European Review for Medical and Pharmacological Sciences 2018; 22: 1554-1568

Effect of FGF2 on the activity of SPRYs/DUSP6/ERK signaling pathway in endometrial glandular epithelial cells of endometriosis

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Abstract. – OBJECTIVE: We aimed at exploring the positive feedback loop in eutopic and ectopic endometrial glandular epithelial cells (EuECs and EECs) in endometriosis.

MATERIALS AND METHODS: Normal epithelial cells (NECs), EuECs and EECs were treated with fibroblast growth factor (FGF)2, FGF2 neutralizing antibody, mitogen-activated protein kinases (MAPKs) inhibitors U0126 and PD98059. FGF2 protein level was detected by enzyme-linked immunosorbent assay (ELISA). The expressions of FGF2, FGF receptor 1 (FGFR1), extracellular signal-regulated kinase (ERK)1/2/pERK1/2 and Sproutys (SPRYs) (Sprouty1, Sprouty2, Sprouty4) and dual specificity phosphatase 6 (DUSP6) were detected by Western blot. The mRNA levels of FGF2, FGFR1 (FGF receptor 1), SPRYs (Sprouty1, Sprouty2, Sprouty4) and DUSP6 mRNA were detected by RT-PCR.

RESULTS: Among treatment groups, the content of FGF2 in EuECs and EECs was significantly higher than that in NECs ($p < 0.05$). The mRNA and protein levels of FGF2, FGFR1, SPRYs (Sprouty1, Sprouty2, Sprouty4) and DUSP6 in EuECs and EECs were increased after adding FGF2 ($p < 0.05$), but decreased after adding FGF2 neutralizing antibody, no significant change was found in NECs ($p > 0.05$). The inhibitory effect of PD9805 on NECs was not significantly different from that of U0126 ($p > 0.05$); however, the inhibitory effects of PD9805 on EuECs and EECs were significantly lower than those of U0126 ($p < 0.05$).

CONCLUSIONS: The positive feedback loop existed in EuECs and EECs, but maybe not in NECs. The results may provide the guideline to treat endometriosis patients.

Key Words:
FGF2, Endometriosis, SPRYs/DUSP6/ERK signal pathway, Feedback loop.

Abbreviations

FGFs = fibroblast growth factors; FGFR = fibroblast growth factor receptor; PI3K = phosphatidylinositol kinase; MAPK = Mitogen-activated protein kinase; PLCγ = Phospholipase CCγ; STAT = signal transducers and activators of transcription; ERK1/2 = extracellular regulated kinase 1/2; DUSP6 = dual specificity phosphatase 6; SPRY = Sprouty; EuECs = eutopic endometrial glandular epithelial cells; EECs = ectopic endometrial glandular epithelial cells; NECs = normal epithelial cells.

Introduction

The members of fibroblast growth factors, or high fibroblast growth factors (FGFs), are involved in various signaling pathways such as embryonic development and wound healing, conferring to FGFs the pivotal functions in cell proliferation and differentiation[1]. With the involvement of cofactors, FGFs can phosphorylate the intracellular tyrosine kinase domain of fibroblast growth factor receptor (FGFR) to active it. Activated FGFR will interact with a variety of pathways such as phosphatidylinositol kinase (PI3K)-AKT, RAS-Mitogen-activated protein kinase (MAPK), phospholipase CCγ (PLCγ), and signal transducers and activators of transcription (STAT)[2]. FGF2 was found to play critical roles in angiogenesis[3]. Previous studies have shown that FGF2 can increase the expression level of itself through the interacting with extracellular regulated kinase 1/2 (ERK1/2) signaling pathway[4]. FGF can induce the activation of ERK1/2 through a dose-dependent manner and higher FGF levels, leading to higher

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ERK phosphorylation than lower FGF level[5]. ERK1/2 signaling pathway can trigger the phosphorylation of nuclear transcription factors and eventually leads to a variety cell responses such as differentiation, proliferation, caryomitosis, and migration[6]. Therefore, determination of cell fate largely depends on the activity of ERK1/2 signaling.

MAPK can also active the negative regulators of the FGF signaling pathways such as dual specificity phosphatase 6 (DUSP6) and Sprouty (SPRY)[2]. DUSP6, which is also called MAPK phosphatase 3 or MKP-3, is a member of ERK1/2-specific dual-specificity phosphatase[7,8]. With the ability of dephosphorylating both phosphothreonine and phosphotyrosine of ERK1/2 proteins, DUSP6 can inactive ERK1/2 signaling pathway[8]. Thus, DUSP6 plays a role as negative feedback regulator of ERK1/2 and FGF2 signaling pathways to avoid the over-expression of FGF2[9]. Downstream of the FGFRs signaling is also tightly regulated by the regulators of the RAS-MAPK and PI3K-AKT pathways such as SPRY proteins[10-14]. Through interacting with other factors in FGF signaling, SPRYs can not only inhibit the RAS-MAPK pathway, but also can regulate the PI3K-AKT pathway[13,15]. Previous studies[16], which performed gain- and loss-of-function experiments, have shown that FGF signaling can trigger the expression of SPRYs and the overexpression of SPRYs reducing the activity of FGF signaling. Thus, SPRYs plays a role as negative regulator in FGF2 signaling pathway.

The presence and function of FGF2-ERK1/2 signaling pathways have been confirmed in various types of cells, tissues, and biological process. Previous studies have shown that the activated ERK1/2 signaling caused by FGF2 is critical for the migration of endothelial cell[17]. Although FGF2 cannot change the protein level of total ERK1/2, the levels of pERK1/2, which are the activated form of ERK1/2, were increased 15 min after FGF2 treatment. In addition, FGF2 protein level was also increased[18]. The upregulation effect of FGF2 on pERK1/2 was completely abolished by adding U0126 (ERK1/2 inhibitor) and LY294002 (PI3K inhibitor)[18]. The presence and function of FGF2/ERK1/2 positive feedback loop in endometrial glandular epithelial cells of endometriosis are still largely unknown. To investigate the presence of the positive feedback loop in eutopic and ectopic endometrial glandular epithelial cells (EuECs and EECs) in endometriosis, the levels of FGF2 protein normal epithelial (NECs), EuECs and EECs were detected by ELISA after treatment with FGF2, FGF2 neutralizing antibody, MAPKs inhibitors U0126 and PD98059 with non-treatment group as control. In addition, protein levels of FGF2, FGFR1, ERK1/2/pERK1/2, SPRYs and DUSP6 in both treatment and control groups were detected by Western blot. The mRNA level of FGF2, FGFR1, SPRYs and DUSP6 mRNA were also detected by RT-PCR. We found that FGF2 showed a higher expression level in EuECs and EECs than that in NECs. FGF2 can increase the expression of FGF2, FGFR1, SPRYs and DUSP6 in both EuECs and EECs but not in NECs. The upregulation function of FGFs on the expression of FGF2, FGFR1, SPRYs, and DUSP6 was reduced by adding FGF2 neutralizing antibody. In addition, the inhibitory effect of PD9805 on EuECs and EECs was significantly lower than that of U0126 ($p < 0.05$), but no significant difference was found on NECs. All the data were consistent with previous studies[17,18] on the function of FGF2/ERK1/2 signaling pathway in other tissues or organs. Our study proved the presence of FGF2/ERK1/2 positive feedback loop in EuECs and EECs and provided theoretical basis for future studies on the function of FGF2/ERK1/2 positive feedback in EuECs and EECs.

