

Metabolic, Endocrine and Genitourinary Pathobiology

Disrupted Cell Cycle Control in Cultured Endometrial Cells from Patients with Endometriosis Harboring the Progesterone Receptor Polymorphism PROGINS

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Presently, little is understood about how endometriosis is established or maintained, or how genetic factors can predispose women to the disease. Because of the crucial role that the progesterone receptor polymorphism PROGINS plays in predisposing women to the development of endometriosis, we hypothesized that this variant may influence critical steps during endometrial cell metabolism that are involved in the pathogenesis of endometriosis. Eutopic endometria were collected from three sources: women with endometriosis who had a single PROGINS allele (from the progesterone receptor gene); women with endometriosis who had the wild-type progesterone receptor allele; and women without endometriosis who had the wild-type allele. Cells prepared from the eutopic endometria of these women were stimulated with both estradiol and progesterone, and then examined for cell proliferation, viability, and apoptosis. The cells from women with endometriosis that carried the PROGINS allele demonstrated increased proliferation, greater viability, and decreased apoptosis following progesterone treatment. In general, these parameters were very different as compared with those of women with endometriosis but without the PROGINS allele and women in the control group. This result indicates there is a reduced level of progesterone responsiveness in women who carry the PROGINS polymorphism. Because progesterone responsiveness is known to be an important characteristic of

women with endometriosis, these data support the contention that the PROGINS polymorphism enhances the endometriosis phenotype. (Am J Pathol 2009, 175:215–224; DOI: 10.2353/ajpath.2009.080966)

Endometriosis is a chronic inflammatory disease characterized by implantation and growth of endometrial tissue outside of the uterus.¹ It affects 10% to 15% of all women of reproductive age, and it is significantly associated with infertility,² chronic pain,³ and morbidity,⁴ making endometriosis a significant problem for public health. In 1925, Sampson et al⁵ suggested that the transtubarian reflux of viable endometrial cells represents the origin of endometriosis. However, several subsequent studies reported that approximately 90% of women have viable endometrial cells in the peritoneal cavity,^{6,7} disputing the notion that retrograde menstruation theory could explain the cause of the disease. It is also noteworthy that only a small portion of women with retrograde menstruation develops endometriosis.

Environmental, endocrine, immune, and genetic factors have all been related to the pathogenesis of endometriosis. Of note, genetic studies of close relatives suggest that there is a 6% increase in the risk of developing endometriosis.⁸ Several studies also suggest that women

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Some preliminary results of this paper were presented at the 10th World Congress on Endometriosis, held in Melbourne, Australia (in March 2008), and it was honored with the "Best Scientific Poster Award" by the World Endometriosis Society (WES) and the Australian Gynaecological Endoscopy Society (AGES).

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with endometriosis present abnormalities in the eutopic endometrium, raising questions about whether the uterine mucosa is involved in the pathogenesis of the disease.

In that context, modifications of cell cycle control, with increased levels of cell proliferation⁹ and decreased apoptosis,¹⁰ emerge as major mechanisms responsible for endometriosis development. Likewise, enhanced cell adhesion and invasion via increased expression of matrix metalloproteinases and the simultaneous down-regulation of their inhibitors,^{11–13} as well as abnormal sex hormone metabolism,^{14,15} are additional hallmarks of the disease.

Progesterone plays a major role in the processes mentioned above,¹⁶ reinforcing the theory that impaired progesterone function could facilitate the genesis and development of endometriosis.^{17,18} Interestingly, several studies demonstrated that progesterone is able to induce the expression of a large number of genes in the eutopic endometrium. Microarray analysis reported by Burney et al¹⁹ demonstrated that *FOX10*, *MIG6*, and *CYP26A1* expression was significantly modified in the uterine mucosa. In another study, Wang et al used a baboon model of endometriosis and demonstrated that the expression of progesterone responsive factors is altered during the secretory stage of the menstrual cycle, suggesting that progesterone resistance plays a major role in the genesis of endometriosis.²⁰

Several studies have addressed the question of whether genetic mutations contribute to the development of endometriosis. In these studies, several candidate genes and polymorphisms were associated with the development of endometriosis.²¹ One particular candidate, the progesterone receptor (PR) gene variant named PROGINS (NCBI Data Bank accession numbers AF016381 and Z49816), has emerged as an important disease component of endometriosis. PROGINS is characterized by a 306-bp insertion in intron G, which exists in linkage disequilibrium with point mutations in exons 4 and 5.²² Epidemiological studies have shown that women carrying the PROGINS polymorphism have an increased risk for the development of hormone-dependent gynecological disorders, such as endometrial and ovarian carcinomas, recurrent abortions, and uterine fibroids,^{23–28} conditions in which progesterone plays a critical role.

Several researchers, including those in our laboratory, have reported that patients carrying a single PROGINS allele have an increased risk for endometriosis development.^{29–32} In addition, *in vitro* data from Romano et al demonstrated that the PROGINS variant of the PR gene is less responsive to progestins, as compared with wild-type PR, resulting in reduced mRNA stability and protein activity, as well as a diminished ability to efficiently inhibit cell proliferation in rodent ovarian cell lines.³³ Yet the effects of the PROGINS polymorphism on the phenotype of eutopic endometrial cells have never been described. The goal of the present study was to evaluate the influence of the PR variant PROGINS on cell viability, apoptosis, and proliferation in cell cultures of eutopic endometrium from women with and without endometriosis.

Materials and Methods

Study Participants and Sample Collection

Endometrial tissues were obtained from informed volunteers in the Endometriosis Unit/Department of Gynecology at the Federal University of São Paulo (UNIFESP). The Institutional Ethics Review Board (CEP0571/06) had previously approved the study. Women undergoing laparoscopy for routine evaluation of infertility, pelvic pain, or elective tubal sterilization were recruited. All patients had a history of regular menses and were not taking any sex steroids or steroid-modulating medications 3 months before surgery. After general anesthesia and just before the surgical procedure, endometrial tissues were collected using the Nowak's curette. During laparoscopy, a systematic observation of the pelvis was conducted and the patients were assigned to either the endometriosis or the control group. The collected endometrial tissue was separated into two parts: one for histological analysis according to the criteria of Noyes et al³⁴ and the other for cell culture processing. Data for the patients' laparoscopic diagnosis, age, menstrual cycle date, and stage of endometriosis were also recorded (Table 1).

