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The relationship between follicle development and progesterone receptor membrane component-1 (PGRMC1) expression in women undergoing in vitro fertilization

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Abstract

Objective—To determine the relationship between Progesterone Receptor Membrane Component-1 (PGRMC1) expression and the outcome of in vitro fertilization treatment.

Design—A prospective study in which PGRMC1 mRNA levels, methylation status of the *Pgrmc1* promoter, and the presence of point mutations within *Pgrmc1* were obtained from granulosa/luteal cells of women undergoing controlled ovarian hyperstimulation (COH).

Setting—Fertility center/Basic science laboratory

Patients—Eighty-five IVF patients and 10 women, who were undergoing COH for the purpose of oocyte donation, were included in this study.

Interventions—None.

Main Outcome Measures—PGRMC1 measurements were correlated with clinical outcomes, such as number of follicles, number of retrieved oocytes and ongoing pregnancy rates.

Results—PGRMC1 mRNA levels within granulosa/luteal cells of 18% of IVF patients were > 2.25 fold higher than those of oocyte donors. Individuals with elevated PGRMC1 mRNA levels had 30% fewer large follicles and fewer oocytes retrieved. The elevated PGRMC1 mRNA levels were associated with an increase in the methylation of *Pgrmc1* promoter.

Conclusion—In patients with elevated PGRMC1 mRNA levels, gonadotropin-induced follicle development is attenuated, although sufficient numbers of follicles develop to allow for embryo transfer and subsequent pregnancy.

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Keywords

PGRMC1; granulosa/luteal cells; IVF; controlled ovarian hyperstimulation; follicular development

Introduction

The ability to conceive and to ultimately deliver a healthy child is an extremely complex process that can be disrupted by genetic and epigenetic events (1, 2). Because of the importance of these genetic and epigenetic events, numerous studies have been conducted to identify genetic and/or epigenetic biomarkers that might be able to predict a successful in vitro fertilization outcome and/or pregnancy (3-6). One gene that might be useful as a predictor of in vitro fertilization outcome is Progesterone Receptor Membrane Component-1 (*Pgrmc1*). *Pgrmc1* (Gene ID: 10857) is located on the X-chromosome (Xq22-q24) and has three exons that encode a 22 kDa protein (7, 8). Moreover, PGRMC1 is highly expressed in human ovarian granulosa cells (9, 10) and functions to preserve the viability of these cells (9-13).

Consistent with its important role in regulating the viability of granulosa/luteal cells, *Pgrmc1* deletion and point mutations have been observed in some women with impaired ovarian function (8, 14, 15). For example a mother and daughter, who were diagnosed with premature ovarian failure, had a deletion mutation that resulted in a 50% reduction in PGRMC1 expression (8). A point mutation within Exon 3 of *Pgrmc1*, which results in an alteration in amino acid 165, was detected in a third patient with premature ovarian failure (8). This point mutation is important in that it reduced the functional capacity of PGRMC1 (8). Moreover, reduced expression of PGRMC1 was observed in women with polycystic ovarian syndrome (14), further implicating PGRMC1 as a regulator of ovarian function.

Based on these observations, the present series of studies were undertaken to determine whether the PGRMC1 mRNA levels within the granulosa/luteal cells of women undergoing controlled ovarian hyperstimulation protocols could be used to identify a subset of women who response to gonadotropin treatment was altered in a way that would affected the success of their in vitro fertilization treatment. In addition to PGRMC1 mRNA levels, the presence of the point mutation within Exon 3 was assessed in these women. Finally, the methylation status of the PGRMC1 promoter was evaluated, because hypermethylation suppresses transcription, thereby reducing mRNA levels (16). Moreover, gonadotropins regulate the methylation status and thereby the expression of several genes (i.e. *Cyp19A1*, *Hsd3B*, and *Cyp11A1*) whose expression is regulated in an ovarian cell-specific manner (1).

