Involvement of Transcription Factor 21 in the Pathogenesis of Fibrosis in Endometriosis

Umida Ganieva[1], Tomoko Nakamura[1], Satoko Osuka[1,2], Bayasula[3], Natsuki Nakanishi[1], Yukiyo Kasahara[1], Nobuyoshi Takasaki[3], Ayako Muraoka[1], Shotaro Hayashi[1], Takashi Nagai[1], Tomohiko Murase[1], Maki Goto[1], Akira Iwase[4], Fumitaka Kikkawa[1]

[1] Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, 65, Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

[2] Department of Maternal and Perinatal Medicine, Nagoya University Hospital, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

[3] Bell Research Center for Reproductive Health and Cancer; Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

[4] Department of Obstetrics and Gynecology, Gunma University Graduate School of Medicine, 3-39-22, Showa-machi, Maebashi 371-8511, Japan

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Corresponding author:

Tomoko Nakamura

Department of Obstetrics and Gynecology, Nagoya University Graduate School of Medicine, 65, Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

Telephone: 81-52-744-2261

Fax: +81-52-744-2268

E-mail: tomonakamura@med.nagoya-u.ac.jp

Abstract

Repeated tissue injury and repair, and fibrosis play a pivotal role in endometriosis. Fibrotic tissue consists of extracellular matrix proteins, regulated by transcriptional factors promoting cell proliferation and survival. Periostin is one of the putative key extracellular matrix proteins. This study aimed to determine whether transcription factor 21 (*TCF21*) is involved in the development of endometriosis as an upstream regulatory gene of periostin. Formalin-fixed, paraffin-embedded tissue samples (normal endometrium of women without endometriosis, NE; eutopic endometrium of women with endometriosis, EE; ovarian endometriosis, OE; deep infiltrating endometriosis, DIE) and respective cells were analyzed. Basal, transiently stimulated, and knocked down periostin and TCF21 concentrations in stromal cells of women with or without endometriosis were examined. Periostin and TCF21 expressions were undetected in NE, weakly positive in EE, moderately positive in OE, and strongly positive in DIE. Th2 type cytokines (interleukin-4, interleukin-13, and transforming growth factor-β1) increased the mRNA expression of periostin and *TCF21*. These cytokines, periostin, and TCF21 co-localized in the stroma of OE and DIE. siTCF21 suppressed periostin expression. Transfection of *TCF21* plasmid vector into stromal cells of women without endometriosis, which originally expressed neither periostin nor *TCF21*, resulted in *TCF21* and periostin expression. *TCF21* and periostin are involved in the regulation of fibrosis in endometriosis. *TCF21* may be a promising therapeutic target and biomarker in endometriosis.

INTRODUCTION

Repeated tissue injury and repair (ReTIAR) resulting in fibrosis [1], may be one of the many pathological mechanisms involved in endometriosis, which affects up to 15% of reproductive-age women [2]. Endometriosis is often accompanied by a profound worsening of the quality of life, due to concomitant chronic pain and infertility [3]. Although it has been hypothesized that hormonal, immunological, and environmental changes may induce normal endometrial cells to flow retrograde into the intra-peritoneal space and become endometriotic [4], the detailed description of all the abnormalities leading to endometriosis is yet to be elucidated.

Endometriosis is a benign disease going through tumor-like processes to form endometriotic lesions. Tumor-like processes include aggression [5], evasion (epithelial-mesenchymal transition, EMT) [6,7], adhesion (CD10, integrins) [8], invasion (matrix metalloproteinases, MMPs) [9], angiogenesis (vascular endothelial growth factor, VEGF) [10,11], surviving (hormones, aromatase) [12], fibro-proliferation [13], and inflammation (cytokines) [14,15]. Although these mechanisms have been studied for long time, more research is still needed as the current medical therapies have many side effects and are not plausible due to the high recurrence rate after performing conservative surgery [16].

Extracellular matrix (ECM) proteins (fibronectin, tenascin-C, type I collagen, and laminin γ2) play an important role in endometriosis. Periostin, a 90 kDA osteoblast-specific factor-2 from the fasciclin family [17], functions as a scaffold for ECM proteins assembly [18-20]. Upon accumulation in the inflamed sites, periostin leads to fibrosis activating immune and non-immune cells via its matricellular nature, by which it can bind to cell-surface receptors of integrins, cytokines and other downstream signals, further expanding the inflammation. Physiologically, periostin is found at collagen-rich regions in connective tissue, such as periodontal ligament [21], cardiac valve [22], periosteum [23], and lung [24]. Moreover,

periostin is considered an important molecule involved in conditions associated with severe diseases, like fibrosis in asthma [25], scar formation in myocardial infarction [26], cancer cell migration, corneal dystrophy, skin [27], and endometriotic fibrosis [28-32]. Therefore, periostin can be used for evaluation of fibrotic aspects of various tissues and endometriosis samples [25-32]. Although the number of periostin-related papers in ophthalmology, dermatology, otolaryngology, allergology, and oncology is increasing every year, there are only few papers describing its expression and interaction with up- and downstream targets in endometriosis [28-32].

The gene coding for transcription factor 21, *TCF21*, is located on chromosome 6q23. It is a member of the basic helix–loop–helix (bHLH) transcription factor family and is essential for the epithelial cell differentiation. *TCF21* is expressed in numerous tissues, including lung, gut, gonad, urinary tract, spleen, and kidney [33]. In physiological conditions, *TCF21* remains inactivated and only if the tissue needs to be recovered after an injury or a stress, it becomes aberrantly activated [34-36]. The transcription factors of the bHLH family regulate the expression of hundreds of other genes, including those promoting cell proliferation and survival, and EMT via Wnt/β-catenin signaling. Although many studies have focused on the role of Wnt/β-catenin pathway [37, 38] and on its silencing effect in endometriosis [39], up to date, no study has demonstrated the direct interaction between *TCF21* and periostin in endometriosis. Studies on the role of *TCF21* and periostin in myocardial infarction showed that *TCF21* is an upstream regulatory gene of periostin and *TCF21* becomes activated from the resident fibroblasts leading to the overproduction of its downstream target - periostin [34,35].

