

PDE8A genetic variation, polycystic ovary syndrome and androgen levels in women

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ABSTRACT: Polycystic ovary syndrome (PCOS) is characterized by excessive theca cell androgen secretion, dependent upon LH, which acts through the intermediacy of 3',5'-cyclic adenosine monophosphate (cAMP). cAMP signaling pathways are controlled through regulation of its synthesis by adenylyl cyclases, and cAMP degradation by phosphodiesterases (PDEs). PDE8A, a high-affinity cAMP-specific PDE is expressed in the ovary and testis. Leydig cells from mice with a targeted mutation in the *Pde8a* gene are sensitized to the action of LH in terms of testosterone production. These observations led us to evaluate the human *PDE8A* gene as a PCOS candidate gene, and the hypothesis that reduced PDE8A activity or expression would contribute to excessive ovarian androgen production. We identified a rare variant (R136Q; NM_002605.2 c.407G > A) and studied another known single nucleotide polymorphism (SNP) (rs62019510, N401S) in the *PDE8A* coding sequence causing non-synonymous amino acid substitutions, and a new SNP in the promoter region (NT_010274.16:g.490155G > A). Although PDE8A kinetics were consistent with reduced activity in theca cell lysates, study of the expressed variants did not confirm reduced activity in cell-free assays. Sub-cellular localization of the enzyme was also not different among the coding sequence variants. The *PDE8A* promoter SNP and a previously described promoter SNP did not affect promoter activity in *in vitro* assays. The more common coding sequence SNP (N401S), and the promoter SNPs were not associated with PCOS in our transmission/disequilibrium test-based analysis, nor were they associated with total testosterone or dehydroepiandrosterone sulfate levels. These findings exclude a significant role for *PDE8A* as a PCOS candidate gene, and as a major determinant of androgen levels in women.

Key words: PDE8A / polycystic ovary syndrome / androgens / theca / SNP

Introduction

Polycystic ovary syndrome (PCOS) is a common endocrine and metabolic disorder that is characterized by increased circulating androgen levels, anovulatory infertility and frequently, insulin resistance and hyperinsulinemia (Franks, 1995; Legro, 2001; Ehrmann, 2005). A hallmark of PCOS is excessive theca cell androgen secretion, which is directly linked to the symptoms and pathophysiology of PCOS. Adrenal androgen excess (e.g. dehydroepiandrosterone sulfate—DHEAS) is also frequently found in women with PCOS.

Theca cell androgen synthesis is dependent upon LH, which acts through the intermediacy of the second messenger, 3',5'-cyclic

adenosine monophosphate (cAMP). cAMP signaling pathways are controlled through regulation of the synthesis of cAMP by adenylyl cyclases, and cAMP degradation by phosphodiesterases (PDEs) (Schwartz, 2001; Beavo and Brunton, 2002; Vasta *et al.*, 2006; Kamenetsky *et al.*, 2006). Theca cells isolated from ovaries of women with PCOS secrete increased amounts of androgens and progestins compared with normal theca cells in response to stimulation with forskolin, a diterpene activator of adenylyl cyclases, suggesting that they have a heightened response to cAMP (Wood *et al.*, 2003, Wickenheisser *et al.*, 2006). Family based studies strongly suggest that the hyperandrogenemia associated with PCOS is an inherited trait, raising the possibility of a genetic predisposition to excessive ovarian androgen

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production in PCOS, which could be related to genetic differences in cAMP signaling (Legro et al., 1998). Moreover, steroid levels in blood in humans, including androgens, are heritable traits (Hong et al., 2001; Ukkola et al., 2002).

The PDE component of the cAMP signaling pathway, represented by a large superfamily that includes 21 mammalian genes that encode more than 100 isozymes, ensures the proper intensity and spatiotemporal distribution of cAMP signaling (Soderling and Beavo, 2000; Conti, 2002; Nikolaev et al., 2005; Zaccolo et al., 2006). PDEs including PDE3A, PDE4B and PDE4D are present in distinct compartments in the rodent ovary (Tsafriri et al., 1996). The mammalian PDE8 family includes PDE8A, a high-affinity cAMP-specific PDE, that is expressed in ovary, testis and other tissues including liver, kidney and heart (Soderling et al., 1998a, b; Mehats et al., 2002; Ahlström et al., 2005; Bender and Beavo, 2006; Sasseville et al., 2009). Leydig cells from mice with a targeted mutation in the *Pde8a* gene are sensitized to the action of LH in terms of testosterone production. These observations led us to evaluate the human *PDE8A* gene as a PCOS candidate gene, based on the hypothesis that reduced PDE8A activity or expression would contribute to excessive ovarian androgen production. Here we report new polymorphisms in the *PDE8A* coding sequence and promoter. The more common of these variants were not associated with PCOS or with testosterone and DHEAS levels.

Materials and Methods

Definition of PCOS

In this study, as in our previous work, the diagnosis was made by history of oligomenorrhea or amenorrhea (six or less menses per year) and biochemical evidence of hyperandrogenemia (Legro et al., 1998; Urbanek et al., 2005). Probands and their sisters were considered affected if they had six or fewer menses per year and either elevated total testosterone (> 58 ng/dl; 2 nmol/l) or elevated non-sex hormone binding globulin-bound testosterone (> 15 ng/dl; 0.5 nmol/l). Evidence for a PCO ovarian morphology was not required. Potential phenocopies (non-classical 21-hydroxylase deficiency, hyperprolactinemia, androgen-secreting tumors) were ruled out by appropriate tests. Sisters who were unaffected or considered to have an 'unknown' phenotype were not included in this analysis.

Theca cell cultures

The isolation and culture methods for normal and PCOS theca cells utilized in these studies have been previously described (Nelson et al., 1999; Wickenheisser et al., 2000; Wood et al., 2003).

Reverse transcription-PCR and PCR

Reverse transcription was carried out with MMLV-reverse transcriptase using RETROscript (Ambion, Austin, TX, USA) in a two step procedure. Briefly, total RNA (1.5 µg) from five normal theca cell preparations and five PCOS theca cell preparations, each from different subjects, was reverse transcribed in the presence of random decamers. The resulting cDNA was then used to carry out PCR amplification.

