Alterations in the expression, structure and function of progesterone receptor membrane component-1 (PGRMC1) in premature ovarian failure

Mahmoud Reza Mansouri1, Jens Schuster1, Jitendra Badhai1, Eva-Lena Stattin2, Ralf Lösel3, Martin Wehling3, Birgit Carlsson1, Outi Hovatta4, Per Olof Karlström5, Irina Golovleva2, Daniela Toniolo6,7, Silvia Bione6,7, John Peluso8 and Niklas Dahl1,*

1Department of Genetics and Pathology, Uppsala University, 751 85 Uppsala, Sweden, 2Medical and Clinical Genetics, Department of Medical Biosciences, Umeå University, 901 85 Umeå, Sweden, 3Clinical Pharmacology Mannheim, University of Heidelberg, 68135 Mannheim, Germany, 4Department of Clinical Science, Intervention and Technology, Karolinska Institutet, 141 86 Stockholm, Sweden, 5Department of Women’s and Children’s Health, Academic Hospital, 751 85 Uppsala, Sweden, 6Institute of Molecular Genetics, Consiglio Nazionale delle Ricerche (CNR), 27100 Pavia, Italy, 7Department of Biotechnological Research (DIBIT), San Raffaele Scientific Institute, 20132 Milano, Italy and 8Department of Cell Biology, University of CT Health Center, Farmington, CT 06030, USA

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Premature ovarian failure (POF) is characterized by hypergonadotropic hypogonadism and amenorrhea before the age of 40. The condition has a heterogeneous background but genetic factors are demonstrated by the occurrence of familial cases. We identified a mother and daughter with POF both of whom carry an X;autosome translocation [t(X;11)(q24;q13)]. RNA expression studies of genes flanking the X-chromosome breakpoint revealed that both patients have reduced expression levels of the gene Progesterone Receptor Membrane Component-1 (PGRMC1). Mutation screening of 67 females with idiopathic POF identified a third patient with a missense mutation (H165R) located in the cytochrome b5 domain of PGRMC1. PGRMC1 mediates the anti-apoptotic action of progesterone in ovarian cells and it acts as a positive regulator of several cytochrome P450 (CYP)-catalyzed reactions. The CYPs are critical for intracellular sterol metabolism, including biosynthesis of steroid hormones. We show that the H165R mutation associated with POF abolishes the binding of cytochrome P450 7A1 (CYP7A1) to PGRMC1. In addition, the missense mutation attenuates PGRMC1’s ability to mediate the anti-apoptotic action of progesterone in ovarian cells. These findings suggest that mutant or reduced levels of PGRMC1 may cause POF through impaired activation of the microsomal cytochrome P450 and increased apoptosis of ovarian cells.

INTRODUCTION

Premature ovarian failure (POF) or premature menopause refers to primary or secondary amenorrhea before the age of 40 years. Women with POF suffer from anovulation, infertility and reduced estrogen levels which results in major health problems and psychosocial consequences (1). Approximately 1% of females are affected at 40 years of age, whereas only 0.1% are affected by the age of 30 (2,3). The diagnosis is based on clinical presentation and the finding of repeatedly elevated FSH levels (4). The pathogenic mechanisms behind POF are heterogeneous and acquired forms may occur in the context of autoimmune disease, infections or after anti-cancer treatment (5,6). However, in the majority of cases, the etiology

*To whom correspondence should be addressed at: Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University, SE-751 85 Uppsala, Sweden. Tel: +46 186112799; Fax: +46 18554025; Email: niklas.dahl@genpat.uu.se

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remains unclear and the underlying mechanisms are unknown (2,3). Genetic factors in POF are well documented due to the occurrence of familial cases and further supported by its association with structural and numerical X-chromosome abnormalities. Partial or complete monosity for chromosome X and X;autosome translocations are well documented causes of primary amenorrhea and ovarian dysfunction (7–10). Many of these rearrangements are located in the chromosome Xq13–q26 region suggesting a POF ‘critical region’ (8,11). It has been hypothesized that one or several genes in this region are required in double dose for normal ovarian function. Balanced X;autosomal translocations in this region could cause a reduction in gene dosage by the direct disruption of an X chromosomal gene which escapes X chromosome inactivation. Alternatively, POF can result from the disruption of X-linked genes required in double dose in oocytes where two active X chromosomes are present throughout female fertile life (10). Another possible mechanism leading to disease is a positional effect of a genomic rearrangement resulting in reduced expression of neighboring genes. Despite the analysis of several genes in the Xq13–q26 region a strong candidate gene for POF remains unidentified.

