Original Article

Dual-specificity Phosphatase 1 Deficiency Induces Endometrioid Adenocarcinoma Progression via Activation of Mitogen-activated Protein Kinase/Extracellular Signal-regulated Kinase Pathway

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Abstract

Background: Previously, we reported that dual-specificity phosphatase 1 (DUSP1) was differentially expressed in endometrioid adenocarcinoma (EEA). However, the role of DUSP1 in EEA progression and the relationship between DUSP1 and medroxyprogesterone (MPA) are still unclear.

Methods: The expression of DUSP1 in EEA specimens was detected by immunohistochemical analysis. The effect of DUSP1 on cell proliferation was analyzed by Cell Counting Kit 8 and colony formation assay, and cell migration was analyzed by transwell assay. MPA-induced DUSP1 expression in EEA cells was measured by Western blot.

Results: DUSP1 expression was deficient in advanced International Federation of Gynecology and Obstetrics stage, high-grade and myometrial invasive EEA. In EEA cell lines (Hec1A, Hec1B, RL952, and Ishikawa), the DUSP1 expression was substantially higher in Ishikawa cells than in other cell lines ($P < 0.05$). Knockdown of DUSP1 promoted Ishikawa cells proliferation, migration, and activation of mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/Erk) pathway. MPA-induced DUSP1 expression and inhibited MAPK/Erk pathway in Ishikawa cells.

Conclusions: Our data suggest that DUSP1 deficiency promotes EEA progression via MAPK/Erk pathway, which may be reversed by MPA, suggesting that DUSP1 may serve as a potential therapeutic target for the treatment of EEA.

Key words: Dual-specificity Phosphatase 1; Endometrioid Adenocarcinoma; Medroxyprogesterone; Phospho-extracellular Signal-regulated Kinase 1/2

Introduction

Endometrial cancer (EC) is one of the most common gynecological malignancies worldwide. It is estimated that 62,000 new cases occur in China annually.[1] The most common histological type of EC is endometrioid adenocarcinoma (EEA), which comprises approximately 85% of all EC cases. EEA is considered to be less aggressive, with a more favorable outcome compared with nonendometrioid carcinoma.[2] However, several studies have demonstrated that EEA outcomes differ.[3,4] Approximately 13–25% of EEA patients, originally thought to have a good outcome, show recurrence and metastasis.[5] Although EEA patients are categorized into low-, intermediate-, and high-risk groups according to age, myometrial invasion, or differentiation grade, the analytical methods are still poorly defined regarding tumor progression and outcome.

Our group studied the heterogeneity of EEA by gene expression profiling since 2005. We found that dual-specificity

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phosphatase 1 (DUSP1) expression varied and was a potential negative prognostic indicator for EEA.[6-8] DUSP1, which is a member of the DUSP1 family, negatively regulates the mitogen-activated protein kinase (MAPK) pathway through dephosphorylation of serine and tyrosine moieties.[9,10] DUSP1 expression and function in cancer varies and depends on the organ studied. In prostate cancer, hepatocellular cancer and lung cancer, DUSP1 expression is elevated in early stage, and the levels decreased in advanced stages.[11-13] In breast cancer, DUSP1 expression is high in poorly differentiated or advanced stages.[14] However, in EEA, the expression and biological functions of DUSP1 in tumor growth and progression remain unclear.

In this study, we analyzed DUSP1 expression in EEA using immunohistochemistry and determined its relationship with clinicopathologic characteristics; the biological effects of DUSP1 short hairpin RNA (shDUSP1) and the signaling pathway in EEA cells were also detected. In addition, we showed that DUSP1 expression level was upregulated by medroxyprogesterone (MPA) to inhibit tumorigenicity in EEA cells. Our data suggest that DUSP1 deficiency promotes EEA progression by activating MAPK/extracellular signal-regulated kinase (Erk) pathway, and is reversed by MPA.