A *PDE8A* full coding sequence was amplified by PCR with a forward primer sequence of 5'-GGATCCCATGGGCTGTGCCCGAGCATC-3' containing a BamHI sequence (underlined) and a reverse primer 5'-GCGGCCGCTCTGGGTGGTGTCTCCCACT-3' containing a NotI sequence, yielding a 2522 bp product. PCR was performed in a 50 µl reaction volume with the Expand long template PCR system (Roche Applied

Science, Mannheim, German) in a Mastercycler ep thermal cycler (Eppendorf). After initial denaturation at 95°C for 3 min, PCR was performed for 35 cycles of denaturation at 95°C for 30 s annealing at 65°C for 30 s and extension at 72°C for 2 min and 45 s followed by a final 10 min elongation at 72°C. The *PDE8A* cDNAs were subjected to DNA sequence analysis. The PCR products were also cloned into the TOPO TA cloning vector (PCR2.1-TOPO, Invitrogen, Carlsbad, CA, USA), and four to five separate clones derived from each subject's PCR amplification were sequenced.

To confirm the G596A (R136Q) variant, we carried out PCR using a forward primer sequence of 5'-GTGTTTACCAAAGAAGATAACCAA TG-3' and a reverse primer 5'-CATTTATAGGCACTTCTCTCTAA CTCC-3' yielding a 547 bp product. After initial denaturation at 95°C for 3 min, PCR was performed for 35 cycles of denaturation at 95°C for 30 s, annealing at 51°C for 30 s and extension at 72°C for 1 min followed by a final 10 min elongation at 72°C. The PCR products were used for restriction fragment length polymorphism (RFLP) analysis with the restriction endonuclease TaqI (New England Biolabs) for genotyping.

Quantitative real-time PCR

RNA isolated from the theca cells were reverse transcribed to cDNA from women who had the coding sequence variants (MC03 and MC09) and from normal women (MC02 and MC31). cDNA diluted 1:10 and aliquots were subjected to quantitative real-time PCR. iTaq™ SYBR® Green Supermix with ROX reagent (Bio-Rad, Hercules, CA, USA) was used to detect amplicons of human *PDE8A* cDNA (forward primer 5'-GCCTGTTTCTGGACAAAC-3' and reverse primer 5'-GCATT CCGACA ACT CTT CTC-3'). The relative abundance of each cDNA were normalized with 18S rRNA gene (RNA primers forward: 5-GG CCCTGTAATTGGAATGAGTC-3' and reverse 5'-CCAAGATCCAAC TACGAGCTT-3'). Before amplification, samples were denatured at 95°C for 2.5 min. The amplification consisted of 40 cycles of denaturation at 95°C 15 s, annealing at the 57°C 30 s, and extension at 68°C 30 s. All reactions were run in triplicate.

Construction of expression plasmids

PCR products were cloned into the pCR2.1 TOPO vector (Invitrogen). The inserts were digested with NotI and BamHI and were subcloned into the pTarget vector.

The QuickChange II XL Site-directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) was used to create cDNAs representing the various *PDE8A* variant sequences.

Construction of plasmids expressing *PDE8A* fused to enhanced green fluorescent protein

Polymerase chain reactions were carried out with the following primers containing restriction enzyme sites (underlined): 5'-CTCGAGCGCCAG CATGGGCTGTGCCCGAGCATCCACATTTT (XhoI) and reverse primer 5'-GGATCCCTTCAGGAGGTGGTCGGAGGTTCCGCAGCT TC-3' (BamHI). The plasmids containing the various *PDE8A* variant sequences were used as templates. After initial denaturation at 95°C for 3 min, PCR was performed for 18 cycles of denaturation at 95°C for 30 s, annealing at 68°C for 30 s and extension at 72°C for 2 min 45 s followed by a final 10 min elongation at 72°C. The PCR products were cloned into pCR2.1 TOPO vector; after sequencing, the inserts were subcloned into the mammalian expression pEGFP-N2 vector.

PDE8A promoter sequence variation

We examined genomic DNA from 20 Caucasian women; 10 with a diagnosis of PCOS and 10 normal women. Their genomic DNA was amplified by PCR to obtain a DNA fragment of the *PDE8A* promoter. Primers to amplify

Table I Primer and probe sequences for Applied Biosystems custom Taqman assays

SNP ID	Forward primer	Reverse primer	VIC probe	FAM probe
352C > G (L55V), rs11540803	CCCCAGGGCCAGAAG	CCGGGCCCTTACCT	AGTCGGAGC TTCGGGAC	AGTCGGAGG TTCGGGAC
596G > A (R136Q)	TTCCTGGACAACATCATGACATTATCA	CTTGGCTTACCTGCACAGT	CAGAAATCCTC GACAGCT	CAGAAATCCTC AACAGCT
1391A > G (N401S), rs62019510	TGAAACTCAAGTATTTCTTTCCATAATGTAGGT	CAGGCATGGGACTACTTTTCT	TCAATATTATCA ATGCTGCC	AATATTATCA GTGCTGCC
-576G > A	CCCCGGCCCCGGATCAC	CGCACCCAGGAGTTGCT	CGGAGCC AGAGCC	CGGAGCC GGGAGCC

the *PDE8A* promoter including 5'-upstream 1297 and 66 bp from the transcription start site, yielding a 1363 bp product were designed using reference sequences from the National Center for Biotechnology Information (GenBank accession NM_002605). The forward primer was 5'-CCACCAAGAAGTTAAGTGACTGGCCC-3' and reverse primer was 5'-CGGGATCTCGCGTCAGGAAACG-3'.

PCR was performed in a 50 µl reaction volume with a GC-rich PCR system (Roche). Amplification conditions were: denaturation at 95°C for 3 min; followed by 10 cycles comprised of 30 s each at 95, 65 and 70 s at 72°C, then additional 25 cycles comprised of 30 s each at 95, 65 and 75 s at 72°C; followed by a final elongation step at 72°C for 7 min. Amplification products were run on 1% agarose and purified using QIAquick Gel extraction kit (Qiagen, Valencia, CA, USA). The amplified promoter fragments were ligated into a TA cloning vector (Invitrogen). The PCR products were then subjected to sequence analysis.

Promoter reporter plasmid construction

The *PDE8A* promoter fragments were amplified by PCR with a forward primer: 5'-GGTACCCCAAGAAGTTAAGTGACTGGCCC-3' (KpnI) and a reverse primer 5'-CTCGAGGCGGATCTCGCGTCAGGAAACG-3' (XhoI). The amplified promoter fragments were ligated into a TA cloning vector and then subcloned into the luciferase reporter PGL3 basic vector (Promega, Madison, WI, USA) after KpnI and XhoI digestion. The DNA sequences of the PCR template and clones were confirmed.

