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3,6-dihydroxyflavone suppresses the epithelial-mesenchymal transition, migration and invasion in endometrial stromal cells by inhibiting the Notch signaling pathway

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Abstract. – OBJECTIVE: Endometriosis is a common disease in women of reproductive age. Characteristics of endometriosis include invasion, metastasis, and recurrence, which are similar to those of malignant tumors. However, the etiology and pathogenesis of endometriosis are still not clear. This study aims to explore the mechanism of 3,6-dihydroxyflavone (3,6-DHF) in the development of endometriosis.

PATIENTS AND METHODS: Primary cultured ovarian ectopic endometrial stromal cells (OvESCs) were utilized as the *in vitro* model of endometriosis. OvESCs were treated with different concentrations of 3,6-DHF. The expressions of proteins related to epithelial-mesenchymal transition (EMT) and Notch signal pathway were detected by Western blot. The mRNA expressions of related genes were detected by quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The viability of treated cells was detected by transwell assay. The impact of 3,6-DHF on ectopic lesions was explored after the animal model of endometriosis was successfully established.

RESULTS: With the increased concentration of 3,6-DHF in OvESCs, the protein and mRNA expressions of E-cadherin were gradually increased, while the protein and mRNA expressions of N-cadherin, Twist, Snail, and Slug were decreased. 3,6-DHF treatment inhibited the migration and invasion ability of OvESCs in a dose-dependent manner. In the endometriosis model of severe combined immunodeficient (SCID) mice, lesions in the 3,6-DHF treated group were significantly smaller than those of the control group. The same changes were found in the endometriosis model of Sprague Dawley (SD) rats. Protein expressions of Notch1, NICD, and Hes-1 in OvESCs were inhibited by 3,6-DHF in a dose-dependent manner. 3,6-DHF can inhibit the binding of NICD-CSL-MAML complex in OvESCs, thereby inhibiting the expressions of proteins related to Notch signaling pathway *in vitro*.

CONCLUSIONS: 3,6-DHF can inhibit the development of EMT, migration, and invasion of endometrial stromal cells by inhibiting the Notch signaling pathway.

Key Words

Endometriosis, 3,6-dihydroxyflavone, Notch, Epithelial-mesenchymal transition.

Introduction

Endometriosis is a condition in which the layer of tissue (glands and mesenchyme) that normally covers the inside of the uterus grows and infiltrates outside of it. Repeated bleeding caused by endometriosis leads to pelvic pain, infertility, and masses. Endometriosis is a common disease in women of childbearing age, the incidence of which is about 10%-15%. Among endometriosis patients, 40%-50% are infertile to get pregnant[1-3]. As more and more molecular mechanisms of diseases are elucidated, the research focus on how endometriosis has evolved from a single disease to a complex systemic disease[4]. The main characteristics of endometriosis include wide lesions, diverse forms, high aggression, and sex hormone dependence[5,6]. Although some theories and hypotheses have been proposed and argued, the exact pathogenesis of endometriosis remains unknown.

Studies have found that adhesion, invasion, and growth of endometriosis are regulated by multiple factors. Interaction of epithelial and interstitial cells, especially epithelial-to-mesenchymal transition (EMT), may exert an essential role in the complex biological process of endometriosis[7]. EMT is a process by which epithelial cells are transformed into cells with mesenchymal phenotype and biological function through a spe-

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cific program. Alterations of cells in the process of EMT include disappearance of cell polarity, loss of connection with the basement membrane, increase of invasion, migration, anti-apoptotic and extracellular matrix degradation abilities, and decrease of cell adhesion[8,9]. Researches have shown that EMT is active in ectopic endometriotic tissue compared with those of non-endometriosis tissue and eutopic endometriotic tissue[10]. Further studies illustrated that the signaling pathways involved in the development and regulation of EMT include Notch signaling pathway, TGF-β/Smad signaling pathway, etc.[11].

