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Transcription factor 21 regulates expression of ERβ and SF-1 via upstream stimulatory factor-2 in endometriotic tissues

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ABSTRACT

Steroidogenic factor-1 (SF-1, encoded by *NR5A1*) and estrogen receptor beta (ERβ, encoded by *ESR2*), which are highly expressed in endometriotic stromal cells (ESCs), contribute to the pathogenesis of endometriosis, but the regulation mechanism remains largely unknown. Transcription factor 21 (TCF21) belongs to the helix-loop-helix (bHLH) family characterized by regulating gene expression via binding to E-box element. Here, we attempted to determine the molecular mechanism of TCF21 on SF-1 and ERβ expression in endometriosis. We found that TCF21 expression in ESCs was higher than that in endometrial stromal cells (EMs), and positively correlated with SF-1 and ERβ expression in ESCs. Since the importance of E-box element for *NR5A1* promoter activity has been previously reported, we performed site-mutation and luciferase assay, revealing that the E-box sequence in the *ESR2* promoter is also a critical element modulating ERβ expression. Upstream stimulatory factor 2 (USF2) is another bHLH factor implicated in transcriptional regulation. Further analyses elucidated that it is not TCF21, but USF2 exhibited higher binding affinities in ESCs to *NR5A1* and *ESR2* promoters than in EMs. Additionally, TCF21 knockdown significantly decreased the binding activities of USF2 to *NR5A1* and *ESR2* promoters via disruption of the TCF21-USF2 complex. Meanwhile, manipulating TCF21 expression significantly affected MMP9 and cyclinD1 expression, as wells as proliferation and invasion of ESCs. Moreover, TCF21 depletion in endometriotic xenografts reduced SF-1 and ERβ expression, abrogating ectopic lesion growth in mice. Cumulatively, a critical role of TCF21 in the pathogenesis of endometriosis is demonstrated, suggesting a potential druggable target for future therapy.

1. Introduction

Endometriosis is an estrogen-dependent disease characterized by the growth of the endometrium outside the uterine cavity [1]. This disease affects up to 10% of women of reproductive age [2]. Despite considerable investigations have been undertaken to better understand the pathogenesis of endometriosis, the molecular mechanisms underlying development of the disease remain unclear. Upon hormone therapy or conservative surgery, only approximately 50% of women diagnosed with endometriosis can achieve pain relief [1]. Thus, there is an urgent need to elucidate the underlying mechanisms and develop novel and effective therapies to combat endometriosis.

Several studies have established the role of aberrant estrogen biosynthesis in facilitating the persistence and growth of endometriotic tissue [3,4]. Compared with normal endometrium, endometriotic lesions show higher estradiol biosynthesis. Series of steroidogenic proteins are involved in catalyzing and giving rise to local estrogen production in endometriosis. The two rate-limiting steps in the estrogen biosynthesis pathway are facilitated by steroidogenic acute regulatory protein (StAR) and aromatase (encoded by the *CYP19A1* gene) [5–8]. The expression of both StAR and aromatase is regulated by a nuclear receptor, steroidogenic factor-1 (SF-1), which is encoded by the *NR5A1* gene in humans [9,10]. SF-1, which is highly expressed in endometriotic tissues but not in eutopic endometrium, is the key transcription factor that transactivates the promoters of both *StAR* and *CYP19A1* in endometriotic stromal cells (ESCs) [11]. Whereas, the effects of estrogen are primarily mediated by nuclear estrogen receptors, including ERα and ERβ, which are encoded by *ESR1* and *ESR2*, respectively [12]. Research has demonstrated that lower levels of ERα and higher levels of ERβ are present in human endometriotic tissues

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and primary ESCs compared to eutopic endometrium and endometrial stromal cells (EMs) [13]. ERβ interacts with the inflammasome complex and cytoplasmic apoptotic machinery to enhance proliferation and adhesion activities of endometriotic tissues and prevent TNF-α-induced cell death [14]. In addition, studies suggest that ERβ-selective agonists might exert therapeutic effects on an experimentally induced model of endometriosis [15,16]. Although accumulating evidence suggests that SF-1 and ERβ play critical roles in the pathogenesis of endometriosis, the underlying regulatory mechanisms remain to be elucidated.

Transcription factor 21 (TCF21) is a member of the basic helix-loop-helix (bHLH) transcription factor family characterized by a helix-loop-helix domain and can form heterodimers or homodimers, which binds to the conserved E-box domain (CANNTG) to promote cell-specific gene expression [17–19]. TCF21 has been shown to influence cellular differentiation and proliferation. A loss of or reduction in the expression of TCF21 has been observed in different types of human cancer, influencing cell proliferation, migration, and invasion [20–27]. Since enhanced proliferation, migration and local invasion of endometriotic cells occurs at ectopic sites, it is a reasonable assumption that endometriosis, although a benign disease, could share some tumor characteristics. However, comprehensive study to functionally evaluate the roles of TCF21 in endometriosis is still lacking. The proximal promoter region of *NR5A1* (−110 bp 5′-flanking region) is considered to be highly conserved among many species and contains several *cis*-acting elements that are essential for its activity. Using deletion mutation and luciferase assay, previous study has identified an E-box element existing in the *NR5A1* promoter region. Moreover, in human adrenocortical tumor cells, studies have revealed that TCF21 binds the E-box sequence of the *NR5A1* promoter to inhibit the expression of SF-1 [28]. Furthermore, upstream stimulatory factors 2 (USF2), which is expressed at higher levels in ESCs than in EMs in subjects with endometriosis, is another helix-loop-helix transcription factor and has been reported to regulate SF-1 expression by binding to the E-box element of the *NR5A1* promoter [29]. However, until now, few article about the effect of TCF21 on ERβ expression has been reported, and the role of TCF21 in regulation of SF-1 expression in endometriosis remains poorly understood.