Methods

Patients and tissue samples

All clinical specimens used in this study were obtained between January 2006 and December 2008 from 113 surgically treated patients with histologically confirmed EEA at the Department of Obstetrics and Gynecology, Peking University People's Hospital. The specimens included 15 benign lesions in the endometrial secretory phase and 15 cases of endometrial hyperplasia serving as control. Informed consent was obtained from all the patients. The diagnosis was confirmed pathologically. Patient demographics and clinicopathological features are listed in Table 1.

Immunohistochemistry

We used 4 μm thick tissue sections of EEA for immunohistochemical analysis. Deparaffinized sections after antigen retrieval were incubated with rabbit polyclonal anti-DUSP1 antibody (1:100, Santa, USA) overnight at 4°C, followed by biotinylated secondary antibody staining. Positive and negative controls were included in each experiment. The detailed protocol was described previously.[13] Immunostaining was evaluated by two independent pathologists, who were blinded to clinical characteristics.

Cell culture and reagents

Human EEA cell lines Hec1A, Hec1B, RL952, and Ishikawa cells were obtained from our laboratory stocks. Hec1A and Hec1B were cultured in Dulbecco's modified Eagle's medium (DMEM, Hyclone, USA) containing 10% fetal bovine serum (FBS, Gibco, USA) and 100 mg/ml penicillin/streptomycin at 37°C in 5% CO_2. RL952 and Ishikawa cells were cultured in DMEM/F12 (Hyclone) containing 10% fetal bovine serum (Gibco) and 100 mg/ml penicillin/streptomycin at 37°C in 5% CO_2. The medium was replenished every day. Pre- and post-transfection cells were treated with 10 μmol/L MPA (Sigma, USA), which was dissolved in 100% dimethyl sulfoxide (DMSO) at concentrations not exceeding 0.1%. Vehicle treated with DMSO alone as control.

Stable dual-specificity phosphatase 1 knockdown Ishikawa cell line

The short hairpin RNA (shRNA) was synthesized by Gene Pharma, China, sequences against DUSP1, 5'-CCACCATCTGCCTTG CTTACCTTAT-3' (sense) and 5'-ATAAGGTAAGCAA GGCAGATGGTGG-3' (antisense). Negative control shRNA sequences included 5'-GTTCTTCCGAACGTGTCACGT-3' (sense) and 5'-ACGTGACACGT TCGGAAGAAC-3' (antisense). Positive control shRNA sequences were 5'-GTATGACAA CAGCCTCAAG-3' (sense) and 5'-CTTGAGGCTG

Table 1: DUSP1 expression and clinicopathologic characteristics in patients with EEA

Characteristics	All patients (n = 113)	DUSP1 expression, n (%)		χ^2	P
		Positive	Negative		
FIGO stages					
I–II	93	72 (88.9)	21 (67.7)	8.522	0.004
III–IV	20	9 (11.1)	11 (32.3)		
Grades					
G1–G2	88	77 (95.1)	11 (34.4)	49.032	0.000
G3	25	4 (4.9)	21 (65.6)		
Myometrial invasion					
<1/2	72	57 (70.4)	15 (46.9)	5.477	0.019
≥1/2	41	24 (29.6)	17 (53.1)		
Lymph node metastasis					
No	100	74 (91.4)	26 (82.3)	2.302	0.129
Yes	13	7 (8.6)	6 (18.7)		

FIGO: International Federation of Gynecology and Obstetrics; DUSP1: Dual-specificity phosphatase 1; EEA:endometrioid adenocarcinoma.

TTGTCATAC-3' (antisense). The target nucleotide sequence was in *DUSP1* coding domain (NM_004417.3) from 796 bp to 821 bp. Ishikawa cells (1×10^5) grown in 6-well plates were transfected with shRNA using lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. Cells were selected using DMEM/F12 (10% FBS) supplemented with 700 μg/ml G418 (Sigma, USA) for 14 days and maintained under selective pressure. Knockdown was confirmed by real-time polymerase chain reaction (PCR) and Western blot.

Cell proliferation assay

A number of 1000 cells were plated in 96-well plates, after incubation for 24 h, on days 0, 1, 2, 3, 4, and 5, followed by addition of 10 μl Cell Counting Kit 8 (CCK8) solution (Dojindo, Japan) to each well. Color intensity was measured by a microplate reader (Tecan Infinite M 200 PRO, Switzerland) at 490 nm to obtain cell growth curves. Experiments were performed in triplicate, and repeated 3 times or more.