Methods and Materials

Patients

This study included 85 patients who underwent controlled ovarian hyperstimulation as part of their IVF treatment between March 2010 and November 2010. This study was approved by The University of Connecticut Institutional Review Board. Patients in this study were undergoing either their first or second IVF cycle. No patients contributed data from more than one cycle in this study. Patients were ≤ 38 years, with normal basal serum FSH, LH and E_2 obtained on day 3 and no prior history of low response to ovarian stimulation. A low ovarian response was defined as a peak serum E_2 level of 850 pg/ml or less and/or retrieval of < 5 oocytes. Severe male factor requiring surgical sperm harvest and patients with polycystic ovarian syndrome were excluded. Patients were stimulated with either a flexible antagonist or GnRH agonist protocols as described by Engmann et al (17). The assignment

of the patients to either protocol was made at their physician discretion. In addition, 10 women undergoing controlled ovarian hyperstimulation for the purpose of oocyte donation were included as controls.

Stimulation Protocols

Briefly, patients were treated with recombinant FSH (Gonal F; Serono, Rockland, MA) and purified urinary hMG (Menopur; Ferring Pharmaceuticals, Tarrytown, NY). In patients undergoing flexible antagonist protocol, ganirelix acetate (0.25 mg s.c., Ganirelix, Organon Pharmaceuticals, Roseland, NJ) was started when either the lead follicle measured ≥ 13 mm or E_2 was >300 pg/mL. Ganirelix acetate was continued until the day of hCG administration. hCG (Profasi, Serono, Rockland, MA) was administered s.c. when at least three follicles had attained or exceeded a mean diameter of 17 mm. Ultrasound imaging was conducted throughout the stimulation cycle to monitor follicle growth and blood samples were periodically taken to determine serum E_2 and P levels as previously published (17). Transvaginal oocyte retrieval was performed approximately 36 hours after hCG administration and the granulosa/luteal cells were obtained from the follicular aspirate.

Isolation of Granulosa/Luteal Cells

After the oocytes were removed, follicular aspirates were pooled and centrifuged at $250 \times g$ for 10 min. The cell pellet was suspended in PBS, layered on Histopaque-1077 (Sigma Chemical Co., St. Louis, MO) and centrifuged for 30 minutes at $400 \times g$. After centrifugation, the opaque interface containing the granulosa/luteal cells was carefully aspirated and transferred into a 15-ml sterile conical centrifuge tube. The cells were then suspended in 12 ml PBS and centrifuged at $250 \times g$ for 10 minutes. This was repeated an additional time (9, 10).

Analysis of Exon 3 for point mutations

Genomic DNA was extracted from granulosa/luteal cells using AllPrep DNA/RNA Micro kit from QIAGEN (Valencia, CA) and used in a PCR protocol to amplify Exon 3 of Human PGRMC1. This protocol used the following primers: Forward: 5'-TTGCAGGCCTCTAATAAATG-3'; Reverse: 5'-CAGCACTGCAGTTCACCTTC-3'. The PCR reaction consisted of 35 cycles of a denaturation phase of 30 sec at 94 C, an annealing phase of 30 sec at 55 C, and an extension phase of 60 sec at 68 C. The PCR product was then run on 1.2% of agarose gel and the 415 bp band extracted and purified using the QIAquick gel extraction kit from Qiagen. The purified PCR product was then send to Agencourt Bioscience Corp (Beverly, MA) for sequencing. The DNA sequence was then analyzed for the presence of any mutation using 4Peaks software program (<http://www.mekentosj.com/science/4peaks>).

Real-time PCR for PGRMC1 mRNA measurements

Total RNA was also extracted from granulosa/luteal cells using AllPrep DNA/RNA Micro kit from QIAGEN (Valencia, CA). This RNA preparation was used in a real time PCR protocol using CFX96 real time PCR system. RNA samples (1 ng) were resuspended in 25 μ l of the SsoFast Probes Supermix (Bio-Rad) containing 300 nm of each PGRMC1 primer: Forward 5'-GGTGTTCGATGTGACCAAAG-3' and Reverse 5'-GATGCATCTCTTCCAGCAAA-3' and the probe: CGCAAATTCTACGGGCCCCGA (5' FAM -> 3' BHQ1). Actin was used as an internal control and was detected using the following primers: Forward 5'-CACTCTTCCAGCCTTCCTTC-3' and Reverse 5'-GGATGTCCACGTCACACTTC-3' and the probe: TGCCACAGGACTCCATGCCC (5' CAL Gold 540 -> 3' BHQ1). Gene expression was evaluated with Bio-Rad iCycler software

and the PGRMC1 mRNA levels expressed relative to the average value obtained from the oocyte donor samples.

