Molecular and Cellular Endocrinology 376 (2013) 60–69

Contents lists available at SciVerse ScienceDirect

Molecular and Cellular Endocrinology

journal homepage: www.elsevier.com/locate/mce

Dual-specificity phosphatase 6 (Dusp6), a negative regulator of FGF2/ERK1/2 signaling, enhances 17β-estrodial-induced cell growth in endometrial adenocarcinoma cell

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ARTICLE INFO

Article history:
Received 26 August 2012
Received in revised form 6 February 2013
Accepted 8 February 2013
Available online 16 February 2013

Keywords:
Dusp6
FGF2/MAPK/ERK1/2
17β-Estradiol
Progesterone
Endometrial adenocarcinoma

ABSTRACT

Dual-specificity phosphatase 6 (Dusp6) is a negative feedback mechanism of fibroblast growth factors (FGFs)/mitogen-activated protein kinase (MAPK)/ERK1/2 signaling. The aim of this study was to explore the expression of Dusp6 in human endometrial adenocarcinomas and the role of Dusp6 expression in the growth regulation of endometrial adenocarcinoma cell. We found that Dusp6 was over-expressed in human endometrial adenocarcinomas. In Ishikawa cells, plasmid-driven Dusp6 expression efficiently blocked the activity of FGF2-induced MAPK/ERK1/2 signaling. Unexpectedly, Dusp6 expression significantly enhanced the growth of Ishikawa cells. In Dusp6 forced-expression cells, 17β-estradiol stimulation increased the cell growth by all most threefolds. In addition, progesterone treatment reduced the cell growth to about half both in Ishikawa cells with and without forced-Dusp6-expression. Dusp6 over-expression is involved in the pathogenesis and development of human endometrial adenocarcinomas. Dusp6 functions as a negative regulator of FGF2/ERK1/2 signaling but enhances the growth and 17β-estradiol-induced cell growth in endometrial adenocarcinoma cell.

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1. Introduction

Endometrial cancer remains one of the leading diagnosed gynecologic malignancies (Wild et al., 2012), while endometrial adenocarcinoma, originating from epithelial cells, is the most common subtype. Despite the progressing diagnosis and therapeutic interventions of endometrial cancers, further investigation of the tumorigenesis mechanisms is expected.

Fibroblast growth factor 2 (FGF2) is one of the first confirmed angiogenesis-related growth factors (Shing et al., 1984), and is involved in the neovascularization of series of human tumors. Aberrant FGF2 gene copy number is present in human endometrial cancer (Schmitt et al., 1996). The expression of FGF2 is upregulated in endometrial adenocarcinomas (Gold et al., 1994; Fujimoto et al., 1995, 1996). The serum level of FGF2 is elevated significantly in patients with endometrial cancer (Chopra et al., 1997). In *in vivo* studies, FGF2 promotes endometrial cancer cell growth and neovascularization in autocrine and paracrine manners (Coltrini et al., 1995). In addition, FGF2 can promote FGF2 expression in endometrial adenocarcinoma cells via FGF2/extracellular signal-regulated kinase 1/2 (ERK1/2) signaling pathway (Sales et al., 2007), establishing a positive feedback loop leading to the persistent activity of mitogen-activated protein kinase (MAPK)/ERK1/2 signaling. These data suggest the essential role of FGF2 signaling in the pathogenesis and development of endometrial cancer.

ERK1/2 are considered to be classic MAPKs and their activation leads to the phosphorylation of nuclear transcription factors and finally triggers a series of responses in target cells, including caryomitosis, differentiation, proliferation and migration (Pearson et al., 2001). The tight control of ERK1/2 signaling is crucial in deciding cell fate. Dual-specificity phosphatase 6 (Dusp6, also called MAPK phosphatase 3 or MKP-3), belonging to the family of dual-specificity phosphatases, acts as a ERK1/2-specific dual-specificity phosphatase (Mourey et al., 1996; Muda et al., 1996a). Dusp6 inactivates ERK1/2 activity selectively via dephosphorylation of both phosphothreonine and phosphotyrosine (Muda et al., 1996b), and efficient function of Dusp6 depends on ERK-induced conformational change within the catalytic domain of Dusp6 (Farooq et al., 2001). Thus, Dusp6 acts as a negative feedback regulator of MAPKs/ERK1/2 signaling, and definitely avoids the over-activity of the signaling (Zeliadt et al., 2008).