Our aim was to investigate whether *TCF21* is involved in the regulation of periostin in endometriosis. We demonstrate how *TCF21* regulation affects periostin expression and therefore, the pathogenesis of endometriosis. This study provides an insight into the

expression of *TCF21*, periostin, and cytokines, and their interaction in the samples from women with or without endometriosis, and further substantiates the value of *TCF21* as therapeutic target and putative biomarker in endometriosis.

MATERIALS AND METHODS

Reagents

Anti-periostin antibody (ab92460), anti-TCF21 antibody-ChIP Grade (ab32981), anti-IL4 antibody [EPR1118Y] (ab62351), anti-TGF-β1 antibody (ab92486) (Abcam, Cambridge, UK), anti-IL13 antibody (F-6) (sc-390676) (Santa Cruz Biotechnology, Inc., Dallas, TX), anti-β actin antibody (Wako Pure Chemical Industries, Ltd., Osaka, Japan), clone OTI4C5 anti-DDK (Flag) monoclonal antibody (TA50011-100) (OriGene Technologies, Inc., Rockville, MD), Opal Multiplex IHC Detection Kit (PerkinElmer, Waltham, MA); recombinant cytokines (PeproTech, Rocky Hill, CT): human IL-4 (catalog#AF-200-04), human IL-13 (catalog#AF-200-13), and human TGF-β1 (catalog#AF-100-21C); Lipofectamine 3000 and Lipofectamine RNAiMAX (Invitrogen, Waltham, MA); *TCF21* silencer pool (Ambion, Life Technologies, USA) (s13904, s13906, s224713, s13905), non-specific control siRNA (Invitrogen); *TCF21* (NM_198392) Human cDNA ORF Clone (OriGene Technologies, Inc.) and empty plasmid (Asia-vector Biotechnology). GFP (green fluorescent protein)- and Flag-tagged *TCF21* vector plasmids were generated for the transfection into normal endometrial cells. Protein level and cytological localization of TCF21 and periostin were evaluated by western blot (WB) and immunocytochemical (ICC) analysis.

Tissue samples

A total of 50 formalin-fixed, paraffin-embedded (FFPE) tissue samples of women with or without endometriosis (5 μm sections) were used: NE, normal endometrium without endometriosis, n=14; EE, eutopic endometrium with endometriosis, n=11; OE, ovarian endometriosis, n=16; DIE, deep infiltrating endometriosis, n=9). The ethical committee of Nagoya University Graduate School of Medicine (Nagoya, Japan) approved the experiments. Written informed consent was obtained from each patient before sampling.

Cell culture

Stromal cells were obtained from control endometrium (n=6) of patients undergoing surgery for fibroid or other benign gynecological diseases; from eutopic (n=7) and ectopic endometrium of women with OE (n=7) and DIE (n=3). The cells were confirmed by a pathologist to be normal endometrial (endometrial stromal cell, ESC), eutopic (eutopic endometrial stromal cell, eESC), and ectopic stromal cells (chocolate cyst stromal cells, CSC and deep infiltrating endometriosis stromal cells, DSC). The patients did not receive any hormonal treatment at least six months prior to surgery. Briefly, biopsy specimens were rinsed by PBS, minced into $1mm^3$ pieces, and digested with 5% dispase and collagenase (2 mg/mL, Gibco Invitrogen, Cergy Pontoise, France) for one hour at 37 °C, with serial filtration for separation. Hypotonic lysis buffer was used to remove red blood cells. Undigested tissue was removed using 100 μm sieves. The stromal cells were further enriched by separation of epithelial cells with a 40 μm sieve. Isolated stromal cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD), 100 IU/mL of penicillin, 100 μg/mL of streptomycin, and 0.025 mg/mL of amphotericin B, at 37 °C in 5% (v/v) CO_2 in air. To confirm the correct stromal phenotype, the isolated cells were checked for their positivity for vimentin (1:50 anti-vimentin, Cell Signaling Technology, Danvers, MA) and for their negative epithelial phenotype using cytokeratin (1:25 anti-cytokeratin, Cell Signaling

Technology) by cell immunofluorescence (IF). The monolayer culture cells, after the third passage, were >99% pure (the proportion of stromal cells were evaluated in five random pictures using a 20X magnification). Each experiment was performed in triplicate and repeated at least three times with cells isolated from separate patients.

Immunohistochemistry (IHC)

Formalin-fixed 5 µm human specimens were deparaffinized, blocked in methanol/0.3% H_2O_2, and incubated with polyclonal primary antibodies against periostin and TCF21 (1:500, Abcam, Cambridge, UK) overnight at 4 ºC, followed by incubation with horse-radish peroxidase (HRP)-conjugated secondary antibody. Then, all specimens were incubated with 3,3'-diaminobenzidine tetrahydrochloride (DAB, Dako, Denmark), counterstained with hematoxylin, dehydrated, and mounted. Positive and negative control slides were incorporated in each staining. Negative control slides were incubated with phosphate-buffered saline replacing the primary antibody. For semi-quantification, each specimen was analyzed by counting periostin- and TCF21-positive cells in the epithelium and in the stroma, by using Zeiss ZEN 2 (blue edition) microscope and software. The H-score (histo-score) was calculated assigning a four-point scale to the intensity of the staining in 10 random fields (0, negative; 1, weak; 2, moderate; 3, strong); the percentage of cells at each intensity level was calculated by the formula:

$$[1 \times (\%\ \text{cells}\ 1^{+}) + 2 \times (\%\ \text{cells}\ 2^{+}) + 3 \times (\%\ \text{cells}\ 3^{+})]$$ [28].