Analysis of methylation of the *Pgrmc1* promoter

A one kilobase segment of DNA that was rich in cytosine nucleotides that were next to a guanine nucleotide separated by a phosphate (i.e. CpG) was identified within the *Pgrmc1* promoter (18). This CpG island spanned the transcriptional start site of PGRMC1. Since the methylation of these cytosine residues can suppress gene transcription, Methyl-Profiler DNA Methylation qPCR assay kit was used to determine the methylation status of this CpG island. Briefly, genomic DNA was aliquoted into four equal portions and subjected to either a mock digestion; a methylation sensitive digestion; a methylation dependent digestion or a double digestion. After restriction digestion, PCR reaction was run using primers designed by SABiosciences to amplify this CpG island. The percentage of hypermethylated DNA was then calculated using the software provided by SABiosciences.

Clinical Outcomes

In vitro insemination or intracytoplasmic sperm injection (ICSI) was performed on retrieved oocytes. Of the 85 patients, 93% opted for the ICSI protocol. Eighteen hours after insemination or sperm injection, the oocytes were examined for the presence of pronuclei and those oocytes that possessed 2 pronuclei were considered fertilized. The number of good quality embryos was assessed after 3 days of culture. Only embryos with ≥ 6 cells and $\leq 20\%$ fragmentation were classified as good quality embryos. The implantation rate was defined as the number of sacs on transvaginal ultrasound divided by the number of embryos transferred. The ongoing pregnancy rate per started cycle was assessed after 20 weeks of gestation.

Statistical analysis

PGRMC1 mRNA levels obtained from granulosa/luteal cells of oocyte donors were used to establish a normal range as defined as the mean ± 2 standard deviations (SD). Using the values from the oocyte donors, PGRMC1 mRNA levels from IVF patients were classified as either being within or outside the normal range. Statistical comparison of end-points for IVF patients with normal and elevated PGRMC1 mRNA levels was performed using the statistical package for the Social Sciences (Release 17.0, SPSS Inc., Chicago). Student's *t*-test was used for comparison of continuous variables, while Fisher's exact test was used for comparison of proportions. A power analysis for Student "t" test and Fisher's exact test revealed a power value of 0.82 and 81, respectively. The relationship between PGRMC1 mRNA levels and serum steroid levels was assessed by Spearman correlation analysis. Regardless of the statistical analysis used, $P < 0.05$ was considered statistically significant.

Results

The levels of PGRMC1 mRNA in granulosa/luteal cells obtained from IVF patients after gonadotropin treatment were not normally distributed and significantly skewed to the right as determined by Kolmogorov-Smirnov test for normality ($p < 0.0001$). To define a normal range, PGRMC1 mRNA levels obtained from oocyte donors were used. This analysis revealed that the donor values were normally distributed with the 95% lower and upper limits being defined by the mean ± 2 SD (**Bracketed line in Figure 1**). Of the 85 IVF patients, one had a PGRMC1 mRNA level that was less than the lower limit, 69 (82%) had PGRMC1 mRNA levels that were within the normal range and 15 (18%) had PGRMC1 mRNA levels that were greater than the upper limit of the oocyte donor population. Interestingly, IVF patients with elevated PGRMC1 mRNA levels had demographic and

clinical characteristics comparable to those of IVF patients with PGRMC1 mRNA levels within the normal range (**Table 1**).

One factor that could account for an increase in PGRMC1 mRNA levels is the hypermethylation status of the *Pgrmc1* promoter. In patients with normal levels of PGRMC1 mRNA, $21 \pm 0.02\%$ of the cytosine residues within the CpG island of the *Pgrmc1* promoter was hypermethylated. In contrast only $16 \pm 0.01\%$ of the cytosine residues within the CpG island of the *Pgrmc1* promoter was hypermethylated in those patients with elevated PGRMC1 mRNA levels. In addition, a point mutation at amino acid 165 was not observed in any of the IVF patients, including all of the patients with elevated PGRMC1 mRNA levels.