Colony formation assay

Cells seeded in 6-well plates (500 cells/well) were incubated in normal growth conditions supplemented with 700 μg/ml G418 for 15 days. The cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. Cells numbering 50 or more were recorded as colonies. Experiments were performed in triplicate, and repeated 3 times or more.

Transwell migration assay

In the transwell assay, 1×10^5 cells in 100 μl DMEM/F12 without FBS were seeded in the upper chamber of Costar transwell culture plates (24-well plates, 8 μm, noncoated membrane), followed by addition of 600 μl of DMEM/F12 containing 10% FBS to the lower chamber. After incubation for 24 h, the chamber was washed 3 times with phosphate-buffered saline. The membrane in the lower chamber was fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The cells in the upper membrane that failed to migrate were wiped with a cotton swab. The number of migrated cells was counted microscopically in five high-power fields (original magnification, ×200). Experiments were performed in triplicate and repeated 3 times or more.

Western blot

Ishikawa cells were harvested and lysed. Total protein concentration was determined by Bradford method. Cell lysates (20 μg) were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Merck Millipore Ltd., German), which were blocked with 5% nonfat milk and incubated with primary antibodies against DUSP1 (1:1000, Santa, USA), phospho-extracellular signal-regulated kinase 1/2 (p-Erk1/2, 1:1000, Cell Signaling, USA), Erk1/2 (1:1000, Cell Signaling, USA), phospho-c-Jun N-terminal kinase 1/2 (p-JNK1/2, 1:1000, Cell Signaling, USA) and JNK1/2 (1:1000, Cell Signaling, USA) at 4°C overnight. The goat anti-rabbit secondary antibody (ZSGB-BIO, Beijing) was incubated for 2 h at room temperature. The enhanced chemiluminescent substrate was used to detect their expression. The band intensities were determined using the Bio-Rad imaging system (Hercules, CA, USA).

Statistical analysis

SPSS 19.0 software package (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5 Software (SanDiego, CA, USA) were used for all statistical analyses. Continuous variables were presented as means and standard deviations or as medians and interquartile ranges, and categorical variables were presented as proportions. One-way analysis of variance (ANOVA) and independent-sample *t*-tests were used to compare continuous variables and Chi-square tests to compare categorical variables. Further two-group comparison using multiple comparison Tukey's test. A $P < 0.05$ was considered to indicate statistical significance, and all *P* values were two-sided.

Results

Expression of dual-specificity phosphatase 1 in endometrioid adenocarcinoma and its relationship with clinicopathological factors

Positive immunohistochemical staining was observed for DUSP1 expression in all benign endometrium in the cytoplasm and nucleus [Figure 1a]. As shown in Table 1, in 113 EEA cases, 81 (71.7%) cases, DUSP1 protein staining did not differ compared with benign endometrium. However, 32 cases (28.3%) exhibited negative or

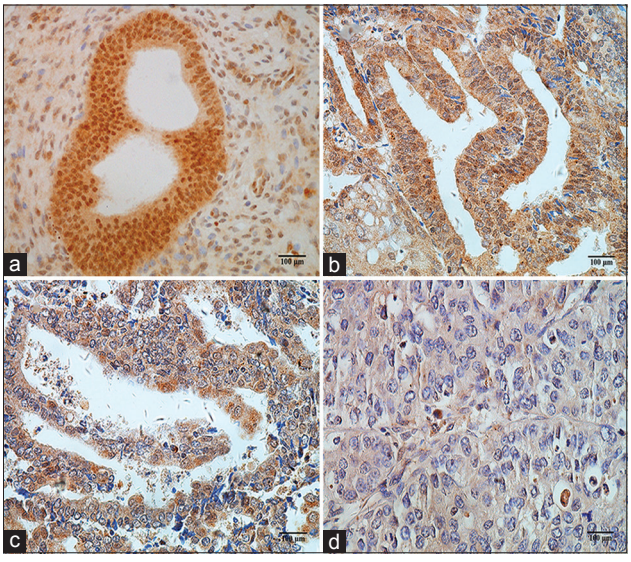

Figure 1: Representative images of DUSP1 immunohistochemical staining in endometrial tissue and endometrioid adenocarcinoma tissue (original magnification, ×400). (a) endometrial tissue of benign lesion. (b) I stage G1 endometrioid adenocarcinoma. (c) II stage G2 endometrioid adenocarcinoma. (d) III stage G3 endometrioid adenocarcinoma. DUSP1: Dual-specificity phosphatase 1.