PROGINS Genotyping

Before the endometrial biopsy procedure, peripheral blood samples were obtained from the patients. Genomic DNA (gDNA) was extracted and purified using the GFX DNA extraction kit (GE Healthcare, Little Chalfont Buckinghamshire, UK). Genotyping of the PROGINS polymorphism was performed by PCR as described previously.²⁹ Endometriosis and control groups were subdivided based on detection of PROGINS: control (CP-Alu), endometriosis wild-type homozygous (E-Alu), and endometriosis PROGINS heterozygous (E+Alu).

Tissue Isolation and Culture

Specimens were transported to the laboratory in culture medium supplemented with antibiotics and antimycotics. Tissues were minced into small pieces and treated with collagenase IA (Life Technologies, Inc., Grand Island, NY), as previously described.³⁵ No procedures were performed to isolate epithelial or stromal cells. Cells from each individual specimen were plated in 100-mm diameter culture dishes (TPP, Trasadingen, Switzerland). After 16 hours of incubation, non-adherent cells were washed away. The typical yield of stromal cells using this technique is 90%.³⁶ Cells were grown in phenol-free Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO) containing 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Rockville, MD), 100 IU/ml penicillin, 100 mg/ml streptomycin (Sigma-Aldrich), and 250 µg/ml amphotericin-B (Cultilab, Campinas, Brazil) in a humidified incubator at 37°C with 5% CO₂. Adherent cells were characterized by immunofluorescence using specific monoclonal antibodies against cytokeratin (Santa

Table 1. Characteristics of Samples Used in the Study

Groups	Samples	Age (yr)	Cycle date	Stage of endometriosis*	Laparoscopic diagnose	PROGINS genotype	Sample selected for an experiment [+]/Passage no. used								
							IF	qPCR	B/AB	Ct	Tm	CC	V/A	A5	CM
Endometriosis (E-Alu)	1	41	Proliferative	Stage I	Endometriosis	T1/1	+/1, 4-7	+/5	+/5	-	-	+/6	+/7	+/7	-
	2	34	Secretory	Stage I	Endometriosis	T1/1	+/1, 4-7	-	-	-	-	+/6	-	+/7	-
	3	23	Proliferative	Stage II	Endometriosis	T1/1	+/1, 4-7	+/5	+/5	+/5	+/5	+/6	+/7	+/7	-
	4	26	Proliferative	Stage IV	Endometriosis	T1/1	+/1, 4-7	+/5	+/5	-	-	+/6	+/7	+/7	+/5
	5	42	Secretory	Stage I	Endometriosis	T1/1	+/1, 4-7	-	+/5	-	-	-	+/7	-	-
	6	33	Proliferative	Stage I	Endometriosis	T1/1	+/1, 4-7	-	-	-	-	-	+/7	-	-
	7	33	Proliferative	Stage III	Endometriosis	T1/1	+/1, 4-7	-	+/5	+/5	+/5	-	-	-	+/5
	8	35	Proliferative	Stage III	Endometriosis	T1/1	+/1, 4-7	+/5	-	+/5	+/5	+/6	+/7	+/7	-
	9	33	Secretory	Stage II	Endometriosis	T1/1	+/1, 4-7	+/5	+/5	+/5	+/5	+/6	+/7	+/7	+/5
	10	22	Secretory	Stage I	Endometriosis	T1/1	+/1, 4-7	+/5	-	+/5	+/5	+/6	-	+/7	+/5
Endometriosis (E+Alu)	11	24	Proliferative	Stage I	Endometriosis	T1/2	+/1, 4-7	-	+/5	+/5	+/5	+/6	+/7	+/7	+/5
	12	36	Secretory	Stage IV	Endometriosis	T1/2	+/1, 4-7	-	+/5	+/5	+/5	+/6	+/7	+/7	+/5
	13	35	Secretory	Stage I	Endometriosis	T1/2	+/1, 4-7	-	+/5	+/5	+/5	+/6	+/7	+/7	+/5
	14	37	Proliferative	Stage I	Endometriosis	T1/2	+/1, 4-7	-	+/5	+/5	+/5	+/6	+/7	+/7	+/5
	15	28	Secretory	Stage I	Endometriosis	T1/2	+/1, 4-7	-	+/5	+/5	+/5	+/6	+/7	+/7	+/5
	16	29	Proliferative	Stage IV	Endometriosis	T1/2	+/1, 4-7	-	+/5	-	-	-	-	-	-
Control (CP-Alu)	17	31	Secretory	N/A	Normal pelvis	T1/1	+/1, 4-7	-	-	+/5	+/5	-	+/7	-	+/5
	18	38	Secretory	N/A	Ovarian cyst	T1/1	+/1, 4-7	-	+/5	+/5	+/5	-	-	-	+/5
	19	30	Secretory	N/A	Pelvic adhesions	T1/1	+/1, 4-7	+/5	+/5	+/5	+/5	+/6	+/7	+/7	-
	20	26	Proliferative	N/A	Adnexal cyst	T1/1	+/1, 4-7	+/5	+/5	+/5	+/5	+/6	-	+/7	-
	21	36	Proliferative	N/A	Normal pelvis	T1/1	+/1, 4-7	-	-	+/5	-	-	+/7	-	-
	22	33	Proliferative	N/A	Adnexal cyst	T1/1	+/1, 4-7	+/5	+/5	-	-	+/6	+/7	+/7	-
	23	31	Proliferative	N/A	Pelvic adhesions	T1/1	+/1, 4-7	+/5	-	-	-	+/6	+/7	+/7	-
	24	25	Secretory	N/A	Ovarian cyst	T1/1	+/1, 4-7	-	-	-	-	-	+/7	-	+/5
	25	28	Proliferative	N/A	Normal pelvis	T1/1	+/1, 4-7	-	-	-	-	-	+/7	-	+/5
	26	34	Proliferative	N/A	Normal pelvis	T1/1	+/1, 4-7	+/5	+/5	-	-	+/6	+/7	+/7	-

Eutopic endometrium was obtained from control patients (CP-Alu) without the PROGINS allele, and from women with endometriosis with (E+Alu) and without (E-Alu) the single PROGINS allele.

