma. The significance of PTEN in cancer and its role as a transcriptional factor are known. However, the role of PTEN in cellular metabolism in cancer has been poorly defined. Our study therefore, investigated the relationship between PTEN and the metabolic enzyme MDH2 to further correlate the expression of PTEN with metabolism in endometrial carcinoma.

**Table 1.** The Difference of MDH2 Expression Between Carcinoma and Normal Endometrial Tissues

Tissue	MDH2		P value
	Positive	Negative	
Carcinoma	25	9	.001
Normal	8	18	

Chi-square test,  $P = .001$ .

Firstly, MDH2 is overexpressed in endometrial carcinoma. In our study, MDH2 was expressed in 74% of the endometrial carcinoma samples compared with 31% in normal tissues. Furthermore, MDH2 was positively associated with the histological grade of endometrial carcinoma, suggesting the possible correlation between the expressions of MDH2 with poor prognosis.

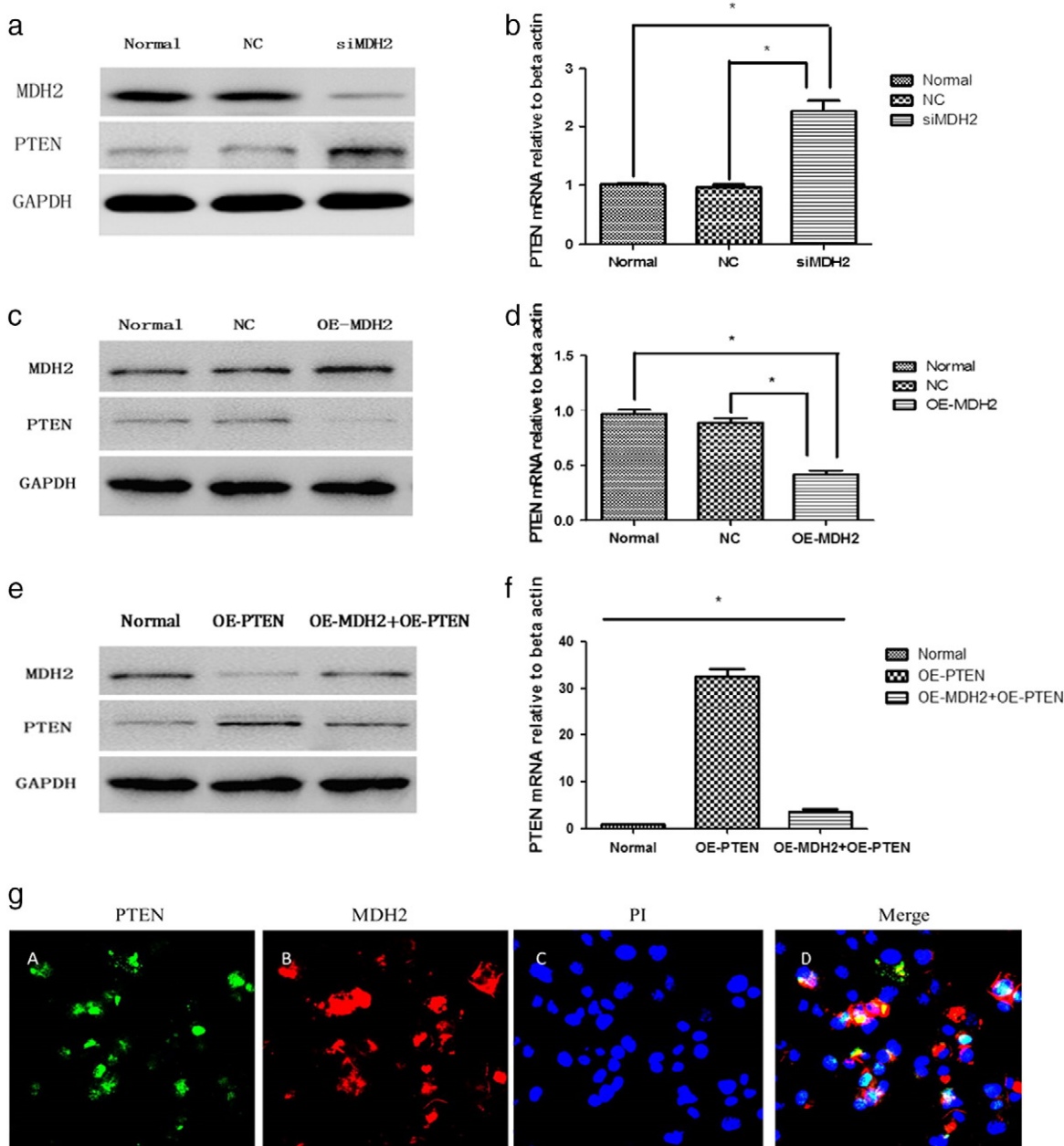
Secondly, our research detected that the expression of MDH2 inhibited the expression of PTEN. Our results also suggested that siRNA-MDH2 upregulated the expression of PTEN, and the overexpression of MDH2 inhibited the expression of PTEN. The overexpression of PTEN in HEC-1-A transfected with overexpression plasmid of GV219-PTEN was inhibited by MDH2. In fact, PTEN also inhibited the expression of MDH2. Knockdown of PTEN upregulated the expression of MDH2, and the overexpression of PTEN suppressed the expression of MDH2. Not only that, MDH2, playing a role different from the tumor suppressor PTEN, promoted the proliferation, migration and invasion but suppressed the apoptosis of endometrial cell line. The suppression of cell proliferation, migration, invasion and the enhancement of apoptosis, resulted from the overexpression of PTEN, were restored by MDH2. This proved

