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

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Objective: To determine the relationship between progesterone receptor membrane component-1 (PGRMC1) expression and the outcome of IVF treatment.
Design: A prospective study in which PGRMC1 messenger RNA (mRNA) levels, methylation status of the *Pgrmc1* promoter, and the presence of point mutations within *Pgrmc1* were obtained from granulosa (GC)/luteal cells of women undergoing controlled ovarian hyperstimulation (COH).
Setting: Fertility center/basic science laboratory.
Patient(s): Eighty-five patients undergoing IVF treatment and 10 women who were undergoing COH for the purpose of oocyte donation were included in this study.
Intervention(s): None.
Main Outcome Measure(s): The PGRMC1 measurements were correlated with clinical outcomes, such as number of follicles, number of retrieved oocytes, and ongoing pregnancy rates (PR).
Result(s): The PGRMC1 mRNA levels within GC/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(s): In patients with elevated PGRMC1 mRNA levels, gonadotropin-induced follicle development is attenuated, although sufficient numbers of follicles develop to allow for ET and subsequent pregnancy. (Fertil Steril® 2012;97:572–8. ©2012 by American Society for Reproductive Medicine.)
Key Words: PGRMC1, granulosa/luteal cells, IVF, controlled ovarian hyperstimulation, follicular development

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 IVF outcome and/or pregnancy (3–6). One gene that might be useful as a predictor of IVF 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). In addition, PGRMC1 is highly expressed in human ovarian granulosa cells (GC) (9, 10) and functions to preserve the viability of these cells (9–13).

Consistent with its important role in regulating the viability of GC/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 (POF), 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 POF (8). This point mutation is important in that it reduced the functional capacity of PGRMC1 (8). In addition, reduced expression of PGRMC1 was

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observed in women with polycystic ovarian syndrome (PCOS) (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 messenger RNA (mRNA) levels within the GC/luteal cells of women undergoing controlled ovarian hyperstimulation (COH) protocols could be used to identify a subset of women whose response to gonadotropin treatment was altered in a way that would affected the success of their IVF 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). In addition, 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).

MATERIALS AND METHODS

Patients

This study included 85 patients who underwent COH 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 $\leq$38 years, with normal basal serum FSH, LH, and E_2 levels 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 $\leq$850 pg/mL and/or retrieval of less than five oocytes. Severe male factor infertility requiring surgical sperm harvest and patients with PCOS 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's discretion. In addition, 10 women undergoing COH for the purpose of oocyte donation were included as controls.

Stimulation Protocols

Briefly, patients were treated with recombinant FSH (Gonal F; Serono) and purified urinary hMG (Menopur; Ferring Pharmaceuticals). In patients undergoing flexible antagonist protocol, ganirelix acetate (0.25 mg SC, Ganirelix, Organon Pharmaceuticals) was started when either the lead follicle measured $\geq$13 mm or E_2 level was >300 pg/mL. Ganirelix acetate was continued until the day of hCG administration. The hCG (Profasi, Serono) was administered SC 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 progesterone (P4) levels, as previously published (17). Transvaginal oocyte retrieval was performed approximately 36 hours after hCG administration and the GC/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 minutes. The cell pellet was suspended in phosphate-buffered saline (PBS), layered on Histopaque-1077 (Sigma Chemical Co.) and centrifuged for 30 minutes at 400 $\times$ *g*. After centrifugation, the opaque interface containing the GC/luteal cells was carefully aspirated and transferred into a 15-mL sterile conical centrifuge tube. The cells were then suspended in 12 mL of 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 GC/luteal cells using All-Prep DNA/RNA Micro kit from Qiagen and used in a polymerase chain reaction (PCR) protocol to amplify exon 3 of human PGRMC1. This protocol used the following primers: forward: 5′-TTGCAGGCCTCTA ATAAATG-3′; reverse: 5′-CAGCACTG CAGTTCACTTTC-3′. The PCR reaction consisted of 35 cycles of a denaturation phase of 30 seconds at 94°C, an annealing phase of 30 seconds at 55°C, and an extension phase of 60 seconds 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. for sequencing. The DNA sequence was 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 GC/luteal cells using All-Prep DNA/RNA Micro kit from Qiagen. This RNA preparation was used in a real time PCR protocol using CFX96 real time PCR system. The RNA samples (1 ng) were resuspended in 25 μL of the SsoFast Probes Supermix (Bio-Rad) containing 300 nm of each PGRMC1 primer: forward 5′-GGTGTTCGATG TGACCAAAG-3′and reverse 5′-GATGCA TCTCTTCCAGCAAA-3′ and the probe: CGCAAATTCTACG GGCCCGA (5′ FAM $\rightarrow$ 3′ BHQ1). Actin was used as an internal control and was detected using the following primers: forward 5′-CACTCTTCCAGCCTTCCTTC-3′ and reverse 5′-GGATGTCCACG TCACACTTC-3′ and the probe: TGCCACA GGACTCCATGCCC (5′ CAL Gold 540 $\rightarrow$ 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 1-kb segment of DNA, which 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. Because the methylation of these cytosine