Materials and Methods

Cell Treatment

NECs, EuECs and EECs were divided into 11 groups respectively for different treatments. Those 11 groups include: FGF2 treatment group, cell-conditioned medium (Hyclone, Beijing, China) treatment group, FGF2 and FGF2 neutralizing antibody (RD Systems, Shanghai, China) treatment group, cell-conditioned medium and FGF2 neutralizing antibody treatment group, FGF2 and MAPKs inhibitor U0126 (Sigma-Aldrich, St. Louis, MO, USA) treatment group, cell-conditioned medium and MAPKs inhibitor U0126 treatment group, FGF2 neutralizing antibodies treatment group, MAPKs inhibitor U0126 treatment group, FGF2 and MAPKs inhibitor PD98059 (Sigma-Aldrich, Saint Louis, MO, USA) treatment group, cell-conditioned medium and MAPKs inhibitor PD98059 treatment group, and MAPK inhibitors PD98059 treatment group. Blank control group was also involved.

Detection of FGF2 Protein Level in the Supernatant of Cell Culture by ELISA

The supernatant of each cell culture group was collected and the protein level of FGF2 was detected by double antibody sandwich ELISA according to the instructions (Invitrogen, Carlsbad, CA, USA).

Detection of mRNA Levels of FGF2, FGFR1, SPRYs, and DUSP6 in Each Cell Group by RT-PCR

The cells were collected by centrifugation. TRIzol reagent was used to extract the total RNA from cells. The quality of RNA samples were checked. The reverse transcription reaction kit was used to carry out the reverse transcription reaction. The following primers were used for PCR: 5'-CCACTTCAAGGACCCCAAG-3' (sense) and 5'-ATAGCCAGGTAACGGTTAGC-3' (anti-sense) for FGF2; 5'--CAGTCGACAATCCCTGGGAAGATCTCATTG-3' (sense) and 5'-GGTGGATCCAGGGCCACAAGGTGGACAATCGG-3' (anti-sense) for FGFR1; 5'-AGGGCTATCTTCCTAGCA-3' (sense) and 5'-GTGAGAAGCATGGGGT-3' (anti-sense) for Sprouty1; 5'-GCGATCACGGAGTTCAG-3' (sense) and 5'-GTGGAGTCTCTCGTGT-3' (anti-sense) for Sprouty2; 5'-CCAGACTCTGGTCAACTATGGCAC-3' (sense) and 5'-GTAGCTGTCCGAAAGGCTTGTCGG-3' (anti-sense) for Sprouty4; 5'-GGGGGTGTTAGTAGGTATGTT-3' (sense) and 5'-TACAAAACCAA CAAATAACTTTAAAAAA-3' (anti-sense) for DUSP6; 5'-TCGTGCGTGACATTAAGGAG-3' (sense) and 5'-CTAGAAGCATTTGCGGTGGA-3' (anti-sense) for β-actin. The PCR product was electrophoresed by agarose gel running and the gel was stained with EB solution. The gel was placed in a gel imager (Promega, Madison, WI, USA) and photographed. ImageJ software was used to calculate the relative gray value of band derived from the PCR product of FGF2, FGF2/FGFR1, and SPRYs (Sprouty1, Sprouty2, Sprouty4)/DUSP6 compared with the gray value of the band derived from internal control (β-actin) to reflect the mRNA level of each gene.

Detection of Expressions of FGF2, FGFR1, ERK1/2/ pERK1/2, SPRYs, and DUSP6 by Western Blot

Cells were digested with 0.25% trypsin (Sigma-Aldrich, St. Louis, MO, USA) and collected by centrifugation. Total protein was extracted by mammalian cell extraction kit. 50 μg protein was diluted with 5 × loading buffer and loaded into 10% polyacrylamide gel. The proteins were electrophoresed under the condition of 20 V for 3 h. The proteins were separated according to the molecular weight, and then were transferred to polyvinylidene difluoride (PVDF) membrane by semi-drying method under the condition of 20 V for 50 min. The membrane was washed and blocked with 5% skim milk. Anti-FKR2 antibody, anti-FGFR1 antibody, anti-ERK1/2 antibody, anti-ERK1/2 antibody, anti-Sprouty1 antibody, anti-Sprouty2 antibody, anti-Sprouty4 antibody, anti-DUSP6 antibody (Sigma-Aldrich, St. Louis, MO, USA) were added separately with a dilution ratio of 1:1000 and kept overnight at 4°C. The membrane was washed 3 times and incubated with the second antibody (rabbit anti-human IgG, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C followed by 3 times' washing. Chemiluminescence method was used to detect the signal. The protein expression level was normalized by β-actin, and the band was scanned and gray scale was quantified by software.

Statistical Analysis

Variance analysis was performed for all the data by SPSS 19.0 statistical software (SPSS Inc., Armonk, NY, USA). All analytical data were presented as mean ± standard deviation (SD). One-way analysis of variance was used for the comparison between more than 2 groups. The LSD test was used for the comparison between 2 groups. $p < 0.05$ was considered to be statistically significant.

Results

FGF2 Protein Content in the Supernatant of Each Group

As can be seen from Figure 1, FGF2 content in cell culture medium of NECs was significantly lower than in cell culture medium of EuECs and EECs ($p < 0.05$). The level of FGF2 in cell culture medium of the cells treated with FGF2 was significantly higher than that in control group ($p < 0.05$), and the effect of FGF2 was reduced by adding FGF2 neutralizing antibody. FGF2 level was reduced after adding MAPK pathway inhibitor PD98059. FGF2 level in NECs cell culture medium was significantly lower than that of control group ($p < 0.05$) but showed no significant difference in U0126 treatment group. However, the inhibitory effect of PD98059 on the expression of FGF2 in EuECs and EECs was significantly lower than that of U0126 ($p < 0.05$).