Loss of Dusp6 has been noted in various human carcinomas, including pancreatic carcinomas (Furukawa et al., 1998), lung

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0303-7207/$ - see front matter © 2013 Published by Elsevier Ireland Ltd.
http://dx.doi.org/10.1016/j.mce.2013.02.007

cancer (Okudela et al., 2009), esophageal squamous cell carcinoma and nasopharyngeal carcinoma (Wong et al., 2012). In *in vitro* studies, Dusp6 expression induces apoptosis in pancreatic cancer cells (Furukawa et al., 2003), inhibits ERK1/2 activity and cell growth in Dusp6-deficient ovarian cancer cells, and sensitizes ovarian cancer cells to cisplatin-induced apoptosis *in vitro* and *in vivo* (Chan et al., 2008). Dusp6 knockdown increases ERK activation and cell growth in Dusp6-high lung cancer cells, while Dusp6 expression decreases ERK activity and cell growth in Dusp6-low lung cancer cells (Zhang et al., 2010). Dusp6 expression can also impair epithelial–mesenchymal transition-associated phenotype (Wong et al., 2012).

Despite the involvement of FGF2/ERK1/2 signaling in endometrial carcinomas, little is known regarding the role of Dusp6 in the disease. In the current studies, we investigated the aberrant expression of Dusp6 in human endometrial adenocarcinomas. The effect of Dusp6 expression on ERK1/2 signaling in endometrial cancer cell line was detected. Additionally, the functional impact of Dusp6 in cell growth was explored.

2. Materials and methods

2.1. Tissue collection and immunohistochemistry analysis

Normal endometrial biopsies from 31 women of reproductive age (proliferative phase: $n = 17$; secretory phase: $n = 14$), five women of post-menopausal age and cancer tissues obtained from 31 patients with endometrial adenocarcinoma (mean age: 50.8 ± 11.1) were employed in immunohistochemistry analysis. The detailed information of the patients with endometrial carcinomas was shown in Table 1. Diagnosis was confirmed by histological examination. None of the participants received any hormonal therapy during the 3 months before their operation.

The experiment was performed as described previously (Zhang et al., 2009). Briefly, fresh tissues were washed with PBS and then fixed in 4% paraformaldehyde. After dehydration and paraffin-embedding, samples were cut into 5-μm sections and mounted onto glass slides. Deparaffinated, rehydrated sections were incubated with 3% H2O2 and then antigen retrieval was performed. After blocking, the sections were incubated overnight with rabbit anti-human Dusp6 primary antibody (diluted 1:100 in PBS, Abcam) in a wet chamber at 4 °C. HRP-conjugated goat anti-rabbit IgG was used as second antibody. Sections incubated with non-immune serum instead of primary antibody were used as negative control. The experiments were repeated in duplicate or triplicate.

The immunohistochemical score was evaluated as described previously (Zhang et al., 2009). Two sections per sample were evaluated blind for immunostaining without any knowledge of the clinical or pathologic data.