Since only one IVF patient had abnormally low PGRMC1 mRNA levels, her data were not analyzed further. Accordingly, data of IVF patients with elevated PGRMC1 levels were compared to those with normal levels. Importantly, the stimulation protocols used in both groups were comparable (**Table 2**). There were also no difference in the length of stimulation and the total dose of gonadotropin (**Table 2**). In spite of the fact that 18% of the infertility patients had elevated PGRMC1 mRNA levels, their ability to response to gonadotropin with an increase in serum E₂ levels was neither enhanced nor inhibited. Similarly, the amount of PGRMC1 mRNA expressed by granulosa/luteal cells did not affect the serum levels of E₂ and P observed after hCG administration (**Table 2**). Finally, PGRMC1 mRNA levels were not correlated with serum E₂ or P4 at any time was assessed by Spearman correlation analysis (*r* values ranged from -0.03 to -0.12; all correlations had *P* values > 0.05).

The only aspect of ovarian function that was related to PGRMC1 mRNA levels was follicular growth. Although the initial number of antral follicles was not significantly different, the number of follicles > 14 mm in diameter that developed following gonadotropin stimulation was reduced by 35% in IVF patients with elevated PGRMC1 mRNA levels (**Table 3**). Consistent with this finding, the number of oocytes retrieved and the number of mature and fertilized oocytes were reduced by approximately 33% in this group (**Table 3**). However, the maturation, fertilization and implantation rates were not significantly different between the two groups. The ongoing pregnancy rate per started cycle for women in which PGRMC1 mRNA levels were elevated was lower than those with PGRMC1 mRNA in the normal range but this did not reach statistical significance (**Table 3**). Finally, the number of cryopreserved embryos was lower in patients with elevated levels of PGRMC1 (**Table 3**).

Discussion

The present study is the first to examine the expression of PGRMC1 in granulosa/luteal cells of women undergoing gonadotropin ovarian stimulation as part of their IVF treatment. Although alternations in PGRMC1 mRNA levels were not correlated with fertilization or ongoing pregnancy rates, a large subset of patients (i.e. 18%) respond to gonadotropin treatment by over expressing PGRMC1. The reason for the over expression is not known. In prior animal studies gonadotropin treatment have been shown to increase mRNA and protein levels of PGRMC1 (12, 19), thus it was expected that patients with elevated PGRMC1 mRNA levels would have received higher amounts of gonadotropins. However, in this study over expression of PGRMC1 cannot be explained by excessive gonadotropin stimulation, since neither the amount nor the duration of gonadotropin treatment was different between these two groups. Similarly, there were no differences in the average age of the patients and the stimulation protocols.

One factor that may have influenced the expression of PGRMC1 is the methylation status of the PGRMC1 promoter. The PGRMC1 promoter possesses a single CpG island that is approximately one kilobase and spans the transcriptional start site (18). As such, the methylation status of the cytosine residues within this CpG island may play a role in the transcription of PGRMC1. Our analysis revealed that on average 16% of the cytosine residues within the CpG island were hypermethylated in women with elevated PGRMC1 mRNA levels compared to 21% in women with normal PGRMC1 levels. Since hypermethylation makes the initiation of transcription more difficult (16), it is possible that the lower level of hypermethylation could account in part for the higher expression of PGRMC1. How much of the increased PGRMC1 expression can be attributed to this 5% decrease in methylation of the PGRMC1 promoter remains to be determined experimentally.

In the current study hypermethylation of the PGRMC1 promoter was associated with attenuated follicular growth. Thus, the hypermethylation likely occurs during follicular development that is induced by gonadotropin (i.e. FSH) stimulation. Interestingly, FSH in the presence of cumulus cell-oocyte complexes induces the expression of DNA methyltransferases (20). Since DNA methyltransferases stimulate the methylation of CpG islands (1), defects or alterations in the FSH signal transduction pathway could be responsible for less methylation of the PGRMC1 promoter and subsequently elevated PGRMC1 mRNA levels.