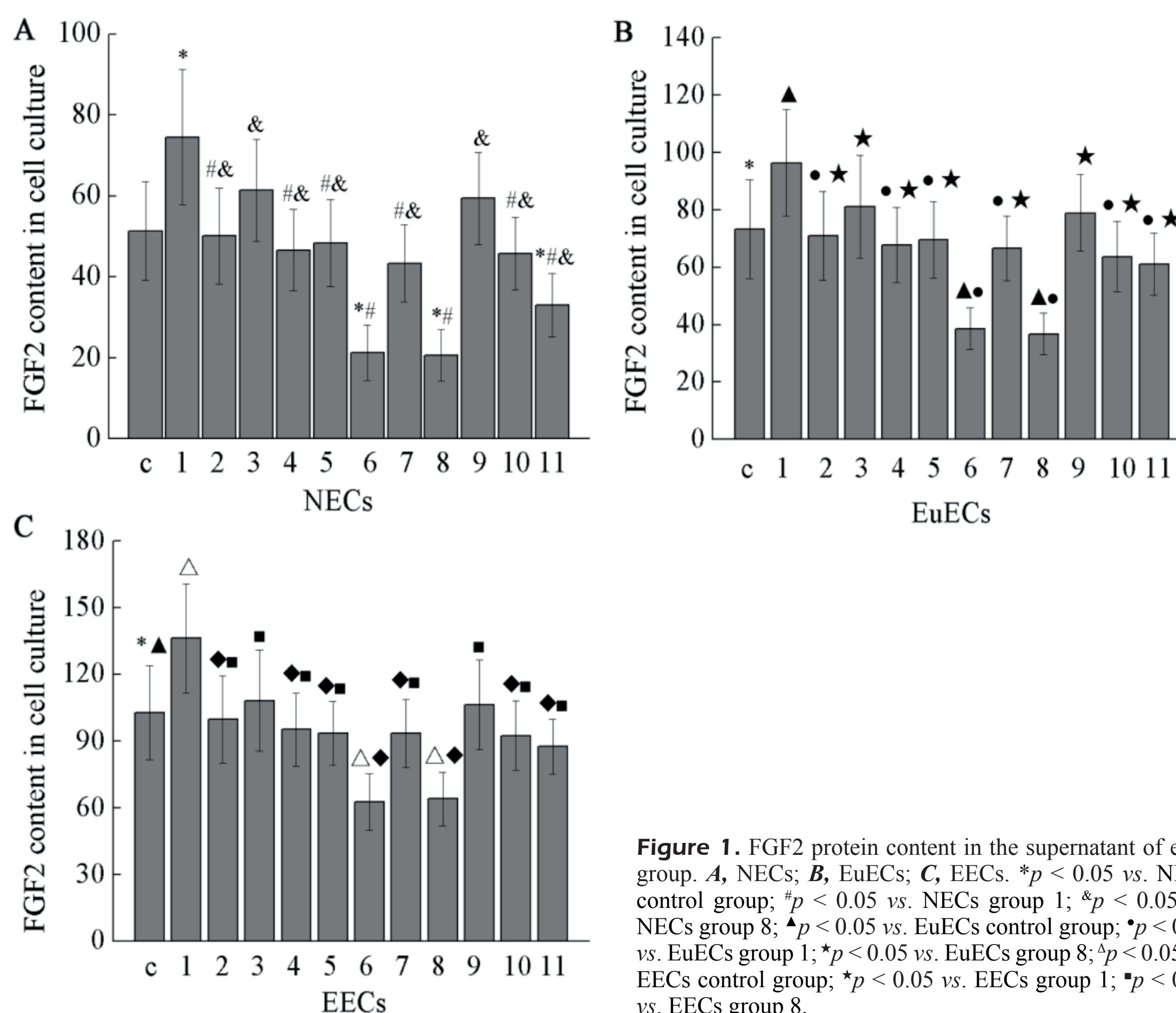

Figure 1. FGF2 protein content in the supernatant of each group. ***A,*** NECs; ***B,*** EuECs; ***C,*** EECs. *$p < 0.05$ *vs*. NECs control group; #$p < 0.05$ *vs*. NECs group 1; &$p < 0.05$ *vs*. NECs group 8; ▲$p < 0.05$ *vs*. EuECs control group; •$p < 0.05$ *vs*. EuECs group 1; ★$p < 0.05$ *vs*. EuECs group 8; △$p < 0.05$ *vs*. EECs control group; ◆$p < 0.05$ *vs*. EECs group 1; ■$p < 0.05$ *vs*. EECs group 8.

The mRNA Levels of FGF2, FGFR1, SPRYs (Sprouty1, Sprouty2, Sprouty4), and DUSP6 in Each Group

As we can see from Figures 2, 3 and 4, EuECs, and EECs showed significantly higher mRNA level of FGF2 and FGFR1 and lower mRNA level of Sprouty1, Sprouty2, Sprouty4 and DUSP6 than that of NECs ($p < 0.05$). The mRNA levels of FGF2, FGFR1, Sprouty1, Sprouty2, Sprouty4 and DUSP6 in NECs were increased after adding FGF2 but showed no significant difference from that of control group ($p > 0.05$). The mRNA levels of FGF2, FGFR1, Sprouty1, Sprouty2, Sprouty4, and DUSP6 in EuECs and EECs were increased after adding FGF2 ($p < 0.05$). Compared with control group, the mRNA levels of FGF2, FGFR1, Sprouty4 and DUSP6 in NECs were significantly reduced after adding FGF2 ($p < 0.05$) but changes in the mRNA levels of Sprouty1 and Sprouty2 were not significant. The mRNA levels of Sprouty1, Sprouty2, Sprouty4, and DUSP6 in EuECs and EECs were decreased significantly ($p < 0.05$), but the decreases in mRNA levels of FGF2 and FGFR1 were not obvious ($p > 0.05$). The inhibitory effect of MAPKs inhibitor PD98059 was significantly lower than that of U0126.

The Protein Levels of FGF2, FGFR1, pERK1/2/ERK1/2, SPRYs (Sprouty1, Sprouty2, Sprouty4), and DUSP6 in Each Group

The protein levels of FGF2, FGFR1, p-ERK1/2 protein in EuECs and EECs were significantly higher than those in NECs ($p < 0.05$) and Sprouty1, Sprouty2, Sprouty4, and DUSP6 protein levels in EuECs and EECs were significantly lower than those in NECs ($p < 0.05$). Compared with control group, the protein levels of FGF2, FGFR1,

Figure 2. The mRNA level of FGF2 and FGFR1 in each group. ***A,*** FGF2 mRNA level in NECs; ***B,*** FGF2 mRNA level in EuECs; **C,** FGF2 mRNA level in EECs; ***D,*** FGFR1 mRNA level in NECs; ***E,*** FGFR1 mRNA level in EuECs; ***F,*** FGFR1 mRNA level in EECs. $^{*}p < 0.05$ *vs*. NECs control group; $^{\#}p < 0.05$ *vs*. NECs group 1; $^{\&}p < 0.05$ *vs*. NECs group 8; $^{\blacktriangle}p < 0.05$ *vs*. EuECs control group; $^{\bullet}p < 0.05$ *vs*. EuECs group 1; $^{\bigstar}p < 0.05$ *vs*. EuECs group 8; $^{\triangle}p < 0.05$ *vs*. EECs control group; $^{\blacklozenge}p < 0.05$ *vs*. EECs group 1; $^{\blacksquare}p < 0.05$ *vs*. EECs group 8.

Figure 3. The mRNA level of Sprouty1 and Sprouty2 in each group. ***A,*** Sprouty1 mRNA level in NECs; ***B,*** Sprouty1 mRNA level in EuECs; **C,** Sprouty1 mRNA level in EECs; ***D,*** Sprouty2 mRNA level in NECs; ***E,*** Sprouty2 mRNA level in EuECs; ***F,*** Sprouty2 mRNA level in EECs. *$p < 0.05$ *vs*. NECs control group; #$p < 0.05$ *vs*. NECs group 1; &$p < 0.05$ *vs*. NECs group 8; ▲$p < 0.05$ *vs*. EuECs control group; •$p < 0.05$ *vs*. EuECs group 1; ★$p < 0.05$ *vs*. EuECs group 8; △$p < 0.05$ *vs*. EECs control group; ◆$p < 0.05$ *vs*. EECs group 1; ■$p < 0.05$ *vs*. EECs group 8.