N/A = Not applicable.

PROGINS genotype: T1/1 = homozygous major allele, T1/2 = PROGINS heterozygous.

[+] = sample selected in an experiment, [-] = sample not used.

IF = immunofluorescence, qPCR = time and dose response quantitative real-time PCR for PR; B/AB = PRB/AB mRNA expression ratio; Ct = Cell Counting; Tm = thymidine incorporation assay; CC = cell cycle analysis; V/A = viability and apoptosis (Viacount assay); A5 = Annexin-V labeling; and CM = chromatin morphology analysis. Experiments were performed between passages 4 and 7.

*Revised American Society for Reproductive Medicine classification of endometriosis.

Cruz Biotechnology, Santa Cruz, CA) and vimentin (Dako Corp., CA). The proportions of cytokeratin-positive cells (endometrial glandular cells) and vimentin-positive cells (endometrial stromal cells) were assessed in each cell culture, as described previously.³⁷ Normal goat serum (Sigma-Aldrich) was used instead of the primary antibody as a negative control. Because the cultures presented high percentages of vimentin-positive cells, human breast ductal carcinoma cells (T47D cell line), which constitutively express cytokeratin in the form of intermediate filaments, were used as positive controls for cytokeratin to ensure the specificity of the method.

Quantitative Real-Time Reverse Transcription PCR

A time-course and dose-response study was conducted with 17β-estradiol (Sigma-Aldrich) to induce optimal progesterone receptor mRNA expression for the cytometric experiments with progesterone.³⁸ Total RNA was extracted from the endometrial cells (RNeasy kit, Qiagen Inc, Hilden, Germany), and the reverse transcriptase reaction (Superscript-III, Invitrogen) was performed with 1 μg of DNase I (G&E Healthcare Biosciences, Upsala, Sweden)-treated

RNA. qPCR was performed using the ABI 7700 thermocycler (Applied Biosystems, Foster City, CA) and the Power SYBr Green Mastermix PCR kit (Applied Biosystems), according to the manufacturer's instructions. PCR primers (IDT, Coralville, IA) were synthesized with the following sequences: total progesterone receptor PR (A+B): forward primer 5'-ACACAAAACCTGACACCTCC-3' and reverse primer 5'-TACAGCATCTGCCCACTGAC-3'; progesterone receptor subunit B (PR-B): forward primer 5'-GCCAGAGAAAAAGTCGGGAG-3' and reverse primer 5'-TGGGGAGCGCAAGAAAAG-3'³⁹; and glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward primer 5'-ACCACAGTCCATGCCATCAC-3' and reverse primer 5'-TCCACCACCCTGTTGCTGTA-3'.⁴⁰ The polymerase chain reaction was performed with the following conditions: one cycle at 95°C for 15 minutes and 35 cycles at 94°C for 15 seconds, 60°C for PR (A+B) and PR-B or 58°C for GAPDH for 30 seconds, and 72°C for 30 seconds. Cycle numbers obtained at the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted control samples. The expression of target PR (A+B) and PR-B genes was normalized to GAPDH mRNA levels, which were measured concurrently.⁴¹ The specificity of each reaction was confirmed by melting curve analysis and agarose gel electrophoresis.

Viability and Apoptosis Analyses by the Viacount Assay

Endometrial cells (2×10^4) seeded in 6-well plates at 50% to 70% confluence were treated with 100 nmol/L 17β -estradiol for 24 hours, followed by 100 nmol/L progesterone for 24 hours. Cells were mixed with Guava Viacount reagent and allowed to stain for 10 minutes (Guava Technologies, Hayward, CA). The Guava System differentiates viable from non-viable cells by detecting fluorescence signals from two fluorescent DNA-binding dyes: one membrane-permeable dye stains all nucleated cells while the second dye enters cells when membrane integrity has been compromised, ie, non-viable cells.⁴² Viable cells were quantified using a Guava Personal Analyzer (PCA) flow cytometer (Guava Technologies), according to the manufacturer's specifications.

Quantification of Apoptotic Cells by Annexin V Labeling

Endometrial cells (2×10^4) seeded in 6-well plates at 50% to 70% confluence were treated with 100 nmol/L 17β -estradiol for 24 hours, followed by 100 nmol/L progesterone for an additional 24 hours. To detect apoptosis, the cells were double stained with annexin V and nexin 7-AAD, according to the manufacturer's recommendations (Guava Nexin Method; Guava Technologies). Cell-associated fluorescence was analyzed using a Guava PCA flow cytometer (Guava Technologies). Results were expressed as the percentage of apoptotic-positive cells. Both early apoptotic (annexin V-positive) and late apoptotic (annexin V and 7-AAD-positive) cells were included in the analysis.⁴³

Quantification of Apoptotic Cells by Chromatin Morphology Analysis

Endometrial cells (3×10^4) were seeded in 6-well plates and cultured until subconfluence (50% to 70%), at which time the cells were serum-deprived for 24 hours. After treating the cells with 100 nmol/L 17β -estradiol for 24 hours followed by 100 nmol/L progesterone stimulation for 24 hours, 0.5 μ g/ml Hoechst 33342 (Sigma-Aldrich) was added to the medium for 30 minutes at 37°C. Attached and non-attached cells were collected and analyzed by UV fluorescence microscopy ($\times 400$). Apoptosis was evaluated based on chromatin morphology.⁴⁴ Two hundred cells per sample were counted. Results are expressed as the percentage of apoptotic cells in the solution.