weak DUSP1 protein expression in the cytoplasm and nucleus [Figure 1b-1d]. The correlation between DUSP1 expression and the clinicopathological parameters of EEA patients was analyzed. EEA patients were divided into DUSP1-positive (n = 81) and DUSP1-negative (n = 32) groups. DUSP1 deficiency was significantly correlated with advanced stage (P = 0.004), higher grade tumor (P < 0.001) and myometrial invasion (P = 0.017). No association with lymph node metastasis was observed (P = 0.129). These data strongly suggested that the deficiency of DUSP1 in EEA tissue may play a role in inhibition of EEA progression.

Dual-specificity phosphatase 1 expression in endometrioid adenocarcinoma cell lines

Hec1A, Hec1B, RL952, and Ishikawa are EEA cell lines, which express different progesterone receptors (PRs). Ishikawa cells are PR-positive while RL952, Hec1A, and Hec1B are PR-negative.[15,16] We evaluated DUSP1 expression levels in Hec1A, Hec1B, RL952, and Ishikawa by real-time PCR and Western blot, respectively. Ishikawa showed higher levels of DUSP1 mRNA and protein expression than RL952, Hec1A and Hec1B [Figure 2a and 2b] (P < 0.05).

Dual-specificity phosphatase 1 knockdown promoted Ishikawa cells proliferation and migration

The high expression of DUSP1 in Ishikawa cells was investigated. DUSP1 was knocked down by transfecting Ishikawa cells with shRNA. The DUSP1 shRNA-transfected cells as well as DUSP1-negative control (NC) cells were assessed by Western blot. DUSP1 expression after shRNA transfection was significantly reduced compared with nontargeting shRNA-transfected cells [Figure 3a] (P < 0.01).

Cell proliferation was evaluated in NC and knockdown cells by CCK8. Interestingly, DUSP1 silencing markedly promoted the proliferation of Ishikawa cells [Figure 3b] (P < 0.01). In addition, DUSP1 shRNA-transfected cells starkly promoted the colony formation ability compared with NC cells [Figure 3c] (P < 0.01).

To assess DUSP1 impact on migration, DUSP1 silenced cells were incubated in transwell systems. After 24 h, a higher cell migration was found in DUSP1 shRNA-transfected cell group compared with NC [Figure 3d] (P < 0.01). These findings indicated that DUSP1 silencing promoted Ishikawa cells proliferation and migration.

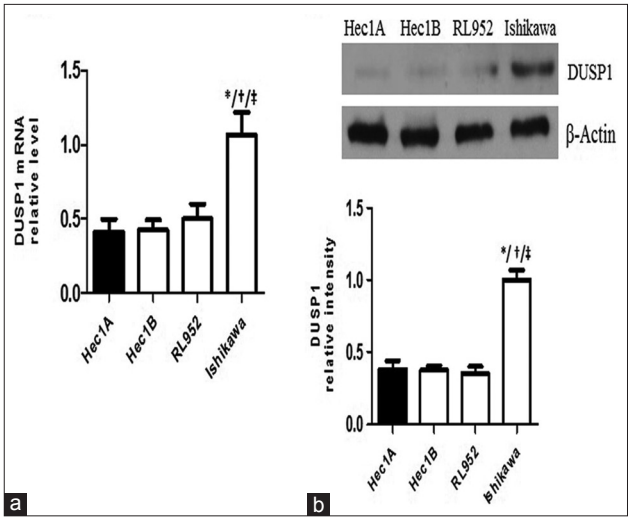

Figure 2: (a and b) DUSP1 mRNA and protein expression in human endometrioid adenocarcinoma cell lines. *P < 0.05, compared with Hec1A. †P < 0.05, compared with Hec1B. ‡P < 0.05, compared with RL952. All data are mean ± standard deviation from three experiments. DUSP1: Dual-specificity phosphatase 1.

