主論文の要旨

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

（子宮内膜症の線維化における Transcription Factor 21 の関与）

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【INTRODUCTION】

Endometriosis is a benign disease going through tumor-like processes to form endometriotic lesions: aggression, evasion (epithelial-mesenchymal transition, EMT), adhesion (CD10, integrins), invasion (matrix metalloproteinases, MMPs), angiogenesis (vascular endothelial growth factor, VEGF), surviving (hormones, aromatase), fibro-proliferation and inflammation (cytokines). Extracellular matrix (ECM) proteins play an important role in endometriosis. Periostin functions as a scaffold for ECM proteins assembly. Upon accumulation in the inflamed sites, it leads to fibrosis activating immune and non-immune cells via its matricellular nature. TCF21 is essential for the epithelial cell differentiation. In physiological conditions, *TCF21* remains inactivated and only if the tissue needs to be recovered after injury or stress, it becomes aberrantly activated. 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. No study has demonstrated the direct interaction between *TCF21* and *periostin* in endometriosis. In the present study, we demonstrated how *TCF21* regulation affects *periostin* expression and therefore, the pathogenesis of endometriosis. This study provides an insight into the expressions of *TCF21*, *periostin* and cytokines, as well as their interactions.

【METHODS AND RESULTS】

Periostin expression

Periostin expression levels were evaluated by immunohistochemistry (IHC), quantitative real-time polymerase chain reaction (qRT-PCR), and western blotting analysis (WB). In normal endometrium (NE), periostin was neither expressed in proliferative nor secretory phases of the menstrual cycle. Weak expression with glandular localization was found in eutopic endometrium (EE). Ovarian endometriosis (OE) and deep infiltrating endometriosis (DIE) showed moderate to strong periostin expression levels in the stroma (Figure 1A). The level of periostin was significantly higher in DIE samples compared with that in OE, EE, and NE ($P<0.0001$) (Figure 1B). The highest level of *periostin* mRNA was observed in DSC with significance between any two groups (Figure 1C). WB analysis revealed that periostin protein level was in line with its mRNA expression level (Figure 1D).

TCF21 expression

TCF21 expression levels in the samples of women with or without endometriosis were evaluated by IHC, qRT-PCR, and WB. In normal endometrium (NE), TCF21 was neither expressed in proliferative nor secretory phases of the menstrual cycle. Weak expression with glandular localization was found in eutopic endometrium (EE). Ovarian endometriosis (OE) and deep infiltrating endometriosis (DIE) showed moderate to strong

TCF21 expression in the stroma (Figure 2A). The TCF21 expression was significantly higher in DIE samples compared with that in OE, EE, and NE ($P<0.0001$) (Figure 2B). The highest level of *TCF21* mRNA was observed in DSC with significance between any two groups (Figure 2C). WB revealed that TCF21 protein level was in line with the mRNA expression level (Figure 2D).

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

Th2 pro-fibrotic cytokines (IL4, IL13, TGF-β1) increased *periostin* and *TCF21* mRNA levels. *Periostin* and *TCF21* were most significantly induced in DSC, with significance between any two cell types (Figure 3A, B). To investigate the interaction between cytokines and *periostin*, cytokines and *TCF21*, and cytokines, *periostin* and *TCF21* expression, we performed immunofluorescence staining (IF). There were no signals of periostin, TCF21and cytokines (Figure 6) in NE samples. In EE, periostin, TCF21 and cytokines were weakly expressed and co-localized. Moderate to strong stromal signal and co-localization were in OE and DIE samples, respectively (Figure 6).

Transient knockdown and overexpression of *TCF21*

TCF21 siRNA transiently knocked down *TCF21* (Figure 7A) and *periostin* expressions in CSC and DSC (Figure 7B) in qRT-PCR. WB analysis revealed similar changes (Figure 7C). qRT-PCR and WB analysis showed that ESC transfected with *TCF21* plasmid, expressed both *TCF21* and *periostin* (Figure 8A, B). Transfected TCF21 localized in the nucleus, and periostin - in the cytoplasm (Figure 8C).

【DISCUSSION】

In the present study, we showed the interaction between *TCF21* and *periostin* 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. For the first time, we analyzed the localization of both, TCF21 and periostin proteins, by separating the samples into NE, EE, OE and DIE, and found a correlation between severe fibrosis and strong expression of both proteins. Both TCF21 and periostin could be involved in the fibrotic proliferation of endometriosis. We then evaluated the interaction between *TCF21* and *periostin*. 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, we focused on Th2-type cytokines which promote fibrosis. These

cytokines increased *TCF21* levels, leading to elevated *periostin* levels, and the former 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. It is worth to mention that periostin is generated inside the cell, even if it is classified as one of the ECM proteins. 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, with retrograde flow, the *TCF21*-activated endometrium could attach to the ectopic tissue, and induce periostin production. Our findings showed that although both ectopic lesions - DIE and OE showed high expressions of TCF21 and periostin, DIE showed higher expressions than OE. It is more 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.

Our present findings showed the importance of *TCF21* in *periostin* regulation *in vitro*. Indeed, *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. Our *in vitro* data suggest that *TCF21* may become a novel preventive and therapeutic target as well as a reliable biomarker in endometriosis.