DNA Synthesis by the [3 H]-Thymidine Incorporation Assay

Endometrial cells (2×10^4) were grown in 24-well plates until 50% to 70% confluence was attained, and then quiescence of the cells was achieved by addition of phenol/serum-free Dulbecco's Modified Eagle's Medium

for 24 hours. Cells were treated with 100 nmol/L 17β -estradiol for 24 hours, followed by 100 nmol/L progesterone for 20 hours. After 14 hours of progesterone exposure, cells were labeled with 1 μ Ci/ml [3 H]-methylthymidine (Amersham Biosciences, Piscataway, NJ) for 6 hours. At the end of this period, the cells were washed in saline solution and methanol, and precipitated with trichloroacetic acid. Samples were dissolved with NaOH and diluted in scintillation buffer. Radioactivity was measured in a Packard Tri-Carb LS β -counter (PerkinElmer, Wellesley, MA).

Cell Number Measurement

Changes in the number of cells were determined by flow cytometry using the Guava Viacount kit (Guava Technologies). Briefly, 1.5×10^4 endometrial cells were plated and grown until a confluence of 50% to 70% was attained. Synchronization of the cell cycle was then induced by serum-starvation for 24 hours. Furthermore, the cells were stimulated with 100 nmol/L 17β -estradiol for 24 hours, followed by another 24 hours of 100 nmol/L progesterone treatment, during which the cells were counted at specified time points (three times per well). Results are expressed as the number of cells/ml.

Cell Cycle Analysis

Endometrial cells (2×10^5) were seeded in 100-mm dishes and grown until a confluence of 50% to 70% was reached. After synchronization of the cell cycle by serum starvation for 24 hours, cells were stimulated with 100 nmol/L 17β -estradiol for 24 hours, followed by treatment with 100 nmol/L progesterone in phenol-free Dulbecco's Modified Eagle's Medium for an additional 24 hours. Cells were fixed with 70% ethanol for 1 hour, stained with propidium iodide, and cell cycle populations were determined using the Guava EasyCyt system, according to the manufacturer's recommendations (Guava Technologies). The Guava Cell Cycle software was used to determine cell populations in the different cell cycle phases, while the amount of propidium iodide was quantified in the G0/G1, S, and G2/M phases.⁴⁵

Statistical Analyses

Statistical analysis was performed with the Statistical Package for Social Sciences (SPSS v14.0). Comparisons between two groups were made using the Student's *t*-test (paired). Analysis of variance was applied whenever differences among three or more groups were analyzed. When a positive global difference between groups was found, *post hoc* Tukey's Honestly Significant Difference test (when variances were homogeneous) or Games Howell (when variances were considered unequal) tests were performed to identify which coupled groups presented significant differences. qPCR values were \log_2 -transformed before the analysis. At least four biological replicates were analyzed in each experimental group. Data are expressed as the ratio between hormonal treat-

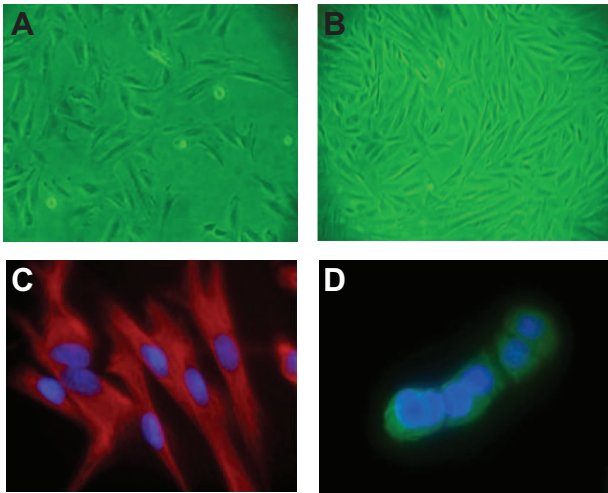


Figure 1. Figures **A** and **B** show nonconfluent and subconfluent endometrial cells from eutopic endometrium used in all experiments. Triple immunofluorescence for vimentin (red), cytokeratin (green), and 4,6-diamidino-2-phenylindole for the nuclei, was performed to characterize percentages of endometrial mesenchymal (**C**) and epithelial constituents in each sample. Figure (**D**) shows T47D cells used as positive controls for cytokeratin staining. Magnification = original $\times 200$ (**A**), $\times 100$ (**B**), and $\times 400$ (**C** and **D**).

ment and basal conditions in the majority of experiments, and $P < 0.05$ was considered as significant.

Results

Experimental Subjects

Endometrial biopsies from 16 patients with endometriosis, age 31.2 ± 4 years (mean \pm SEM), were staged according to the guidelines from the American Society for Reproductive Medicine⁴⁶ and were selected for the study. For comparison, endometrial biopsies were obtained from 10 women (age 31.9 ± 6) submitted for laparoscopy for benign ovarian cysts without signs of endometriosis in the pelvis. Tissues were collected at various times during the menstrual cycle and were dated according to Noyes' criteria.³⁴ No homozygous cases of PROGINS were included in the study, and all of the listed endometrial biopsies yielded viable cell cultures (Table 1).

Endometrial Cell Culture

Cell cultures were established from human endometrial tissues as described by Piva et al.³⁶ Cultures reached 90% to 95% confluence within 3 ± 1 day (mean \pm SEM) of the initial isolation (Figure 1, A and B). Cells were used from passages 3 until 7, and cell characterization was performed for each passage used in the experiments. The results from each passage showed approximately 90% vimentin-positive cells in all samples, as indicated by immunofluorescence microscopy (Figure 1, C and D). Cells were frozen at the third passage, and experiments were performed using cells from subsequent passages to ensure that a homogeneous population of cells was used for each phenotypic analysis (Table 1).