Mitogen-activated protein kinases/extracellular signal-regulated kinase was activated by dual-specificity phosphatase 1 knockdown

MAPK activation was analyzed after DUSP1 knockdown in Ishikawa cells by Western blot. A higher p-Erk1/2 level was obtained after DUSP1 silencing compared with NC. Meanwhile, total Erk1/2, JNK1/2, and p-JNK1/2 levels were unaltered [Figure 4] (P < 0.01).

Medroxyprogesterone inhibited Ishikawa cells proliferation and induced dual-specificity phosphatase 1 expression

MPA showed time- and dose-dependent inhibition of Ishikawa cells growth [Figure 5a and 5b] (P < 0.05). We evaluated the changes in DUSP1 expression induced by MPA. As shown in Figure 5c and 5d, Western blot demonstrated that DUSP1 expression was increased after treatment with 10 μmol/L MPA in a time-dependent (P < 0.05) and dose-dependent manner (P < 0.05), along with decreased p-Erk1/2 level. After DUSP1 silencing in Ishikawa cells, DUSP1 and p-Erk1/2 levels were unaltered following exposure to 10 μmol/L MPA [Figure 5e] (P > 0.05).Taken together, these results indicate that DUSP1 regulated MPA anti-proliferative effects on Ishikawa cells.

Discussion

This study demonstrated that deficiency of DUSP1 expression leads to EEA progression. Although DUSP1 has been studied in many types of cancer,[11-14] the biological function of DUSP1 and its relationship with MPA in EEA have not been reported. Our results show that a lower DUSP1 expression was associated with advanced stage and higher grade, and illustrate the proliferation and migration effects of shDUSP1 on Ishikawa cells. We also show that MPA increased DUSP1 expression in Ishikawa cells. In brief, our study proposes a novel mechanism suggesting that DUSP1 deficiency in EEA, reversed by MPA, promotes proliferation and migration by activating MAPK/Erk [Figure 6].

Dual-specificity family phosphatases have been shown to inactivate MAPK and prevent excessive mitogenic or apoptosis signaling via dephosphorylation at its tyrosine and threonine residues, which is required for cell growth, differentiation, and apoptosis.[17,18] DUSP1 is overexpressed in pancreatic tumor, where it inhibits mitogenic signals