To date, a few X-linked genes located outside the critical interval have been found mutated in rare cases of POF. To date, genes encoded from both chromosome X and autosomes which encode the forkhead transcription factor (FOXL2) (15), the follicle stimulating hormone receptor (FSHR) (16), the luteinizing hormone receptor (LHR) (17), Inhibin (18), galactose-1-phosphate uridylyltransferase (GALT) (19), the eukaryotic initiation factor 2B (EIF2B) (20), newborn ovary homeobox gene (NOBOX) (21) and Factor in germline alpha (FIGLA) (22). A number of candidate genes encoded from both chromosome X and autosomes have been suggested from different studies in a review by Goswami and Conway (7).

We have explored our initial finding of an X;autosome translocation in a mother and daughter both of whom were diagnosed with POF. We observed that the cytogenetic location of the X-chromosome breakpoint coincides with the ‘critical region’ for POF and a detailed characterization of the translocation breakpoint was undertaken in order to identify novel candidate genes and mechanisms for this condition.

**RESULTS**

**Mapping the translocation breakpoints**

Cyto genetic investigation revealed an apparently balanced reciprocal chromosome translocation t(X;11)(q24;q13) in a mother and daughter both of whom were diagnosed with idiopathic hypogonadotropic hypogonadism. We undertook a systematic mapping of the Xq breakpoint using a set of 14 different BAC clones corresponding to Xq24 and fluorescence in situ hybridization (FISH) to metaphase chromosomes from the mother and daughter. A combination of four contiguous genomic BACs allowed us to initially position the chromosome X breakpoint (Fig. 1A) to a 200 kb region.

The Xq24-specific BAC clones RP5-113911 and RP4-555N2 hybridized to the normal chromosome X and to the derivative chromosome X. The two overlapping BAC clones RP3-404F18 and RP11-799J12 hybridized to the normal chromosome X as well as to the two derivative chromosomes der(11) and der(X), which indicates that both clones span the breakpoint. The results allowed us to restrict the breakpoint to within a 70 kb region (Fig. 1B). This region was further narrowed down to 40 kb by Southern blot analysis using four different restriction enzymes and a set of DNA probes ranging from 170 to 350 bp generated from non-redundant sequences in the breakpoint region (data not shown). The detailed mapping positioned the breakpoint just telomeric of the gene LOC203427.

**Gene expression analysis**

Eight genes were found to be located within 300 kb on either side of the Xq24 translocation breakpoint region (Fig. 1A). We performed quantitative real-time RT–PCR on lymphoblastoid cell lines (LCLs) derived from both patients carrying the X;autosome translocation and from seven healthy female controls using SYBR green reagent and forward and reverse primers placed in different exons for each gene. The analysis revealed significantly reduced levels of the PGRMC1 (P < 0.01), LOC203427 (P < 0.001) and UBE2A (P < 0.05) transcripts as well as significantly increased levels for the SLC25A5 (P < 0.001) transcript in both patients compared with the female controls (Fig. 2A). Additionally, we analyzed PGRMC1 protein levels in LCLs from both patients carrying the translocation and four healthy female controls using a polyclonal antibody raised in rabbit against PGRMC1. Western analysis revealed significantly reduced levels of PGRMC1 in LCLs derived from the two translocation carriers compared with controls (P < 0.001) (Fig. 2B).

**DNA methylation analysis and mutational screening**

The X-chromosome inactivation pattern around the human androgen receptor gene was investigated on fresh peripheral nucleated blood cells by restriction cleavage with the methyl ation sensitive enzyme HpaII. A skewed X-inactivation pattern was found in both the mother and daughter. More than 85% of cells retained a specific X chromosome active in both cases suggesting the normal X to be predominantly inactivated (Supplementary Material, Fig. S1). A mutation screening of the PGRMC1 coding sequence in 67 independent cases diagnosed with idiopathic POF revealed one patient with a heterozygous missense mutation (c.494A>G). The mutation predicts a p.H165R amino acid substitution in the intracellular cytochrome b5 domain (Pfam PF00173) of PGRMC1 (Fig. 3A). The patient is a sporadic case with no known metabolic or iatrogenic causes of the conditions. The variant was excluded in 307 control X-chromosomes from healthy blood donors.