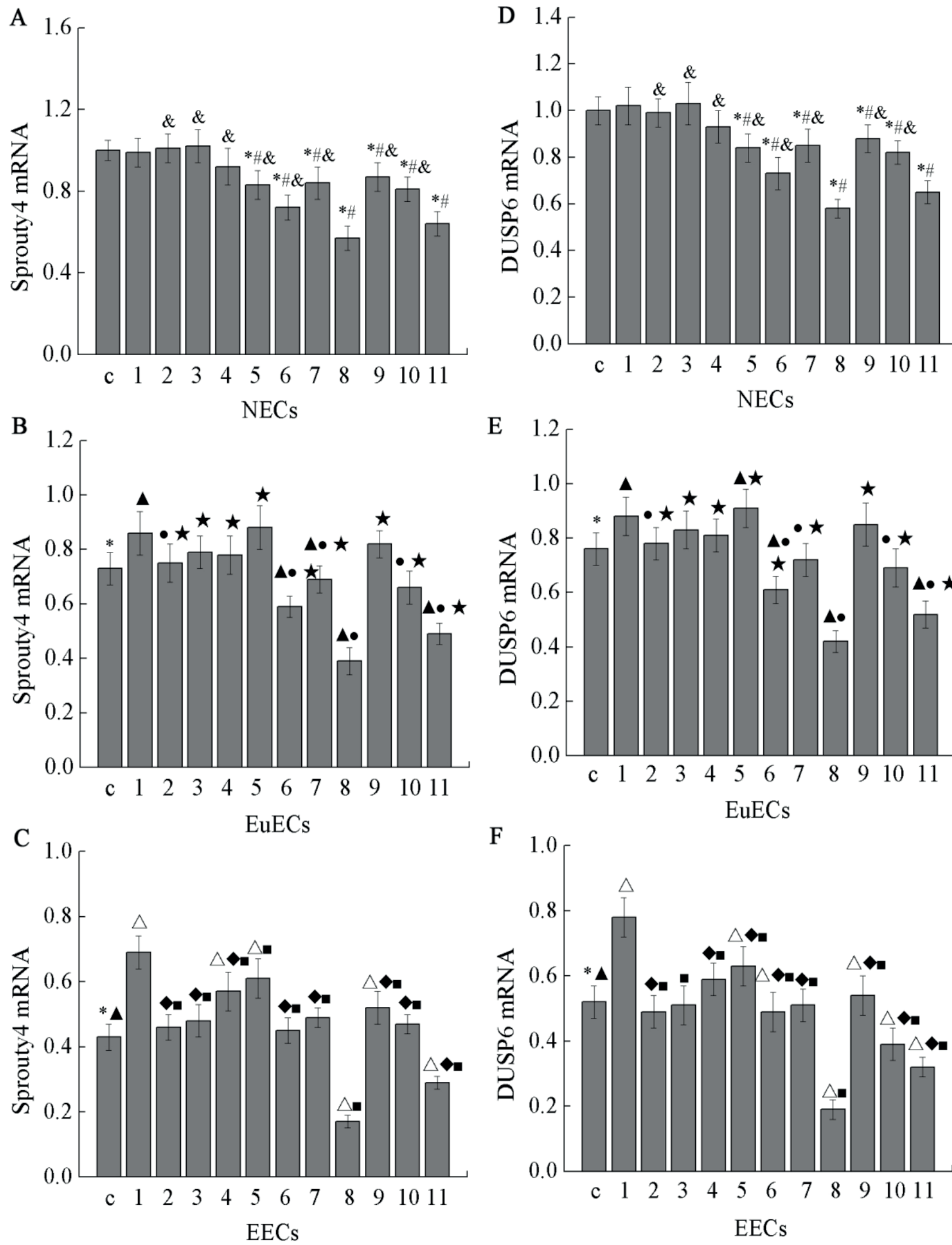

Figure 4. The mRNA level of Sprouty4 and DUSP6 in each group. ***A,*** Sprouty4 mRNA level in NECs; ***B,*** Sprouty4 mRNA level in EuECs; ***C,*** Sprouty4 mRNA level in EECs; ***D,*** DUSP6 mRNA level in NECs; ***E,*** DUSP6 mRNA level in EuECs; ***F,*** DUSP6 mRNA level in EECs. *$p < 0.05$ *vs*. NECs control group; #$p < 0.05$ *vs*. NECs group 1; &$p < 0.05$ *vs*. NECs group 8; ▲$p < 0.05$ *vs*. EuECs control group; •$p < 0.05$ *vs*. EuECs group 1; ★$p < 0.05$ *vs*. EuECs group 8; △$p < 0.05$ *vs*. EECs control group; ◆$p < 0.05$ *vs*. EECs group 1; ■$p < 0.05$ *vs*. EECs group 8.

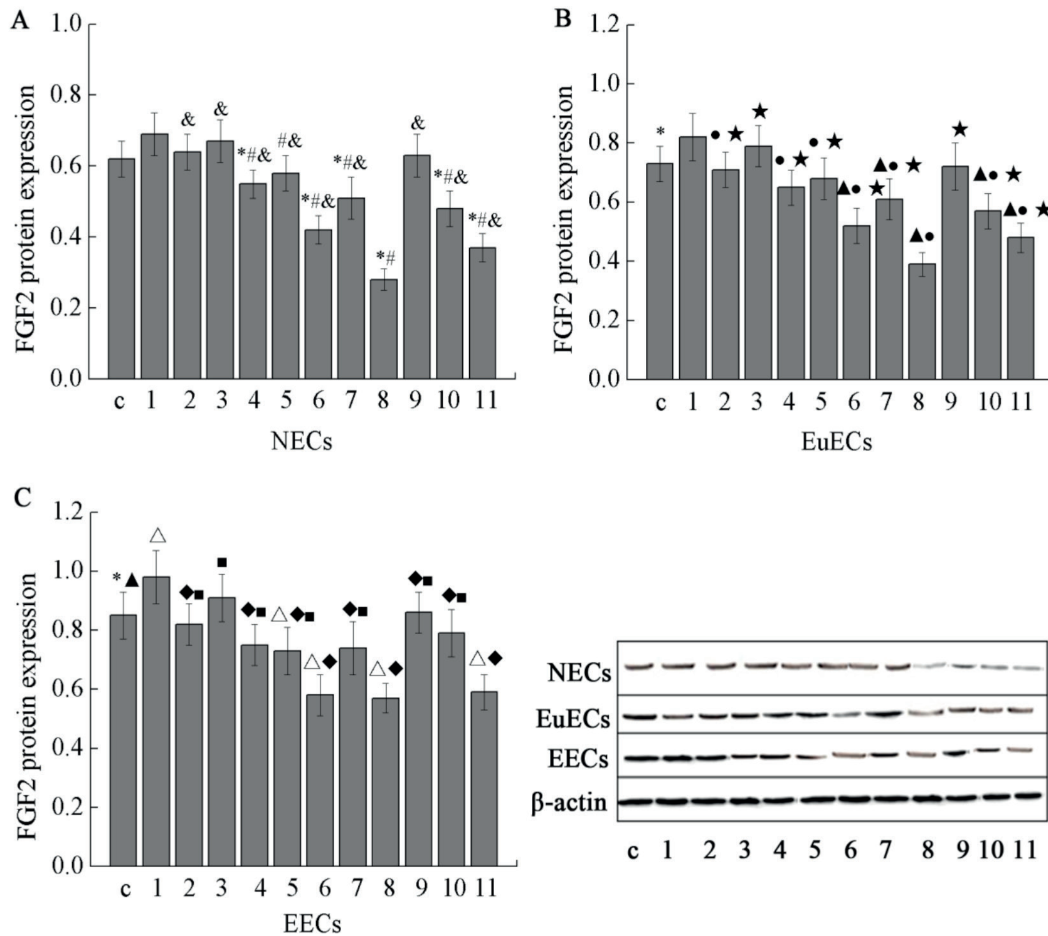

Figure 5. Protein levels of FGF2 in each group. ***A,*** FGF2 protein levels in NECs; ***B,*** FGF2 protein levels in EuECs; **C,** FGF2 protein levels in EECs. *$p < 0.05$ *vs*. NECs control group; #$p < 0.05$ *vs*. NECs group 1; &$p < 0.05$ *vs*. NECs group 8; ▲$p < 0.05$ *vs*. EuECs control group; •$p < 0.05$ *vs*. EuECs group 1; ★$p < 0.05$ *vs*. EuECs group 8; △$p < 0.05$ *vs*. EECs control group; ◆$p < 0.05$ *vs*. EECs group 1; ■$p < 0.05$ *vs*. EECs group 8.