Cell culture and transfection

The day before transfection, COS-1 cells and Leydig tumor cells (MA-10) were seeded into 12-well culture plates. COS-1 cells were cultured in DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum and antibiotics [100 units/ml penicillin G, 100 units/ml streptomycin sulfate (Gibco/BRL, Gaithersburg, MD, USA)]. MA-10 cells kindly provided by Dr Mario Ascoli, University of Iowa, were maintained in Weymouth MB 752/1 medium modified to contain 1.1 g/l of NaHCO₃, 20 mM HEPES, 50 µg/ml of gentamicin, and 15% horse serum (Invitrogen). All cells were maintained at 37°C in a water-saturated atmosphere under 5% CO₂ in air.

Cells were transfected using FuGENE 6 transfection reagent (Roche) with 0.5 µg of plasmids DNA. Empty pGL3 plasmid was transfected as a control. The medium was changed 24 h after transfection. The cells were incubated for an additional 48 h before they were harvested. A Renilla luciferase plasmid was co-transfected to control per transfection efficiency.

Table II *PDE8A* coding sequence variation

Samples	Variation
Normal	MC02 –
	MC11 –
	MC31 –
	MC40 –
	MC50 –
PCOS	MC09 G596A(R136Q), C352G (L55V) (rs11540803)
	MC03 C352G(L55V), A1391G(N401S) (rs62019510)
	MC01
	MC16
	MC23

Three SNPs resulted in non-synonymous amino acid substitutions 596G > A (R136Q), 352C > G (L55V) and 1391A > G (N401S). These were discovered in two different PCOS theca cell preparations. The 55V substitution was present in the theca cells containing both the 136Q and 401S substitutions.

A	Human	120	FLDKHHDIIDHRNP	R	QLDAE	141
	Pan troglodytes	120	FLDKHHDIIDHRNP	R	QLDAE	141
	Macaca mulatta	120	FLDKHHDIIDHRNP	R	QLDAE	141
	Equus caballus	472	FLDKQHDIIIDHRNP	R	QLDAE	493
	Canis familiaris	129	FLDKHHDIVIIDHRNP	R	QLDAE	150
	Mus musculus	116	FQDKLHDIIDHRYP	R	QMDAE	137
	Rattus norvegicus	114	FQDKLHDIIDHRNP	R	QMDAE	135
	Monodelphis domestica	144	FLEKHHDIIDHRHS	R	QLDAE	166
	Bos Taurus	127	FLDKHHDIIDHRNS	R	HLDAE	148
	Gallus gallus	145	FLDKHHEIIIDHRHS	R	YFDAE	166
	Human	390	EAPITKVINII	N	AAQESSMPV	411
	Pan troglodytes	390	EAPITKVINII	N	AAQESSMPV	411
	Macaca mulatta	390	EAPITKVINII	N	AAQESSMPV	411
	Equus caballus	696	EAPITKVINII	N	AAQESSMPV	717
	Canis familiaris	399	EAPITKVINII	N	AAQESSMPV	420
	Mus musculus	386	EAPITKVINII	N	AAQENSPVPV	407
	Rattus norvegicus	384	EAPITKVINIL	S	AAQENSPVSV	405
	Monodelphis domestica	414	EAPITKVINII	N	AAQESSMPV	435
	Bos Taurus	351	EAPITKVINII	N	AAQESSMPV	372
	Gallus gallus	415	EAPITKVINII	N	AAQESSMPV	437

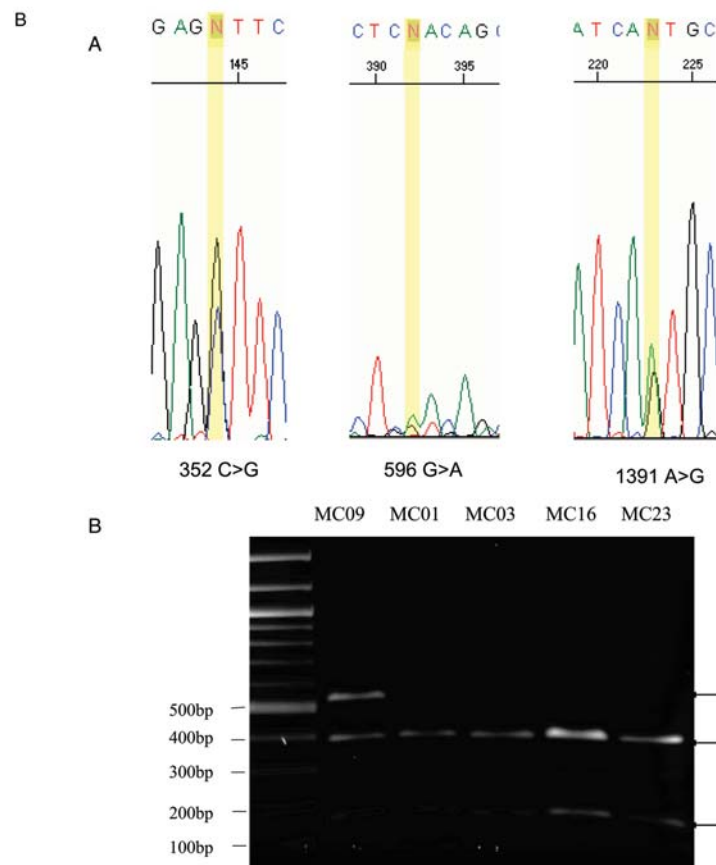


Figure 1 (A) Conservation of R 136 and NHO1 in PDE8A.

The Arg at position 136, or its equivalent position in other species, is conserved in all known PDE8A sequences including the chimpanzee, macaque, cat, cow, mouse, rat chicken and opossum. The Asp at position 401 is conserved in all known PDE8A sequences except in the rat, where it is a Ser. (B-A) Confirmation of *PDE8A* variants. A *PDE8A* full coding sequence was amplified by PCR with a forward primer sequence of 5'-GGATCCCATGGGCTGTGCCCGAGCATC-3' containing a BamHI sequence (underlined) and a reverse primer 5'-GCGGCCGCTCTGGGTGGTGTCTCCCACT-3' containing a NotI sequence, yielding a 2522 bp product. *PDE8A* variants in coding sequences were determined by ABI Prism 3730 Sequence Detection System. Three SNPs, 596G > A, 352C > G and 1391A > G were identified. (B-B) Confirmation of *PDE8A* G596A variants by RFLP. PCR was carried out using a forward primer sequence of 5'-GTGTTTACCAAAGAAGATAACCAATG-3' and a reverse primer 5'-CATTATAGGCATTCTCCTAACTCC-3' yielding a 547 bp product. The G596A variant was identified using the ABI Prism 3730 Sequence Detection System. The G596A variant was confirmed by RFLP. The 547 bp PCR products was digested with the restriction endonuclease *TaqI*, which yielded two fragment with size of 153 and 394 bp for the 596G allele, and one for the 596A allele.