**Overexpression of PGRMC1 and apoptosis**

We then analyzed the anti-apoptotic effect of the missense mutation in response to progesterone by transfecting wild-type
pEGFP-PGRMC1, pEGFP-PGRMC1-H165R and an empty vector (pEGFP) in spontaneously immortalized granulosa cells (SIGCs) derived from rat. Transfection with wild-type pEGFP-PGRMC1 in the presence of progesterone significantly suppressed the rate of apoptosis compared with pEGFP-PGRMC1-H165R and an empty vector control (Fig. 3B). Although the H165R mutation conveyed some protective effects, it was not as effective as the wild-type. The H165R missense mutation did not affect the ability of PGRMC1 to bind progesterone suggesting that the impaired anti-apoptotic ability is downstream of PGRMC1 ligand binding (data not shown).

Figure 1. (A) The chromosome Xq24 region and the chromosome translocation breakpoint region (boxed) associated with POF in a mother and daughter. Genes (black bars) and genomic clones used for FISH (grey bars) are outlined. The Xq24-specific BAC clones RP3-404F18 and RP11-799J12 hybridize to both the normal chromosome X and to the two derivative chromosomes 11 and X, respectively, on metaphase spreads from the translocation carriers. The boxed region corresponds to 40 kb spanning the breakpoint. (B) FISH analysis with the chromosome Xq24 BAC clone RP3-404F18 and metaphase chromosomes from the daughter with the X-autosome translocation. EBV-transformed B lymphocytes derived from the patient were used to prepare chromosomes for FISH analysis. Signals were detected on the normal chromosome X (chr X) and the two derivative chromosomes, der(X) and der(11), which indicate that the BAC clone spans the Xq24 breakpoint.

Figure 2. (A) mRNA levels of genes flanking the X chromosome breakpoint in LCLs as determined by quantitative real-time PCR using SYBR green (Invitrogen). Values are based on means of 3–7 experiments, each performed in triplicate. The patients consist of the mother and daughter with t(X;11). The control group consists of seven healthy female controls. Error bars indicate standard error. Data were analyzed by two-tailed Student’s t-test. * Indicates value differences from the two translocation carriers; ** Corresponds to (P < 0.05) while *** and **** indicate value differences of (P < 0.01 and P < 0.001), respectively. (B) PGRMC1 protein expression in LCL’s from the two patients carrying the t(X;11) and controls (from western blot analysis). Values are based on the mean value from four independent measurements from both patients and four healthy female controls. Error bars indicate standard error and **** denotes the value difference (P < 0.001) from patients.
PGRMC1 and cytochrome P450 interaction

To study the downstream effect of the missense mutation on PGRMC1’s ability to bind cytochrome P450, we transfected HEK293 cells with wild-type pEGFP-PGRMC1, pEGFP-PGRMC1-H165R or the empty vector (pEGFP). The cells were harvested and detergent-solubilized microsomes were subjected to immunoprecipitation using anti-GFP antibody coated magnetic beads (Dynabeads). Bound and unbound proteins were separated and probed with antibodies against CYP7A1. As expected, no interaction was found between the empty construct and CYP7A1. The wild-type pEGFP-PGRMC1 construct was found to bind CYP7A1 while the PGRMC1-CYP7A1 interaction was lost when using the mutant pEGFP-PGRMC1-H165R construct thus showing the H165R mutation to be detrimental for this PGRMC1-CYP interaction (Fig. 3C).