p-ERK1/2 protein in NECs were increased after FGF2 was added but the increases were not significant (Figures 5, 6 and 7) ($p > 0.05$), the protein levels of FGF2, FGFR1 and p-ERK1/2 in EuECs and EECs were significantly increased (Figures 5, 6 and 7) ($p < 0.05$). The protein levels of Sprouty1, Sprouty2, Sprouty4 and DUSP6 protein in NECs were increased after adding FGF2 but the increases were not significant (Figures 8, 9, 10 and 11) ($p > 0.05$), significant increase in protein levels of Sprouty1, Sprouty2, Sprouty4 and DUSP6 were found in EuECs and EECs after FGF2 was added (Figure 8, 9, 10 and 11) ($p < 0.05$). Compared with control group, the protein levels of FGF2, FGFR1 and p-ERK1/2 in NECs were significantly decreased ($p < 0.05$) after FGF2 neutralizing antibody was added (Figures 5, 6 and 7), but no significant difference was found in protein levels of Sprouty1, Sprouty2, Sprouty4 and DUSP6 in NECs (Figures 8, 9, 10 and 11) ($p > 0.05$). After adding FGF2 neutralizing antibody, no significant changes were found in protein levels of FGF2 and FGFR1 (Figures 5 and 6) ($p > 0.05$), but protein levels of p-ERK1/2 Sprouty1, Sprouty2, Sprouty4 and DUSP6 were significantly decreased (Figures 8, 9, 10 and 11) ($p < 0.05$). Compared with control group, the inhibitory effect of PD98059 was lower than that U0126 in all the 3 groups.

Discussion

FGFs play pivotal roles in variety biological procedures including embryonic development,

limb development, neural induction, neural development and wound healing[2,19]. Most of members of FGFs gene family are mitogens, but also have regulatory, morphological, and endocrine functions on multiple cell types[2,20,21]. The function of FGF is important for the development of both vertebrates and invertebrates and irregular function of FGF usually leads to defect in development[22-25]. FGFs mainly perform their function by phosphorylating the intracellular tyrosine kinase domain of FGFR to make activated form of FGFR, the activated FGFR will be coupled with a variety of pathways such as PI3K-AKT, RAS-MAPK, PLCγ, and STAT to play pivotal roles in various biological procedures[2]. The signaling transduction of FGF2 signaling pathway depends on ERK 1/2 signaling pathway[4]. FGF2 can increased the levels of pERK 1/2, which are activated form of ERK 1/2, the increased the levels of pERK 1/2 will further increase the expression level of FGF2 to form a positive feedback for FGF2 signaling pathway. In our study we found that content of FGF2 in EuECs and EECs are both higher than those in NECs (Figure 1), indicating than FGF2 may play more important roles in EuECs and EECs than that in NECs. After treatment with FGF2, both mRNA levels and protein levels of FGF2 and FGFR1 in both EuECs and EECs were increased significantly after adding FGF2 ($p < 0.05$) (Figures 2, 5, and 6); in addition, the protein levels of p-ERK1/2 were also increased (Figure 7), but no significant change was found in normal endometrial glandular epithelial cells ($p > 0.05$), suggesting that the FGF2/ERK1/2 may exist in EuECs and EECs but not in NECs. FGF2 neutralizing antibody can reduce the protein level of

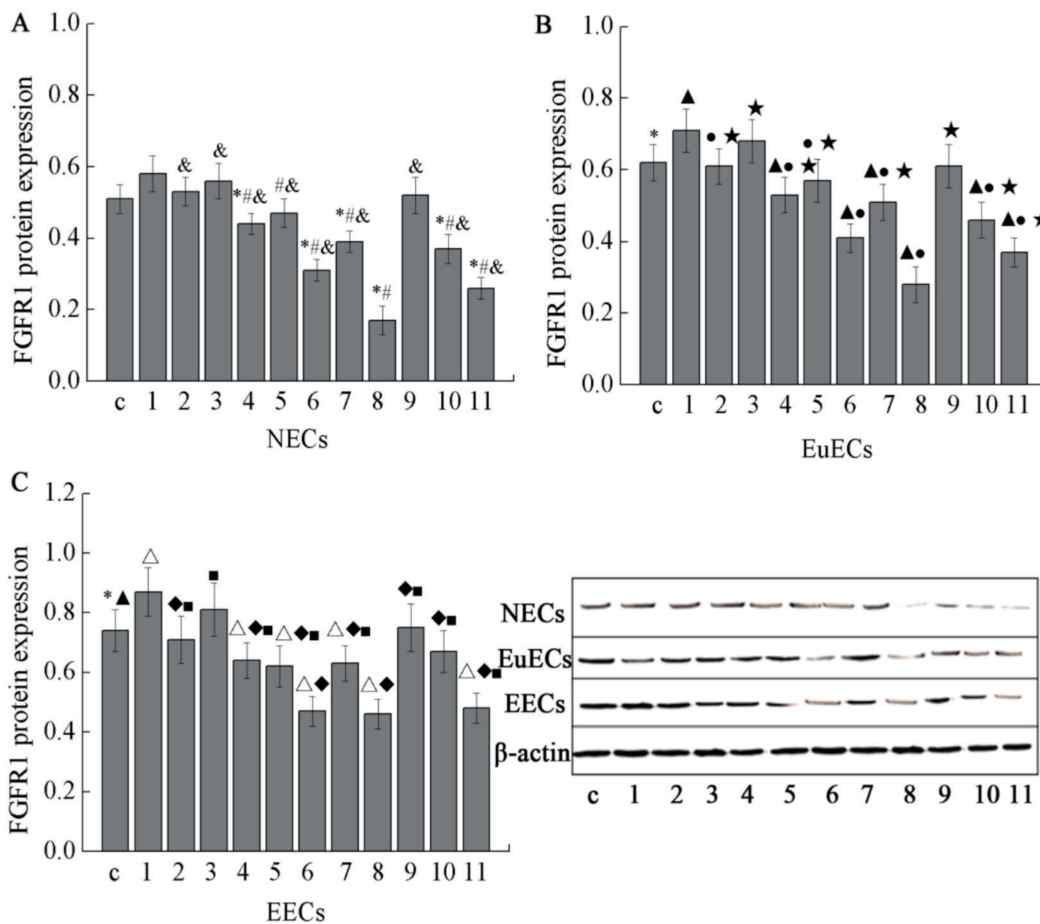

Figure 6. Protein levels of FGFR1 in each group. ***A,*** FGFR1 protein levels in NECs; ***B,*** FGFR1 protein levels in EuECs; ***C,*** FGFR1 protein levels in EECs. *$p < 0.05$ *vs*. NECs control group; #$p < 0.05$ *vs*. NECs group 1; &$p < 0.05$ *vs*. NECs group 8; ▲$p < 0.05$ *vs*. EuECs control group; •$p < 0.05$ *vs*. EuECs group 1; ★$p < 0.05$ *vs*. EuECs group 8; △$p < 0.05$ *vs*. EECs control group; ◆$p < 0.05$ *vs*. EECs group 1; ■$p < 0.05$ *vs*. EECs group 8.