Scholars have demonstrated that Notch signaling pathway can regulate the invasion and metastasis of malignant tumors through EMT[12,13], mainly by down-regulating the expression of Snail, a transcriptional factor binding to E-cadherin promoter[14,15]. Recently, some studies have shown that Notch signaling pathway also exerts an essential role in regulating the occurrence and development of endometriosis. However, there is an inconsistency in the regulation of endometriosis. The up-regulation or down-regulation of Notch signaling pathway may affect the development of endometriosis through modulating the formation of blood vessels and decidualization of the endometrium, respectively[16,17]. As an important signal pathway involved in the regulation of EMT, there is still little research on the correlation between Notch signaling pathway and EMT in endometriosis. Further studies are needed to investigate the underlying mechanism.

Researchers have pointed out that adhesion, invasion, and angiogenesis are the basic pathophysiological processes of endometriosis formed by ectopic implantation of eutopic endometrial cells[18]. Although endometriosis is a benign disease, the main biological characteristics, including aggressive growth and easy relapse, are similar to those of malignant tumors. According to the similarities of life activities, endometriosis is speculated to have something in common with malignancy in the pathogenesis. 3,6-dihydroxyflavone (3,6-DHF) is a flavonoid compound widely found in plant foods such as vegetables and fruits. It is considered to have an effective anti-cancer component that can inhibit proliferation, malignant transformation, invasion, and other pathological processes of tumor cells[19,20]. Studies[21-23] have found that 3,6-DHF can significantly inhibit the development of breast cancer. However, the function of 3,6-DHF has not been reported in endometriosis. Therefore, it is of great significance to study the function of 3,6-DHF in endometriosis.

Patients and Methods

Collection and Processing of Aamples

Fresh samples were taken from endometriosis patients who underwent an oophorocystectomy or endometrial biopsy in our hospital. This study was approved by the Ethics Committee of PLA Nanjing General Hospital. Signed written informed consents were obtained from all participants before the study. Samples were immediately placed in the culture medium and stored in a 4°C incubator. Samples were then transferred to a super-clean bench within 60 min for primary culture.

Isolation and Culture of Ectopic Endometrial Stromal Cells

Isolation and culture of ectopic endometrial stromal cells was performed following instructions produced by Mylonas et al[24] and Tsai et al[25]. Briefly, ectopic endometrial tissues obtained from endometriosis patients were washed three times with phosphate buffered saline (PBS) containing penicillin and streptomycin (100 U/mL each) on a super-clean bench. Tissues were cut into pieces of about 1 mm^3 in size and digested with 0.1% collagenase type II in a 37°C incubator. Precipitated tissues were suspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Rockville, MD, USA) medium containing 5% fetal bovine serum (FBS, Gibco, Rockville, MD, USA), and maintained in a 5% CO_2 incubator at 37°C. Culture medium was replaced based on the cell condition. Cells were passaged and seeded until the cell confluence was up to 80%.

In Vivo Endometriosis Model

21 female severe combined immunodeficient (SCID) mice and 40 female Sprague Dawley (SD) rats that were sexually mature and unmated were selected. Three SCID mice underwent autologous fat transplantation were taken as controls, and the remaining 18 were used in the model of human endometrium transplantation. Autotransplantation of endometrium was performed in SD rats during the estrous cycle. Four SD rats underwent autologous fat transplantation were taken as controls, and the other 36 were used in the autotransplantation model of endometrium. The fresh endometrium tissue taken during the operation was diagonally sutured to the plentiful blood vessels of the abdominal wall. Two weeks after the surgery, intraperitoneal injection of 3,6-DHF was performed every day for two weeks.

24 h after the last injection, lesion size, cyst fluid color and conditions of blood vessels were observed and recorded. The calculation formula of the lesion was $V = a \times b^2/2$ (*a* indicated the widest diameter of the lesion, *b* indicated the vertical diameter of the lesion, $V > 2$ mm^3 indicated that the model was successfully established).