2.2. Cell cultures and treatments

Ishikawa cells, derived from a well-differentiated endometrial adenocarcinoma, were maintained in DMEM supplemented with 5% charcoal-stripped fetal bovine serum (FBS), 100 μ/ml of penicillin and 100 μ/ml of streptomycin at 37 °C in a humidified environment with 5% CO_2 in air. FGF2 (R & D Systems) was dissolved in 1% bovine serum albumin (BSA) at a stock concentration of 1 μmol/L and stored at −80 °C. 17β-estradiol (Sigma) was dissolved in ethanol at a stock concentration of 10 mmol/L and stored at −80 °C. U0126, a specific MAPK/ERK inhibitor, was dissolved in Dimethyl sulfoxide (DMSO) at a stock concentration of 20 mmol/L and stored at 4 °C. 10 nmol/L FGF2, 10 nmol/L 17β-estradiol, and 10 μmol/L U0126 were used in cell treatments. In experiments employing U0126, cells were pretreated with U0126 for 1 h before FGF2 (final concentration: 10 nmol/L) or 17β-estradiol (final concentration: 10 nmol/L) was added into the medium. Mock-treatment with an identical volume of 1% BSA, ethanol or DMSO was used as control.

2.3. Constructs and luciferase reporter assays

pcDNA3.1-Dusp6/WT-V5 plasmid and an enzyme-dead Dusp6 mutant C293S plasmid were kindly provided by Dr. Nelly Pitteloud and Dr. Yisrael Sidis (Centre Hospitalier Universitaire Vaudois, Switzerland). The Dusp6 mutant C293S construct was generated by a 293 Cystenin to Serine point mutation using Site-Directed Mutagenesis Kit (Fig. 3A). Elk1-dependent luciferase reporter plasmids were obtained from Promega, USA. All plasmids were sequence-verified before use. In luciferase assays, Ishikawa cells were plated at 40–50% confluence on 24-well plates and grown overnight in DMEM supplemented with 5% charcoal-stripped FBS, penicillin (100 μ/ml) and streptomycin (100 μ/ml). Transfections were performed using FuGENE6 transfection reagent (Roche) according to the manufacturer's protocol. Briefly, cells were transfected with a total of 200 ng of DNA per well, including 10 ng pFA2-ELK-1 luciferase reporter and different amounts of pcDNA3.1-Dusp6/WT-V5 plasmid (125 ng, 25 ng, 5 ng, and 1 ng). 6 h after transfection, the cells were serum-starved overnight (>12 h) and stimulated with FGF2 (10 nmol/L) for 24 h. After FGF2 treatment, cells extracts were prepared and luciferase assays were performed. The results were expressed as mean ± SD. Luciferase assay using Dusp6/C293S mutant was performed in duplicate to confirm that the inhibition was not due to the DNA transfection.

2.4. Generation of stably transfected cell clones

Ishikawa cells were plated on 24-well plate and maintained in DMEM containing 10% FBS. Cells were transfected with 25 ng

Table 1
The information of patients with endometrial adenocarcinomas.

No.	Age	Surgery–pathological stage	No.	Age	Surgery–pathological stage
1	54	IA, well-differentiated	17	52	IB, well-differentiated
2	61	IA, moderately differentiated	18	63	IB, well-differentiated
3	58	IA, moderately differentiated	19	59	IB, well-differentiated
4	47	IB, poorly differentiated	20	51	IB, poorly differentiated
5	50	IB, well-differentiated	21	29	IB, moderately differentiated
6	60	IB, moderately differentiated	22	56	IB, poorly differentiated
7	39	IB, poorly differentiated	23	43	IB, well-differentiated
8	37	IB, well-differentiated	24	82	IC, moderately differentiated
9	59	IB, moderately differentiated	25	41	IC, well-differentiated
10	63	IB, moderately differentiated	26	53	IC, moderately differentiated
11	36	IB, moderately differentiated	27	45	IIIA, moderately differentiated
12	31	IB, poorly differentiated	28	59	IIIA, moderately differentiated
13	58	IB, well-differentiated	29	46	IIIC, well-differentiated
14	54	IB, moderately differentiated	30	50	IVA, well-differentiated
15	47	IB, moderately differentiated	31	63	IVB, well-differentiated
16	40	IB, poorly differentiated			

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