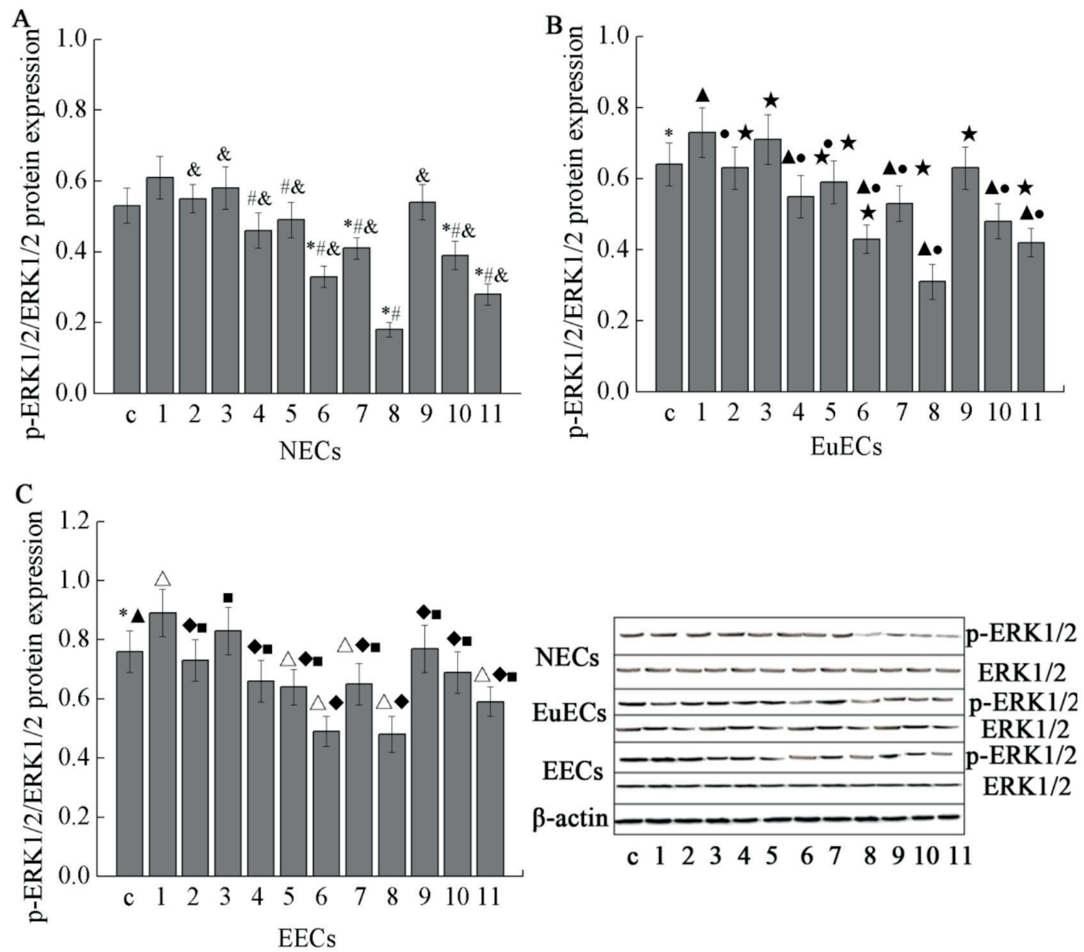

Figure 7. Protein levels of p-ERK1/2/ERK1/2 in each group. ***A,*** p-ERK1/2/ERK1/2 protein levels in NECs; ***B,*** p-ERK1/2/ERK1/2 protein levels in EuECs; ***C,*** p-ERK1/2/ERK1/2 protein levels in EECs. *$p < 0.05$ *vs*. NECs control group; #$p < 0.05$ *vs*. NECs group 1; &$p < 0.05$ *vs*. NECs group 8; ▲$p < 0.05$ *vs*. EuECs control group; •$p < 0.05$ *vs*. EuECs group 1; ★$p < 0.05$ *vs*. EuECs group 8; △$p < 0.05$ *vs*. EECs control group; ◆$p < 0.05$ *vs*. EECs group 1; ■$p < 0.05$ *vs*. EECs group 8.

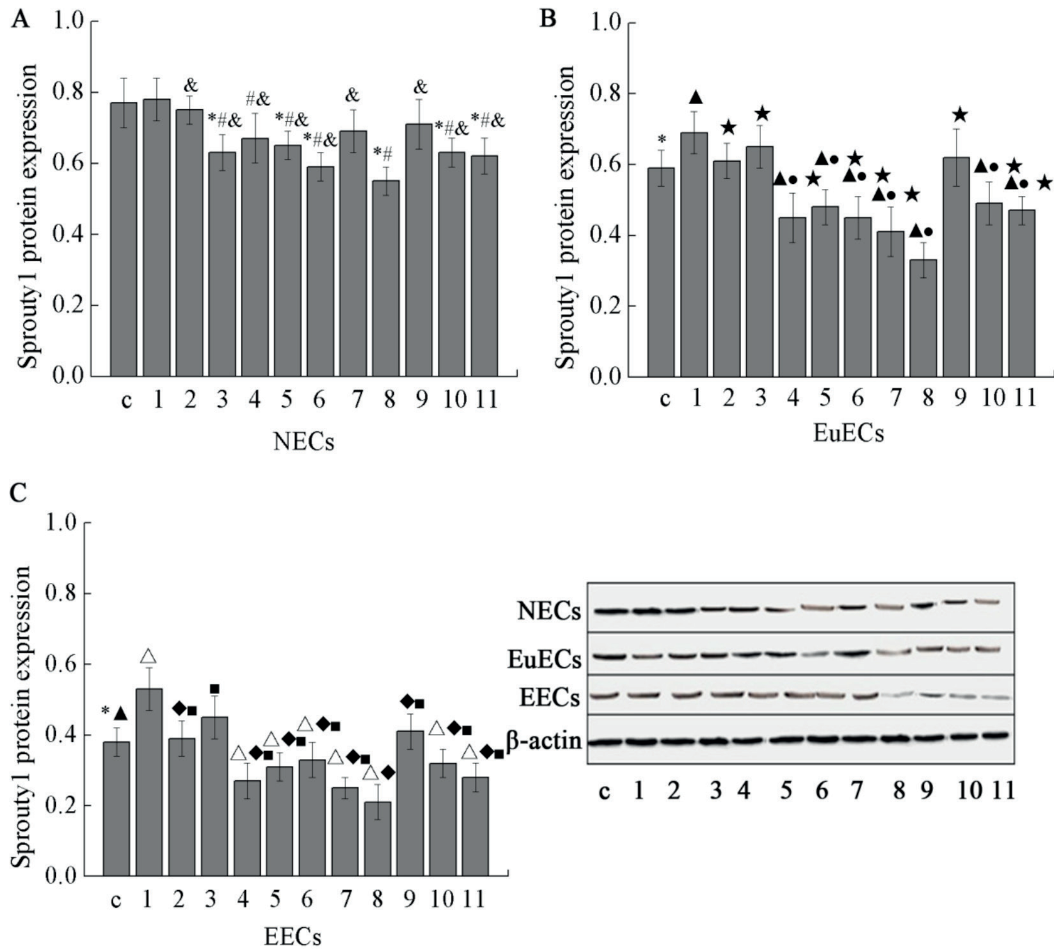

Figure 8. Protein levels of Sprouty1 in each group. ***A*,** Sprouty1 protein levels in NECs; ***B*,** Sprouty1 protein levels in EuECs; ***C*,** Sprouty1 protein levels in EECs. *$p < 0.05$ *vs*. NECs control group; #$p < 0.05$ *vs*. NECs group 1; &$p < 0.05$ *vs*. NECs group 8; ▲$p < 0.05$ *vs*. EuECs control group; •$p < 0.05$ *vs*. EuECs group 1; ★$p < 0.05$ *vs*. EuECs group 8; △$p < 0.05$ *vs*. EECs control group; ◆$p < 0.05$ *vs*. EECs group 1; ■$p < 0.05$ *vs*. EECs group 8.