Identification of the Lesion Tissue by Hematoxylin-Eosin (H&E) Staining

Lesions were immediately fixed with neutral formalin. Paraffin slices were dewaxed with xylene solution. Slices were then stained with hematoxylin and eosin (HE), respectively. Staining results were observed under a microscope.

qRT-PCR

We used TRIzol reagent (Invitrogen, Carlsbad, CA, USA) to extract total RNA for reverse transcription. qRT-PCR was performed to amplify the target genes to detect the differences of mRNA expressions. Primers used in this study were as follows: E-cadherin (Forward) AAGAAGCTGGCTGACATGTACGGA, E-cadherin (Reverse) CCACCAGCAACGTGATTTCTGCAT; N-cadherin (Forward) TGTGGGAATCCGACGAATGGATGA, N-cadherin (Reverse) TGGAGCCACTGCCTTCATAGTCAA; Snail (Forward) TTTCTGGTTCTGTGTCCTCTGCCT, Snail (Reverse) TGAGTCTGTCAGCCTTTGTCCTGT; GAPDH (Forward) AGGAGCGAGATCCCGCCAACA, GAPDH (Reverse) CGGCCGTCACGCCACATCTT.

Detection of Cell Migration by Transwell Assay

Cells were starved overnight with serum-free medium, then seeded in a 24-well plate with 500 ml of medium containing 10% FBS in each well. An appropriate amount of cells were added to the chamber. The chamber was removed and the medium was discarded 24 h later. After cells were rinsed three times with phosphate buffered saline (PBS), they were placed in precooled methanol and stained with 0.1% crystal violet (Sigma-Aldrich, St. Louis, MO, USA). Results were observed under a microscope and images were taken for cell counting.

Detection of Cell Invasion by Transwell Assay

Diluted Matrigel solution was added in the culture medium containing 5% FBS 4 h before the transwell assay. The mixture was fully compounded and added into the chamber. The next steps were as the same as those of transwell assay for detecting cell migration. The cell density applied in this assay was 1×10^5/ml and the culture period was 48 h.

Detection of Protein Expressions by Western Blotting

Total protein was extracted using a cell lysate (RIPA) containing protease after cells were treated with different concentrations of 3,6-DHF for 24-48 h. Protein samples were then separated by electrophoresis and incubated with primary antibodies overnight at 4°C. Primary antibodies included E-cadherin, N-cadherin, Twist, Snail, Slug (CST, 1:1000, Danvers, MA, USA), and Notch1, NICD, Hes-1 (Abcam, 1:1000, Cambridge, MA, USA). After washed with PBS, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (Cell Signaling, goat anti-rabbit IgG, 1:5000, Danvers, MA, USA) for 2 h at room temperature. Enhanced chemiluminescence (ECL) was performed and the integral optical density value of each band was determined by Gel imaging analysis system. β-actin was taken as the loading control.

Immunochemistry

Para-embedded tissues were cut into slices and paraffinized. Slices were blocked with blocking solution for 30 min at room temperature. Primary antibody was used for incubating overnight, and secondary antibody was used for incubation for 1 h at room temperature. Slices were then observed under a light microscope.

Statistical Analysis

Statistical product and service solutions (SPSS22.0, IBM, Armonk, NY, USA) statistical software was used for data analysis, and GraphPad Prism5.0 (La Jolla, CA, USA) was used for image processing. Measurement data was compared using the *t*-test and expressed as mean ± standard deviation ($\bar{x} \pm s$). Classification data was compared using the χ^2-test. $p<0.01$ was considered statistically significant.

Results

3,6-DHF Inhibited the Development of EMT in Ectopic Endometrial Stromal Cells

Decreased expression of E-cadherin, as well as increased expressions of N-cadherin, Twist, Snail, and Slug are important features of EMT.