Western blot analysis

After 48 h, whole cell lysates were collected from transfected COS-1 cells with complete lysis-M buffer (Roche). To detect PDE8A protein in these samples, 25 μg of total protein were separated by SDS-PAGE, transferred to Immobilon P polyvinylidene difluoride membrane (Millipore), and probed with a 1:500 dilution of PDE8A I21AP (C-terminal Ab IgG, 98-102 kDa) antibody (FabGennix). After extensive washing, membranes were incubated with a secondary anti-rabbit horseradish peroxidase-linked whole antibody Ab at 1:2000 (GE healthcare). PDE8A protein was detected using the SuperSignal West Femto Sensitivity reagent (Pierce, Rockford, IL, USA).

PDE8A assays

Cells were harvested with complete lysis-M buffer and centrifuged for 10 min at $13\,000 \times g$ in a microcentrifuge. A total of 10 μl aliquots of the supernatant (normalized to the 100 ng/ μl protein) were assayed for PDE activity in the presence of cAMP as substrate (0.1–10 μM) and 10 nM [^3H]cAMP (Thompson and Appleman, 1971). 3-Isobutyl-methylxanthine (IBMX, 100 μM) was added to inhibit PDEs except PDE8. Kinetic constants were calculated from means of replicate assay points.

Luciferase assays

Luciferase activity were assayed with Dual-Luciferase reporter system (Promega Corp., Madison, WI, USA), and luminescence was determined

using a Lumat LB9507 luminometer (Berthold Systems, Pittsburgh, PA, USA). Promoter activities were expressed as the ratio between *Photinus luciferase* and *Renilla luciferase* activities. Each experiment was carried out in triplicate and each experiment was repeated a minimum of three times.

Cell culture and transfection for confocal microscopy

COS-1 cells were cultured in two-well chamber slides. The cells were transfected with pEGFP-N2 plasmids using FuGENE 6. Forty-eight hours after transfection, some cells were lysed with complete lysis-M buffer (Roche), and western blotting was performed to verify protein expression. A 1:500 dilution of PDE8A I21AP antibody (FabGennix, Frisco, TX, USA) directed against the PDE8A C-terminus and 1:1000 dilutions of anti-green fluorescent protein (anti-GFP, Roche) were used in our studies. Living and fixed transfected cells were visualized with a Leica TCS-SP2 AOBS confocal laser scanning microscope.

Genetic studies

Genotypes of the *PDE8A* single nucleotide polymorphisms (SNPs) were determined using Assay By Design technology and an ABI Prism 3730 Sequence Detection System (Applied Biosystems).

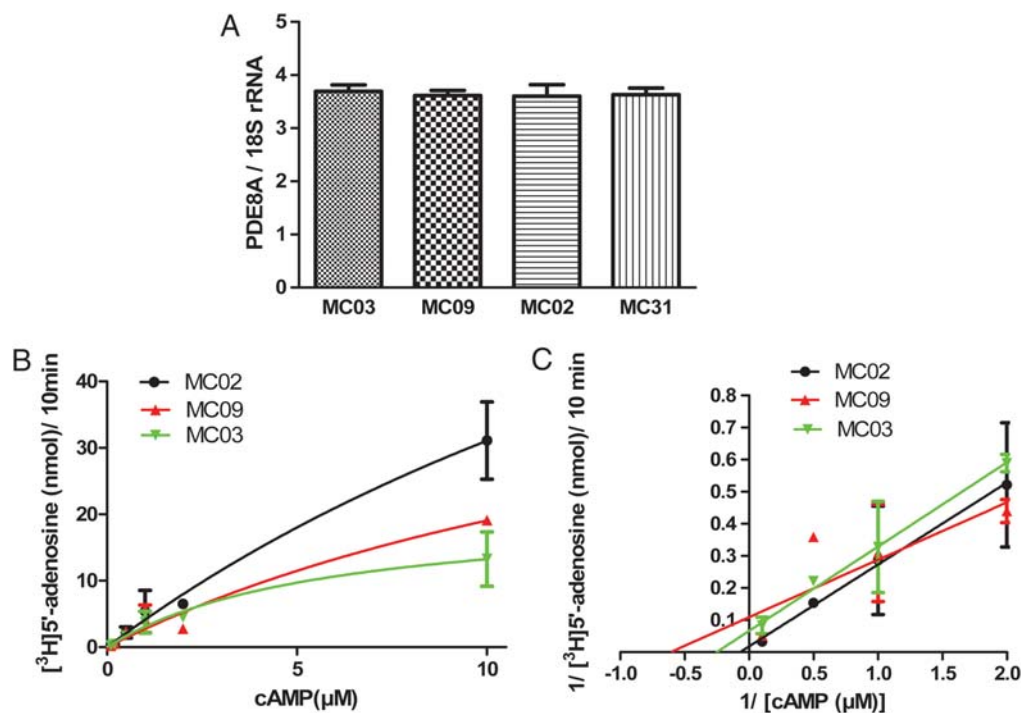


Figure 2 (A) PDE8A mRNA levels in theca cells.

PDE8A mRNA levels were similar in the theca cells from women who had the coding sequence variants (MC03 and MC09) compared with theca cells from normal women (MC02 and MC31) by quantitative real-time PCR. cDNA diluted 1:10 and aliquots were subjected to quantitative real-time PCR. SYBR Green reagent was used to detect amplicons of human PDE8A cDNA. The relative abundance of each cDNA was normalized with 18S rRNA. All reactions were run in triplicate. Values presented are mean \pm SD. (B) PDE8A activity in theca cells. The PDE assay was performed by the radiolabeled nucleotide method as previously described (Thompson and Appleman, 1971). Km and relative Vmax values of the human PDE8A activities were derived from Michaelis–Menten (left) and Lineweaver–Burk plots (right) using cAMP as substrate (0.1–10 μM). The best fit of Km value of PDE8A for MC02, MC03 and MC09 were 26.4, 19.3 and 5.7 μM . Best-fit of Vmax value of PDE8A for MC02, MC03 and MC09 were 113.0, 55.9 and 20.8 nmol/10 min.

