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Levels of Regulatory Proteins Associated With Cell Proliferation in Endometria From Untreated Patients Having Polycystic Ovarian Syndrome With and Without Endometrial Hyperplasia

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Abstract

Polycystic ovarian syndrome (PCOS) has been associated with endometrial hyperplasia and cancer. The aim of this study was to establish whether the expression of proliferation regulatory proteins in the endometria of patients having PCOS, with or without hyperplasia, differs from control women. Control endometria (CE), patients having PCOS without and with endometrial hyperplasia (PCOSE and HPCOSE, respectively), and that of women with endometrial hyperplasia (HE) were used. The phosphorylated estrogen receptor form (pERα), similar to mother against decapentaplegic (SMAD) 2, SMAD3, and SMAD4, vascular epithelial growth factor (VEGF), and phosphorylated SMAD (pSMAD) 2 and pSMAD3 were detected by immunohistochemistry or Western blot. The results show higher levels of pERα in HE versus CE ($P < .05$), while higher VEGF levels were found in PCOSE and HE ($P < .05$) compared to CE; SMAD2 diminished in HE ($P < .05$) versus CE. Consequently, the higher levels of VEGF and pERα in PCOSE could represent early changes in the progression of PCOSE toward hyperplasia and cancer, whereas changes observed in SMAD proteins support the differential origin of the pathologies of HPCOSE and HE.

Keywords

endometria, PCOS, proliferation, SMAD, VEGF, estrogen receptor

Introduction

Polycystic ovarian syndrome (PCOS) is an endocrine-metabolic pathology, with a high prevalence in women of fertile age, affecting between 5% and 10% of this group.[1,2,3] This pathology is related to the development of endometrial hyperplasia and carcinoma.[4,5,6,7] In this regard, PCOS has been associated with the deregulation of endometrial cell proliferation. In fact, higher levels of the cell cycle marker Ki67 have been reported in these tissues compared to control endometria (CE).[8,9]

It is known that endometrial function is importantly regulated by estrogens and that these steroids regulate cell proliferation.[10,11] This action is mediated, principally, by estrogen receptor α (ERα). Interestingly, high levels of ERα, as well as of the coactivators of steroid receptors transcriptional intermediary factor 2 (TIF-2) and amplified in breast cancer-1 (AIB1), are detected in the endometrial tissues of women with PCOS (PCOSE).[12,13,14] Additionally, recent studies have indicated that PCOSE contains elevated concentrations of androstenediol, a molecule with estrogenic activity that is able to stimulate the proliferation of human endometrial cells.[15] The activity of ERα is modified by its phosphorylation, particularly in serine residues, where phosphorylation in Ser118 modulates recruitment of coregulators and increases the transcription of genes under the control of ERα.[16,17] This posttranslational modification has been also described in human endometrial tissue.[18]

The ERα phosphorylation at Ser118 occurs when estrogenic ligands bind its receptor, and additionally it has been observed that this phosphorylation increases by the activity of mitogen-activated protein kinase (MAPK).[16] A group of molecules involved in the control of cellular proliferation and

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ERα phosphorylation are the growth factors,[19] and interestingly MAPK could be activated by growth factors.[16] Besides, reports suggest that vascular epithelial growth factor (VEGF) is involved in cellular proliferation in various cancer models[20,21] and could be participating in the preliminary stages of cancer as the development of hyperplasia. In the present investigation, we determined the relationship between phosphorylation of ERα and VEGF levels.

Another important signaling pathway in the control of endometrial cell proliferation is transforming growth factor β (TGF-β) through SMAD proteins. The TGF-β receptor phosphorylates SMAD2 and SMAD3 and induces the formation of complexes with SMAD4.[22] These complexes enter the nucleus where they can increase the transcription of genes that inhibit cell proliferation, and this molecular process is diminished in endometrial hyperplasia and cancer.[23,24] Importantly, the SMAD pathway interacts with ERα such that estrogens are able to favor the degradation of SMAD2/3 and SMAD4 complex.[22,25] Additionally, it is relevant that in an ovarian carcinoma model where SMAD4 is overexpressed, VEGF levels are decreased,[26] suggesting an interaction between molecules associated with the proliferation process. Nevertheless, other molecules could be involved in the development of endometrial hyperplasia in women with PCOS.

Based on the above-mentioned findings, the objective of the present work was to evaluate whether the endometria of patients having PCOS with or without hyperplasia exhibit a deregulation in the phosphorylation of ERα, as well as, in VEGF and TGF-β signaling pathways that could partially explain the differences in proliferation rate reported for this type of endometria. The experimental design of the present study permits to evaluate the effect of PCOS condition in the development of endometrial hyperplasia.