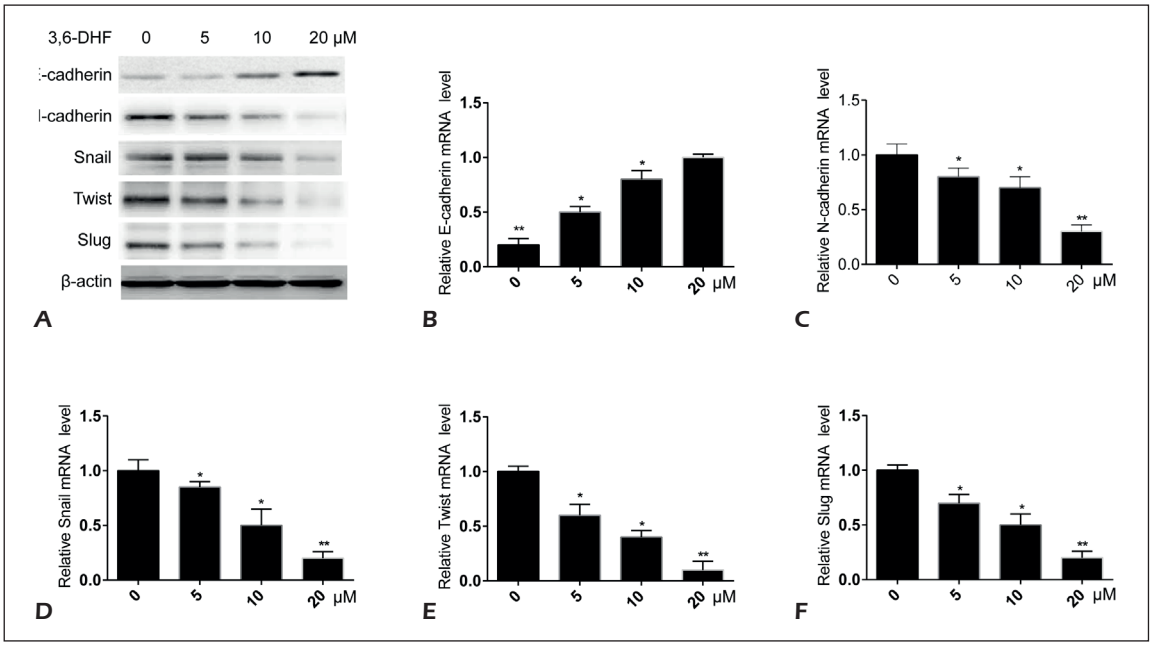

Figure 1. Effect of different concentrations of 3,6-DHF on EMT in ectopic endometrial stromal cells. **A**, Effect of 3,6-DHF on the expressions of E-cadherin, N-cadherin, Snail, Twist, and Slug in OvESCs. **B**-**F**, Effect of 3,6-DHF on the mRNA expressions of E-cadherin, N-cadherin, Snail, Twist, and Slug in eutopic endometrial stromal cells.

In this study, OvESCs were treated with different concentrations of 3,6-DHF (0, 5, 10, and 20 μM, respectively) for 24 h. Expressions of related proteins were detected by Western blot. Results showed that the protein expression of E-cadherin was gradually increased, while the protein expressions of N-cadherin, Twist, Snail, and Slug were decreased in a dose-dependent manner (Figure 1A). The mRNA expressions of EMT-related genes were detected by qRT-qPCR and similar results were obtained (Figure 1B-F). The above data demonstrated that 3,6-DHF can inhibit the development of EMT in OvESCs.

3,6-DHF Inhibited Cell Migration and Invasion in Ectopic Endometrial Stromal Cells

Transwell assay was performed to investigate the effect of 3,6-DHF on the migration and invasion of OvESCs. As shown in Figure 2A and 2B, after treatment with 3,6-DHF for 24 h, the cell number of passing through the reconstituted basement membrane in 3,6-DHF high-dose group was remarkably decreased compared with those of low-dose group and control group (p <0.01). These data suggested that 3,6-DHF treatment can inhibit the migration ability of OvESCs. After treatment for 48 h, the cell number of passing through the reconstituted basement membrane and Matrigel in 3,6-DHF high-dose group was significantly decreased compared with those of low-dose group and control group (p <0.01), indicating that 3,6-DHF can attenuate the invasive ability of OvESCs. To detect whether metalloproteinases (MMPs) play a role in this biological process, the expression of the MMP2 protein was detected by Western blot. Results indicated that no significant differences in MMPs expressions were found among the three group (p> 0.05). However, the protein expression of MMP9 was significantly down-regulated in the 3,6-DHF group in a dose-dependent manner (p <0.01) (Figure 2E and F), implying a potential role of MMP9 in EMT of OvESCs.