**Table 2.** The Correlation Between MDH2 Protein and Clinic-Pathological Factors in Endometrial Carcinoma Tissues (n = 34)

Tissue	MDH2		P value
	Positive	Negative	
Age(years)			.480 <sup>1</sup>
$\leq 50$	5	2	
$> 50$	19	8	
Grade			.038 <sup>2</sup>
I	6	0	
II	13	6	
III	5	4	
Stage			.090 <sup>3</sup>
I	13	4	
II	5	1	
III	6	3	
IV	0	2	
Lymph node metastasis			.078 <sup>4</sup>
Yes	6	5	
No	18	5	

Analyzed by Redit analysis:  $P^1 = .480$ ,  $P^2 = .038$ ,  $P^3 = .090$ .

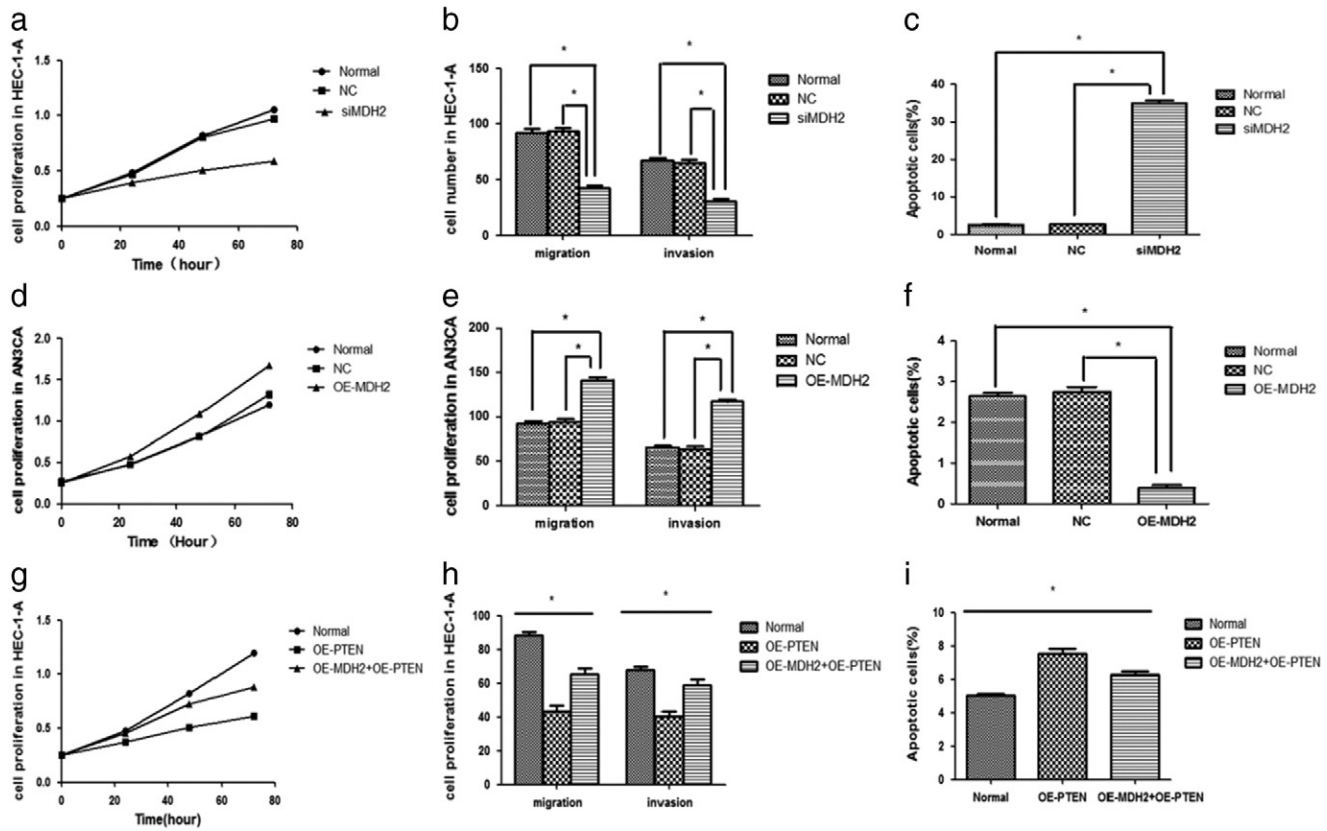
Analyzed by Chi-square test:  $P^4 = .078$ .



