Expression of Nodal, Cripto, SMAD3, Phosphorylated SMAD3, and SMAD4 in the Proliferative Endometrium of Women With Endometriosis

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Abstract

Background: Nodal is a growth factor of the transforming growth factor β superfamily that is expressed in high turnover tissues, such as the human endometrium, and in several malignancies. The effects of Nodal are modulated by the coreceptor Cripto and mediated by SMAD proteins. This study evaluated the gene and protein expression of Nodal, Cripto, total and phosphorylated (p) SMAD3, and SMAD4 in the proliferative endometrium of women with and without endometriosis. **Method:** Total RNA was isolated and complementary DNA synthesized from eutopic endometrium of women with ($n = 15$) and without ($n = 12$) endometriosis, followed by quantitative real-time polymerase chain reaction (PCR) to evaluate the gene expression of Nodal, Cripto, SMAD3, and SMAD4. Western blot was used to evaluate the protein levels of Nodal and Cripto, and immunohistochemistry was performed to localize SMAD3, pSMAD3, and SMAD4. **Results:** Although Nodal expression was unchanged in women with endometriosis, real-time PCR indicated lower gene expression of Cripto (fold change 0.27, $P < .05$) in the endometriosis group. This difference, however, was not maintained at protein expression level as assessed by Western blot. The immunostaining of total SMAD3 was reduced in the endometriosis group ($P < .01$), but the localization of pSMAD3 and the nuclear staining of SMAD4 were unchanged. **Conclusion:** These findings suggest that the Nodal signaling pathway has subtle changes in the endometrium of women with endometriosis, but this imbalance may not cause functional damage as it seems not to affect the nuclear expression of SMAD4.

Keywords

endometrium, endometriosis, Nodal, Cripto, SMADs

Introduction

Superfamily members of transforming growth factor β (TGF- β) are growth factors dynamically expressed in human endometrium, and their actions are related to cell proliferation, decidualization, apoptosis, and tissue remodeling after menstruation. These mechanisms are critical for the human reproductive cycle and for the establishment of pregnancy.¹ However, studies indicate that altered expression of members of this family in the uterus could be associated with manifestations of various diseases such as endometriosis, abnormal uterine bleeding, and endometrial cancer.²⁻⁴

Nodal is a member of the TGF- β family, which regulates early embryonic formation in vertebrates.^{5,6} Nodal plays numerous biological activities during embryogenesis such as the induction of mesoderm and endoderm and the formation of the right-left axis.⁷ In this regard, Nodal signaling is

essential for maintaining the embryonic cells of mice and human in their undifferentiated state.⁷⁻⁹ The expression of Nodal was initially thought to be restricted to the embryo, but subsequent studies have shown that it may also occur in some adult tissues such as the lactating mammary gland and pancreatic cells.^{10,11}

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Like other members of the TGF- β superfamily, Nodal starts its signaling by binding simultaneously 2 types of serine–threonine kinase receptors, known as activin receptors type I and type II.^{12,13} Upon Nodal binding to both receptors, the type I receptor (ALK4 or ALK7) is phosphorylated and recruits signal-transducing proteins known as SMAD2 and SMAD3. When these proteins form an SMAD2/3 complex and undergo phosphorylation, they recruit a common SMAD4 which is translocated to the nucleus to activate gene transcription.^{5,7,14} If the subtype of type I receptor involved in Nodal binding is ALK4, the coreceptor Cripto is recruited and becomes essential for its signaling.^{15–17} When Nodal instead binds ALK7, the receptor is activated by a Cripto-independent pathway.

Endometriosis is an estrogen-dependent gynecological disease characterized by the presence of endometrial tissue outside the uterine cavity, associated with high degree of morbidity due to pelvic pain and infertility. It is estimated that between 5% and 10% of women have endometriosis and between 35% and 50% of them have symptoms such as pain and infertility.^{18–20} This disease is likely to be polygenic and multifactorial, but the exact pathogenic mechanisms are still not entirely clear.^{21–23}

Studies have correlated the altered expression of Nodal with the development, progression, and aggressiveness of various cancers.^{7,24–26} We have previously observed reduced Cripto messenger RNA (mRNA) levels in endometriotic lesions as well as in the eutopic endometrium of affected women.^{3,27} However, no previous study has evaluated the protein levels of Nodal and Cripto and the SMADs involved in their signaling pathway in women with endometriosis. Therefore, the aim of this study was to assess the gene and protein expression of Nodal and Cripto and to evaluate the immunolocalization of SMAD3, phosphorylated (p) SMAD3, and SMAD4 in endometrial samples from women with and without endometriosis.