3,6-DHF Reduced the Ectopic Lesion Size in the In Vivo Endometriosis Model

Intraperitoneal injection of 3,6-DHF was performed in SCID mice after the animal model was successfully established for two weeks. Ectopic lesions in SCID mice were harvested and cyst fluid was removed. Glands, epithelial and interstitial cells were found by H&E staining, indicating that the animal model was successfully established. Ectopic lesions in SCID mice were identified by immunohistochemistry (Figure 3A and B). Lesion size before and after treatment was compared. Results demonstrated that the le-

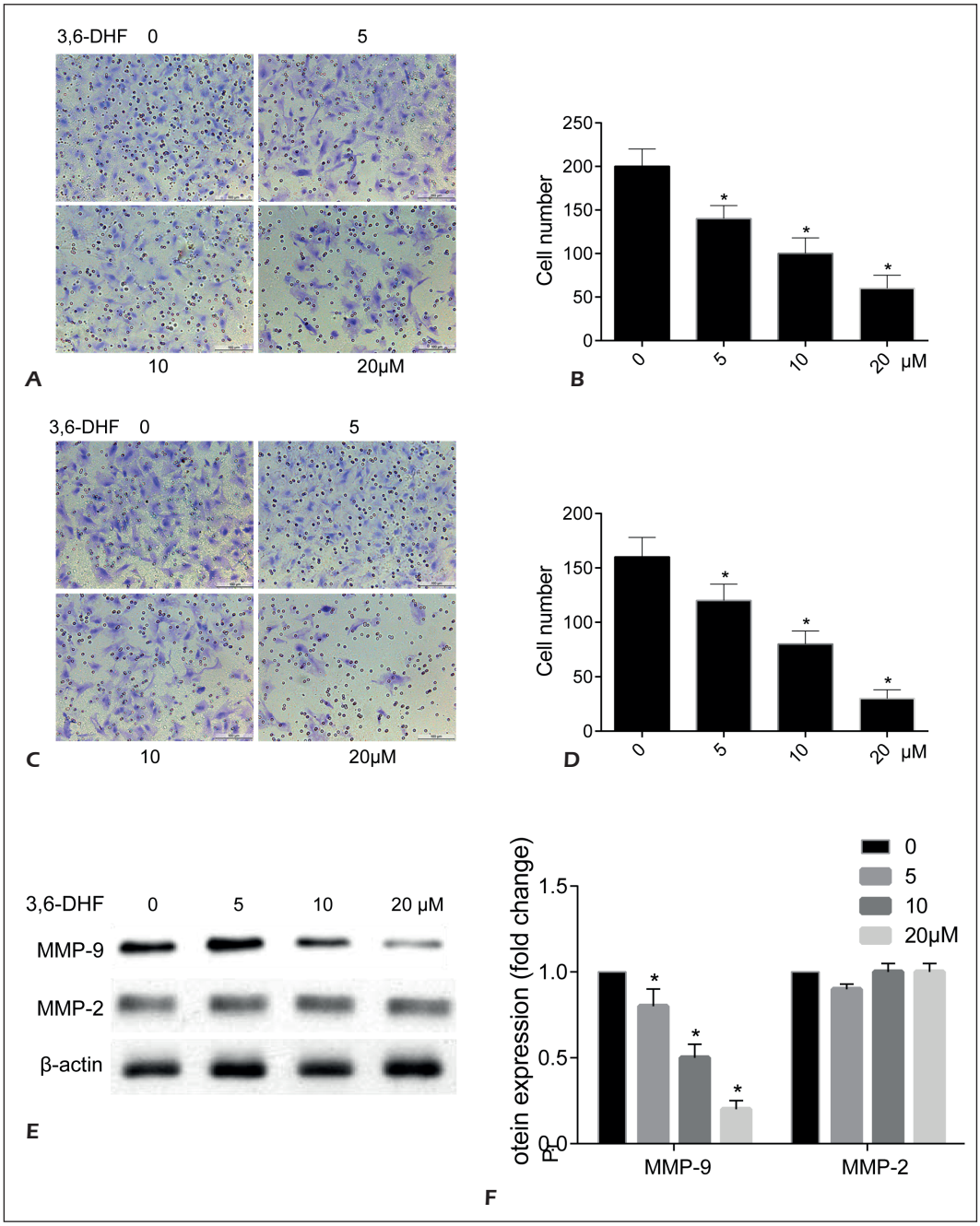

Figure 2. Effect of 3,6-DHF on cell migration and invasion of ectopic endometrial stromal cells. **A**, and **B**, The migration ability of OvESCs was detected by transwell assay. After 24 h, the chamber was removed and the cells were counted under a light microscope (200×). **C**, and **D**, The invasive ability of OvESCs was detected by transwell assay. **E**, and **F**, The expressions of MMP2 and MMP9 in OvESCs were detected by Western blot (*p<0.01).