**Figure 3.** MDH2 inhibited the expression of PTEN and was co-localized with PTEN. Western-blot and real-time PCR were used to explore the relationship between MDH2 and PTEN on the level of protein and mRNA in endometrial cancer line HEC-1-A. Immunofluorescent staining was performed to investigate the localization of MDH2 and PTEN. (a and b): siRNA-MDH2 to knockdown of MDH2 upregulated the expression of PTEN. (c and d): The overexpression of MDH2 downregulated the expression of PTEN. (e and f) The overexpression of PTEN was inhibited by MDH2. (g) MDH2 was co-localized with PTEN in endometrial cell line HEC-1-A. \* $P < .05$ , One-way ANOVA.

that MDH2 enhanced the proliferation, migration and invasion but blocked the apoptosis of endometrial carcinoma cells through suppressing PTEN. Furthermore, our study demonstrated that MDH2 was co-localized with PTEN in cytoplasm, which not only reinforced the correlation of PTEN with MDH2, but also established that PTEN acted by combining with MDH2 directly. Taken together, we found MDH2 might play as a transcriptional regulator in promoting the formation and development of endometrial carcinoma through inhibiting the tumor suppressor PTEN. Metabolic reprogramming theory suggested that the inactivation of tumor suppressor genes and activation of

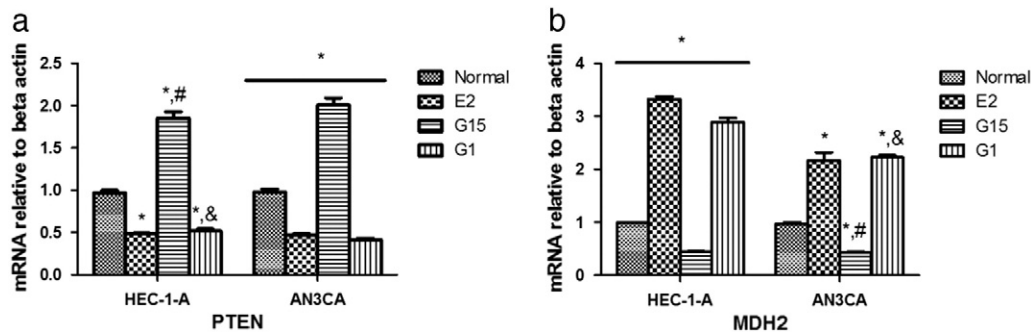
oncogenes was mediated by reprogramming of cellular metabolism [14,15]. In other words, we found a possible mechanism of PTEN in regulation of endometrial carcinoma through MDH2-related cellular metabolism. Our research aimed to reveal the role of MDH2 in PTEN-related regulation of endometrial cancer, thus we focused on the impact of MDH2 on PTEN from the angle of expression and biological behavior. As a matter of fact, PTEN regulated the expression of MDH2 as well. There might be a complicated network between MDH2 and PTEN. The exact roles of MDH2 and PTEN played in this network and how they regulated each other required further research.