Materials and Methods

Hormone determinations were assayed using commercial kits: serum testosterone, androstenedione, and progesterone by radioimmunoassay (RIA; Diagnostic System Laboratories, Webster, Texas); sex hormone-binding globulin (SHBG) concentration by RIA (DPC, Los Angeles, California); and estradiol by electrochemiluminescence (Roche, Basel, Switzerland). The utilized antibodies for immunohistochemistry and/or Western blot were ERα (sc-8005, Santa Cruz Biotechnology, California), antibody for ERα phosphorylated in Ser118 (pERα; 07-487, Upstate, New York), VEGF (ab1316, Abcam [Abcam, Cambridge, UK]), SMAD2 and SMAD3 (610842, BD Biosciences, Pharmigen [Pharmigen, San Diego, CA, USA]), β-actin (A 5441, Sigma [Sigma, USA]), pSMAD2 (566415, Calbiochem [Calbiochem, San Diego, CA, USA]), pSMAD3 (9514, Cell Signaling Technology, Danvers, Massachusetts), and SMAD4 (sc-7966, Santa Cruz Biotechnology).

Participants

This is a case–control study with 4 experimental groups: PCOSE, PCOSE with hyperplasia (HPCOSE), CE, and HE. The PCOSE samples were obtained with a pipelle suction curette from the corpus of the uteri from 28 women diagnosed as having PCOS. From the 28 biopsies analyzed, 10 corresponded to HPCOSE. The diagnosis of PCOS was made according to the Rötterdam Consensus,[27] considering 2 criteria of the 3 (oligo and/or anovulation, clinical and/or biochemical signs of hyperandrogenism, and polycystic ovaries to ultrasonography). Hyperprolactinemia, androgen-secreting tumors, Cushing syndrome, congenital adrenal hyperplasia, attenuated 21-hydroxylase deficiency, and thyroid disease were excluded by appropriate tests. This investigation was approved by the School of Medicine and Clinical Hospitals Ethical Committees of University of Chile, and informed written consent was obtained from all participants. The CE samples were obtained by hysterectomy from 23 healthy women with nonneoplastic pathology. None of these women had taken oral contraceptives or other medications for at least 3 months before starting the study. On the basis of histological dating and classification according to Noyes criteria[28] by an experienced histopathologist, the endometrial samples were selected in the proliferation phase. The HE from patients without PCOS (n = 7) were obtained by hysterectomy. The diagnosis of endometrial hyperplasia in patients with and without PCOS was established according to Kurman et al criteria[29] by an experienced histopathologist.

Tissue Preparation

Endometrial tissue samples were divided into 3 or more pieces. Two pieces of each sample were frozen in liquid nitrogen and maintained at −80°C for Western blotting, and 1 piece was embedded in paraffin for immunohistochemistry.

Western Blotting

The Western blot was assessed as reported previously.[8] In brief, the fresh tissue specimens were homogenized and lysed on ice using a cell lysis buffer consisting of 20 mmol L^{-1} hepes, 2 mmol L^{-1} EDTA, 2 mmol L^{-1} EGTA, 1 v/v triton, 5 mmol L^{-1} phenylmethanesulfonylfluoride (PMSF), and Na_3VO_4 50 μmol L^{-1} containing protease inhibitor cocktail (Roche). After centrifugation at 10 000*g* for 20 minutes at 4°C, protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce, Illinois). Total proteins (50 μg from each sample) were denatured in Laemmli buffer, fractionated using 7.5 v/v 1-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and transferred to nitrocellulose membrane (BioRad, California). Blots were blocked for 2 hours in TBST (20 mmol L^{-1} Tris, pH 7.6, 137 mmol L^{-1} NaCl, and 0.1 v/v Tween 20) containing 10 v/v nonfat dry milk. Subsequently, the blots were washed 3 times for 7 minutes each in TBST and then incubated for 2 hours at room temperature with polyclonal antibodies against the phosphorylate ERα in Ser118 (pERα; 1:500); and mouse antibodies against ERα (1:300), SMAD2 and SMAD3 (1:350) overnight with rocking at 4°C, and β-ACTIN monoclonal antibody (1:15 000) were

incubated for 1 hour at room temperature. The blots were washed 3 times for 7 minutes each with TBST, followed by incubation for 1 hour at room temperature with anti-mouse immunoglobulin (Ig) G, peroxidase-linked species-specific F (ab')2 fragment (1:2000), or anti-rabbit IgG peroxidase-linked species-specific whole antibody (1:5000) while rocking. After washing 3 times for 7 minutes each with TBST, the bound antibodies were detected with an enhanced chemiluminescence system (Amersham International, GE Healthcare, United Kingdom). Band intensities were quantified by scanning densitometry utilizing the UN-SCAN-IT software gel 6.1. The results were expressed as a ratio of the housekeeping gene β-ACTIN.