Material and Methods

Patients

This study included 15 women with endometriosis and 12 without endometriosis, who underwent diagnostic hysteroscopy at the Division of Human Reproduction of Universidade Federal de Minas Gerais in Belo Horizonte, Brazil, from November 2010 to October 2012. The age of the volunteers ranged from 22 to 43 years and was similar between the groups of women with and without endometriosis (36.0 ± 4.7 years and 33.1 ± 4.9 years, respectively, $P = .128$). Patients with pathological conditions such as polyps or fibroids and who had received steroid treatment during the previous 6 months were excluded from the study. Histological analysis of the endometrium excluded the presence of endometritis, hyperplasia, or cancer in all samples. In all cases of endometriosis, the disease had been previously confirmed by laparoscopy and pathological examination. Informed consent was obtained from each patient, and the study was approved by the local ethics committee.

Sample Collection

Endometrial samples were obtained from biopsy using Pipelle (Unimar Inc, Prodimed, Neuilly-en-Thelle, France) in the early proliferative phase of the menstrual cycle, immediately after the completion of hysteroscopy. Two portions of the endometrial tissue were immediately frozen in liquid nitrogen for mRNA extraction (real-time reverse transcription [RT] polymerase chain reaction [RT-PCR]) and protein analysis by Western blot, and another portion was fixed at Histochoice (Amresco), embedded in paraffin and processed for immunohistochemistry.

RNA Extraction, RT, and Real-Time PCR

Samples were disrupted, homogenized, and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, California). All samples were pretreated with DNase I for 15 minutes (Invitrogen) to remove undesired genomic DNA contamination. RNA was quantified by ultraviolet absorption, and 1 μ g was reverse transcribed to prepare complementary DNA (cDNA). Synthesis of cDNA from total RNA (1 μ g) was carried out in a reaction volume of 20 μ L using SuperScript III reverse transcriptase kit (Invitrogen). The RNA was initially denatured at 65°C for 5 minutes. The reaction mixture was then added, and RT was performed at 25°C for 10 minutes, 50°C for 50 minutes, and the reaction was stopped by denaturing the enzyme at 85°C for 5 minutes. The cDNA was subsequently subjected to real-time RT-PCR using the SYBR Green Master Mix (Life Technologies, Invitrogen, Carlsbad, California). The reaction conditions consisted of 1 step at 95°C for 10 minutes, followed by 40 cycles of 2 steps: 15 seconds at 95°C (denaturation) and 60 seconds at 60°C (annealing). The melting curves were obtained, which showed a single amplicon and no primer dimers. All values of the target gene (Nodal, Cripto, SMAD3, and SMAD4) were normalized to the expression of the reference gene S26. Primers were designed with the NCBI Primer-BLAST online tool and the primer sequences are available in the supplemental material.

Western Blot Analysis

The endometrial samples were lysed in a radioimmunoprecipitation assay lysis buffer (comprised of 50 mmol/L Tris, 150 mmol/L NaCl buffer, 1% Triton-100, 1% deoxycholate, 0.1% sodium dodecyl sulfate, 1 mmol/L phenylmethylsulfonyl fluoride, 10.28 mmol/L sodium orthovanadate, 10.09 mmol/L pyrophosphate sodium, 100 mmol/L sodium fluoride), prepared manually, associated with a tablet SigmaFast (Sigma; 2 mmol/L AEBSF, 1 mmol/L EDTA, Bestatin 130 μ mol/L, 14 μ mol/L E-64, 1 μ mol/L Leupeptin, 0.3 mmol/L Aprotinin). The extraction buffer was prepared at the time of use, and the samples were homogenized in ice. After extraction, the samples were centrifuged at 12 000g for 20 minutes at 4°C, the supernatant discarded, and the pellet was maintained. Total proteins of 40 μ g were incubated at 95°C for 10 minutes for protein

Figure 1. Real-time polymerase chain reaction (PCR) analysis of Nodal, Cripto, SMAD3, and SMAD4 messenger RNA (mRNA) in endometrial samples of women with and without endometriosis. Data are means $\pm$ standard error of relative gene expression. * $P < .05$ (Mann-Whitney U test).

denaturation before being loaded onto 12.5% polyacrylamide gels. After transfer to nitrocellulose membranes using a semi-dry system (BioRad), the membranes were blocked with 5% skimmed milk in phosphate-buffered (PBS) and incubated with antibodies to human Nodal (1:200), Cripto (1:200), and β -actin (1:500; all from Santa Cruz Biotechnology), followed by horse radish peroxidase-conjugated secondary antibody (1:100). The immunoreactive bands were detected using diaminobenzidine (DAB; Sigma) and 4-chloro-1-naphthol (Sigma). The densities of the immunoreactive bands were normalized to the β -actin signal, and the analyses were conducted using Image J software (National Institutes of Health, Bethesda, Maryland).