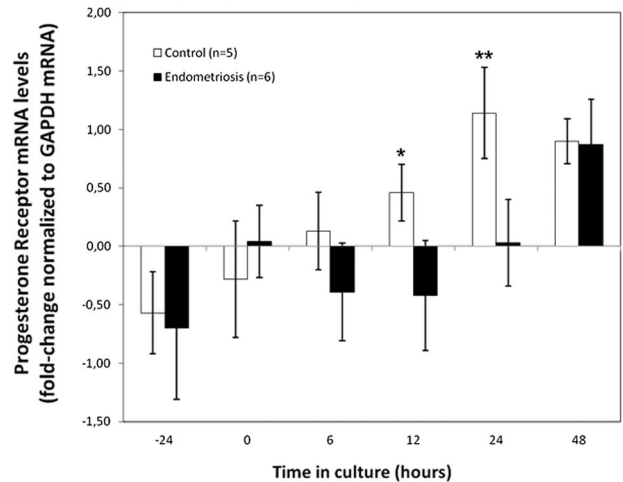


Figure 2. Quantitative real-time PCR analysis for progesterone receptor (PR) was performed from samples of eutopic endometrium with cDNA from controls and endometriosis without the PROGINS allele. Time course induction of PR by 100 nmol/L 17 β -estradiol for 6 hours, 12 hours, 24 hours, and 48 hours of exposure was applied to determine optimum concentration of progesterone used as treatment for cytometric analyses. PR was also detected in basal conditions (Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum and serum-free medium) before estradiol exposure (indicated as -24 hours and 0). Control ($n = 5$) vs. endometriosis ($n = 6$). Differences among studied groups appeared after 12 hours ($*P = 0.05$), and reached highest significance at 24 hours ($**P = 0.047$). Expression levels were normalized by GAPDH and compared with baseline. The 100 nmol/L E2 treatment for 24 hours followed by the same regimen with progesterone was the chosen procedure for all cytometric studies.

Induction of PR mRNA Expression by 17 β -Estradiol and PRB/PRAB Ratio Analysis

Endometrial cell cultures were treated with 100 nmol/L 17 β -estradiol (E2) for different time periods, and total PR mRNA levels were analyzed by real-time PCR. PR showed a time-dependent modulation for both groups (Figure 2), but demonstrated more significant results for the controls (6 hours to 12 hours, $P = 0.44$; 6 hours to 24 hours, $P = 0.04$; 6 hours to 48 hours, $P = 0.007$) when compared with the endometriosis group (6 hours to 12 hours, $P = 0.91$; 6 hours to 24 hours, $P = 0.23$; 6 hours to 48 hours, $P = 0.04$). This pattern was also noticed 24 hours before the time course study, when the cells were maintained in phenol-free Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (-24 hours) immediately before serum-deprivation for 24 hours (0 hours) and E2 treatment (6 to 48 hours). Administration of 1 nmol/L or 10 nmol/L E2 did not modulate PR expression levels (data not shown). Significant differences among groups began to appear only after 12 hours of treatment ($P = 0.05$), and increased after 24 hours ($P = 0.047$). Interestingly, up-regulation of PR in the endometriosis group was noticed only 48 hours after E2 treatment, at which time it reached the same levels displayed by the control cells ($P = 0.94$). This delayed PR gene transcription could suggest a resistance mechanism in endometrial cells from women with endometriosis (Figure 2). Analysis of cells carrying the PROGINS polymorphism and consideration of the individual expression levels of PR subunits during the 24 hours treatment (Figure 3) revealed higher PRB mRNA expression levels in the con-

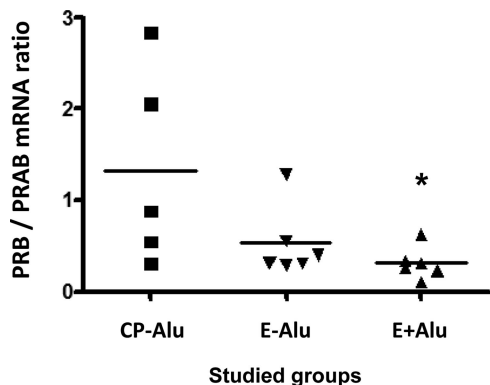


Figure 3. Analysis of the PRB/PRAB balance at transcriptional levels by quantitative real-time PCR using total RNA from samples of endometrial cells from CP-Alu ($n = 5$), E-Alu ($n = 6$), and E+Alu ($n = 6$) groups, after treatment with 100 nmol/L 17 β -estradiol for 24 hours. * $P = 0.0485$ (CP-Alu versus E+Alu). GAPDH was used to normalize the values. Experiments were performed in triplicate. The statistical significance was determined using Student's *t*-test.

trol group (0.82 ± 0.410) compared with the endometriosis E-Alu (0.31 ± 0.047) and E+Alu (0.16 ± 0.049) groups. The mean ratio of PRB to PRAB (PRA+PRB) mRNA expression was decreased in the endometriosis group consisting of PROGINS carriers (0.31 ± 0.070) as compared with the endometriosis group of non-carriers (0.53 ± 0.158) and the controls (1.32 ± 0.483). However, the mean ratio was also significantly lower when the E+Alu and CP-Alu groups were compared ($P = 0.048$), whereas the CP-Alu versus E-Alu ($P = 0.12$) and E-Alu versus E+Alu ($P = 0.24$) comparisons were not significant (Figure 3).

Effects of Progesterone on Cell Viability and Apoptosis

Analysis of cell culture phenotypes revealed sustained viability and decreased apoptosis in E+Alu cells compared with the CP-Alu ($P < 0.05$) and E-Alu ($P < 0.05$) groups (Figure 4, A and B). Lower percentages of apoptotic cells were observed in the E+Alu group compared with CP-Alu and E-Alu based on chromatin morphology analysis after progesterone administration (Figure 5A, CP-Alu $6.8\% \pm 0.7$, E-Alu $5.7\% \pm 1.3$, E+Alu $2\% \pm 0.3$, E+Alu versus CP-Alu, $P = 0.001$; E+Alu versus E-Alu, $P = 0.006$). Annexin-V labeling (Figures 4B and 5B) permitted identification of viable (CP-Alu $7.2\% \pm 0.9$, E-Alu $8.7\% \pm 0.3$, E+Alu $10.8\% \pm 0.6$), early apoptotic (CP-Alu $14.7\% \pm 4.1$, E-Alu $13.5\% \pm 1.8$, E+Alu $8.9\% \pm 1.2$), and late apoptotic cells (CP-Alu $32.2\% \pm 5.3$, E-Alu $16.5\% \pm 1.2$, E+Alu $7.8\% \pm 1.7$). The decrease in cell viability and induction of apoptosis were monitored by flow cytometry with the Viacount kit under basal conditions and after a pulse of 100 nmol/L progesterone for 24 hours (Figure 5, C and D).