Family material

SNPs were genotyped in 454 families with PCOS: 44 multiplex families (parents and two or more affected daughters) and 410 simplex families (one affected daughter and parents). There were a total of 454 affected probands plus 49 affected sisters. The self-identified ethnicities of probands in the 454 families were: 90% white, 3% Hispanic, 2% black and 5% other or unknown. This study was approved by the institutional review boards of the University of Pennsylvania, Pennsylvania State University College of Medicine, Brigham and Women's Hospital and Northwestern University. Written informed consent was obtained from all adult subjects and from a parent or guardian for minor subjects.

Genotyping and analysis

The five SNPs identified in this study plus four additional SNPs in *PDE8A* were genotyped using Applied Biosystems Taqman SNP Genotyping Assays. Four of these assays, 596G > A (R136Q), 352C > G (L55V), 1391A > G (N401S) and -576G > A used Taqman Custom Genotyping Assays (see Table I for primer and probe sequences). Allelic PCR products

were separated using the Applied Biosystems 7900HT Sequence Detection System with SDS 2.2 software. Genotypes were auto-called by SDS 2.2 software with quality value set at 0.95. Two individuals from the Centre d'Etude du Polymorphisme Humain (CEPH, Dausset et al., 1990) collection were typed on each of 16 96-well plates; no discrepancies were found for any of the seven SNPs.

Error-checking of genotypes was performed with Merlin software (Abecasis et al., 2002) and families with one or more Mendelian discrepancies for a marker were excluded in analysis of that marker. Linkage disequilibrium between SNPs and PCOS was tested with the transmission/disequilibrium test (TDT, Spielman et al., 1993). The principle of the TDT is the following: under the null hypothesis of no association and no linkage between diseases and candidate gene, the two alleles in the heterozygote for a candidate gene will be transmitted to affected offspring with equal frequency. If there are unaffected offspring they are ignored, but multiple affected (sibs) can be included, provided the test is interpreted as a test of linkage.

The quantitative TDT (QTDT version 2.4.6; Abecasis et al., 2000) was used to test for association of the SNPs in *PDE8A* with PCOS-related

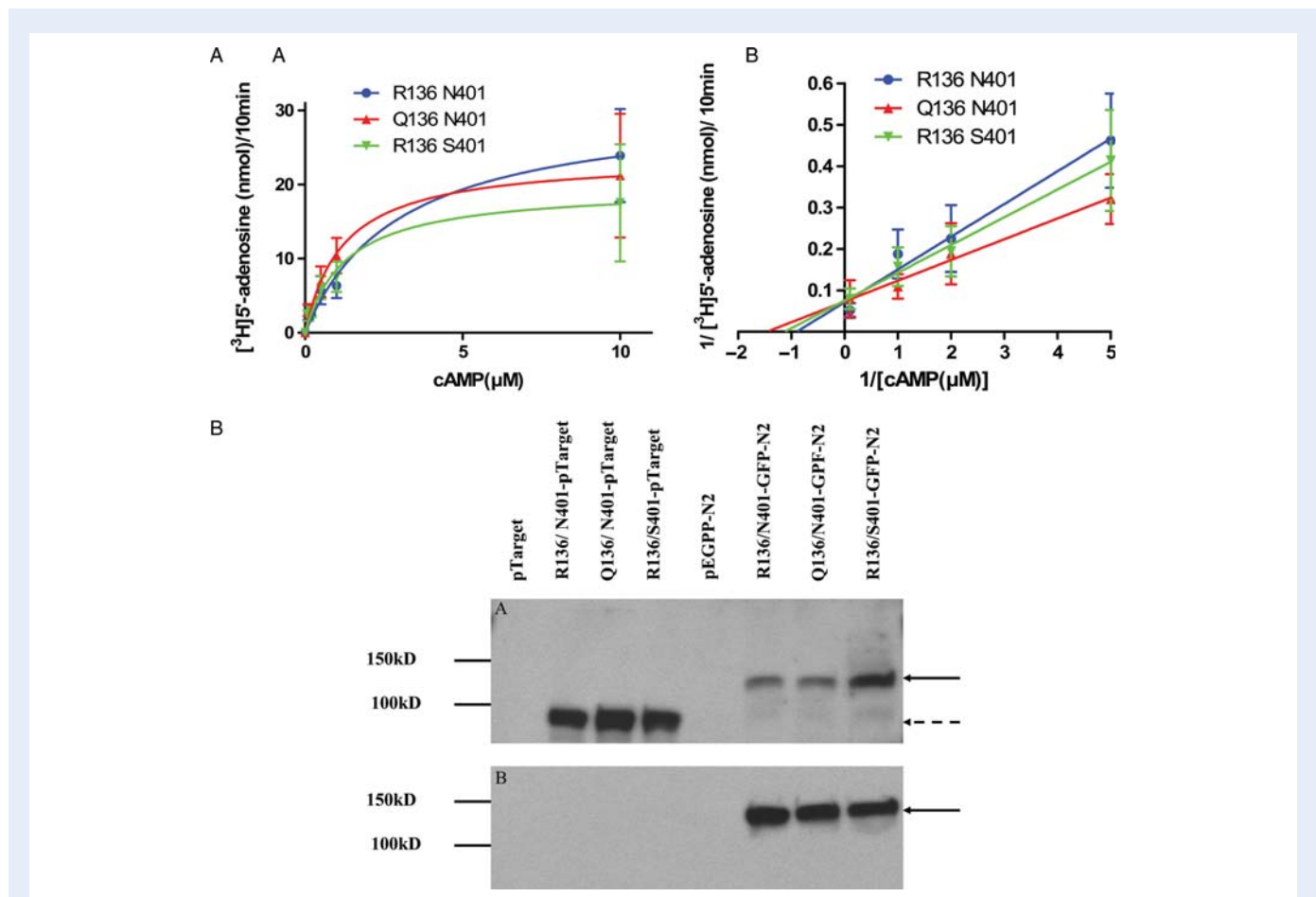


Figure 3 (A) PDE8A activity of coding variants.