sion size in the treatment group was significantly smaller than the control group after treatment. It was also found that the lesion size was decreased in a dose-dependent manner (Figure 3C). SD rats were injected intraperitoneally after the animal model was successfully established for two weeks. Laparotomy was performed to observe the morphological changes after two weeks of injection. Ectopic lesions in SD rats were identified by immunohistochemistry and the larger ectopic lesion was observed in the control group (Figure 3D and E). Intraperitoneal injection of high-dose of 3,6-DHF, however, significantly inhibited the growth of ectopic lesions in SD rats (Figure 3F).

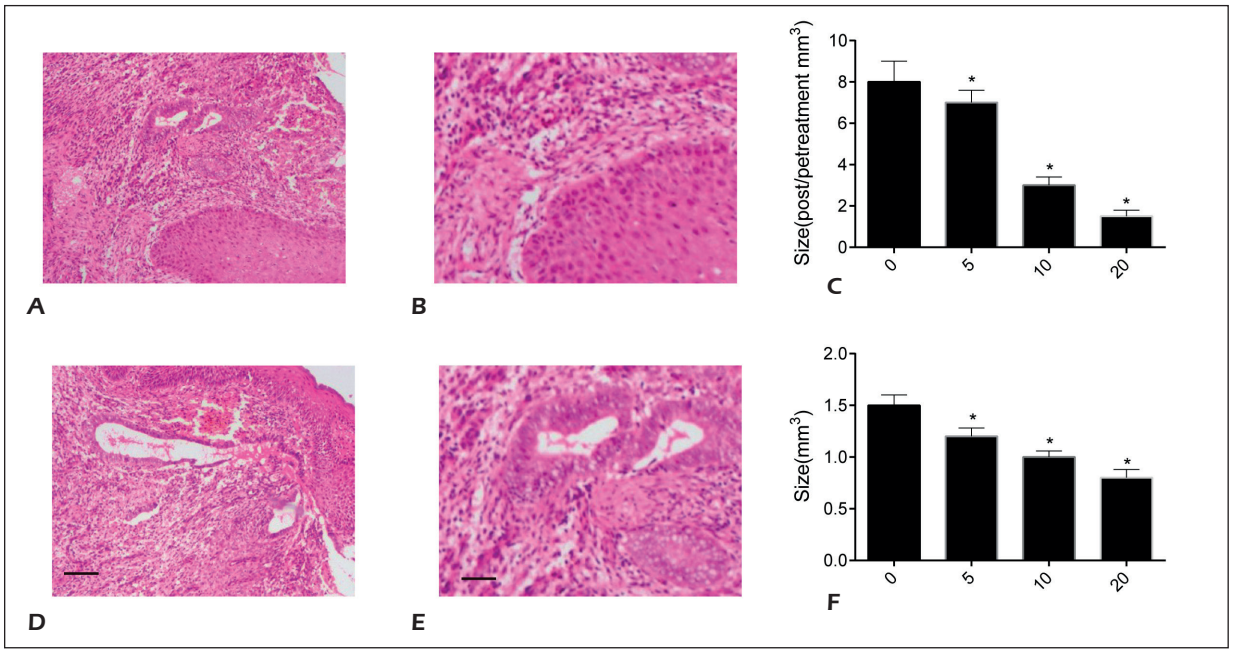

Figure 3. 3,6-DHF reduced the size of ectopic lesions in endometriosis model. Immunohistochemistry showed different lesions with 200 × (**A**) and 400 × (**B**) in SCID mice. C, Comparison of lesion sizes in 3 groups of SCID mice after treatment (*p<0.01). Immunohistochemistry confirmed the ectopic lesions in SD rats with 200 × (**D**), 400 × (**E**). **F**, Changes in the size of the ectopic lesion in SD rats after intraperitoneal administration (*p<0.01).