Immunohistochemistry

Immunostaining was performed on 5-μm sections of formalin-fixed, paraffin-embedded endometrial biopsies. The assay was performed as reported previously.[30] Briefly, tissue sections were deparaffinized in xylene and hydrated in a series of graded alcohols. Endogenous peroxidase activity was prevented by incubating the samples in 3 v/v hydrogen peroxide for 5 minutes. The sections were incubated in antigen retrieval solution (10 mmol L^{-1} sodium citrate buffer) at 95°C for 20 minutes. Nonspecific antibody binding was prevented with 4 v/v phosphate-buffered saline–bovine serum albumin for 1 hour. The samples were incubated for 18 hours at 4°C with the specific antibody: VEGF (1:1000), pSMAD2 (1:1000), pSMAD3 (1:150), and SMAD4 (1:1600). Negative controls were analyzed on adjacent sections incubated without the primary antibody and using nonimmune species-specific antisera. The second antibody was a biotinylated broad-spectrum antibody (Zymed Laboratories Inc). The reaction was developed by the streptavidin-peroxidase system, and 3,3′-diaminobenzidine (DAB #K3467, Dako, Carpinteria, California) was used as the chromogen; counterstaining was carried out with hematoxylin. The slides were evaluated in a Nikon optical microscope (Nikon Inc, Melville, New York). For VEGF, the evaluation was made detecting the intensity grade (i) of the staining and evaluated as strong (grade 3), moderate (grade 2), or weak (grade 1), and integrated in a semiquantitative parameter[31,32] named the histochemical score (HSCORE = [% of positive cells × (i 1)]/100). The results were expressed as H-SCORE (for VEGF) or percentage of positive cells (for other proteins). Each protein was evaluated by 3 independent observers, and the positive staining was assessed in at least 3000 cells per sample.

Statistical Evaluation

The number of participants in this study was calculated assuming $\alpha = .05$ and $\beta = 20\%$, a difference between means of 0.2 and standard deviation of 0.165 according to our previous studies.[8,14] Comparisons between groups were performed by nonparametric Mann Whitney *U* test. *P* values <.05 were considered significant. Statistical tests were performed using Graph Pad Prism 6.

Results

Clinical and Endocrine Characteristics

The clinical and endocrine characteristics of women participants in this study are summarized in Table 1. The control group shows a higher range of age than women with PCOS. The higher body mass index observed in the group of women with PCOS is inherent to the syndrome, which is consistent with the fact that around 60% to 70% of patients with PCOS are obese.[33] In addition, all women with PCOS presented hyperandrogenism, and the excessive ovarian androgen production, besides the decreased SHBG blood level, leads to a significantly higher free androgen index in women with PCOS.

Protein Levels of the ERα Phosphorylated in Ser118

The phosphorylation of ERα is well accepted as an important positive modulator of the transcriptional activity of the receptor. The results showed that the ratio of pERα to ERα was greater in endometria of women with hyperplasia without PCOS (HE; 500%, $P = .03$) than in CE (Figure 1). Additionally, PCOSE and HPCOSE exhibited increased levels compared to CE (72% and 168%, respectively, $P > .05$; Figure 1).

Immunodetection of VEGF

As reported, VEGF is highly related to uncontrolled proliferation pathologies, such as cancer.[20,21] We determined that VEGF levels in both epithelial and stromal compartments are significantly higher in PCOSE ($P = .002$ and $P = .004$, respectively) compared to CE. The immunostaining was less intense in CE than in HE ($P = .01$ and $P = .02$, respectively). Additionally, the levels of expression of the protein VEGF were greater in PCOSE than in HPCOSE epithelia ($P = .02$; Figure 2 and Table 2).