Immunohistochemistry

The paraffin-embedded specimens were cut at 4 μ m. Tissue sections were deparaffinized with xylene and hydrated with graded ethanol and PBS. The slides were microwaved in EDTA buffer for 5 minutes for better exposure of the epitopes of interest.

All steps of immunohistochemistry were performed using reagents of the amplification system for nonbiotinylated polymers Novolink (Novocastra, New Castle Upon Tyne, United Kingdom). The endogenous peroxidase activity was blocked with peroxidase block for 5 minutes. To reduce background

staining, the sections were incubated for 5 minutes with the protein block solution included in the kit. The sections were then incubated overnight at 4°C with primary rabbit polyclonal antibodies to human SMAD3, pSMAD3, and SMAD4 purchased from Abcam (Cambridge, United Kingdom) diluted 1:50. After incubation, each slide received the postprimary block for 30 minutes and then the reactant polymer was applied for 30 minutes. The reactions were developed with DAB, and the sections were counterstained with Harris hematoxylin. Negative control reactions consisted of omitting the primary antibody step from the protocol. The slides were photographed using a Zeiss optical microscope, and the digital images were analyzed using ImageJ software²⁸ by an expert investigator blinded to the source of the samples. The immunostaining was graded by the software as 0 to 10 according with the color intensity and 0 to 10 according to the proportion of labeled cells, and the final immunostaining score was automatically calculated by a built-in formula as the sum of both the grades.

Statistical Analysis

The results of real-time PCR were expressed as Δ Ct, which is the cycle threshold (Ct) of the target gene minus the Ct of the reference gene, S26. The relative gene expression was

Figure 2. Protein expression levels of Nodal, Cripto, SMAD3 (total and phosphorylated), and SMAD4 in endometrial samples of women with and without endometriosis. Data are means $\pm$ standard error of protein band density in Western blot (Nodal and Cripto) and immunostaining score (SMADS). * $P < .05$ (Mann-Whitney U test).

calculated as fold change using the formula $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t$ is the ΔC_t of the endometriosis group minus the ΔC_t of the control group.²⁹ The statistical analysis was carried out using GraphPad Prism 6. The sample size was calculated to detect a minimal difference of 1.0 standard deviation (SD) between the study groups with power = 0.80 and $\alpha = .05$. Data distribution was evaluated using the Kolmogorov-Smirnov test and did not differ significantly from normal distribution, so the results are summarized as mean $\pm$ SD. The 2 groups were compared using the Mann-Whitney U test, and $P < .05$ was considered statistically significant.

Results

The relative gene expression of Nodal, Cripto, SMAD3, and SMAD4 is shown in Figure 1. No significant difference was found in the mRNA levels of Nodal between the endometriosis and the control groups. The eutopic endometrium of women with endometriosis had significantly reduced gene expression of Cripto (fold change 0.45, $P < .05$) and SMAD 3 (fold change 0.39, $P < .05$). However, no difference was found in SMAD4 gene expression in the endometrial samples from women with and without endometriosis (Figure 1).

Figure 2 shows the protein expression levels of Nodal and Cripto, quantified by Western blot, and the immunostaining scores of SMAD3, pSMAD3, and SMAD4 in the same endometrial samples from both the groups. The protein levels of Nodal and Cripto were very similar in the endometrial samples from controls and patients with endometriosis. Total SMAD3 immunostaining score was weaker in the endometriosis group (5.0 ± 1.6) than in the controls (11.7 ± 1.9 , $P < .01$), but pSMAD3 was comparable in the 2 groups (endometriosis, 4.7 ± 1.7 ; control, 6.2 ± 1.3 , $P = .28$). Accordingly, the distribution/intensity of SMAD4 protein staining was similar in the endometriosis and control groups (5.9 ± 1.1 and 5.6 ± 0.9 , respectively, $P = .70$, Figure 2).