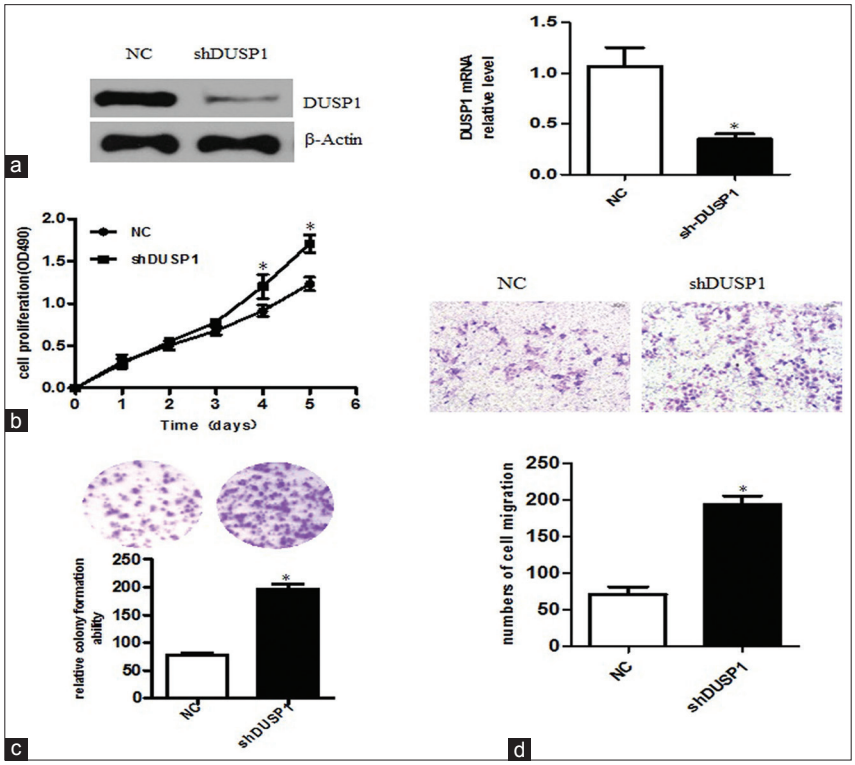

Figure 3: DUSP1 knockdown promoted Ishikawa cells proliferation and migration. (a) DUSP1 expression was significantly reduced after transfection with DUSP1 shRNA compared with NC. (b) Cell proliferation was assessed with CCK8 between shDUSP1 and NC. (c) Colony formation assay in Ishikawa cells after DUSP1 knockdown with shRNA (0.5% crystal violet staining, original magnification, ×100). (d) DUSP1 knockdown increased Ishikawa cell migration (0.5% crystal violet staining, original magnification, ×200). All data are mean ± standard deviation from three experiments. *$P < 0.01$, compared with NC group; NC: Negative control; DUSP1: Dual-specificity phosphatase 1; shDUSP1: Cells knockdown with DUSP1 shRNA; shRNA: Short hairpin RNA; CCK8: Cell Counting Kit 8.

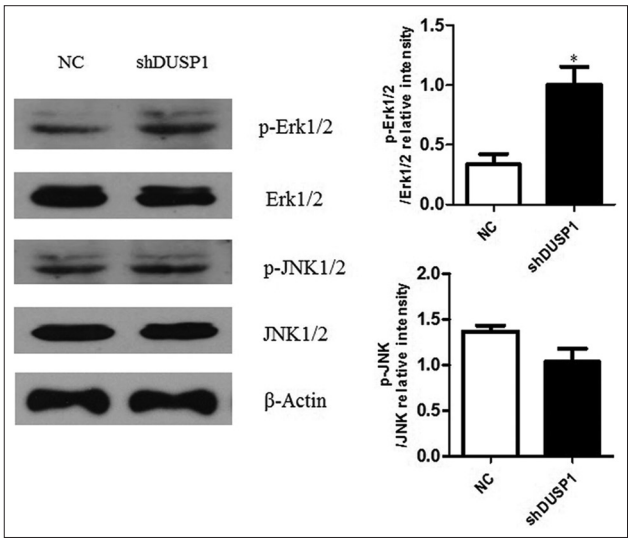

Figure 4: DUSP1 knockdown activated MAPK/Erk pathway. The p-Erk1/2 was upregulated after DUSP1 knockdown (shDUSP1) compared with NC. *$P < 0.01$ versus NC group, all data are mean ± standard deviation from three experiments. NC: Negative control; DUSP1: Dual-specificity phosphatase 1; shDUSP1: Cells knockdown with DUSP1 shRNA; p-: Phospho-; Erk1/2: Extracellular signal-regulated kinase 1/2; JNK: c-Jun N-terminal kinase 1/2. MAPK: Mitogen-activated protein kinase; shRNA: Short hairpin RNA.

induced via Erk pathway.[19] In early lung cancer, DUSP1 expression levels independently predicted improved survival through JNK activation whereas late-stage cancer was correlated with Erk activation.[20] Furthermore, DUSP1 expression was low or even absent in prostate cancer. DUSP1 promoted apoptosis of prostate cancer cells via inhibition of p38 MAPK.[21] These data show that DUSP1 levels were increased in response to mitogenic factors or reduced apoptotic signals in different cancers.[22,23] Our results involving EEA were similar to DUSP1 deficiency in late stages and Erk activity was uncontrolled during hepatocellular carcinoma progression.[24] DUSP1 silencing in Ishikawa cells significantly increased the response to mitogenic growth factors p-Erk1/2 compared with control. However, no response to the apoptotic factors JNK1/2 was observed. Therefore, down-regulation of DUSP1 promotes EEA tumorigenicity by activating MAPK/Erk rather than MAPK/JNK.