Protein Levels of SMAD2, SMAD3, and SMAD4 and Their Phosphorylated Forms

It is well known that the proliferative process can be importantly regulated by the SMAD protein pathway. The results showed a nonstatistically significant reduction in the protein expression levels of SMAD2 in PCOSE (34%, $P = .5$) compared to CE, whereas the difference between HE and CE was significant (73%, $P = .03$; Figure 3). On the other hand, the immunodetection of the phosphorylated (active) forms of SMAD2 in the different endometrial groups showed nuclear staining and a diminution in stroma of HPCOSE compared to PCOSE ($P = .04$). Furthermore, HPCOSE showed less intense immunostaining than HE at the stromal and epithelial compartments ($P = .03$; Figure 2 and Table 2). In the case of pSMAD3, a lower nuclear staining than pSMAD2 was observed in all studied endometria and fundamentally of glandular location (Figure 2). Besides, a diminution of pSMAD3 from HPCOSE compared to PCOSE (in epithelial cells; $P = .02$) and compared to HE (at stromal compartment; $P = .03$) was also

Table 1. Clinical and Hormonal Characteristics of Patients.[a]

	C	PCOS	HPCOS	H
N	23	18	10	7
Age	37.1 ± 1,5	26.6 ± 1.0[b]	29.4 ± 1.4[b]	42.1 ± 2.9
BMI, kg/m^2	25.6 ± 1.0	31.4 ± 1.6[b]	31.9 ± 2.0[b]	27.2 ± 1.8
P_4, ng/mL	0.9 ± 0.5	1.0 ± 0.3	1.55 ± 1.1	0.61 ± 0.1
E_2, pg/mL	53.7 ± 13	60.4 ± 5.4	74.4 ± 8.3	98.4 ± 30.6
A_4, ng/mL	1.7 ± 0.3	4.5 ± 1.6[b]	3.2 ± 0.7	1.2 ± 0.3
T, ng/mL	0.4 ± 0.1	0.9 ± 0.1[b]	0.8 ± 0.0[b]	0.55 ± 0.02
SHBG, nmol/L	60.4 ± 16.1	23.8 ± 3.1[b]	25.7 ± 5.5[b]	46.9 ± 0.8
FAI	2.7 ± 0.7	15.0 ± 2.5[b]	11.9 ± 2.3[b]	4.0 ± 0.2

Abbreviations: C, control women; PCOS, women with polycystic ovarian syndrome without endometrial hyperplasia; HPCOS, women with polycystic ovarian syndrome with endometrial hyperplasia (HPCOS); H, women with endometrial hyperplasia; n, number of women included in the study; BMI, body mass index; P_4, progesterone; E_2, estradiol; A_4, androstenedione; T, testosterone; SHBG, sex hormone binding globulin; FAI, free androgen index; SEM, standard error of the mean.
[a]The values are expressed as mean ± SEM.
[b]$P < .05$ versus C.

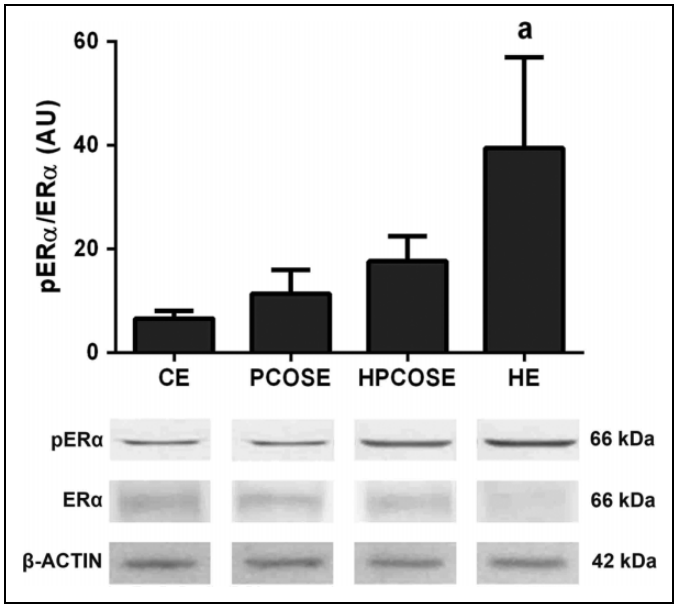

Figure 1. Phosphorylation of estrogen receptor α (pERα) in Ser118. The phosphorylation was detected by Western blot in total protein extracts of different groups of tissues: Control proliferate endometria (CE), endometria of women with polycystic ovarian syndrome without hyperplasia (PCOSE) and with endometrial hyperplasia (HPCOSE), endometria of patients without polycystic ovarian syndrome and with endometrial hyperplasia (HE). The results were normalized with respect to estrogen receptor α (ERα). β-actin is included in the representative photography. The number of women evaluated was n = 8 for CE, PCOSE, and HPOSE and n = 6 for HE. [a]$P < .05$ versus CE.

obtained (Figure 2 and Table 2). For SMAD4, the positive immunostaining was observed at the nuclear level and to a lesser extent in the cytoplasm (Figure 2). The semiquantitative analysis showed that the level of SMAD4 diminished in HPCOSE nuclei of stromal cells compared with HE ($P = .01$). Meanwhile, epithelia of PCOSE showed less positive nuclei than HPCOSE ($P = .02$; Figure 2 and Table 2).