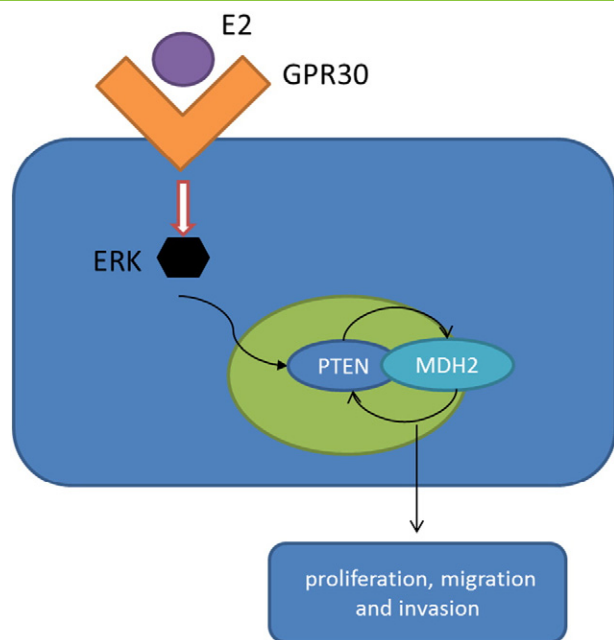
**Figure 4.** MDH2 enhanced the proliferation, migration and invasion but inhibited the apoptosis of endometrial cancer cell line through suppressing PTEN. To explore the role of MDH2 in biological behavior of endometrial cancer cell line, MDH2 RNAi (siMDH2) and overexpression plasmid of MDH2-plasmid GV219-MDH2 (OE-MDH2) was transfected into HEC-1-A and AN3CA respectively. (a-c): siRNA-MDH2 to knockdown of MDH2 inhibited the proliferation, migration and invasion but promoted the apoptosis of HEC-1-A. (d-f): The overexpression of MDH2 promoted the proliferation, migration and invasion but suppressed the apoptosis of AN3CA. (g-i) The suppression of cell proliferation, migration, invasion and the enhancement of apoptosis resulted from the overexpression of PTEN were inhibited by addition of overexpressed MDH2.\*  $P < .05$ , One-way ANOVA.

Finally, to our knowledge, estrogen is a key factor in the pathophysiology of endometrial carcinoma [16]. In the classical mechanism, estrogen combines with ER to activate the estrogen response element located in target genes and trigger a series of physiological responses [12,17]. In recent years, additional evidence

supported the rapid and estrogen-independent mechanism regulated by GPR30, a member of the seven- transmembrane GPCR family [17,18]. Our previous data demonstrated that E2 and G1, an agonist of GPR30, stimulated the proliferation of endometrial carcinoma cells via MEK/ERK/MAPK pathway [12]. Interestingly, in the present



**Figure 5.** E2 might inhibit the expression level of PTEN but enhanced MDH2 through GPR30-related pathway. (a) The stimulation of estrogen and G1, an agonist of GPR30, downregulated the mRNA level of PTEN, while the stimulation of G15, an antagonist of GPR30, upregulated the mRNA level of PTEN in both HEC-1-A (ER positive, GPR30 positive) and AN3CA (ER negative, GPR30 positive). (b) The stimulation of estrogen and G1, upregulated the mRNA level of MDH2, but G15 downregulated the mRNA level of MDH2 in both HEC-1-A and AN3CA. One-way ANOVA was used. \* $P < .05$  versus Normal; # $P < .05$  versus E2; & $P > .05$  versus E2.



**Figure 6.** E2 might modulate PTEN and MDH2 in endometrial carcinoma via GPR30-dependent mechanisms. Our previous data in 2009 has demonstrated that E2 stimulated the proliferation of endometrial carcinoma cells via MEK/ERK/MAPK pathway. In the present study, we revealed that E2 might promote the proliferation, migration and invasion but inhibited apoptosis in endometrial carcinoma through upregulating the expression of MDH2 and downregulating the expression of PTEN via GPR30-dependent pathway. There might be a complicated regulation network between MDH2 and PTEN.

study, in both HEC-1-A (ER positive and GPR30 positive, data not shown) and AN3CA (ER negative and GPR30 positive, data not shown) we found that E2 and G1 downregulated the expression of PTEN but upregulated MDH2. In addition, G15 acted as an antagonist to GPR30, by upregulating PTEN but downregulating MDH2. Therefore, we speculated that E2 might modulate PTEN and MDH2 in endometrial carcinoma via GPR30-dependent mechanisms (Figure 6). Of course, the exact pathway where E2 stimulated the expression of MDH2 through GPR30 was still unclear.

In summary, we found the overexpression of MDH2 in endometrial carcinoma and its close interaction with tumor suppressor PTEN. Furthermore, MDH2, stimulated by estrogen via GPR30-dependent pathway, promoted the cell proliferation of endometrial cell line through inhibiting the expression of tumor suppressor PTEN. This made a very interesting correlation between cell metabolism and gene modulation, as well as hormones and cell metabolism in endometrial cancer. To our knowledge, this is the first report investigating the correlation of PTEN with the metabolic enzyme MDH2. However, the precise role played by MDH2 in PTEN-mediated regulation is still unclear. Estrogen stimulation of the proliferation of endometrial carcinoma cells via the estrogen-MDH2-PTEN pathway is elusive. Further studies will be conducted to determine the

importance of PTEN and MDH2 modulated by E2 in cellular metabolism and their roles in the pathophysiology of endometrial carcinoma.

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