Effects of Progesterone on Cell Proliferation

DNA synthesis analysis by [3 H]-thymidine incorporation revealed that both the CP-Alu and E-Alu groups significantly differed from the E+Alu group (Figure 6A; CP-Alu $146 \pm 40\%$, E-Alu $224 \pm 9\%$, E+Alu $490 \pm 21\%$, CP-Alu versus E-Alu, $P = 0.27$; CP-Alu versus E+Alu, $P = 0.002$), suggesting that the presence of PROGINS can increase the proliferation of cells treated with 17 β -estradiol and progesterone. This data were confirmed by cell proliferation analysis, in which the number of cells relative to the

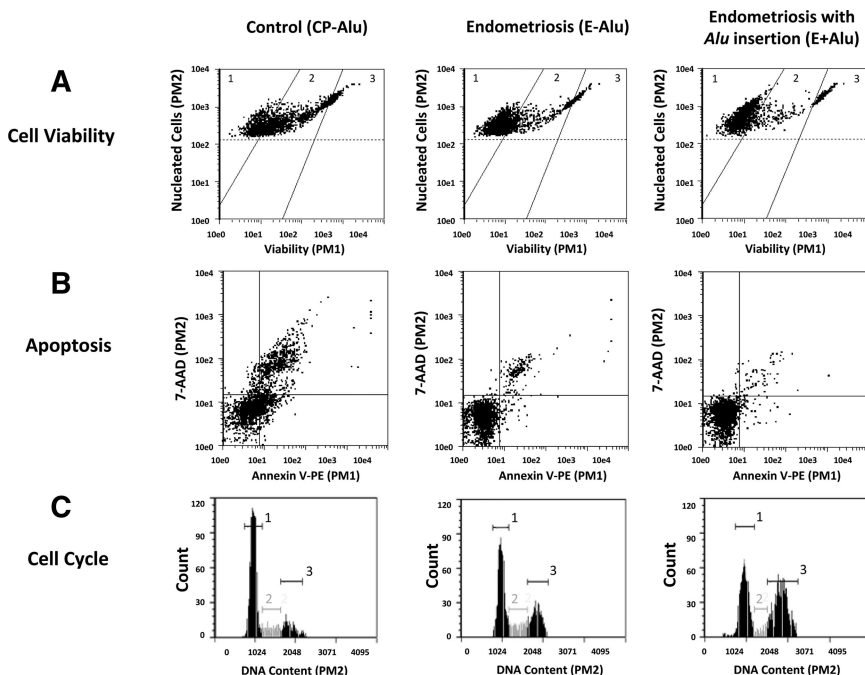


Figure 4. Flow cytometry diagrams of representative experiments with progesterone acquired and analyzed by the Guava CytoSoft (v2.0) Software. Line (A) represents the cell viability analysis representing the distinction of viable (1), apoptotic (2) and dead (3), cells dependent on the incorporation of the Viacount dyes. Line (B) shows percentages of apoptotic cells determined by Annexin-V labeling with standard quadrant gating: annexin-V negative/7-AAD negative (alive), annexin-V positive/7-AAD negative (early apoptotic), annexin-V positive/7-AAD positive (late apoptotic) and annexin-V negative/7-AAD positive (necrotic) cells. In line (C) the cell cycle analysis was applied to determine percentages of cell populations in different phases of the cell cycle, and propidium iodide was quantified from the G0/G1 (1), S (2), and G2/M (3) stages.