Discussion

It is known that steroid receptors undergo posttranslational modifications, such as acetylation and phosphorylation and that these modifications influence their activity,[16,34] particularly phosphorylation on Ser118 ERα increases the activity of this receptor.[16,35] Moreover, greater sensitivity to estrogen action exhibited by different tissues, including the endometrium, could be associated with the phosphorylated status of steroid receptors.[18] The present work reports that the fraction of pERα related to total ERα exhibits a significant increase in endometria that present hyperplasia in HE and HPCOSE. Although not significant, it is also important to note the 72% increase in the phosphorylated fraction in PCOSE compared to CE (Figure 1). These results allow us to suggest that the increase in pERα could be associated with a higher transcriptional activity of the receptor in pathological endometria. Interestingly, high levels of the ERα and coactivators described in PCOSE[13] could also contribute to a high sensitivity to estrogen action. This phenomenon would be essential for the progression of proliferative processes toward hyperplasia. Along these lines, the hyperplastic endometrium could have even more estrogenic activity associated with high phosphorylation of the ERα[16] as it is described in this article (Figure 1).

It is most likely that the deregulation in the transcription of genes involved in the control of the cell cycle could potentially induce endometrial development of hyperplasia in women with PCOS. As known, the endometria of women with oligo or anovulatory PCOS are continuously exposed to estrogen action for larger periods of time than those from normal women, in the presence of very low concentrations of progesterone. In addition, recent results indicate that in endometria of women with PCOS exists a high metabolism of dehydroepiandrosterone to androstenediol[32] and increased concentrations of androstenediol.[15] Androstenediol is structurally an androgen although functions as an estrogen, activating nuclear receptors.[36,37,38] It has been described that androstenediol exhibits similar effects to 17β-estradiol in human endometrial cell lines. Androstenediol modulates molecules involved in favoring cell cycle progression, which would increase cellular proliferation of endometrial cell lines and tissues.[15] Furthermore, the endocrine status of PCOS, where estrogen action at the endometrial level is enhanced,[13,14] could induce a deregulation of the cellular cycle in endometrial cells.[9,39]

The mechanism by which increased ERα phosphorylation at Ser118 could be activated is through the binding of the ligand.[16] As reported, in endometrium of women with PCOS, androstenediol could also contribute to the hyperestrogenic condition.[15] However, another pathway that favors phosphorylation of ERα on Ser118 is MAPK.[16] The MAPK pathway can be activated by growth factors, such as VEGF. In fact, high protein levels of VEGF present in endometrial tissue with hyperplasia and/or in women with PCOS (Figure 2) allow us to