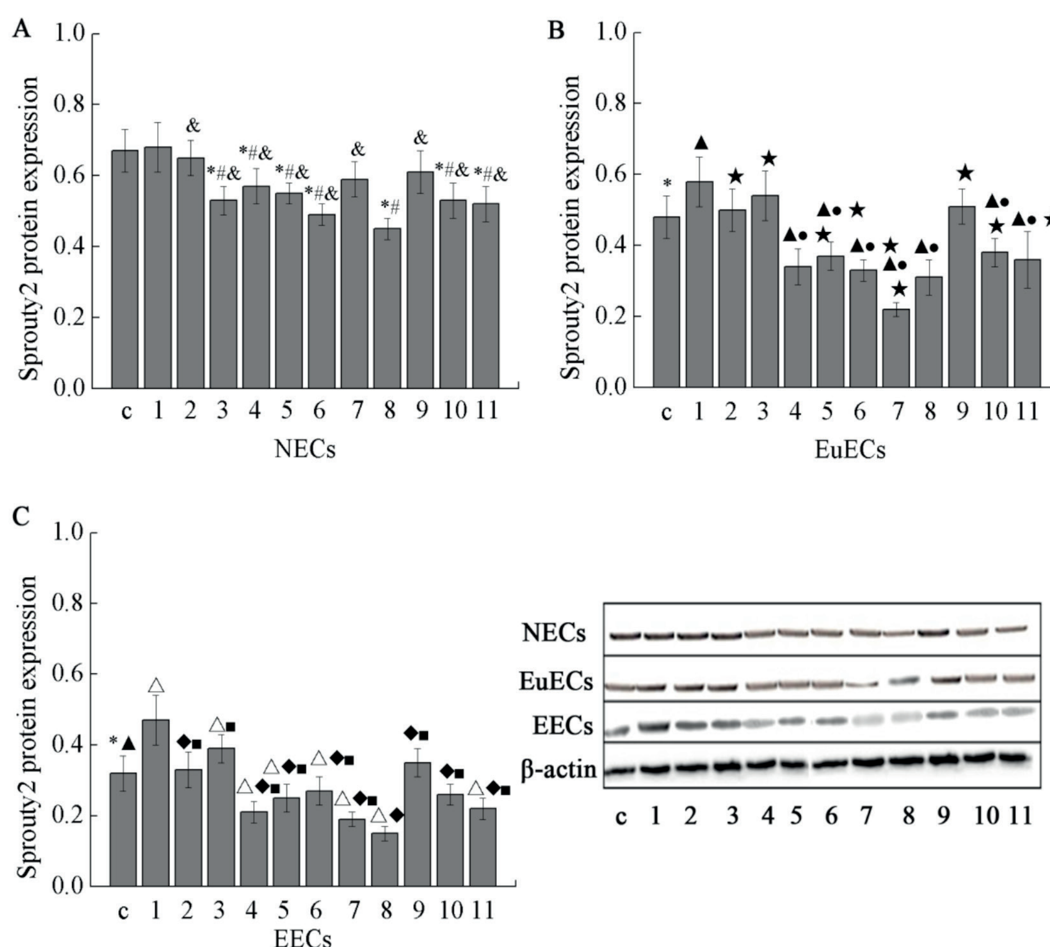

Figure 9. Protein levels of Sprouty2 in each group. ***A,*** Sprouty2 protein levels in NECs; ***B,*** Sprouty2 protein levels in EuECs; ***C,*** Sprouty2 protein levels in EECs. *$p < 0.05$ *vs*. NECs control group; #$p < 0.05$ *vs*. NECs group 1; &$p < 0.05$ *vs*. NECs group 8; ▲$p < 0.05$ *vs*. EuECs control group; •$p < 0.05$ *vs*. EuECs group 1; ★$p < 0.05$ *vs*. EuECs group 8; △$p < 0.05$ *vs*. EECs control group; ◆$p < 0.05$ *vs*. EECs group 1; ■$p < 0.05$ *vs*. EECs group 8.

FGF2 and FGFR1 by neutralizing FGF2, which confirms the interaction between FGF2 and FGF2 neutralizing antibody (Figures 5 and 6). The inhibitory effects of MAPKs inhibitors PD9805 and U0126 on FGF2 signaling pathway were reflected by the decreased mRNA and protein level of FGF2 and FGFR1 (Figures 2, 5 and 6). The inhibitory effect of PD9805 on EuECs and EECs was significantly lower than that of U0126. This can be explained by the fact that PD9805 and U0126 have different targets in the signaling pathway, leading to the different inhibitory effects.

Once the FGF2/ERK1/2 positive feedback loop was started, the increased activity of FGF2 signaling pathway will follow. However, FGF2 signaling pathway was also regulated by negative regulator to avoid the over-activated signaling transduction. The activated MAPK pathway can also increase the activity of the negative regulators of FGF2 signaling pathway such as DUSP6 and SPRY[2]. As a member of ERK1/2-specific dual-specificity phosphatase[7,8], DUSP6 can dephosphorylate both phosphothreonine and phosphotyrosine of ERK1/2 proteins to inactive FGF2/ERK1/2 signaling pathways. In addition, the downstream of FGF2 signaling pathway is tightly controlled by various factors including PRY proteins[10-14]. Through the interactions with other factors in FGF signaling, Sprys can not only inhibit the RAS-MAPK pathway, but also regulate

the PI3K-AKT pathway and eventually reduce the activity of FGF2 signaling pathway[13,15,16]. In this study we found that the mRNA and protein levels of DUSP6 and three SPRYs, Sprouty1 Sprouty2 and Sprouty4 in both EuECs and EECs, were significantly increased after adding FGF2 ($p < 0.05$), but no significant change was found in NECs ($p > 0.05$) (Figures 2, 3, 8, 9, 10 and 11). After adding FGF2 neutralizing antibody, the protein level of DUSP6 Sprouty1, Sprouty2 and Sprouty4 in EuECs and EECs were decreased. The inhibitory effects of MAPKs inhibitors PD9805 and U0126 on FGF2 signaling pathway was reflected by the decreased mRNA and protein level of DUSP6, Sprouty1, Sprouty2 and Sprouty4 (Figures 2, 3, 8, 9, 10 and 11). All those data support the presence of FGF2/ERK1/2 positive feedback loop in EuECs and EECs but not in NECs.

Conclusions

The effect of FGF2 on NECs was not obvious. However, the expression of FGF2, FGFR1, SPRYs and DUSP6 in the EuECs and EECs were all affected by FGF2. The mRNA and protein level of FGFR1, SPRYs and DUSP6 were decreased after adding MAPKs inhibitors PD9805 and U0126.