Expression plasmids representing each allele were constructed with pTarget vector and transfected in COS-1 cells. The cell lysates were used to perform the PDE assay by the radiolabeled nucleotide method using cAMP as substrate (0.1–10 μM). Km and relative Vmax values of PDE8A activities were derived from Michaelis–Menten (A) and Lineweaver–Burk plots (B). The best fit of Km value of PDE8A for the major allele R136 N401, Q136 N401 (I36Q variant) and R136 S401 (401S variant) were 3.0, 1.2 and 1.3 μM . The best-fit of Vmax value for R136 N401, Q136 N401 and R136 S401 were 31, 24 and 20 nmol/10 min. (B) Western blot analysis were detected with 25 μg of total protein in each sample probed with a 1:500 dilution of PDE8A 12IAP antibody (A) upper panel and 1:1000 dilution of anti-GFP (B) lower panel. A single band at 98 kDa was observed with pTarget vector and 140 kDa with the GFP-N2 vector.

quantitative traits. Unbound testosterone, total testosterone and DHEAS were tested in families using the quantitative TDT.

Statistical analysis

GraphPad Prism 5 software (GraphPad, San Diego, CA, USA) was used for statistical analyses. Comparisons among multiple groups were made using Tukey's multiple comparison test with $P < 0.05$ being considered statistically significant.

Results

Polymorphism in the *PDE8A* coding sequence

In an analysis of *PDE8A* coding sequences from five normal theca cell preparations and five PCOS theca cell preparations from different subjects, we identified three SNPs that resulted in non-synonymous amino acid substitutions 596G > A (R136Q, NM_002605.2

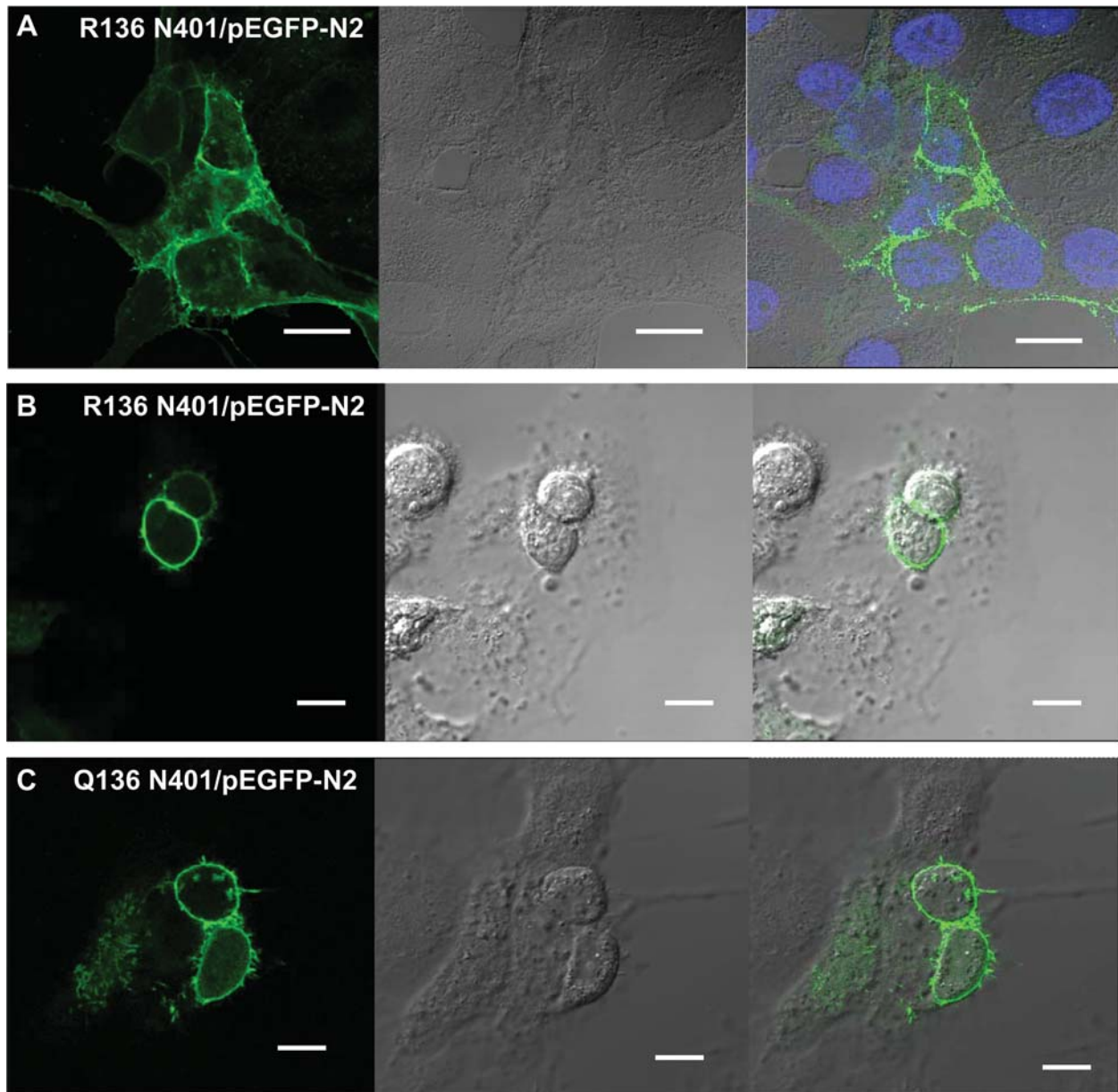


Figure 4 Localization of PDE8A variants in COS-1 cells.

The expression plasmids for the different PDE8A variants coupled to GFP at the protein N-terminus were transfected into COS-1 cells. Forty-eight hours after transfection, living transfected cells and fixed transfected cells were visualized with a Leica TCS-SP2 AOBS confocal laser scanning microscope. Living COS-1 cell transfected images with R136 N401/pEGFP-N2 plasmids were captured by confocal microscopy and stained with DAPI (**A**) ($\times 2.5$ magnification, bar = 20 μm). Forty-eight hours after transfection, COS-1 cells transfected with R136 N401/pEGFP-N2 plasmids (**B**) and Q136 N401/pEGFP-N2 (**C**) were fixed with methanol and captured by confocal microscopy. ($\times 2.5$ magnification, bar = 16 μm). The GFP-tagged proteins were localized to the cell periphery at the plasma membrane. There appeared to be no differences in distribution among the variants.