Measurement of the protein expression of periostin and TCF21

The cells were lyzed in a radioimmunoprecipitation buffer (10 mmol/L Tris-HCl, pH 7-4, 150 mmol/L NaCl, 1 % Nonidet P- 40, 5 mmol/L EDTA, 1% sodium deoxycholate, o.1% sodium dodecyl sulfate [SDS], 1.2% aprotinin, 5 μmol/L leupeptine, 4 μmol/L antipain, 1 mmol/L phenylmethylsulfonylfluoride, and 0,1 mmol/L Na_3VO_4). The cell lysates were clarified by centrifugation at 13,000g at 4 °C for 15 min, for protein extraction. Equal amounts of proteins were mixed with 2x sample buffer (4% SDS), 10% beta-mercaptoethanol, and 20% glycerol in 0.125 M Tris, pH 6.8) containing bromophenol blue and boiled for 5 min. Equal amounts of proteins (30μg) were loaded and separated by 10% SDS-polyacrilamide gel electrophoresis for the analysis of periostin and TCF21 protein level. Blocking was done with 5% skimmed milk for one hour at room temperature. Then the membranes were incubated overnight at 4 °C with anti-periostin (1:1000), anti-TCF21 antibody - ChIP Grade ab32981 (0.5 μg/mL), and anti-β actin antibody (1:4000) in 5% non-fat dry milk/0.05% PBS-Tween. The binding of the specific antibodies was detected using a 1:1000 dilution of HRP-conjugated goat anti-rabbit (anti-periostin and anti-TCF21 antibodies) and goat anti-mouse (anti-β actin antibody) IgG secondary antibody (Dako, Denmark). β-actin was used as a loading control. The membranes were visualized by ImageQuant LAS 4000 (GE Healthcare) after reaction with Amersham ECL WB detecting reagent (GE Healthcare, Buckinghamshire, UK). Optical densities (OD) were measured using ImageQuantTL (GE Healthcare). Ratios between periostin OD and β-actin OD or TCF21 OD and β-actin OD were calculated in each case.

RNA extraction and quantitative real-time PCR (RT-qPCR)

Total RNA was extracted from ESC, eESC, CSC, and DSC samples, using RNeasy Mini Kit (Qiagen, Tokyo, Japan). The RNA was also isolated from the same cells after stimulation with 20 ng/mL of cytokines, to verify whether they affect periostin and *TCF21* mRNA expression (Th2 type/ pro-fibrotic - IL4, IL13, and TGF-β1). The RNA (10 ng) was then

retro-transcribed using ReverTrace qPCR RT Master Mix (TOYOBO, Osaka, Japan) and the cDNA was used for performing RT-qPCR, which was performed in triplicate using LightCycler 480 (Roche Diagnostics, Penzberg, Germany) and KOD SYBR qPCR Mix (TOYOBO, Osaka, Japan). The primers used for periostin were: forward (5′-AATCCAAGTTGTCCCAAGCC-3′) and reverse (5′-GCACTCTGGGCATCGTGGGA-3′); for *TCF21*: forward (5′-TCCTGGCTAACGACAAATACGA-3′) and reverse (5′-TTTCCCGGCCACCATAAAGG-3′); and for *Gapdh*: forward (5′-CAGCCTCAAGATCATCAGCA-3′) and reverse (5′-GTCTTCTGGGTGGCAGTGAT-3′) (Thermo Fisher Scientific, Waltham, MA). *Gapdh* was used as internal control. Periostin and *TCF21* expression levels were calculated relative to *Gapdh* applying the ΔΔCT method and the data are presented as the percentage of periostin/*Gapdh* and *TCF21/Gapdh* of eESC, CSC, and DSC relative to that of ESC.

Multiplexed IHC/ IF

Multiplexed staining was performed using Opal Multiplex IHC Detection Kit (PerkinElmer, Waltham, MA) following the manufacturer's protocol. Briefly, the slides were first deparaffinized in xylene and then rehydrated in ethanol, following antigen retrieval in citrate buffer (pH 6.0) using a microwave. Primary antibody for periostin (1:500) was incubated for one hour in a humidified chamber at room temperature, followed by rabbit Opal Polymer HRP mouse + rabbit secondary antibody working solution. Visualization of periostin was performed using opal 520 (GFP-B) TSA Plus (1:100), followed by treatment with citrate buffer (pH 6.0) and heating using a microwave. Each slide was then incubated in a serial manner, with primary rabbit antibodies for IL-4 (1:500), TGF-β1(1:100) and mouse antibody for IL-13 (1:100) for one hour in a humidified chamber, at room temperature, followed by detection using the Opal Polymer HRP mouse + rabbit secondary antibody working solution. The expression of the cytokines IL-4, TGF-β1, and IL-13 was visualized using opal 570

(TxRed), 690 (Cy5), and spectral DAPI (DAPI-B) TSA Plus (1:100) fluorophores, respectively. The same procedure was performed with TCF21 (opal 520 fluorophore) (5.0 µg/mL), IL-4 (opal 570 fluorophore), IL-13 (opal 690 fluorophore), and TGF-β1 (spectral DAPI fluorophore) primary antibodies in a serial manner, on the same slides. A third visualization was performed after applying TCF21 (opal 520 fluorophore), periostin (opal 570 fluorophore), IL-4 (opal 690 fluorophore), and IL-13 (spectral DAPI fluorophore) on the same slides, in a serial mode. In all experiments, nuclei were subsequently visualized with DAPI working solution, and the sections were cover-slipped using Dako Fluorescence mounting medium (CA). The advantage of using the Opal method is that it allows applying four primary antibodies sequentially on a single slide.