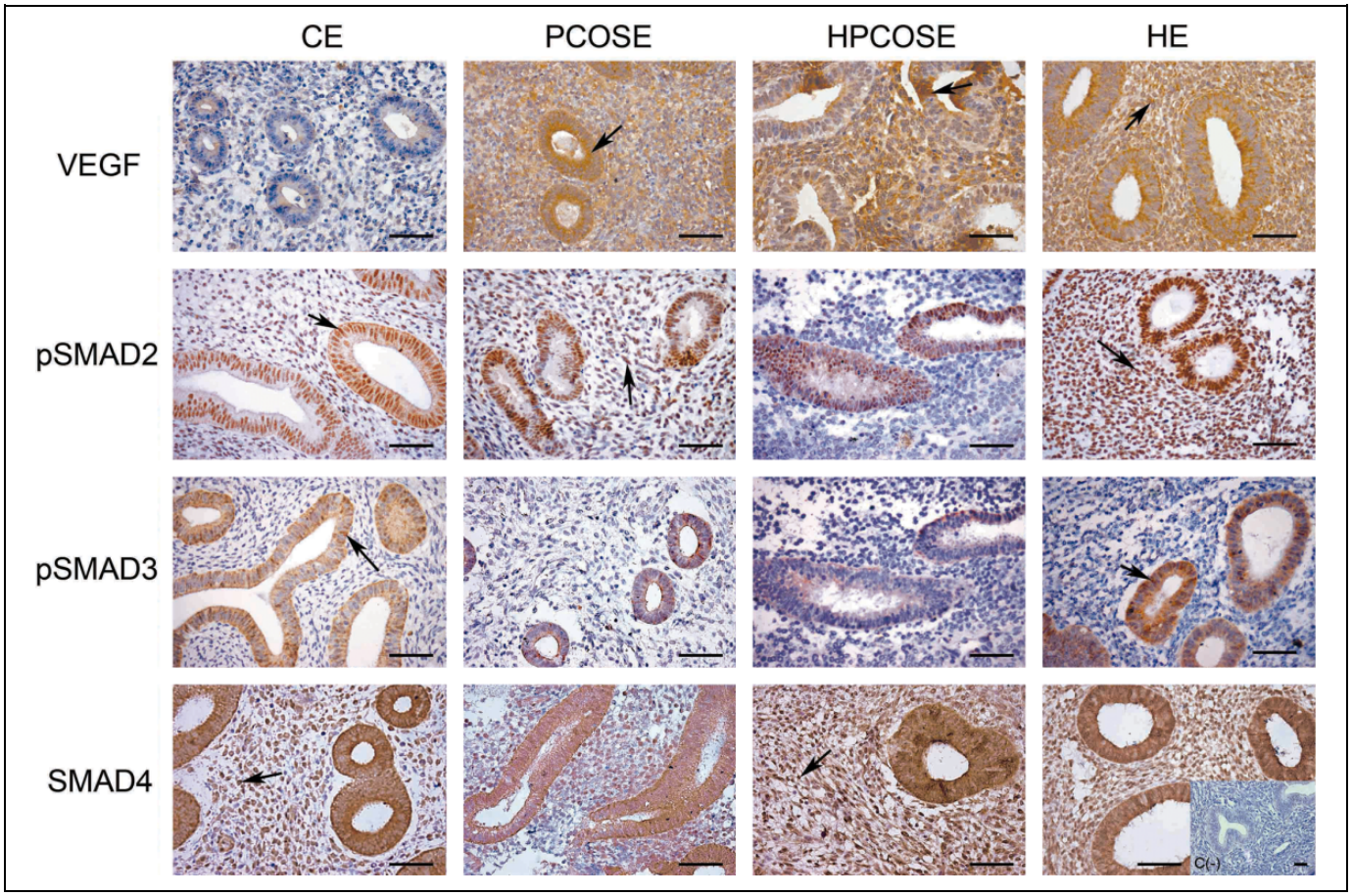

Figure 2. Immunohistochemistry of VEGF, pSMAD2, pSMAD3, and SMAD4 in endometrial tissue. The immunostaining was evaluated in control proliferate endometrium (CE), polycystic ovarian syndrome endometria without (PCOSE) and with endometrial hyperplasia (HPCOSE), endometria of patients without polycystic ovarian syndrome and with endometrial hyperplasia (HE). The bar represents 50 μm and the arrows indicate positive staining. Insert: C (−): negative control.

Table 2. Semiquantitative Analysis of VEGF by HSCORE, pSMAD2, pSMAD3, and SMAD4 by Percentage of Positive Nuclei in Human Endometrial Tissue.[a]

		CE	PCOSE	HPCOSE	HE
VEGF	EEC	1.8 ± 0.2	3.7 ± 0.1[b]	3.3 ± 0.1[c]	3.0 ± 0.1[b]
	ESC	1.6 ± 0.2	3.4 ± 0.1[b]	3.2 ± 0.03	3.0 ± 0.2[b]
pSMAD2	EEC	95.7 ± 2.0	90.4 ± 5.3	80.4 ± 7.9	98.5 ± 1.2[d]
	ESC	83.6 ± 6.0	81.7 ± 5.8	46.3 ± 14.4[c]	91.9 ± 6.6[d]
pSMAD3	EEC	18.6 ± 5.6	22.8 ± 6.3	0.7 ± 0.6[c]	28.2 ± 12.9
	ESC	1.8 ± 0.9	0.3 ± 0.2	0.01 ± 0.0	7.0 ± 3.2[d]
SMAD4	EEC	74.0 ± 3.8	62.0 ± 6.4	87.7 ± 6.6[c]	89.2 ± 5.7
	ESC	71.0 ± 3.0	57.8 ± 5.7	61.3 ± 6.0	87.1 ± 2.4[b,d]

Abbreviations: EEC, epithelial cells; ESC, stromal cells of endometrium; CE, control proliferate endometrium; PCOSE, polycystic ovarian syndrome endometria without endometrial hyperplasia; HPCOSE, polycystic ovarian syndrome endometria with endometrial hyperplasia; HE, endometria of patients without polycystic ovarian syndrome and with endometrial hyperplasia; SEM, standard error of the mean

[a]The results are expressed as the mean of the percentage of positive cells ± SEM.

[b]$P < .05$ vs CE.

[c]$P < .05$ vs PCOSE.