3,6-DHF Inhibited Proteins Related to Notch Signaling Pathway and Formation of NICD-CSL-MAML Transcriptional Complex in Ectopic Endometrial Cells

The continuous activation of Notch signaling pathway is a significant cause of EMT. Here, we examined the effect of 3,6-DHF on the expressions of the relative proteins in Notch signaling pathway, including Notch1, NICD, and Hes-1 in ectopic endometrial cells. Western blot results illustrated that 3,6-DHF significantly inhibited the expressions of Notch1, NICD, and Hes-1 in ectopic endometrial cells, and the inhibitory effect was enhanced in a dose-dependent manner (Figure 4A-D).

NICD (the activated intracellular domain of Notch protein) binds to CSL protein and recruits MAML, the nuclear transcriptional activator protein family. NICD-CSL-MAML complex is then formed to facilitate the transcription of the target genes, which exerts a crucial role in the activation of the Notch signaling pathway. We next observed the effect of 3,6-DHF on the NICD-CSL-MAML complex by immunoprecipitation and Western blot. The results showed that the concentration of 3,6-DHF increased and the expression of NICD gradually decreased when the immunoprecipitation proteins were CSL and MAML (Figure 4E). It was suggested that 3,6-DHF inhibits the binding of NICD-CSL-MAML complex in ectopic endometrial cells, thereby inhibiting the expressions of proteins related to Notch signaling pathway *in vitro*.

Discussion

EMT is a process in which epithelial cells lose their polarity and connection with the basement membrane. EMT endows cells with migratory and invasive properties, increases the abilities of invasion and viability, prevents apoptosis and senescence, and contributes to immunosuppression[26]. Compared with eutopic endometrium, the expression of E-cadherin in the ectopic endometrium is significantly decreased, while the expressions of N-cadherin, Twist, and Snail are up-regulated. The expression of N-cadherin is confirmed to be negatively correlated with the expression of E-cadherin, but positively correlated with the expression of Twist[27,28]. Proestling et al[29] also showed that E-cadherin expression is decreased in ectopic endometrium compared with that of eutopic endometrium[30,31]. Moreover, scholars[32-34] have observed that Notch signaling