Here, we found that the expression of TCF21 is significantly elevated in ESCs compared to EMs. The aim of this study was to investigate the molecular mechanisms by which TCF21 regulates the expression of SF-1 and ERβ in endometriosis. We propose that the elevated levels of TCF21 in endometriotic tissues enhanced SF-1 and ERβ expression via the formation of heterodimers with USF2, which bound to the E-box sequences of the *NR5A1* and *ESR2* promoters. Meanwhile, positive correlations between TCF21 expression and proliferation and invasion were observed both in vitro and in vivo, suggesting that TCF21 represents a potential druggable target for future therapies.

2. Materials and methods

2.1. Subjects and primary cell culture

Eutopic endometrial tissues and ectopic endometrial tissues from the cyst walls of ovarian endometriomas were obtained from 19 subjects immediately after surgery, composing 19 self-controlled pairs. All patients (age range, 23–40 years) had regular menstrual cycles and none had received hormonal therapy. The diagnoses for all samples were histologically confirmed, and the phase of the menstrual cycle was determined based on a preoperative history and histological examination. Half of the tissue samples were in the proliferative phase and the other half were in the secretory phase. The experimental protocol was approved by the Institutional Review Board of Peking University (No. 2014[789]), and informed consent forms were signed by each patient before samples were used. Human EMs and ESCs were isolated from the collected tissues using a protocol previously described by Ryan et al. with minor modifications [30]. Briefly, the tissues were washed with phosphate-buffered saline (PBS) and minced into small pieces of 1 mm^3. After the enzymatic digestion of minced tissues with collagenase (1 mg/mL) (Sigma, St. Louis, MO, USA) and DNase (0.04 mg/mL) (Sigma) for 1 h at 37 °C, dissociated tissues were sequentially filtered through a 70-μm and a 20-μm nylon mesh to remove epithelial cells. The filtered cells were centrifuged, suspended in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL, Grand Island, NY, USA), 100 U/mL penicillin (Lonza, Basel, Switzerland), 100 U/mL streptomycin (Lonza, Basel, Switzerland), and 250 ng/mL amphotericin B (Lonza, Basel, Switzerland), and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Stromal cells were plated and allowed to adhere to plastic dishes for approximately 30 min, after which blood cells and debris were removed with PBS rinses. The purity of the stromal cell was > 98%, as judged by positive cellular staining for vimentin, a specific marker of stromal cells. All the experiments were conducted before the third passage of the cultures.

2.2. RNA extraction and quantitative analysis by real-time quantitative PCR

Total RNA was extracted from ESCs and EMs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 2 μg of total RNA was converted into cDNAs using an ABI High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Real-time (RT) quantitative PCR (qPCR) was performed using an ABI 7500 Sequence Detection system and an ABI Power SYBR Green gene expression system (Applied Biosystems) to quantify glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *TCF21*, *SF-1*, *ERβ*, matrix metalloproteinase 9 (*MMP9*), and *cyclinD1* mRNA expression The primers were as follows: *ESR2*, forward 5′-ATGATCAGCTGGGCCAAGAA-3′, reverse 5′-CCACATCAGCCCCATCATTAA-3′; *SF-1*, forward 5′-CTGGAG CCGGATGAGGAC-3′, reverse 5′-ACCTGGCGGTAGATGTGGT-3′; *TCF21*, forward 5′-AGCTACATCGCCCACTTGAG-3′, reverse 5′-CGGTCACCACT TCTTTCAGG-3′; *MMP9*, forward 5′-AGACCTGGGCAGATTCCAAAC-3′, reverse, 5′-CGGCAAGTCTTCCGAGTAGT-3′; *cyclinD1*, forward 5′-CAAT GACCCCGCACGATTTC-3′, reverse 5′-CATGGAGGGCGGATTGGAA-3′; *GAPDH*, forward: 5′-GAAGGTGAAGGTCGGAGTC-3′, reverse 5′-GAAG ATGGTGATGGGATTTC-3′. The human *GAPDH* mRNA was used as a control for normalization. Relative quantification of gene expression was calculated using the comparative ΔΔCT method. Independent experiments were performed using cultured cells from at least three different subjects and repeated three times.

2.3. Western blotting

ESCs and EMs were washed and lysed in RIPA buffer (KeyGen Biotech, Nanjing, China) supplemented with 1% protease inhibitor cocktail (Amresco, Solon, OH). A micro-BCA protein assay kit (KeyGen) was used to determine protein concentrations. Briefly, equal amounts of protein (at least 30 μg) were separated using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), transferred onto nitrocellulose membranes, and incubated with the following antibodies: anti-TCF21 (1:1000; Abcam; ab49475), anti-ERβ (1:1000; Merck Millipore; 04-824), anti-SF-1 (1:500; Abcam; ab65815), anti-MMP9 (1:1000; Abcam; ab76003), and anti-cyclinD1 (1:1000; Cell Signaling Technology; 92G2). Anti-GAPDH antibody (1:1000; ZSGB-BIO; TA-08) was used as a loading control. Protein bands were visualized by ECL (enhanced chemiluminescence) solution (Syngene). Intensities of western blot bands were analyzed using AlphaEaseFC software and normalized with respect to GAPDH. Independent experiments were performed using cultured cells from at least three different subjects and repeated three times.

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