Another interesting finding in our study was that serum E₂ and P levels were not correlated with and PGRMC1 mRNA levels. PGRMC1 is thought to enhance steroidogenesis by increasing cholesterol synthesis through two distinct mechanisms. First, PGRMC1 can directly bind two proteins, insulin-induced gene-1 (INSIG-1) and sterol regulatory element binding protein cleavage-activating protein (SCAP) (21), and this interaction increases the transcriptional action of Sterol Regulatory Element Binding Protein (SREBP), and ultimately the expression of Steroidogenic Acute Regulatory Protein (StAR) (22), an essential factor in steroid synthesis. The second mechanism by which PGRMC1 could influence steroidogenesis, is through its ability to activate the cytochrome P450 enzymes (23) such as cytochrome P450 21, which plays an essential role in steroid synthesis (24). Apparently, the elevated PGRMC1 mRNA observed in some IVF patients was not sufficient to activate either of these two mechanisms to the point that affects serum steroid levels.

In addition to changes in mRNA levels, the biological activity of PGRMC1 can be influenced by the presence of a point mutation at amino acid 165. Specifically, mutated PGRMC1, which has an arginine instead of a histidine at amino acid 165, cannot bind to cytochrome P450 7A1 (CYP7A1), a key member of the P450 family that catalyzes a rate-limiting step in cholesterol metabolism (25). If present, this would have likely reduced the overall functional capacity of PGRMC1 in IVF patients with elevated PGRMC1 mRNA. However, this mutation was not observed in any of the IVF patients, including those with elevated PGRMC1 mRNA levels. Thus, the reason why elevated PGRMC1 did not influence steroidogenesis remains to be determined.

Although without an affect on serum steroid levels, the elevations in PGRMC1 expression adversely affected gonadotropin-induced follicle growth. This is illustrated by the fact that those patients with elevated PGRMC1 mRNA levels develop fewer large follicles after gonadotropin treatment. PGRMC1 has numerous sites of action that could influence follicular growth (26, 27). The most likely mechanism relates to PGRMC1's ability to interact with the mitotic spindle apparatus of granulosa cells (28). In order for granulosa cells to undergo mitosis and thereby contribute to follicular growth, PGRMC1 intermittently associates and dissociates with the mitotic spindle (28). Elevations in PGRMC1 could disrupt this dynamic interaction with the mitotic spindle and thus slow or inhibit granulosa

cell mitosis and in turn follicle development. Moreover, prolonging mitosis could trigger a “mitotic catastrophe” and result in granulosa cell apoptosis. It is important to appreciate that PGRMC1 depletion in granulosa cells also leads to granulosa cell apoptosis (11). Thus it appears that PGRMC1 must be maintained at an optimal level that is sufficient to preserve cell viability and allow for mitotic events to occur in a timely coordinated manner.

The reduction in follicular growth is also reflected in fewer oocytes retrieved, fewer mature oocytes, fewer fertilized oocytes and fewer embryos available for transfer. Importantly, oocytes retrieved from women with elevated PGRMC1 levels have the same capability to mature, to be fertilized, to undergo embryonic development in vitro and to implant and ultimately develop into viable embryos, as do those derived from women with normal levels.

Although these findings may cast doubt on an association between PGRMC1 expression and IVF success, this conclusion may not be completely justified. Patients with elevated PGRMC1 mRNA levels had fewer embryos available for transfer than patients with normal levels. Moreover, the number of cryopreserved embryos in this group was significantly less than patients with normal levels. Thus, the resulting cumulative pregnancy rate per retrieval might be reduced in patients with elevated PGRMC1 mRNA levels.

Finally, the present study revealed that patients with elevated PGRMC1 mRNA levels respond to gonadotropin with a normal increase in serum E₂ but a reduced number of large antral follicles and retrieved oocytes. Interestingly, the number of oocytes retrieved from “poor responders” is similar to that of IVF patients with elevated PGRMC1 mRNA levels (29). Moreover, serum E₂ is increased in “poor responders” to the same level as those IVF patients in the present study, although more gonadotropin is required to induce both serum E₂ levels and follicular development (29). These observations are consistent with the idea that follicular growth and steroid secretion are independent responses to gonadotropin treatment and that elevated PGRMC1 mRNA levels is only associated with attenuated follicular development. If so, then the elevated level of PGRMC1 mRNA might be a genetic biomarker of an IVF patient who either is or will become a “poor responder”. This possibility merits further consideration.

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Figure 1.