Transient knockdown and over-expression of TCF21 expression and transfection

TCF21 was transiently knocked down by transfecting 10nM of small interfering RNA (siRNA) against human *TCF21* gene (siTCF21 ID#s13904) and non-specific control siRNA (Invitrogen) into CSCs and DSCs for 48 hours using Lipofectamine RNAiMAX, according to the manufacturer's instructions. For transient over-expression of *TCF21*, ESCs were transfected with 5 µg Flag-tagged *TCF21* plasmid and 5 µg empty plasmid serving as control (Asia-vector Biotechnology) for 48 hours using Lipofectamine 3000, according to the manufacturer's instructions.

Immunocytochemistry (ICC)

ESCs were cultured on coverslips, transfected with *TCF21*-Flag-tagged vector plasmid or empty plasmid serving as control for 48 hours, fixed in 4% paraformaldehyde for 15 min at room temperature and permeabilized with 0.5% TritonX-100 for 10 min. After blocking with 3% bovine serum albumin (BSA) for 30 min at room temperature, the cells were incubated with the primary antibodies (anti-Periostin, anti-TCF21, anti-DDK) at 4 °C overnight. Then,

the cells were incubated with goat anti-rabbit 568 and 488 (Alexa Fluor, abcam) (in case of anti-periostin and anti-TCF21 antibodies) and goat anti-mouse 488 (Alexa Fluor, abcam) (in case of anti-DDK antibody) secondary antibodies for one hour at room temperature. Hoechst 33342 was used for nuclei staining. Visualization was done by a confocal Laser Scanning Microscope (TiE-A1R, Nikon).

Statistical analysis

Data were expressed as mean ±SEM (standard error of the mean) or the median and all values. The level of significance between groups was determined by Student's *t*-test or Mann-Whitney U test. The Mann-Whitney U test was applied instead of the Student's *t*-test when the variables were not normally distributed. Statistical analyses were performed using SPSS software version 24.0 (SPSS Inc., Chicago, IL). H-score was analyzed by GraphPad Prism software version 7.01 (San Diego, CA). A *P*-value of less than 0.05 was considered statistically significant. All the experiments were performed at least in triplicate.

RESULTS

Periostin expression

To understand the role of periostin in endometriosis, its protein expression levels were assessed in the samples of women with or without endometriosis by IHC. In NE, periostin was neither expressed in proliferative nor secretory phases of the menstrual cycle (Fig.1A). Weak expression with glandular localization was found in EE throughout the menstrual cycle (Fig.1A). OE and DIE samples showed moderate to strong periostin expression levels in the stroma (Fig.1A). To analyze the levels of significance, H-score was calculated (Fig.1B). The level of periostin was significantly higher in DIE samples compared with that in OE, EE, and NE ($P < 0.0001$); negative in NE, whereas there was significance between any two groups (*P*

< 0.001). To evaluate the expression of periostin mRNA level in the samples of women with or without endometriosis, RT-qPCR was performed with the mRNA samples obtained from the cells cultured for several passages. The highest level of periostin mRNA was observed in DSC with significance between any two groups (Fig.1C). WB analysis revealed that periostin protein level was in line with the mRNA expression level (Fig.1D). These findings suggest that periostin is exclusively featured in women with endometriosis. Typically, more fibrosis was observed in OE compared with EE, and further fibrosis was encountered in DIE compared with OE [40].

TCF21 expression

Previous studies have reported the interaction between *TCF21* and periostin [34, 35]. To assess this interaction in endometriosis, TCF21 protein distribution was analyzed by IHC in NE, EE, OE, and DIE samples. In NE, TCF21 was expressed neither in proliferative nor in secretory phases of menstrual cycle (Fig.2A). Weak expression with mainly glandular localization was found in EE in both phases of the menstrual cycle (Fig.2A). In OE, moderate expression of TCF21 protein was found in the stroma (Fig.2A). Similarly, DIE showed mainly stromal pattern of TCF21 protein distribution, however the expression level was stronger (Fig.2A). H-score showed significant differences ($P < 0.001$) in the levels of TCF21 within any two groups with the highest level in DIE compared with OE, EE, and NE ($P < 0.0001$) (Fig.2B). RT-qPCR with the mRNA samples obtained from the cell cultures was performed to assess the relative expression of *TCF21* mRNA in the samples of women with or without endometriosis. Similar to periostin, the highest level of *TCF21* mRNA was observed in DSC compared with CSC, eESC, and ESC, with significance between any two groups (Fig.2C). These findings are in line with the results of WB analysis of the levels of TCF21 protein (Fig.2D). These data suggest that TCF21 is uniquely expressed in women with endometriosis.