Figure 3 shows representative samples of endometrial biopsies processed by immunohistochemistry for SMAD proteins. All samples analyzed from controls and patients with endometriosis expressed SMAD3 in the cytoplasm and in the nucleus of endometrial cells. SMAD3 was found in the luminal epithelium, in the endometrial glands, and in the stroma. Also, pSMAD3 was expressed in the surface epithelium, glandular epithelium, and stroma of all but 2 samples, both from the endometriosis group. As expected, the localization of pSMAD3 staining was cytoplasmic and nuclear. SMAD4

Figure 3. Immunohistochemistry for SMAD3, pSMAD3, and SMAD4 in endometrial samples from the control group (C) and endometriosis group (E). Negative controls (N) are shown in the bottom. The arrows indicate examples of immunostained cells. Original magnification: 200 $\times$.

protein was localized in all endometrial tissue compartments, with clear predominance of nuclear staining in all samples evaluated (Figure 3).

Discussion

The present study investigated for the first time the mRNA and protein expression of Nodal and Cripto in the same endometrial samples from women with and without endometriosis during the proliferative phase of menstrual cycle. Our data regarding the mRNA expression of Nodal and Cripto corroborate the findings of previous studies, in which both gene transcripts were evaluated and only Cripto had lower expression in endometriosis.^{3,27} However, the resulting protein levels of Nodal and Cripto were similar between endometriosis and controls, suggesting that the endometrium of women with endometriosis does not undergo the aberrant activation of the Nodal cascade that may lead to malignant transformation.

Although endometriosis is considered to be a benign condition, it does share common characteristics with malignant cells.³⁰ Endometriosis, like cancer, can be both locally and distantly metastatic, and it can attach to other tissues, invade, and damage them.³¹ In several types of cancer, the acquisition of Nodal expression is associated with increased tumorigenesis, invasion and metastasis.^{2,6,32-34} Endometrial stromal cells express the Activin/Nodal receptor subtypes ALK 4, ActRIIA, and ActRIIB with maximum levels in the early secretory phase,³⁵ but the potential functions of Nodal signaling in the endometrial stroma remain unknown. Extrapolation from its effects on embryogenesis suggests that Nodal stimulation induces the cells to adopt features as proliferation and migration,¹¹ which are key steps in the regeneration of the endometrium.²

The actions of TGF- β family members start in the cell membrane and reach the nucleus through the activation of specific intracellular mediators, the SMAD proteins.^{36,37} SMADs are downregulated in breast cancer,^{38,39} and inactivation of the

SMAD4 gene has been correlated with the progression of pancreatic and colorectal cancer.⁴⁰ Therefore, we assessed the endometrial gene expression of SMAD3 and SMAD4 by real-time PCR and localized the respective proteins by immunohistochemistry. Despite the decrease in the mRNA levels of SMAD 3 and in the total SMAD3 protein, the levels of pSMAD3 and the mRNA and protein expression of SMAD4 were similar in patients with and without endometriosis. This dissociation between total SMAD3 and pSMAD3 levels reinforces the concept that SMAD3 phosphorylation is finely regulated by specific mechanisms,⁴¹ mainly by the ligand activation of TGF β /activin receptors.¹² We have previously shown increased activin A gene expression in proliferative endometrium of women with endometriosis.²⁷ If activin A is increased in the same cell where total SMAD3 is reduced, activin A binding to its receptor could activate SMAD3 phosphorylation and equalize the pSMAD3 levels. However, only colocalization studies could accurately test this mechanism. The relationship between SMADs and endometriosis has been poorly understood, and 1 study assessed the endometrial immunolocalization of SMADs 2-4 without significant changes in endometriosis.³⁹

Our findings are restricted to the proliferative phase of menstrual cycle and limited by the lack of subgroup analyses or adjustment of results for different endometriotic lesions and disease stages. This would only be possible with much larger numbers of women recently diagnosed with endometriosis. This limitation, however, does not preclude the characterization of molecular pathways in normal proliferative endometrium nor its comparison with a group affected by endometriosis.

In summary, proliferative endometrium of women with endometriosis shows a decrease in gene expression of Cripto and SMAD 3 and no alterations in gene and protein analysis of Nodal, as well as in the immunostaining of pSMAD3 and SMAD4, suggesting that the downregulation of Cripto mRNA is compensated downstream in the signaling pathway. Thus, our data suggest that the Nodal signaling pathway has subtle changes in the endometrium of women with endometriosis, but this imbalance may not cause functional damage as it seems not to affect the nuclear expression of SMAD4. Nonetheless, this signaling system might provide candidate molecular targets to modulate endometrial cell functions in women with and without endometriosis.

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Declaration of Conflicting Interests

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Supplemental Material

The online supplemental material are available at <http://rs.sagepub.com/supplemental>.

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