Table III *PDE8A* promoter sequences variation

Samples	Variation	
Normal	1	
	2	
	3	
	4	
	5	
	6	
	7	-576G > A, -1235G > A (rs12900078)
	8	-576G > A, -1235G > A (rs12900078)
	9	
	10	
PCOS	1	-576G > A, -1235G > A (rs12900078)
	2	
	3	-576G > A, -1235G > A (rs12900078)
	4	
	5	
	6	
	7	-576G > A, -1235G > A (rs12900078)
	8	
	9	
	10	-576G > A, -1235G > A (rs12900078)

Two variants (-576G > A, -1235G > A) occurred in four PCOS and two unaffected subjects. The -1235G > A SNP has been previously identified (rs12900078).

c.407G > A), 352C > G (L55V) and 1391A > G (N401S) (Table II). These were discovered in two different PCOS theca cell preparations by sequence analysis of PCR products and confirmed by RFLP in the case of the G596A SNP (Fig. 1B). The 55 V substitution, which results from a previously identified SNP (rs11540803), was present in the theca cells containing both the I36Q and 401S (rs62019510) substitutions, but it was not found on the same chromosome based on DNA sequence analysis of cloned PCR products.

The Arg at position 136, or its equivalent position in other species, is conserved in all known *PDE8A* sequences including the chimpanzee, macaque, cat, cow, mouse, rat chicken and opossum (Fig. 1A top). The Asp at position 401 is conserved except in the rat, where it is a Ser (Fig. 1A bottom). The Leu at position 55 is not conserved, and is a Pro in the macaque, cow and rat. Confirmation of *PDE8A* sequences were performed by PCR and RFLP (Fig. 1B) An examination of genomic DNA from 69 other subjects, 66 females with the diagnosis of PCOS and three CEPH subjects revealed the presence of Arg at position 136 in all samples. Combined with the theca cell analysis, 156 chromosomes were interrogated, and the estimated allele frequency of the 596A allele was 0.0064. Thus, the 596A allele is a rare variant, or possibly a new mutation. The 1391G causing the N401S substitution allele was found in four PCOS women and one CEPH DNA sample and combined with the one PCOS theca sample gives an allele frequency of 0.038 in the 156 chromosomes examined.

We measured the steady-state levels of *PDE8A* mRNA in the theca cell preparations that we used to identify the coding sequence variants by quantitative real-time PCR and found that they were similar, suggesting that the sequence variation does not have a major effect on *PDE8A* gene transcription, RNA processing or mRNA turnover (Fig. 2A). We then assayed *PDE8A* activity in lysates of the cells in which the two coding sequence variants were identified and compared activity to that in

a theca cell preparation from a normal woman. All other *PDE* activity was inhibited by the addition of IBMX, which does not inhibit *PDE8A*. We found that the *PDE8A* activity (V_{max}) was reduced in the I36Q (MC09) and 401S (MCO3) variant-containing cells compared with the normal theca cells (MC02) without these variants (Fig. 2B).

To further investigate the apparent functional differences, we prepared expression plasmids representing each of the R136Q and N401S alleles and tested the expressed proteins for *PDE* activity in COS-1 cells, which do not express this gene (Fig. 3A). In cells expressing equivalent amounts of *PDE8A* protein, as assessed by western blot analysis, we found that the different enzyme proteins had essentially similar activity (Fig. 3B). This finding was contrary to the expectations based on the analysis of activity in cell lysates and the notion that the two variants (I36Q, 401S) would contribute to PCOS as a result of diminished activity. Collectively, these observations demonstrate that the two coding sequence variants display kinetic properties that would reduce cAMP catabolism in the context of human theca cells, which could be a reflection of the presence of another coding sequence variant (55 V) on the other chromosome. However, when studied in isolation in a different cell context, these kinetic differences are not evident so we must conclude that the I36Q and 401S alleles do not unto themselves impair enzyme function in a cell-free system. We did not examine the L55V alleles in this study because our genetic analysis, described below, indicated that this variant is not associated with PCOS.

To determine if the minor alleles influenced cellular compartmentalization, we studied protein localization in COS-1 cells using tagged enzyme molecules. When expression plasmids for the different *PDE8A* variants coupled to GFP at the protein N-terminus were transfected into COS-1 cells, the GFP-tagged proteins were localized to the cell periphery at the plasma membrane. There appeared to be no differences in distribution among the variants (Fig. 4). This localization pattern, which was previously unknown, positions *PDE8A* to control cAMP levels generated in response to tropic stimulation of cell surface receptors coupled to adenylate cyclase.

Polymorphism in the *PDE8A* promoter sequence

An analysis of the *PDE8A* promoter sequence of genomic DNA from PCOS and normal women revealed two variants (-1235G > A, -576G > A, NT_010274.16:g.490155G > A) (Table III and Fig. 5A), which occurred in four PCOS and two unaffected subjects. The -1235 SNP has been previously identified (rs12900078). These variants did not have a significant impact on function of the *PDE8A* promoter when tested in COS-1 and MA-10 cell hosts (Fig. 5B). The promoter activities in human theca cells with the -576G/-1235G, -576G/-1235A and -576A/-1235G alleles were 0.23 ± 0.03 , 0.28 ± 0.05 and 0.30 ± 0.05 (means \pm SE, $N = 3$) based on luciferase assays normalized with β -galactosidase. The -576G/-1235A and -576A/-1235G alleles were $118 \pm 16\%$ and $129 \pm 25\%$, respectively, of the promoter -576G/-1235G allele.

Lack of association of common variants with PCOS

We used the TDT to test for linkage and association between PCOS and each SNP described above as risk alleles for PCOS (Table IV). The coding sequence variant 596A > G (R136Q) was not polymorphic in