The effect of cytokines on the expression of periostin and *TCF21*

The fibrotic aspect of endometriosis were studied so far; inflammation also greatly contributes to fibrosis. Therefore, the influence of the Th2 pro-fibrotic cytokines (IL4, IL13, TGF-β1) was examined on periostin and *TCF21* expression. These cytokines increased periostin and *TCF21* mRNA levels (Fig.3A, B). Specifically, periostin and *TCF21* were most significantly induced in DSC, with significance between any two cell types. Furthermore, to investigate the interaction between cytokines and periostin, cytokines and *TCF21*, and cytokines, periostin, and *TCF21* expression, IF staining was performed. As hypothesized, there was no signal of periostin and cytokines (Fig.4), TCF21 and cytokines (Fig.5), and periostin, TCF21, and cytokines (Fig.6) in NE samples, either in secretory or in proliferative phases of the menstrual cycle. In EE, cytokines, periostin and TCF21 were weakly expressed in both phases and they were co-localized in the areas of their expression which was evaluated based on the color change on the merged images (Figs. 4, 5, 6). Moderate to strong stromal signal and co-localization were detected in OE and DIE samples, respectively (Figs. 4, 5, 6). The signals of periostin, TCF21, and cytokines in OE and DIE were more intensive and more abundant than in EE and NE samples. Therefore, they were evaluated as being moderate and strong signals. These results suggest that cytokines, periostin, and TCF21 expression increased with their co-localization.

Transient knockdown and overexpression of *TCF21*

Co-localization of TCF21 and periostin (Fig.6) suggested that *TCF21* may be an upstream regulatory gene of periostin in the pathogenesis of endometriosis. Thus, siRNA-mediated knockdown of *TCF21* was performed to check its possible influence on periostin retention. As hypothesized, RT-qPCR showed that *TCF21* siRNA transiently knocked down not only *TCF21* (Fig.7A) but also periostin mRNA expression in CSC and DSC (Fig.7B). WB analysis revealed similar changes in the periostin expression levels of CSC and DSC

(Fig.7C). Moreover, siTCF21 significantly suppressed IL4-induced elevation of periostin and *TCF21* (Fig.7D). Successful knockdown of periostin by *TCF21* siRNA suggested that *TCF21* transfection in ESC, which was negative for both TCF21 and periostin, may evoke periostin expression. RT-qPCR and WB analysis showed that ESC transfected with *TCF21* plasmid, expressed both *TCF21* and periostin (Fig.8A, B). Transfected TCF21 was found to be localized in the nucleus, whereas periostin was found in the cytoplasm of ESC (Fig.8C). These findings inferred that *TCF21* can effectively regulate periostin during the development of endometriosis.

DISCUSSION

Endometriosis is one of the major causes of infertility among women in reproductive age. Despite continuous biomedical research efforts, the pathogenesis of endometriosis remains unclear. Fibro-genesis and fibro-proliferation are important stages for the formation of endometriotic lesions. Some studies have already emphasized the consistent presence of fibrosis and myofibroblasts in endometriotic lesions and their importance in the pathogenesis of the disease [41-43]. Myofibroblasts are usually activated in response to injury. Their intent is to repair damaged ECM. Myofibroblasts can be dispatched from various cellular lineages, including endothelial cells undergoing endothelial-to-mesenchymal transition, tissue resident fibroblasts, vascular smooth muscle cells, and epithelial cells after EMT [44]. Periostin acts as a "switch" in the assembly of all ECM proteins, participating in cell adhesion, differentiation, and organization of ECM in case of tissue damage [45]. Particularly, its direct binding to fibronectin in the endoplasmic reticulum of fibroblastic cells, supports the suggested role of periostin in the protein secretory pathway [19]. There is a detailed description of the role of periostin in ophthalmology, dermatology, respiratory processes, otolaryngology, and oncology, where it is activated in response to local inflammation triggered by cytokines, supported by integrins and downstream signals [45]. However, the

possible down-and upstream regulatory genes of periostin in endometriosis are not fully described yet. Hence, periostin is gaining a great interest in relation to fibrotic processes in endometriosis.

In the present study, the interaction between *TCF21* and periostin was clearly demonstrated in the samples of women with or without endometriosis. TCF21 and periostin levels were elevated in the samples from patients with OE and DIE, supporting their role in the pathogenesis of endometriosis. In previous reports demonstrating the distribution of periostin, the endometriotic samples were not considered separately as OE/CSC and DIE/DSC types [5, 28, 36]. For the first time, the localization of both, TCF21 and periostin proteins, was analyzed by separating the samples into NE, EE, OE, and DIE, and a correlation was found between severe fibrosis and strong expression of both proteins [40]. Both TCF21 and periostin could be involved in the fibrotic proliferation of endometriosis. The interaction between *TCF21* and periostin was then studied. Treatment with *siTCF21* significantly decreased the expression of periostin. After transfection of ESC, formally negative for TCF21 and periostin, with DDK tagged *TCF21* vector plasmid, ESC expressed both proteins. These findings suggested that periostin may be regulated by *TCF21* in endometriosis, once *TCF21* is activated.

The pathogenesis of endometriosis consists of the vicious cycle of inflammation and fibrosis. Therefore, Th2-type cytokines which promote fibrosis were studied. Increased level of periostin is induced by Th2-type anti-inflammatory cytokines, mainly IL-4, IL-13, and TGF-β in asthma, myocardial infarction, and some types of tumors [46]. As expected, in this research, the same cytokines increased *TCF21* levels, leading to elevated periostin levels. These pro-fibrotic cytokines were found to be closely localized with both TCF21 and periostin in endometriotic tissues, and they were not expressed in the tissue samples of women without endometriosis. These results suggest that *TCF21* may be activated in normal

endometrium before the pathological process leading to endometriosis starts. However, it is not clear whether the activation of cytokines precedes the trigger of *TCF21* or whether *TCF21* attracts the cytokines once it is switched on, followed by a vicious cycle of co-activation (Fig.9). Although in other cell types TCF21 and periostin have nuclear and cytoplasmic localization, respectively [33, 17], there are no previous studies showing the cellular localization of these proteins in endometriosis. Periostin is generated inside the cell, even if it is classified as one of the ECM proteins [47]. In our findings, periostin was localized in the cytoplasm of stromal cells, very close to TCF21 which is situated in the nuclear compartment of the cells.