[d]$P < .05$ vs HPCOSE.

consider that this growth factor may be involved in favoring phosphorylation of ERα (Figure 4). The VEGF, through receptor tyrosine kinases, can activate the MAPK pathway[40] and thereby increase phosphorylation of this nuclear receptor.[16] Additionally, it is important to consider that estrogens increase VEGF expression, since its promoter region has an estrogen

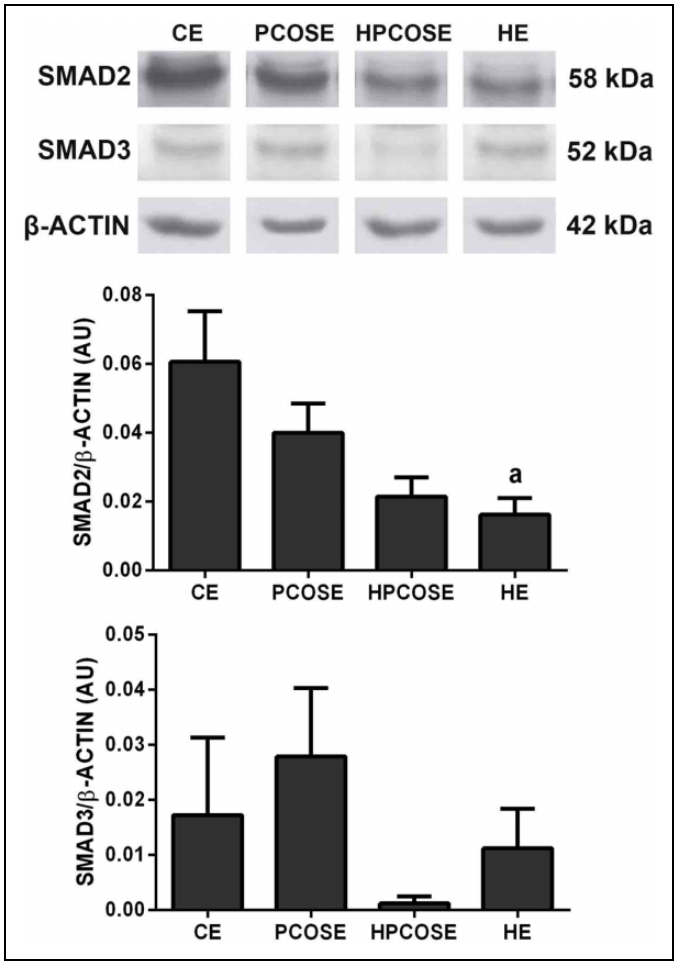

Figure 3. SMAD proteins levels were evaluated by Western blot. The studied groups were endometria from controls (CE; n = 8), from patients with polycystic ovarian syndrome without (PCOSE; n = 8) and with endometrial hyperplasia (HPCOSE; n = 8), and endometria with hyperplasia not associated to polycystic ovarian syndrome (HE; n = 6). SMAD2 and SMAD3 were normalized versus β-actin. The results were expressed as mean ± standard error of the mean (SEM). [a]$P < .05$ versus CE.

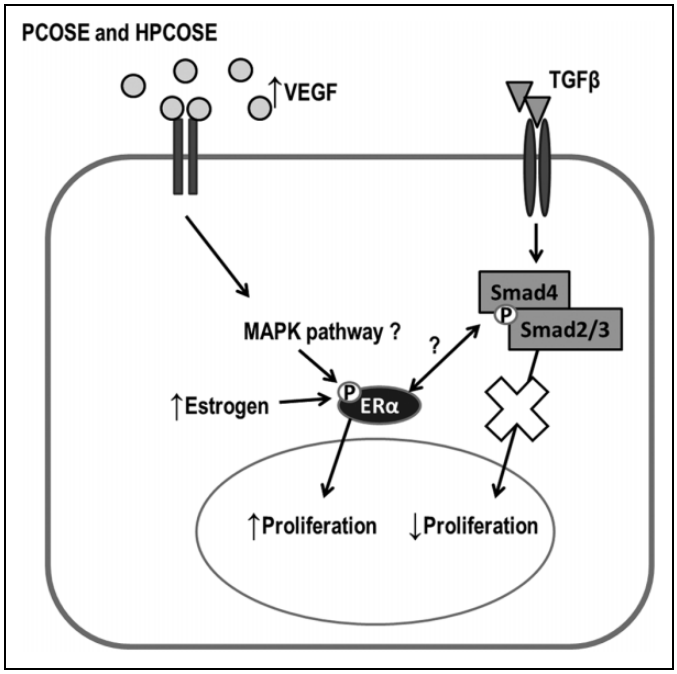

Figure 4. Molecular model proposed for endometrium of women with polycystic ovary syndrome without hyperplasia (PCOSE) or with hyperplasia (HPCOSE). High levels of VEGF could activate the MAPK pathway. It is known that this pathway promotes estrogen receptor α (ERα) phosphorylation in Ser118. Additionally, high levels of steroids with estrogenic activity in these tissues have been described. Therefore, high levels of phosphorylation and ligands would enhance the activity of ERα. Meanwhile, the TGFβ-SMADs pathway is decreased, which could be caused by the action of ERα. This model could explain the high PCOSE tissues proliferative activity, allowing progression to the development of hyperplasia.