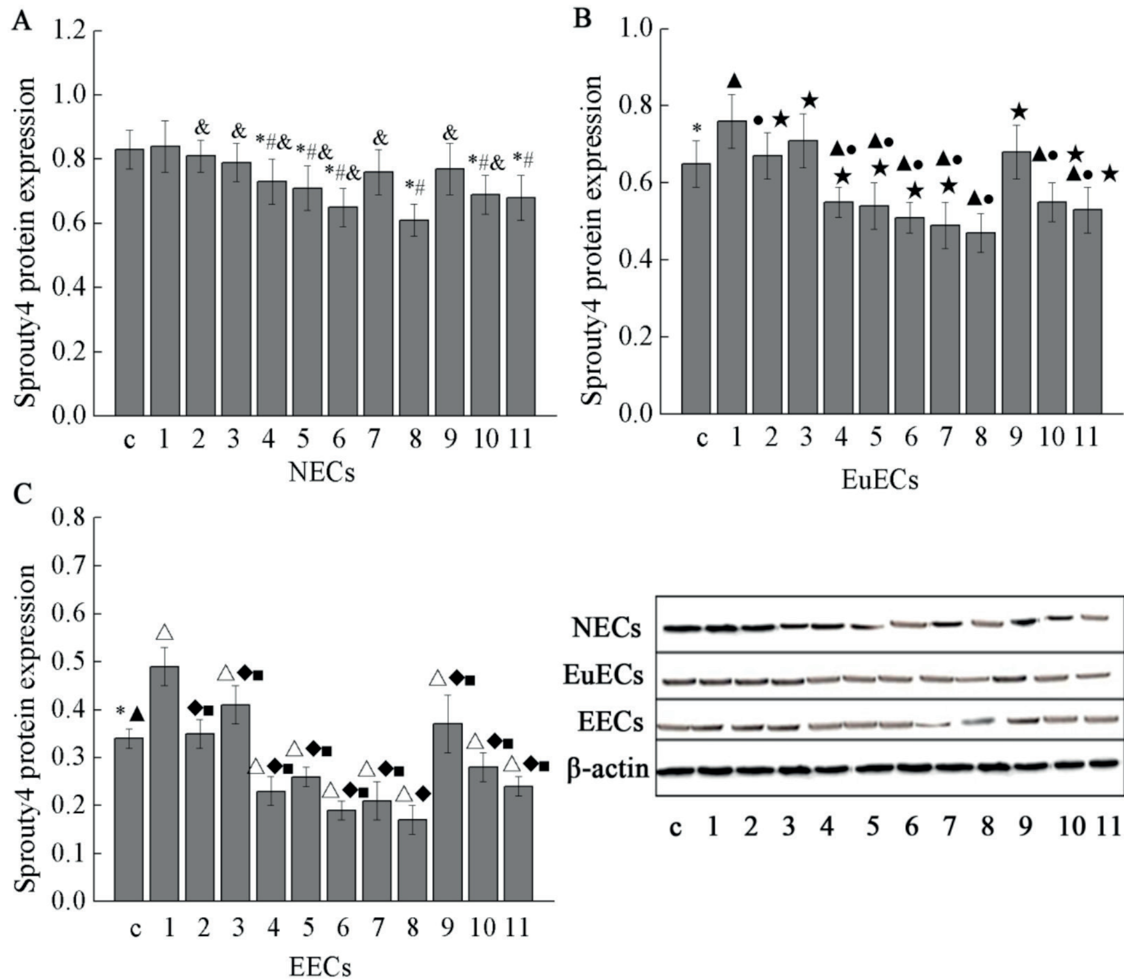

Figure 10. Protein levels of Sprouty4 in each group. ***A,*** Sprouty4 protein levels in NECs; ***B,*** Sprouty4 protein levels in EuECs; ***C,*** Sprouty4 protein levels in EECs. *$p < 0.05$ *vs*. NECs control group; #$p < 0.05$ *vs*. NECs group 1; &$p < 0.05$ *vs*. NECs group 8; ▲$p < 0.05$ *vs*. EuECs control group; •$p < 0.05$ *vs*. EuECs group 1; ★$p < 0.05$ *vs*. EuECs group 8; △$p < 0.05$ *vs*. EECs control group; ◆$p < 0.05$ *vs*. EECs group 1; ■$p < 0.05$ *vs*. EECs group 8.

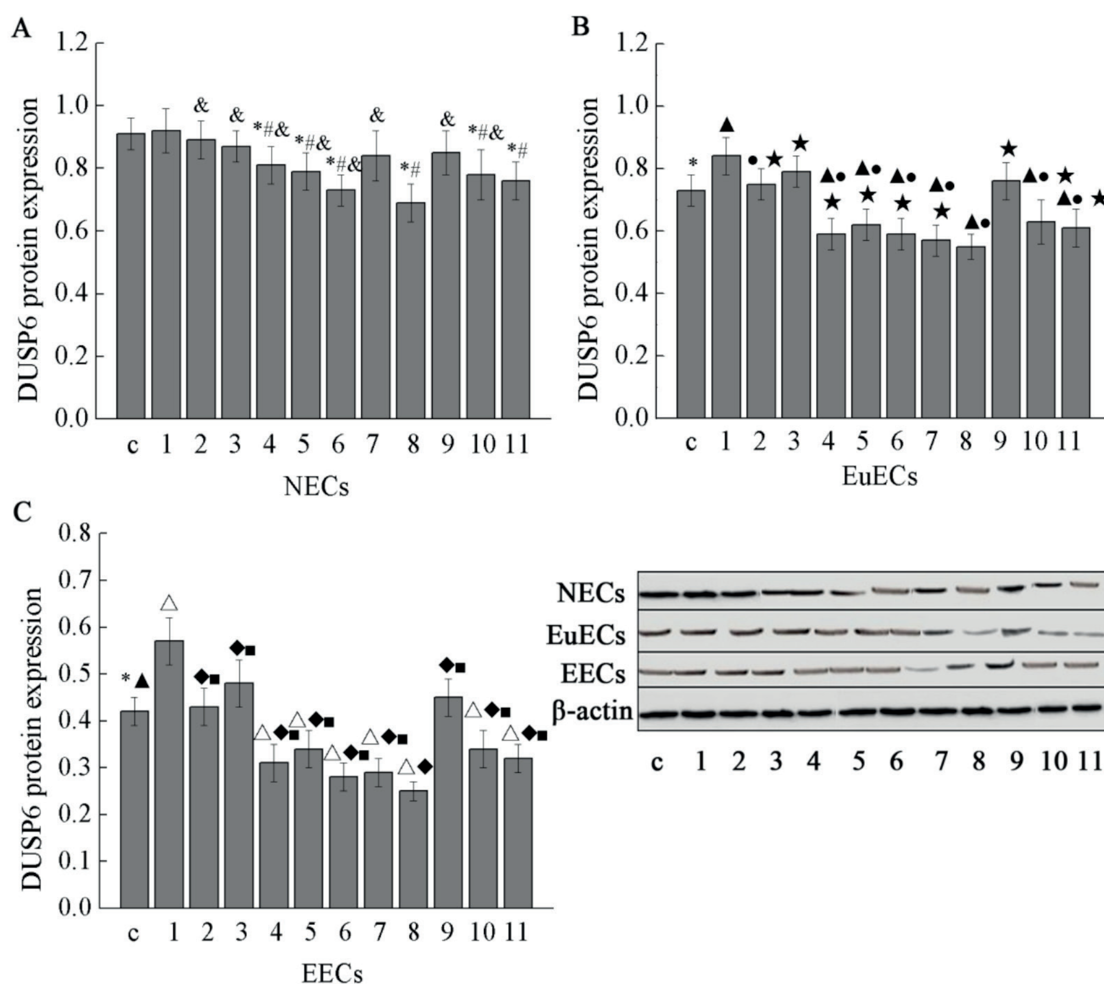

Figure 11. Protein levels of DUSP6 in each group. ***A,*** DUSP6 protein levels in NECs; ***B,*** DUSP6 protein levels in EuECs; ***C,*** DUSP6 protein levels in EECs. $^{*}p < 0.05$ *vs*. NECs control group; $^{\#}p < 0.05$ *vs*. NECs group 1; $^{\&}p < 0.05$ *vs*. NECs group 8; $^{\blacktriangle}p < 0.05$ *vs*. EuECs control group; $^{\bullet}p < 0.05$ *vs*. EuECs group 1; $^{\star}p < 0.05$ *vs*. EuECs group 8; $^{\triangle}p < 0.05$ *vs*. EECs control group; $^{\blacklozenge}p < 0.05$ *vs*. EECs group 1; $^{\blacksquare}p < 0.05$ *vs*. EECs group 8.

The positive feedback loop existed in EuECs and EECs but not in NECs, high FGF2 levels caused an increase in the activity of SPRYs/DUSP6/ERK signaling pathway, which in turn maintains the presence of high FGF2 levels. The results may provide the guideline to treatment endometriosis patients.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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