TCF21 is ubiquitously expressed in all cells and remains inactive under normal conditions. Only after injury or stress, it becomes aberrantly activated [34-36]. Xiang et al demonstrated that stress caused by myocardial infarction induced the activation of *TCF21* in cardiac fibroblasts [34]. The activated *TCF21* then regulates the expression of genes promoting proliferation, survival, and EMT [34-36]. The same injury or stress could happen in the uterus. Bacterial endotoxin (lipopolysaccharide, LPS) has been reported to regulate the pro-inflammatory response in the pelvis and growth of endometriosis via the LPS/TLR4 (toll-like receptor 4) cascade [48]. Cincinelli et al reported that intrauterine microbes causing chronic endometritis may be the initial factor for developing endometriosis [49]. The increased prevalence of endometriosis reported in women with Herlyn-Werner-Wunderlich syndrome with menstrual blood outflow obstruction has been suggested to be caused by the inability of the immune response to remove the debris [50]. Such infectious injury and immune response stress may contribute to the activation of *TCF21* in the normal uterus, leading to endometriotic predisposition. With retrograde flow, the *TCF21*-activated endometrium could attach to the ectopic tissue, leading to a stromal invasion of *TCF21* activated endometrium and its induction of periostin production. According to some disputes regarding

endometriosis development, DIE is not formed via retrograde menstruation like other endometriotic lesions. Our findings showed that although both ectopic lesions–DIE and OE—showed high expressions of TCF21 and periostin, DIE showed higher expressions than OE. Therefore, all types of lesions might have retrograde menstruation as an initial triggering factor that should necessarily be accompanied by favorable endocrine and metabolic environment, EMT, altered immunity, and inflammatory responses [51].

Along with the need to better understand the pathophysiology of endometriosis, the need for a reliable diagnostic biomarker for endometriosis is imperative. It is highly likely that instead of a single biomarker, a group of biomarkers including *TCF21* and periostin will provide improved diagnostic performance and minimize false positive results during differential diagnosis.

Protein and mRNA analyses were performed on stromal cells that were cultured from the tissue samples. Therefore, there might be a change in gene expression during *in vitro* culture. However, such changes were not examined in this study.

CONCLUSIONS

This study highlights the importance of *TCF21* in periostin regulation *in vitro*. *TCF21* may be a key regulator for switching off periostin, which retains the scaffold of all ECM proteins necessary to cause fibrosis in endometriotic lesions. *In vitro* data suggest that *TCF21* may become a novel preventive and therapeutic target as well as a reliable biomarker in endometriosis.

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Figure legends

Figure 1. Levels of periostin in the samples from women with and without endometriosis. A: Immunohistochemical analysis of the levels of periostin protein expression (Σn=50). **B:** H-score of the levels of periostin protein found in immunohistochemical analysis. **C:** Levels of periostin mRNA (48 hours, Σn=12). **D:** Western blot analysis of the levels of periostin protein (48 hours, Σn=12). The mean OD ratio for periostin/β-actin was calculated for each endometriotic cell type and compared with the OD ratio of the same proteins in normal endometrial cells. Data were expressed as mean ±SEM (standard error of the mean) or the median. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. H-score, "histo" score. OD, optical density. Magnifications were 5X and 20X, and the scale bars indicate 200 µm and 50 µm, respectively.

Figure 2. Levels of TCF21 in the samples from women with and without endometriosis. A: Immunohistochemical analysis of the levels of TCF21 protein expression (Σn=50). **B:** H-score of the levels of TCF21 protein found in immunohistochemical analysis. **C:** Levels of *TCF21* mRNA (48 hours, Σn=12). **D:** Western blot analysis of the levels of TCF21 protein (48 hours, Σn=12). The mean OD ratio for TCF21/β-actin was calculated for each endometriotic cell type and compared with the OD ratio of the same proteins in normal endometrial cells. Data were expressed as mean ±SEM (standard error of the mean) or the median. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. H-score, "histo" score. OD, optical density. Magnifications were 5X and 20X, and the scale bars indicate 200 µm and 50 µm, respectively.

Figure 3. Effect of cytokines on periostin and *TCF21* mRNAs. A: Levels of periostin mRNA in the samples from women with and without endometriosis after stimulation with cytokines (48 hours, Σn=12). **B.** Levels of *TCF21* mRNA in the samples from women with and without endometriosis after stimulation with cytokines (48 hours, Σn=12). Data were expressed as mean ±SEM (standard error of the mean). ***P* < 0.01, ****P* < 0.001.

Figure 4. Expression and co-localization of periostin and cytokines shown by immunofluorescence. Expression and co-localization of periostin and cytokines found in immunofluorescence (Σn=50). All magnifications were at 40X, scale bars indicate 200 µm.

Figure 5. Expression and co-localization of TCF21 and cytokines shown by immunofluorescence. Expression and co-localization of TCF21 and cytokines found in immunofluorescence (Σn=50). All magnifications were at 40X, scale bars indicate 200 µm.