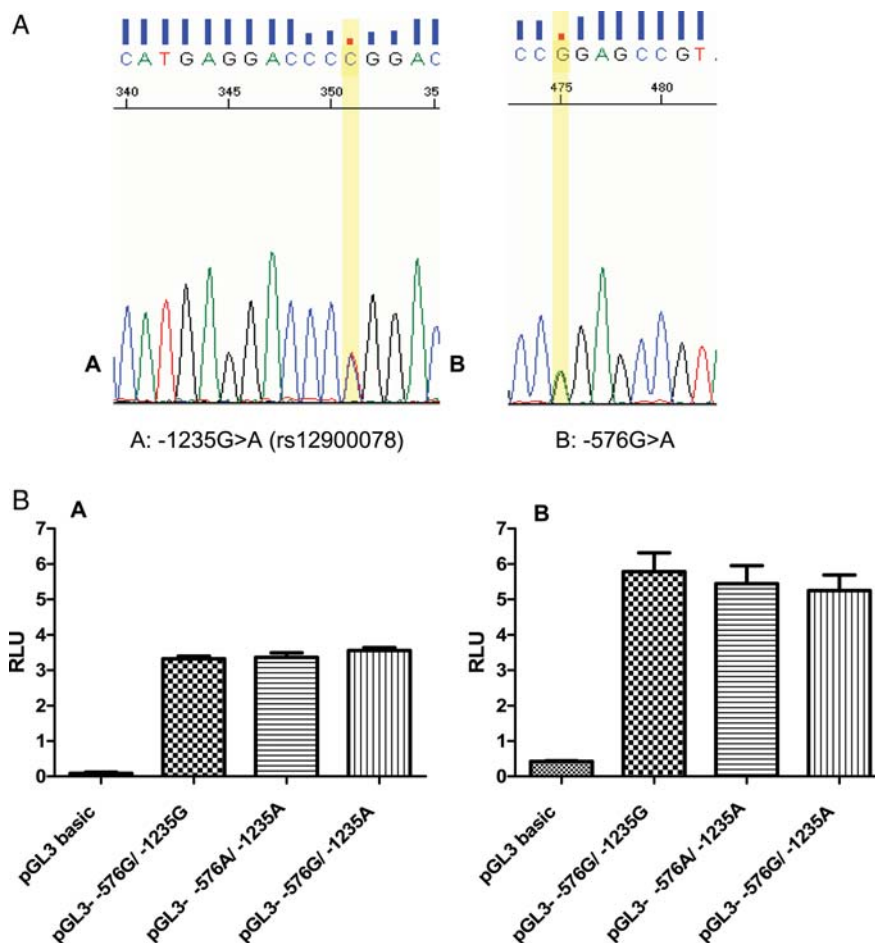


Figure 5 Two SNPs were identified in the *PDE8A* promoter sequence of genomic DNA from four PCOS and two normal women. **(A-A)** The -1235G > A SNP is shown in the reverse strand, which has been previously identified (rs12900078). **(A-B)** A new SNP -576G > A (-576 from genomic reference sequence, NT_010274.16:g.490155G > A) was identified (Forward strand). **(B)** Functional activity of PDE8A promoters in COS-1 and MA-10 cells. COS-1 cells and MA-10 cell were transfected with reporter plasmids containing PDE8A promoter fragments representing the -1235G(g.489496G) and -576G(g.490155G) alleles. The relative *Photinus* luciferase activities ($n = 3$ separate experiments performed with triplicate wells for each cell host) standardized to *Renilla* luciferase. Luciferase activities were compared using the Tukey–Kramer test. These variants did not have a significant impact on function of the *PDE8A* promoter when tested in COS-1 cell (B-A) and MA-10 cell (B-B) hosts.

our sample families and therefore was not evaluated. The two promoter SNPs -1235G > A and -576G > A were in near total linkage disequilibrium in a subset of the probands; therefore only -1235G > A (rs12900078) was genotyped in all the families. We also typed four additional SNPs in *PDE8A*: rs1109065 located ~10 kb upstream of the gene, rs930716 in intron 1, and two SNPs near a region of splice site variation, rs8032301 and rs2304418. None of these *PDE8A* SNPs was significantly associated with PCOS. These genetic studies rule out a significant contribution of the 401S coding sequence variant to PCOS as well as the promoter SNPs.

Lack of association of *PDE8A* variants with PCOS or with the level of testosterone and DHEAS

We used the quantitative TDT (Abecasis *et al.*, 2000) to test whether there was an association between any of the *PDE8A* SNPs and the

level of testosterone (total and unbound) and DHEAS in the 454 PCOS families. None of the SNPs were significantly associated with hormone levels, with all P -values being ≥ 0.13 .

Discussion

Although *PDE8A* appeared to be a viable candidate gene for PCOS, and new variants in the *PDE8A* coding sequence and promoter were identified, these variants do not appear to be functionally significant and a genetic analysis using the TDT did not reveal any evidence for significant association/linkage with PCOS. The fact that the 136Q variant is extremely rare also makes it highly unlikely that it is a significant risk allele as well, although we cannot exclude it from having a pathophysiological role. It should be noted that our findings do not exclude a potential role for variation in the activities of other PDEs, including members of the PDE4 family which are expressed in the rodent ovary (Tsafiri *et al.*, 1996) in the pathophysiology of PCOS.

Table IV TDT results in 454 families having at least one offspring with PCOS

SNP location	SNP ID	MAF ^a	Over-transmitted allele	T ^b	not T	Total: T + not T	Fraction T	c ²	P-value
352C > G (L55V)	rs11540803 ^c	G: 0.10	G	94	91	185	0.508	0.049	0.82
596G > A (R136Q)	NA ^c			Monomorphic					
1391A > G (N401S)	rs62019510 ^c	G: 0.03	G	30	26	56	0.536	0.286	0.59
Promoter -576G > A	NA ^c			Near-complete LD with rs12900078					
Promoter -1235G > A	rs12900078	A: 0.13	G	106	94	200	0.530	0.720	0.4
~10 kb upstream	rs1109065	C: 0.34	T	213	194	407	0.523	0.887	0.35
Intron I	rs930716	A: 0.41	G	229	210	439	0.522	0.822	0.36
Near sites of splice variation	rs8032301	C: 0.41	T	253	218	471	0.537	2.601	0.11
	rs2304418	C: 0.26	T	192	183	375	0.512	0.216	0.64

^aMinor allele frequency (MAF) calculated from SNP genotypes in parents.

^bT: number of transmissions in TDT analysis.

^cSee Table I for Taqman assay primer and probe sequences.

Although there is evidence for heritability of androgen levels in man (Hong et al., 2001), *PDE8A* variants were not associated with either testosterone or DHEAS levels in our study. No evidence for linkage on chromosome 15q was detected by performing a genome-wide linkage scan to identify loci affecting steroid concentrations (Ukkola et al., 2002), which is consistent with a lack of strong influence of the *PDE8A* gene on androgen levels. We, therefore, conclude that variation in the *PDE8A* gene is not a major contributor to the PCOS phenotype or serum androgen levels in women. This study has, however, identified new *PDE8A* variants and documented that *PDE8A* is expressed in human theca cells and that it is localized at a strategic position in the cell, the plasma membrane, to control cAMP levels. Despite the lack of evidence for genetic variation in *PDE8A* as a major risk factor for PCOS, pharmacologic interventions that increase either expression or activity of *PDE8A* might be of value in reducing hyperandrogenemia of ovarian origin.

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