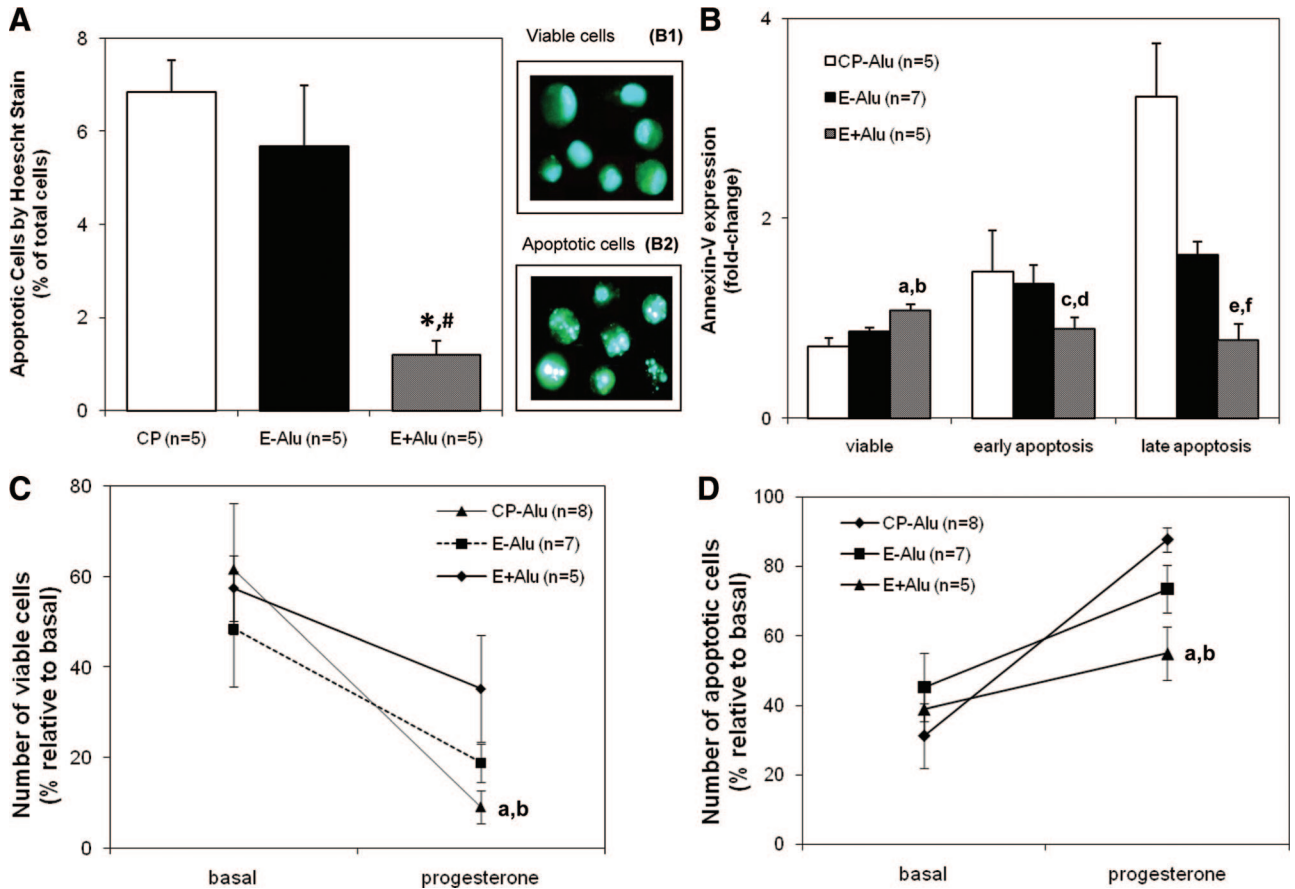


Figure 5. Progesterone fails to promote death in endometrial cells with PROGINs. Endometrial cells were serum-deprived and treated with 100 nmol/L progesterone for 24 hours. Apoptosis was measured by flow cytometry with the annexin-V labeling kit (A) which allows the detection of viable (^aE+Alu versus CP-Alu and ^bE+Alu versus E-Alu: ^a*P* = 0.002, ^b*P* = 0.05), early apoptotic (^cE+Alu versus CP-Alu and ^dE+Alu versus E-Alu: ^c*P* = 0.42, ^d*P* = 0.13); and late apoptotic cells (^eE+Alu versus CP-Alu and ^fE+Alu versus E-Alu: ^e*P* = 0.02, ^f*P* = 0.01). Apoptosis was detected morphologically with Hoechst 3342 staining (B), where viable (B1) and apoptotic nuclei (B2) were distinguished: ^aE+Alu versus CP-Alu (*P* = 0.001), and ^bE+Alu versus E-Alu (*P* = 0.006); and the Viacount assay (C): which monitors apoptosis (^aE+Alu versus CP-Alu, and ^bE+Alu versus E-Alu: ^a*P* < 0.0001, ^b*P* = 0.003); and cell viability (D): (^aE+Alu versus CP-Alu, and ^bE+Alu versus E-Alu: ^a*P* = 0.04, ^b*P* = 0.15).

corresponding number under basal conditions was counted (Figure 6B; CP-Alu 1.5 ± 0.10 -fold-change, E-Alu 1.71 ± 0.11 -fold-change, E+Alu 2.19 ± 0.16 -fold-change; CP-Alu versus E-Alu, *P* = 0.47; CP-Alu versus E+Alu, *P* < 0.01). Both methods demonstrated that cells

carrying the PROGINs polymorphism exhibit higher proliferation rates than CP-Alu and E-Alu cells (Spearman Correlation of 0.554, *P* = 0.04). A cell cycle analysis (Figure 4C) also confirmed this tendency, revealing exuberant progression to S and G2/M phases in the E+Alu

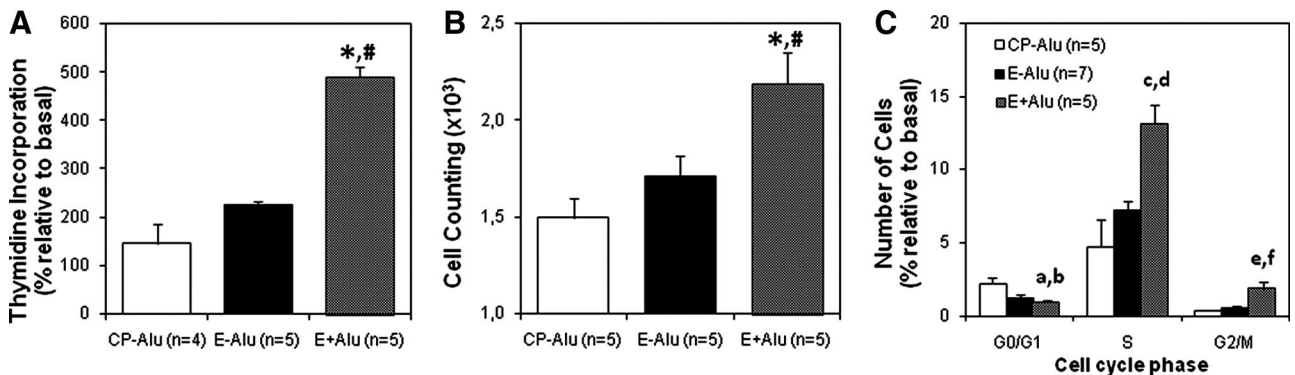


Figure 6. Cell proliferation was monitored by the [³H]-thymidine incorporation assay (A), after synchronization with serum starvation, followed by consecutive treatments with 100 nmol/L 17β-estradiol and progesterone both for 24 hours. ^aE+Alu versus CP-Alu (*P* = 0.002), and ^bE+Alu versus E-Alu (*P* = 0.0001). Similar hormonal stimuli were used to monitor cell counting (B): 6-well plates were seeded with 5×10^3 cells at day 0 and counted again after treatment. Electronic cell counts were performed in triplicate. Cell cycle analysis (C) showed progression of E+Alu (in relation to CP-Alu and E-Alu) throughout the cell cycle into S and G2/M phases. ^aE+Alu versus CP-Alu (*P* = 0.01), and ^bE+Alu versus E-Alu (*P* = 0.04). G0/G1 (^a*P* = 0.07, ^b*P* = 0.15), S (^c*P* = 0.001, ^d*P* = 0.01), and G2/M (^e*P* = 0.05, ^f*P* = 0.14).

group compared with the CP-Alu ($P = 0.001$) and E-Alu ($P = 0.01$) groups (Figure 6C).