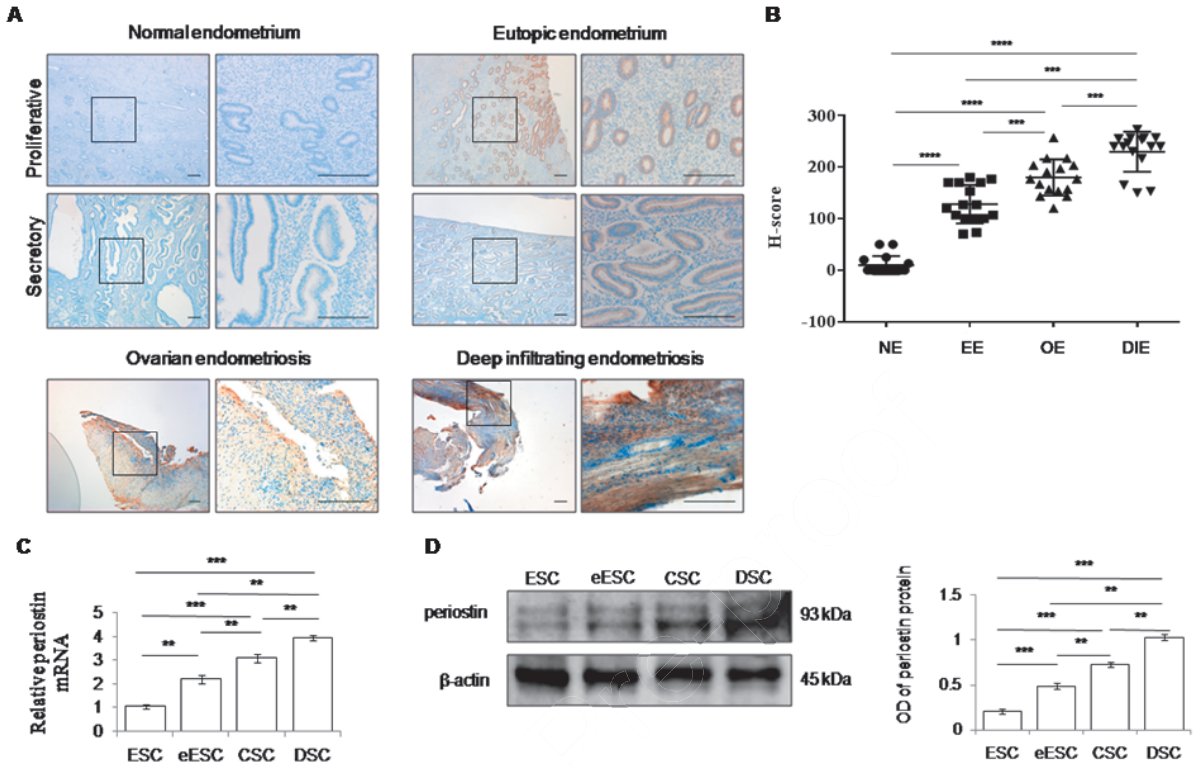
Figure 6. Expression and co-localization of TCF21, periostin, and cytokines shown by immunofluorescence. Expression and co-localization of TCF21, periostin, and cytokines found in immunofluorescence (Σn=50). All magnifications were at 40X, scale bars indicate 200 µm.

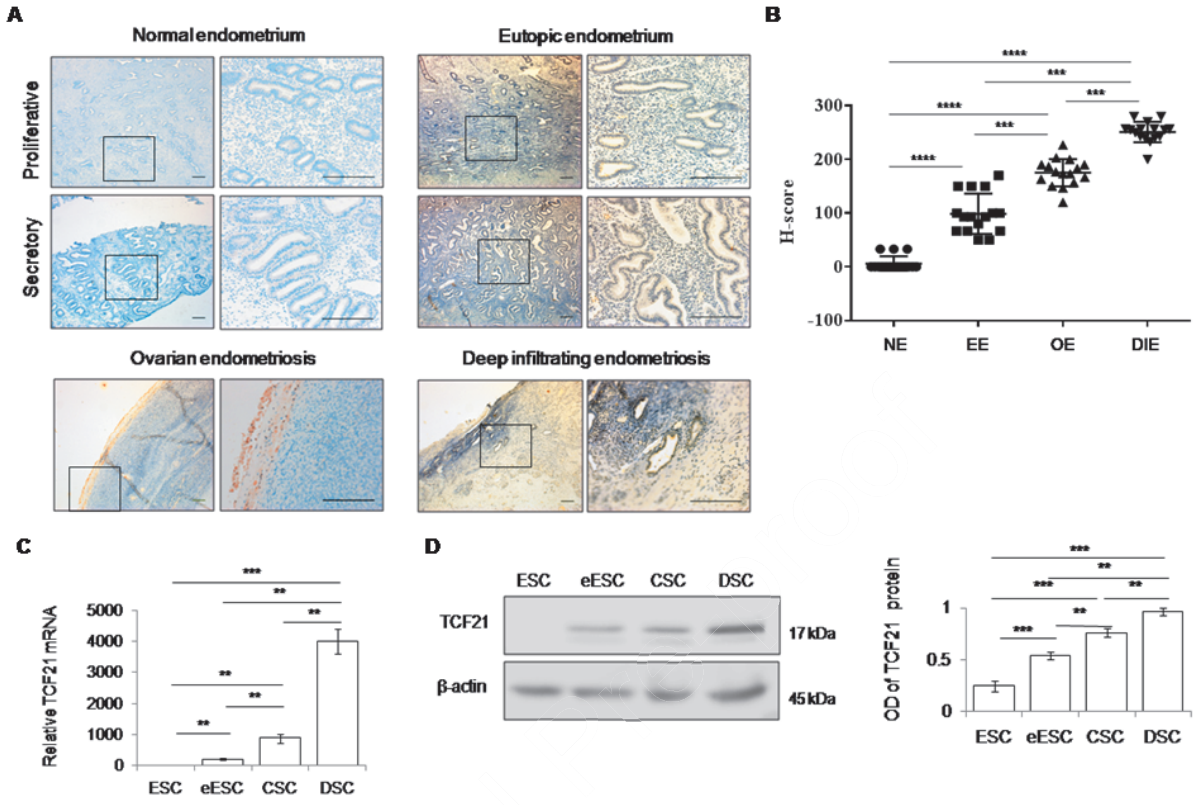
Figure 7. Effect of *TCF21* siRNA on TCF21 and periostin. A: mRNA levels of *TCF21* in chocolate cyst stromal cells (CSC; n=3) and deep infiltrating endometriosis stromal cells (DSC; n=3) after transfection with *TCF21* siRNA (48 hours). **B:** mRNA levels of periostin in CSC (n=3) and DSC (n=3) after transfection with *TCF21* siRNA (48 hours). **C:** Western blot analysis of the levels of periostin protein after *TCF21* siRNA transfection (48 hours, Σn=6). **D:** mRNA levels of periostin in CSC (n=3) and DSC (n=3) after *TCF21* siRNA transfection