Distribution of relative PGRMC1 mRNA levels obtained from granulosa/luteal cell of patients undergoing ovulation induction for the treatment of infertility. Values are shown relative to the mean values obtained from oocyte donors. Values obtained from infertility patients were considered to be in the normal range if they were within the 95% confidence interval of oocyte donors. This confidence interval is shown as a bracketed line.

Table 1

Demographic and clinical characteristics of infertility patients with normal and elevated PGRMC1 mRNA levels within their granulosa/luteal cells obtained after gonadotropin stimulation.

	Normal (n=69)	Elevated (n=15)	P value
Age (years)	34.6 ± 3.9	35.3 ± 2.6	0.35
Body Mass Index (kg/m ²)	26.4 ± 6.3	25.2 ± 6.8	0.52
Day 3 FSH (mIU/mL)	6.5 ± 2.4	7.8 ± 2.4	0.08
Day 3 LH (mIU/mL)	4.8 ± 2.4	4.6 ± 2.1	0.79
Day 3 E ₂ (pg/mL)	47.5 ± 17.4	38.7 ± 16.8	0.08
Parity	0.29 ± 0.55	0.27 ± 0.59	0.87
Etiology of Infertility			
Male Factor (%)	32	33	1.0
Tubo-peritoneal Factor (%)	19	20	1.0
Unexplained (%)	23	27	0.74
Endometriosis (%)	12	7	1.0
Ovulation disorders (%)	14	13	1.0

Note: values are expressed as either a mean ± standard deviation or as a percentage. For those values expressed as a mean ± standard deviation, statistical comparisons were made using a Student "t" test. A Fisher's exact test was used to compare percentage values.

Table 2

Gonadotropin stimulation and resulting steroid levels of infertility patients with normal and elevated PGRMC1 mRNA levels within their granulosa/luteal cells obtained after gonadotropin stimulation.

	Normal (n=69)	Elevated (n=15)	P value
Stimulation Protocol			
Antagonist (% of patients)	64	53	0.56
Agonist (% of patients)	36	47	0.56
No. of Stimulation Days	10.19 ± 1.6	10.73 ± 2.2	0.37
Total Gonadotropins (U)	3201 ± 1817	4125 ± 2247	0.15
Peak E ₂ (pg/ml)	1934 ± 947	1918 ± 935	0.95
Peak E ₂ 1 day after hCG (pg/ml)	2445 ± 1074	2185 ± 1174	0.44
P 1 day after hCG (ng/ml)	3.7 ± 2.0	3.1 ± 2.0	0.26

Note: values are expressed as either a mean ± standard deviation or as a percentage. For those values expressed as a mean ± standard deviation, statistical comparisons were made using a Student “t” test. A Fisher’s exact test was used to compare percentage values.

Table 3

Follicle growth and oocyte characteristics of infertility patients with normal and elevated PGRMC1 mRNA levels within their granulosa/luteal cells obtained after gonadotropin stimulation.

	Normal (n=69)	Elevated (n=15)	P value
No. Antral Follicles at Day 3	10.9 ± 5.0	12.3 ± 6.4	0.49
No. Follicles > 14mm on day of hCG	11.6 ± 6.0	7.5 ± 2.7	0.004
No. Oocytes Retrieved	13.4 ± 6.9	9.2 ± 3.7	0.002
No. Mature Oocytes	10.0 ± 5.6	7.1 ± 3.9	0.02
Maturation Rate (%)	76	77	0.83
No. Oocytes with 2 Pronuclei	7.3 ± 4.8	4.5 ± 3.4	0.01
Fertilization Rate (%)	69	61	0.36
No. Available Embryos for Transfer on Day 3 of Culture	6.3 ± 4.5	3.9 ± 3.2	0.02
No. Good Quality Embryos	1.8 ± 2.2	1.3 ± 1.8	0.4
No. Embryos Transferred	2.2 ± 0.7	1.9 ± 1.2	0.46
Implantation rate (%)	34	36	0.83
Ongoing Pregnancies/Started cycle (%)	52	33	0.19
No. Embryos Frozen	1.0 ± 0.2	0.3 ± 0.2	0.02

Note: values are expressed as either a mean ± standard deviation or as a percentage. For those values expressed as a mean ± standard deviation, statistical comparisons were made using a Student "t" test. A Fisher's exact test was used to compare percentage values.