response element,[20,41] therefore this process could be self-exacerbated. In order to better understand the regulatory mechanisms of endometrial cell survival, we evaluated the participation of the SMAD pathway in normal and pathological tissues. In the present work, it was found that there were diminished levels of SMAD2 in HE compared to CE (Figure 3). Additionally, pSMAD3 in PCOSE and HPCOSE was found at lower levels than their respective controls, CE and HE (Figure 2). These data are relevant in that SMAD proteins constitute regulatory molecules of cellular proliferation and apoptosis. In fact, it has been indicated that the TGF-β signaling pathway is a tumor suppressor pathway whose activity can be lost in many types of cancer, including the endometrial cancer.[42,43] Moreover, it has been reported that the activation of this pathway is responsible for the translocation of p27 from cyclin D1/cyclin-dependent kinase (CDK) 4 and 6 complex toward cyclin E/CDK2 complex and, consequently, for the inhibition of the cellular cycle progression.[44] Therefore, the results of the present study could indicate that in endometria of women with PCOS exists a diminution of the suppressor activity of the transition from G1 to S phase of the cellular cycle through the action of SMAD proteins, inducing the progression of the cellular cycle.[22,45,46] The deregulation of SMAD proteins is probably associated with high estrogenic sensitivity and activity characterized in PCOSE and especially in HPCOSE.

Meanwhile, in previous studies from our laboratory, it was determined that cyclin D1, a positive regulator of the cell cycle, is increased in PCOSE.[15] Likewise, recent studies have demonstrated an inverse relationship between cyclin D1 and molecules of the TGF-β pathway.[47] Consequently, the greater levels of cyclin D1 observed in the cellular nuclei together with the decreased levels of SMAD2 and pSMAD3 in PCOSE (Figures 2 and 3) could contribute to the greater number of cells in the proliferation cycle found in these endometria as we have reported previously.[9]

Consequently, the differences found in the levels of the SMAD proteins in the studied groups could alter the activation of steroid receptors and, thus, tissue homeostasis. In fact, a coordinated function of the SMAD signaling pathway with the nuclear receptors has been reported,[48] that is, the SMAD proteins are able to regulate the transactivation of steroid

receptors, and the steroid receptors are able to regulate the activity of the SMAD proteins. In this context, it has been published in prostate cancer that SMAD3 is physically bound to the androgen receptor modulating its transactivation activity.[48]

Interestingly, the results of the higher levels of pSMAD2, pSMAD3, and SMAD4 detected in HE compared to CE or HPCOSE (Figure 2) were unexpected. However, it is noteworthy that in some conditions, the TGF-β pathway, rather than acting to suppress cell proliferation, could have the opposite effect, given that molecules can associate with MAPK/extracellular signal-regulated kinases or phosphatidylinositol 3′-kinase/Akt, classical proproliferation pathways.[49]

Endometria from patients with PCOS, with or without hyperplasia, behave similarly regarding p-ERα, VEGF, pSMAD2, pSMAD3, and SMAD4 protein levels; however, it cannot be ruled out the possibility that the PCOS condition could have a role in the genesis of hyperplasia (Figures 1 and 2).[4,5,6,7] However, endometria of patients with hyperplasia and without PCOS present increased SMAD4 levels (Figure 2). This probably indicates that the molecular origin of hyperplasia in both cases may be different although with similar clinical manifestation.

Based on the different results found in the present investigation for the analyzed groups of endometria, and despite the similar morphologic characteristics of HPCOSE and HE, and the similar hormone microenvironment in which PCOSE and HPCOSE are developed, the data strongly suggest that these 3 types of endometria exhibit a different molecular behavior. Thus, the molecular mechanisms that govern the development of endometrial hyperplasia in women with PCOS could be different from those of patients without PCOS, constituting this issue an important fact for the therapeutic decisions that must be taken in both groups of patients. Even more, the data of this work indicate that these pathological endometria which are accompanied by important molecular alterations still have regulatory mechanisms of their tissue homeostasis that resists the tendency to a greater proliferation. Therefore, additional studies are necessary to understand how these endometria restrain, for a variable period of time, their malignant transformation.

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Declaration of Conflicting Interests

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