DISCUSSION

In this study, we have reported the molecular characterization of an X;autosome translocation t(X;11)(q24;q13.4) in a mother and daughter with POF. Rearrangements of the X-chromosome including X;autosome translocations are well documented in the etiology of ovarian dysfunction (7–10). The cytogenetic breakpoints are usually located on the long arm of the X-chromosome corresponding to the Xq13–q26 interval which consequently is defined as a ‘critical region’ for normal ovarian function. Despite the characterization of many X-chromosome breakpoints associated with POF no strong candidate gene has yet been identified that could explain the disease in these patients (10). It has been shown that genomic rearrangements may convey position effects on the expression of genes that flank the breakpoints (23). Such a long range transcriptional silencing may be caused by genes being relocated in the neighborhood of heterochromatin by chromosomal rearrangements, i.e. position effect variegation (24). X-linked genes with only one active allele are particularly sensitive to genomic rearrangements when the structurally normal X chromosome is inactivated. In such cases, gene expression levels from the allele on the derivative chromosome can be significantly altered due to heterochromatization of regions flanking the breakpoint or by the separation of the promoter and the transcription units from a distant cis-acting regulatory element as a result of the rearrangement. In our study, the mother and daughter with the translocation display a skewed X-inactivation pattern with a ratio between the two X chromosomes of approximately 6:1. This suggests the structurally normal chromosome X to be predominantly inactive, thus making the genes in the vicinity of the Xq breakpoint region valid candidates for further analysis. Furthermore, the X-chromosomal breakpoint in the t(X;11) translocation coincides with the cytogenetic breakpoint identified in several previously recorded cases with POF (8,11).

Our detailed mapping of the chromosome X breakpoint revealed no gross loss or gain of X-chromosomal DNA, although we cannot exclude minor rearrangements in a 40 kb region across the breakpoint. Eight genes are located in the immediate vicinity of the rearrangement and within 300 kb on each side of the breakpoint; KIAA1210, PGRMC1, LOC203427, SLC25A5, Cxorf56, UBE2A, NKRFP2 and SEPT6. Six of these are expressed in lymphocytes which allowed us to analyze the effect of the translocation on the mRNA expression and protein levels in lymphoblastoid cells from both mother and daughter. Four of the genes were found to have significantly altered mRNA levels in both translocation carriers when compared with control samples. Three of these were found downregulated (PGRMC1, LOC203427 and UBE2A), whereas SLC25A5 was found to be upregulated.

Functional predictions and previous knowledge of these genes and their products directed our focus to PGRMC1. We confirmed that PGRMC1 protein levels in LCLs derived from the two translocation carriers were significantly reduced compared with controls. PGRMC1 encodes a

Figure 3. (A) DNA sequence chromatogram from exon 3 in the PGRMC1 gene illustrating the missense mutation in the sporadic patient with POF. The arrow shows the heterozygous mutation c.494A>G resulting in p.H165R. (B) The H165R mutation reduces the anti-apoptotic capacity of PGRMC1 in the presence of 1 nM progesterone (P4). SIGCs were transfected with either of the construct wild-type pEGFP-PGRMC1, pEGFP-PGRMC1-H165R or the empty vector (pEGFP). SIGCs were placed in serum-free medium with 1 nM progesterone for 5 h and then assessed for apoptosis. Values are based on means of four separate experiments. Error bars indicate standard errors. Data were analyzed by a one-way ANOVA followed by a Fisher’s PLSD post hoc test. Asterisks indicate value differences from those obtained with the empty vector pEGFP; * corresponds to (P<0.05) while ** corresponds to (P<0.01). The value difference between pEGFP-PGRMC1-wild-type and PGRMC1-H165R was found significant (P<0.01). (C) The H165R mutation abolishes PGRMC1’s ability to bind CYP7A1. HEK293 cells were transfected with either of the constructs pEGFP-PGRMC1-wild-type, pEGFP-PGRMC1-H165R or the empty vector (pEGFP). Microsomes were immunoprecipitated using anti-GFP antibody coated magnetic beads. Bound (B) and unbound (U) proteins were separated and hybridized with antibodies against CYP7A1 (repeated twice).
broadly expressed and highly conserved 22 kDa transmembrane protein that is part of a progesterone binding complex (25,26). It has a short extracellular amino terminus and an intracellular domain which contains a heme binding motif (27). The protein is expressed in both granulosa and luteal cells and its levels and subcellular localization are regulated by gonadotropins (28). Studies in granulosa and luteal cells have shown that antibodies against PGRMC1 abrogate the protective effect of progesterone. The anti-apoptotic action of progesterone is dependent on PGRMC1 in both human (29) and rodent ovarian cells (30,31). PGRMC1 is a component of the membrane complex that functions as a receptor for progesterone (P4) and siRNA knock down of its gene product results in a 60–80% decline in P4 binding with a loss of P4’s anti-apoptotic action in SIGCs (30). This suggests that PGRMC1 accounts for an important part of progesterone’s action in these cells. Over-expression of PGRMC1 in cultured cells increases hydroxylation of progesterone by the cytochrome P450 Cyp21 which suggested an intracellular down-stream effect of PGRMC1 (33). This was verified by the finding that PGRMC1 binds and regulates several P450 cytochrome oxidases (34). The P450 enzymes participate in the biosynthesis of cholesterol which is essential for intracellular sterol metabolism and steroidogenesis. Consequently, the RNAi mediated knock down of PGRMC1 reduces activity of cytochrome P450 enzymes thereby blocking these intracellular cholesterol pathways (34).