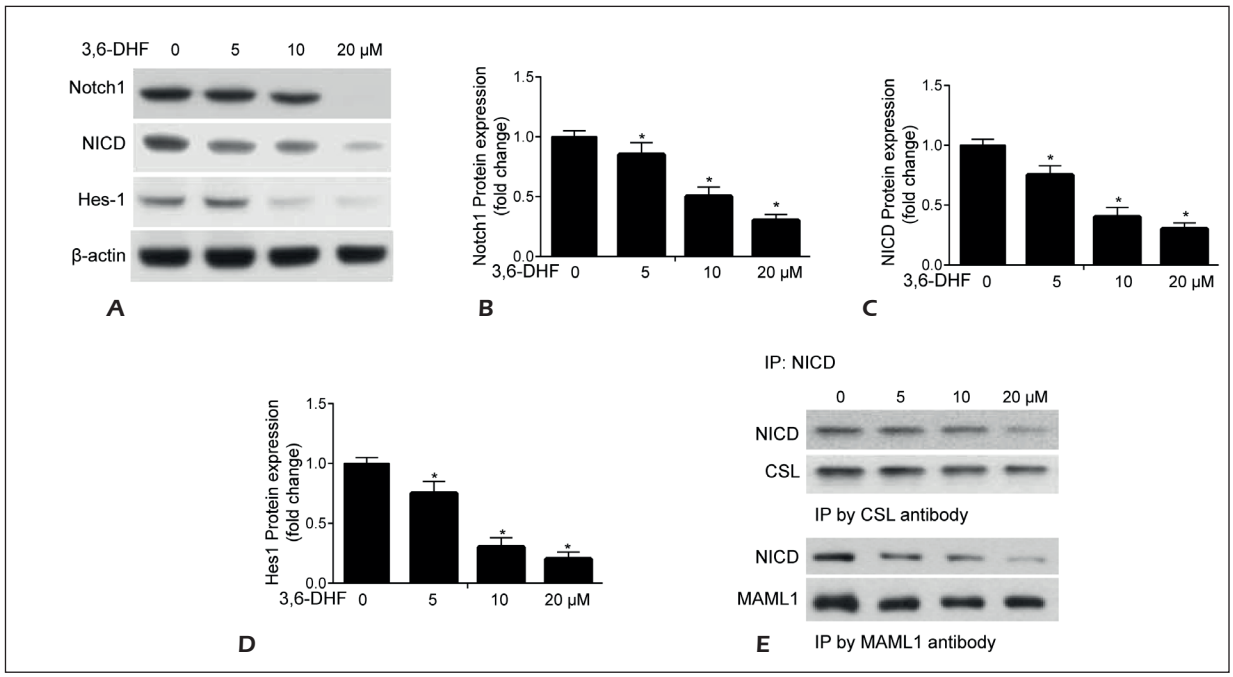

Figure 4. Effect of 3,6-DHF on the expressions proteins related to Notch signaling pathway in ectopic stromal cells. ***A-D***, Effect of 3,6-DHF on protein expressions of Notch1, NICD, Hes-1, and c-Myc in ectopic endometrial stromal cells. **E**, Effect of 3,6-DHF on the expression of NICD-CSL-MAML complex in ectopic endometrial stromal cells.

pathway is involved in the regulation of various EMT processes, the invasion of malignant tumors, lymph node metastasis, and tumor recurrence. Timmerman et al[15] found that in endothelial cells, Notch1 can up-regulate the expression of Snail and decrease the expression of E-cadherin, thereby promoting the EMT process. Bradford et al[35] have shown that abnormally activated Notch signaling pathway is involved in the development of endometriosis. Furthermore, Krüppel-like factor (KLF) was proved to promote the formation of endometriosis lesions by jointly upregulating Notch signaling, somatostatin receptor-related signaling, and hedgehog signaling pathways.

As a benign and common gynecological disease, endometriosis has malignant biological features such as proliferation, invasion, and adhesion. Ovarian endometriosis presents a more frequent prevalence in females. However, stable and reliable cell model is lacked for biological research of endometriosis. In our study, primary cells were selected for *in vitro* model of endometriosis. 3,6-DHF is proved to exert a strong anti-tumor activity, which can significantly inhibit the development of breast cancer. Here, different concentrations of 3,6-DHF were used to treat ovarian ectopic stromal cells. The data suggested that 3,6-DHF can promote the protein expression of E-cadherin, but reduce the protein expressions of N-cadherin, Twist, Snail, and Slug. Taken together, these results indicated that 3,6-DHF can inhibit the development of EMT in ectopic endometrial stromal cells.

Proteolytic enzymes, such as MMPs, are considered to be related to the pathogenesis of many metastatic tumors, including endometriosis[36]. It has been found that expressions of MMP2 and MMP9 in endometriosis patients are increased[37,38]. The high expressions of MMP2 and MMP9 in endometriosis patients are associated with some infertility symptoms, such as poorly developed oocytes and embryos requiring assisted reproductive technology[39]. We found that 3,6-DHF can affect the expression of MMP9, but not MMP2, and change the invasion and migration abilities. Our results provide new insight into the development and pathological mechanism of endometriosis, especially in ovarian endometriosis.

In this study, growth inhibition of ectopic lesions and decreased vascular density were found in 3,6-DHF high-dose group in both SCID mouse model and SD rat model. This may be explained by effusions or immune-inflammatory responses

due to the previous laparotomy. On the other hand, the intraperitoneal injection of medication may have a certain dispersion effect, thus weakening the medication efficacy. In this work, the results of the two experimental animal models were consistent. Ectopic lesions were significantly smaller in 3,6-DHF groups compared with those of control group. In addition, we demonstrated that 3,6-DHF inhibits the binding of NICD-CSL-MAML complex in ectopic endometrial cells, thereby inhibiting the expressions of proteins related to Notch signaling pathway *in vitro*.

Taken together, 3,6-DHF inhibits the development of EMT, invasion and migration of ectopic endometrial stromal cells by regulating Notch signaling pathway. We provide a scientific basis for targeted therapy of endometriosis. However, the biological mechanism of endometriosis is very complicated and further investigations are urgently needed.

Conclusions

We showed that 3,6-DHF can inhibit the development of EMT, migration, and invasion of ectopic endometrial stromal cells via inhibiting the Notch signaling pathway.

Conflict of interest

The authors declared no conflict of interest.

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