and cytokine stimulation (48 hours). Data were expressed as mean ±SEM (standard error of the mean) or the median and all values. $^{**}P < 0.01$, $^{***}P < 0.001$.

Figure 8. Effect of *TCF21* overexpression on periostin. A: mRNA levels of periostin in endometrial stromal cell (ESC) after transfection with *TCF21*/DDK-tagged plasmid (48 hours, n=3). **B:** Western blot analysis of the levels of periostin protein in ESC after *TCF21*/DDK-tagged plasmid transfection (48 hours, n=3). **C:** Immunocytochemical analysis of the co-localization of TCF21 and periostin after *TCF21*/DDK-tagged plasmid transfection in ESC (n=3). White arrows indicate *TCF21*-transfected cells with induced production of periostin. Data were expressed as mean ±SEM (standard error of the mean) or the median and all values. $^{*}P < 0.05$, $^{**}P < 0.01$. All magnifications were at 100X, scale bar indicates 20 μm.

Figure 9. Schematic view of possible interaction between cytokines, TCF21, and periostin in a vicious cycle of co-activation in endometriosis. *TCF21* is ubiquitously expressed in all cells and only after injury or stress, it becomes aberrantly activated. The activated *TCF21* then regulates the expression of genes promoting proliferation, survival, and epithelial-mesenchymal transition (EMT). With retrograde flow, the *TCF21*-activated endometrium may attach to the ectopic tissue, leading to a stromal invasion of *TCF21*-activated endometrium and its induction of periostin production. Periostin contributes to fibrosis and activates immune cells to produce pro-inflammatory cytokines (eg, IL4) to further expand inflammation. The resulting fibrosis and inflammation construct the vicious cycle of the pathogenesis of endometriosis.

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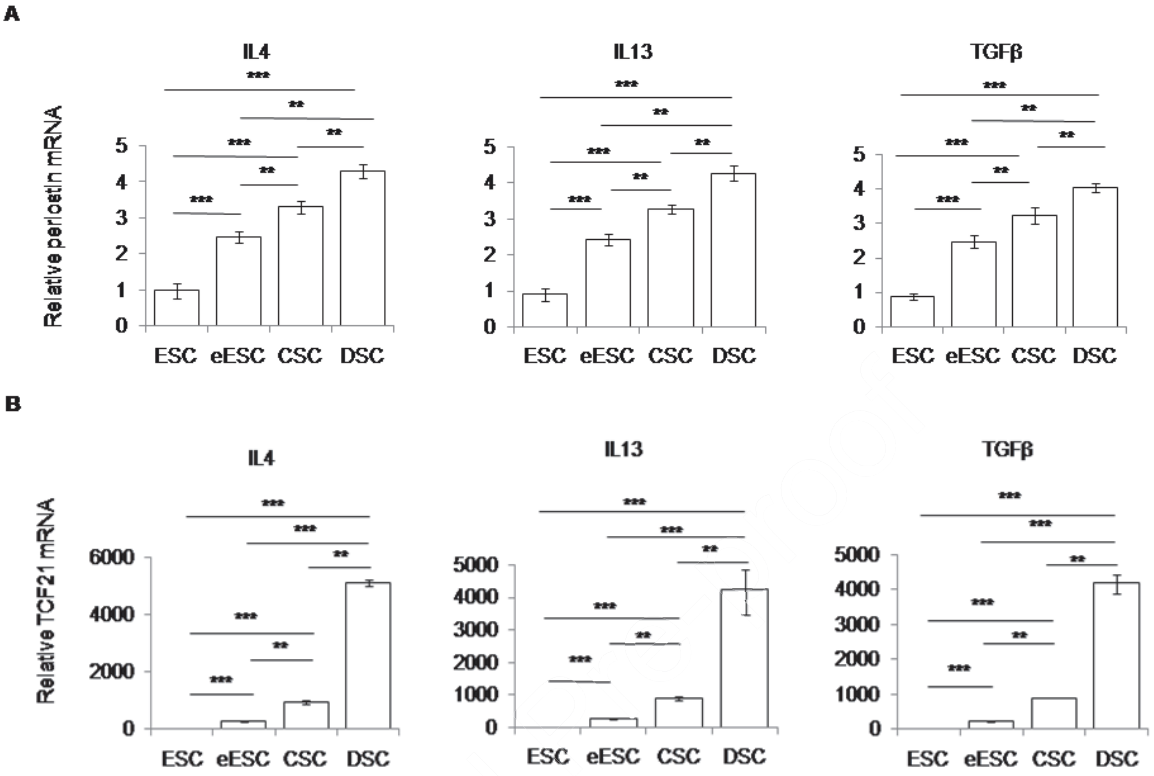
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