The observed downregulation of PGRMC1 mRNA and protein levels in the two translocation carriers, possibly through a positional effect, led us to hypothesize that a similar phenotypic effect may be caused by structural mutations in PGRMC1. We performed mutational screening of the entire gene on a cohort of 67 independent women with idiopathic POF. The patients were diagnosed after exclusion of iatrogenic or metabolic factors as a cause of their condition. Sequence analysis revealed one sporadic patient that is heterozygous for a single missense mutation in PGRMC1. The mutation is located in the intracellular C-terminus and within the cytochrome b5 domain which is essential for heme-binding and the regulation of P450s (33,35,36). The effect of the missense mutation was first analyzed in SIGCs. We show that expression of the mutation significantly increases the rate of apoptosis in SIGCs when compared with SIGCs transfected with the wild-type PGRMC1 construct. This supports the concept that the missense mutation in PGRMC1 could mediate premature ovarian failure through altered apoptosis and thereby cause a more rapid loss of ovarian follicles. Progesterone influences the rate at which the oocytes interact with granulosa cells to form follicles in the developing ovary (37). Thus, failure to transduce any of progesterone’s anti-apoptotic action in the developing ovary may result in the premature loss of ovarian follicles and, ultimately, in ovarian dysfunction.

We then analyzed the direct interaction between mutant PGRMC1 and one of its down-stream targets CYP7A1 which is a key member of the P450 family of enzymes. The CYP7A1 catalyzes the rate limiting step in cholesterol metabolism (38) and it is one of several P450 enzymes that has been shown to bind PGRMC1 (34). We show that the missense mutation abolishes the binding capacity of PGRMC1 to CYP7A1. We also studied the effect of the missense mutation on PGRMC1’s ability to bind CYP3A4 and CYP21A2, previously reported to bind PGRMC1 (34), in HEK293 cells. However, the endogenous expression of these enzymes was too low to determine any interaction with wild-type PGRMC1, using a pull-down assay (data not shown).

There are several indications of a direct role for PGRMC1 in steroidogenesis. The 21-hydroxylation of progesterone resulting in the synthesis of deoxycorticosterone is increased by exogenous PGRMC1 in the presence of high concentrations of progesterone (39). Moreover, an antibody against PGRMC1 has been shown to inhibit the 21-hydroxylation of progesterone and 18-hydroxylation of deoxycorticosterone in a dose-dependent manner (40). It has also been shown that mutations in the cytochrome P450 regulatory enzyme NADPH cytochrome P450 oxidoreductase, which is an electron donor involved in the catalytic activity of cytochrome P450 enzymes, cause Antley-Bixler syndrome characterized by the accumulation of steroid metabolites, adrenal hyperplasia and amenorrhea (41). PGRMC1 interacts with several members of the P450 complex and it is reasonable that a mutation in PGRMC1 may have an effect on the levels of several sterol metabolites. The sensitivity of different cytochrome P450 enzymes to altered PGRMC1 activity may be related to particular enzymes expressed in a tissue-specific manner. Consequently, other important functions of cytochrome P450 enzymes, e.g. in drug metabolism and detoxification processes, could remain unchanged despite subnormal levels or activity of PGRMC1. The ovaries may be more sensitive to a reduction in PGRMC1 transducing capacity and related to the CYPs expressed therein. Thus, altered PGRMC1 could result in a demise of the follicles at a faster rate than normal and, consequently, in an early menopause.

In summary, we present several lines of evidence for altered PGRMC1 function resulting in premature ovarian failure. First, we identified a mother and daughter with POF and an X chromosomal breakpoint adjacent to PGRMC1 which is found to be down-regulated in both carriers. Secondly, a sporadic POF patient carries a missense mutation in the cytochrome b5 binding domain of PGRMC1. Thirdly, the missense mutation attenuates PGRMC1’s ability to transduce progesterone’s anti-apoptotic action in granulosa cells and finally, we show that the missense mutation abolishes the interaction between PGRMC1 and the P450 enzyme CYP7A1.