MPA is an established therapy for advanced or recurrent cases of EEA and the response to MPA is an important factor determining EEA patient outcomes.[25,26] The mechanisms of MPA in EEA may relate to altered downstream molecular signaling to inhibit cell proliferation and induce apoptosis, mediated by cycline-dependent kinase, transforming growth factor-β1 (TGF-β1), p27 and survivin.[27] In this study, DUSP1 expression modulated by MPA was consistent with progesterone-induced DUSP1 expression in human breast cancer cells and similar to

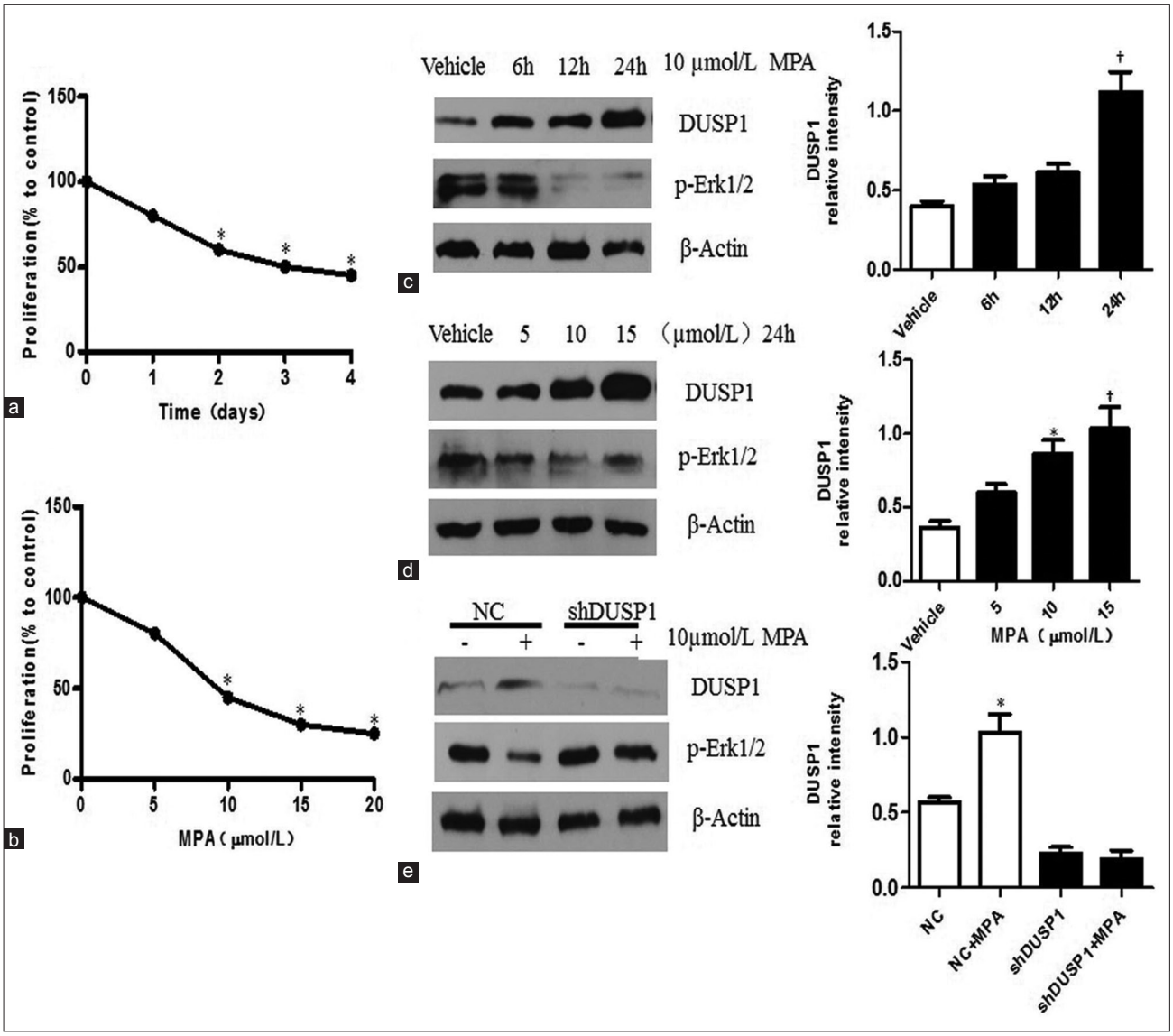

Figure 5: MPA inhibited Ishikawa cells proliferation and induced DUSP1 expression. (a and b) MPA inhibited Ishikawa proliferation in time-dependent and dose-dependent manner. *$P < 0.05$, compared with control. (c) DUSP1 expression was increased by 10 μmol/L MPA in time-dependent manner. †$P < 0.01$, compared with vehicle. (d) DUSP1 expression was increased by MPA in dose-dependent manner. *$P < 0.05$, compared with vehicle, and †$P < 0.01$, compared with vehicle. (e) DUSP1 protein level in Ishikawa cells with or without 10 μmol/L MPA. *$P < 0.05$, compared with NC. DUSP1: Dual-specificity phosphatase 1; NC: Negative control; MPA: Medroxyprogesterone; p-: Phospho-; Erk1/2: Extracellular signal-regulated kinase 1/2.