Discussion

The etiology of endometriosis has been exhaustively studied over recent decades. Several studies have focused at the endometrial cellular and molecular levels to understand the pathogenesis of ectopic endometriosis.⁴⁷ Some of the proposed mechanisms point to progesterone as the main effector in the process. An increasing number of studies describe the differential PR gene expression and signaling that occur in patients with endometriosis, suggesting that progesterone resistance in the endometrium⁴⁸ may cause this differential response, purported to be one of the mechanisms responsible for the development of endometriosis.

Progesterone signaling depends on interactions between the hormone and its receptors.⁴⁹ It was recently shown that progesterone signaling is blocked in cells carrying the PROGINS allele and the V660L mutation on exon 4 of the PR gene. Indeed, comparisons with rodent ovarian (CHO) cells carrying common polymorphic variants for PR gene demonstrated that cells with the PROGINS allele display diminished PR mRNA stability, altered post-translational modifications (such as phosphorylation), and modified protein degradation patterns, which may account for the disrupted response to progesterone.³³

The present study demonstrates that there are significant alterations in the cellular phenotype of women with endometriosis carrying the PROGINS polymorphism using an *in vitro* model consisting of endometrial cell cultures exposed to progesterone. It suggests that the PROGINS polymorphism promotes changes in the cellular phenotype that lead to disrupted cell cycle control in the endometrium, reinforcing the hypothesis that endometrial resistance to progesterone could have deleterious effects and contribute to the genesis of endometriosis.⁵⁰

Analysis of the cell culture phenotype of eutopic endometrium from women diagnosed with endometriosis allowed us to perform a comprehensive study of cell proliferation, viability, and apoptosis. This approach provided more reliable results, illustrating that the *in vitro* model is appropriate for such a comprehensive study. Because the cells were not manipulated to establish pure cultures of stromal or epithelial cells, possible sources of stress that could trigger cell differentiation, signaling mechanisms, or other kinds of perturbations were avoided.

One limitation of the study is the absence of any homozygous PROGINS samples, mainly because of their low frequency in our population.²⁹ Another issue is the absence of disease-free women with a single PROGINS allele. Such a group would be helpful in proving the relationship between PROGINS and the cell cycle deregulation observed in endometriosis.

Progesterone signaling is mediated by two functionally distinct receptor isoforms (PRA and PRB) that are expressed from a single gene at which transcription can

occur from two alternative promoters.⁵¹ Some studies have revealed that the mRNA ratio of PRA to PRB is critical for the cellular response to progesterone. In addition, the relative expression of PRA and PRB is species-dependent and differs in each cellular context.⁵²⁻⁵⁵ In human gynecological cancers, especially ovarian cancers, the dominant expression of PRB mRNA is a marker for a malignant phenotype.⁵⁶ With respect to endometriosis, Misao et al⁵⁷ showed that PRB mRNA is highly expressed in endometrioma. A recent study from Wu et al⁵⁸ demonstrated how knockdown of PRB can promote cell proliferation in immortalized endometrial stromal cells. In the present study, we observed that endometrial cells carrying the PROGINS polymorphism exhibit a decreased expression ratio of PRB to PRAB mRNA when compared with E-Alu and CP-Alu samples. This evidence suggests that PRA and PRB mRNA expression in endometriosis might be altered in the presence of PROGINS, which could lead to a disruption in the control of progesterone effects and responses to sex steroid-mediated growth regulation.

Progesterone is described as an inhibitor of endometrial cell proliferation.⁵⁹ In cultured endometrial cells from women with endometriosis carrying the PROGINS allele, a significant increase in cell proliferation after treatment with progesterone was observed compared with controls and cells from non-carrier women with endometriosis. This finding could indicate an impaired ability of progesterone to inhibit cell proliferation, an expected effect of this hormone.⁶⁰

Cell proliferation is, in fact, a balance between cells that progress through the cell cycle and those that undergo cell death. For this reason, we also evaluated the effect of PROGINS on cell apoptosis and cell cycle progression. Interestingly, apoptosis is less pronounced in the eutopic and ectopic endometrium of patients with endometriosis.⁶¹ Our results show impaired induction of cell death by apoptosis after progesterone stimulation of cells from the endometriosis group with PROGINS, in contrast to the non-carriers and controls. An analogous pattern was observed for cell viability, as evidenced by endometriosis cells presenting sustained viability after progesterone stimulation; especially those with PROGINS. This information clearly shows that progesterone was unable to induce death by apoptosis in cells carrying the PROGINS allele. Additionally, the disparity between both endometriosis groups may explain why some women with endometriosis respond differently than others to progesterone-based therapies and why such therapies fail to treat the disease. Nonetheless, studies examining larger numbers of samples from different stages will be necessary to test the role of this polymorphism in the progression of the disease.

Our results show that the presence of PROGINS significantly influenced the behavior of cultured endometrial cells. Our results suggest that this polymorphism may be involved in the pathogenesis of the disease, and that further studies are necessary to clarify the role of PROGINS in different aspects of endometriosis.

The identification of genetic markers related to the genesis of endometriosis is critical for developing pre-

ventive strategies. Considering our results, further *in vivo* and epidemiological studies are required to better understand the relevance of PROGINS to the cellular response to progesterone, and its role in the development of endometriosis. Such information would be vital to developing novel therapeutic approaches or to using traditional therapies in a more patient-specific manner.

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