Our combined data suggest that PGRMC1, an important regulator of the cytochrome P450 enzymes, is critical for ovarian function and maintained fertility. This illustrates the complexity of PGRMC1 and warrants further investigation to clarify its regulation, mechanism of action and specific effects on microsomal P450s.

MATERIALS AND METHODS

Patients

We identified a mother and daughter with POF both of whom carry a balanced X;autosome translocation [t(X;11)
(q24;q13.4)]. Cytogenetic analysis was performed using standard G-banding technique on peripheral blood samples.

The mother had menarche at 12 years of age followed by irregular and sparse menstruation cycles with menopause at 27 years of age. She is otherwise healthy. Autoimmune disease, infections or drugs were excluded as possible causes of her condition. Repeated analysis of gonadotropins showed elevated values of FSH (>45 u/l) and LH (>25 u/l) indicating hypergonadotropic hypogonadism. Abdominal ultrasound at age 37 years revealed ovaries of subnormal size without cystic changes. The daughter had menarche at 14 years of age and only one additional menstruation cycle at 16 years of age. She was diagnosed with hypergonadotropic hypogonadism at age 19 years (FSH values >70 u/l, LH values >40 u/l). Laparoscopy showed that the ovaries were of a quarter of normal size.

Peripheral blood samples were collected from 67 independent patients diagnosed with 'idiopathic' POF for mutational analysis. The patients were mostly sporadic cases of Swedish or Italian origin. This study was approved by the regional ethical committee of Uppsala.

**FISH and Southern blot analysis**

Metaphase chromosomes from LCLs from the patients, 14 chromosome 11q specific BAC clones and 14 BAC clones specific for Xq were used for FISH analysis as described (42). A complete list of clones is available upon request. The clones were labeled with either digoxigenin-16-dUTP or biotin-16-dUTP (Roche) by nick-translation. After hybridization and washing, the digoxigenin labeled probes were detected by rhodamine labeled anti-digoxigenin antibody and the biotin labeled probes were detected by fluorescein isothiocyanate labeled anti-biotin antibody (Roche). The chromosomes were counterstained with DAPI and antifade (Vector labs) and the slides analyzed with a Zeiss Axioscope microscope using a cooled CCD camera (Photometrics) and Smart Capture software.

DNA from both patients and controls was prepared from frozen B-lymphocytes and Southern blotting was performed as previously described (43). Briefly, following overnight restriction endonuclease digestion, DNA was separated overnight by agarose gel electrophoresis and transferred to a nylon membrane (Amersham) in 5 × SSC. Probes were generated by PCR, labeled with \[^{32}P\]dCTP using Megaprime DNA labeling system (Amersham) and hybridized to membranes in a hybridization buffer (7 × SSC, 0.5% SDS, 5.5 × Denhardt solution and 11% dextran sulphate) supplemented with salmon sperm DNA. Blots were washed in 2 × SSC/0.1% SDS before exposure to radiographic film.

**Gene expression analysis**

Quantitative real-time RT–PCR using SYBR green (Invitrogen) was performed on total RNA prepared by TRIZOL® (Invitrogen) from LCLs derived from the patients carrying the X;autosome translocation and seven female controls in 3–7 independent experiments, each performed in triplicate measurements, in order to investigate the expression of the eight genes found to be located within 300 kb on either side of the Xq24 translocation breakpoint region (KIAA1210, PGRMC1, LOC203427, SLC25A5, CXorf56,UBE2A,NKRF and SEPT6). β-actin was used as an internal control. Additionally, we prepared proteins from patient-derived LCLs by TRIZOL® and analyzed PGRMC1 protein levels by western blot. Proteins were separated in a 10% NuPage Bis/Tris gel (Invitrogen) and subsequently transferred to an Immobilon™-FL transfer membrane (Millipore) according to manufacturer’s recommendations. PGRMC1 protein levels in the patients carrying the X;autosome translocation and four female controls were assayed using a polyclonal antibody raised in rabbit against a peptide matching the 15 C-terminal amino acids of PGRMC1 (DEEPEKDESARKND) according to standard protocol (Odyssey system; LiCor).