DUSP6 was proposed as a potential molecular marker for progestin therapy of atypical endometrial hyperplasia and earlier endometrial carcinomas.[28,29] This study may demonstrate that the molecular mechanisms of DUSP1 in MPA-induced Ishikawa cells proliferation. Furthermore, PR expression serves as an independent marker of progesterone therapy and disease-free survival.[30] Our data showed that DUSP1 was upregulated by MPA in PR-positive Ishikawa cells, suggesting the PR-specific correlation of DUSP1 with MPA. Increased DUSP1 protein levels may be related to MPA interaction with PR to induce p-Erk1/2 down-regulation. Additional studies are needed to explore the possible correlation of DUSP1 and PR.

In conclusion, DUSP1 deficiency accelerates EEA progression and is modulated by MPA. The mechanism of DUSP1 provides further insight into the pathogenesis of EEA. DUSP1 is a biomarker and potential therapeutic target for MPA.

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Conflicts of interest

There are no conflicts of interest.

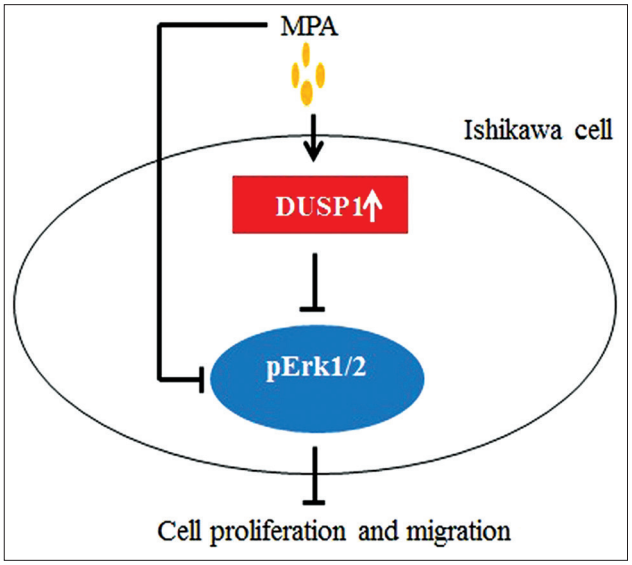

Figure 6: DUSP1 mediates MPA-induced cell proliferation and migration in Ishikawa cell. The large circle represents a single Ishikawa cell. The orange small circles represent MPA. The red rectangle denotes DUSP1 and the blue one stands for pErk1/2 protein. MPA: Medroxyprogesterone; DUSP1: Dual-specificity phosphatase 1; p-Erk1/2: Phospho-extracellular signal-regulated kinase 1/2.

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