**DNA methylation analysis and mutational screening**

Genomic DNA was extracted from fresh blood samples derived from both patients carrying the X; autosome translocation and digested with the methylation-sensitive restriction enzyme HpaII. After digestion the DNA samples were amplified using primers flanking the HpaII restriction site and the polymorphic CAG repeat in the human androgen receptor gene as described (44). PCR products were separated by size and the X-chromosome inactivation pattern determined by calculating the area under the peak for the unmethylated allele divided by the total peak area for both alleles.

Sequencing was performed on templates generated from genomic DNA using PCR. The amplicons were generated with three primer pairs flanking the coding sequences of the three PGRMC1 exons (primer sequences available upon request). Sequences were obtained from the entire coding regions, exon–intron boundaries, the 5' -UTR and the first 195 bp of the 3' -UTR of the PGRMC1 gene (RefSeq NM_006667). The reverse primer for exon 3 was positioned in the 3' -UTR. Sequencing was performed using BigDye Terminator Chemistry and an ABI 3700 DNA Analyzer (PE- Applied Biosystems) as previously described (43).

**Overexpression of PGRMC1 and apoptosis**

SIGCs from rat were cotransfected with 1 μg/dish of vector DNA (pEGFP-PGRMC1, pEGFP-PGRMC1-H165R and empty vector pEGFP). After 24 h the serum-supplemented medium was removed and the cells placed in serum-free medium supplemented with 1 nM P4. After 5 h, the cells were washed with Krebs/HEPES buffer and stained with rhodium fluoride (3.5 μg/ml Krebs/HEPES buffer) for 15 min at room temperature in the dark. The cells were washed again and apoptosis was assessed as previously described (31). Approximately 400 cells transfected with the empty vector pEGFP, 400 cells transfected with pEGFP-PGRMC1 and 500 cell transfected with pEGFP-PGRMC1-H165R were evaluated for apoptosis.

**PGRMC1 and cytochrome P450 interaction**

HEK-293 and CHO cells were grown in DMEM medium supplemented with 10% FBS, 1% PEST. Cells were transfected in OptiMEM Medium (Invitrogen) with 5 μg vector DNA pEGFP-N1, pEGFP-PGRMC1 and pEGFP-PGRMC1(H165R),
respectively] using Lipofectamine™2000 (Invitrogen) according to manufacturer’s recommendations. After 24 h, cells were assayed for expression of recombinant eGFP protein by fluorescence microscopy, washed with PBS, harvested by centrifugation, and stored at −20°C until further analysis. HEK-293 or CHO cells were thawed for 5 min at 37°C and resuspended in 400 µl buffer H (50 mM Tris/Cl, pH 7.9; 0.25 mM sucrose; 200 mM PMSF) supplemented with protease inhibitor cocktail (Sigma). Cells were lysed by repeated passage through a G27 needle and particulate material was removed by centrifugation (1000g, 5 min, 4°C). The supernatant was centrifuged (12 000g, 10 min, 4°C) and microsomes were collected from the remaining supernatant by ultracentrifugation (100 000g, 1 h, 4°C). Microsomal proteins were dissolved in 100 µl buffer H and stored at 4°C.

Tosylated Dynabeads™ (Invitrogen) were coated with Living Colors® A.v. α-GFP Monoclonal Antibody (IL-8; Clontech) according to manufacturer’s protocols. Fifty micrograms microsomal protein was incubated with 5 × 10⁻⁴ α-GFP coated Dynabeads™ in buffer IDP (50 mM Tris/Cl, pH 7.9; 150 mM NaCl; 10% w/v Glycerol; 0.05% NP-40) for 60 min at 4°C. Unbound proteins were precipitated with acetone and resuspended in SDS-loading dye. Beads were washed twice with buffer IDP. Bound proteins were resuspended in SDS-loading dye and eluted by denaturation (5 min, 100°C). Proteins were separated on a NuPage 10% Bis/Tris Gel Proteins were separated on a NuPage 10% Bis/Tris Gel (Invitrogen) and transferred to an Immobilon™-FL transfer membrane (Millipore) according to manufacturer’s recommendations. Recombinant eGFP fusion proteins and co-immunoprecipitated CYP7A1 proteins were detected using a-Mouse and IRD800 labeled a-rabbit (LiCor) secondary antibodies. Western blots were analyzed using an Odyssey scanner (Westburg).

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SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

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Conflict of Interest statement. None declared.

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