Overexpression of a DENND1A isoform produces a polycystic ovary syndrome theca phenotype

Jan M. McAllister[a,b,1], Bhavi Modi[c], Bruce A. Miller[a], Jessica Biegler[a], Richard Bruggeman[a], Richard S. Legro[b], and Jerome F. Strauss III[c]

Departments of [a]Pathology and [b]Obstetrics and Gynecology, Pennsylvania State College of Medicine, Hershey, PA 17033; and [c]Department of Obstetrics and Gynecology and Human and Molecular Genetics, Virginia Commonwealth University, Richmond, VA 23298

Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved March 7, 2014 (received for review January 13, 2014)

Polycystic ovary syndrome (PCOS), characterized by increased ovarian androgen biosynthesis, anovulation, and infertility, affects 5–7% of reproductive-age women. Genome-wide association studies identified PCOS candidate loci that were replicated in subsequent reports, including *DENND1A*, which encodes a protein associated with clathrin-coated pits where cell-surface receptors reside. However, these studies provided no information about functional roles for *DENND1A* in the pathogenesis of PCOS. DENND1A protein was located in the cytoplasm as well as nuclei of theca cells, suggesting a possible role in gene regulation. DENND1A immunostaining was more intense in the theca of PCOS ovaries. Using theca cells isolated and propagated from normal cycling and PCOS women, we found that DENND1A variant 2 (DENND1A.V2) protein and mRNA levels are increased in PCOS theca cells. Exosomal DENND1A.V2 RNA was significantly elevated in urine from PCOS women compared with normal cycling women. Forced overexpression of DENND1A.V2 in normal theca cells resulted in a PCOS phenotype of augmented *CYP17A1* and *CYP11A1* gene transcription, mRNA abundance, and androgen biosynthesis. Knock-down of DENND1A.V2 in PCOS theca cells reduced androgen biosynthesis and *CYP17A1 and CYP11A1* gene transcription. An IgG specific to DENND1A.V2 also reduced androgen biosynthesis and *CYP17* and *CYP11A1* mRNA when added to the medium of cultured PCOS theca cells. We conclude that the PCOS candidate gene, *DENND1A*, plays a key role in the hyperandrogenemia associated with PCOS. These observations have both diagnostic and therapeutic implications for this common disorder.

Polycystic ovary syndrome (PCOS) is one of the most common endocrinopathies affecting 5–7% of reproductive age women world-wide (1). PCOS is associated with hyperandrogenemia/hyperandrogenism, anovulation, infertility, and a characteristic ovarian morphology consisting of multiple small subcortical follicular "cysts" embedded in bilaterally enlarged ovaries (2–5). The presence of elevated circulating concentrations of testosterone results primarily from increased production of androgens by the ovaries, and is a classic endocrine phenotype of women with PCOS. Although there has been debate about the diagnostic criteria for PCOS, hyperandrogenemia/hyperandrogenism and anovulation, not explained by other causes, is a hallmark of the disorder, and is included as a key element in all "consensus" diagnosis schemes (6–10).

There is consensus that the ovarian theca cells are the primary source of excess androgen biosynthesis in women with PCOS (11–13). Studies on freshly isolated thecal tissue or cultures of theca cells derived from normal and PCOS women have demonstrated that PCOS theca secretes greater amounts of androgen than theca tissue or cells from regularly ovulating women (12, 14–19). Increased androgen biosynthesis in PCOS theca cells results from increased expression of the key enzymes involved in androgen biosynthesis, steroid-17-α-hydroxylase/17,20 lyase (encoded by the *CYP17A1* gene) and cholesterol side-chain cleavage enzyme, mitochondrial (encoded by the *CYP11A1* gene) (15–17, 20).

PCOS is a heterogeneous disorder that shows evidence of genetic predisposition among affected individuals (21, 22). Despite the semblance to an autosomal dominant inheritance, an oligogenic/polygenic model most likely contributes to the underlying pathophysiology (23, 24). Incomplete penetrance, epigenetic modification, and environmental contributions have hindered attempts to clarify the underlying model of inheritance. Despite advances in genetic technologies, very few PCOS susceptibility genes have been validated. Numerous candidate gene-association studies have been conducted, but few have yielded statistically significant associations that have been replicated (25).

The first genome-wide association studies (GWAS) and subsequent follow up performed on Han Chinese populations identified the following PCOS candidate loci: *DENND1A*, insulin receptor (*INSR*), *YAP1*, *C9orf3*, *RAB5B*, *HMGA2*, *TOX3*, *SUMO1P1/ZNF217*, *THADA*, follicle stimulating hormone receptor (*FSHR*), luteinizing hormone/choriogonadotropin receptor (*LHCGR*) (23, 24). The identification of the *DENND1A* locus at 9q22.32 has been replicated in both Asian and European populations (26–29), hence *DENND1A* has gained recognition as a strong PCOS susceptibility gene (30). *DENND1A* is a member of a family of 18 human genes, termed "connecdenns." These proteins contain differentially expressed in normal and neoplastic

Significance

Family-based studies revealed that polycystic ovary syndrome (PCOS), a common endocrinopathy of women, has a genetic basis. Genome-wide association studies identified *DENND1A* as a PCOS locus, but its role in PCOS was unknown. We report that an alternatively spliced form of *DENND1A* (DENND1A.V2) is increased in PCOS theca cells, the source of the excess androgens that characterizes PCOS. Forced expression of DENND1A.V2 in normal theca cells increased expression of genes encoding steroidogenic enzymes, leading to augmented androgen biosynthesis, whereas silencing of DENND1A.V2 in PCOS theca cells reverts them to a normal phenotype. Our findings establish that increased DENND1A.V2 expression is sufficient to promote a PCOS phenotype in human theca cells, information that can inform development of diagnostic tests as well as novel therapeutic interventions.

Author contributions: J.M.M. and J.F.S. designed research; J.M.M., B.M., B.A.M., J.B., R.B., and R.S.L. performed research; J.M.M., B.M., B.A.M., J.B., R.B., R.S.L., and J.F.S. analyzed data; and J.M.M., B.M., B.A.M., R.S.L., and J.F.S. wrote the paper.

Conflict of interest statement: J.M.M. and J.F.S. have patents pending related to this material. J.F.S. is also a consultant to Takeda on topics of infertility treatment. R.S.L. consults for Euroscreen and AstraZeneca, and obtains research funding from Ferring.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The consensus DNA sequence for this polymorphism was obtained from separate, overlapping sequences and has been deposited as SNPs to the NCBI dsSNP Build 139 database (rs4115, rs3842, and rs3895).

[1]To whom correspondence should be addressed. E-mail: jmcallister@psu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1400574111/-/DCSupplemental.

cells domains (DENN domains). The DENN domain is tripartite, consisting of a u-DENN (upstream DENN domain), DENN (core DENN domain), and d-DENN (downstream DENN domain) separated by "linker" sequences. The DENN domains function as Rab-specific guanine nucleotide-exchange factors (31, 32). The *DENND1A* gene consists of 22 exons extending over 500,000 bases, and encodes protein connecdenn 1, which has a clathrin-binding domain and is thought to facilitate endocytosis and receptor-mediated turnover (31, 33, 34). Connecdenn 1 is a guanine nucleotide-exchange factor that interacts with members of the Rab family of small GTPases, which are involved in membrane trafficking (31). Connecdenn 1 is associated with lipids, particularly phosphoinsitol-3-phosphate and other endocytosis/endosome proteins (31). *DENND1A* encodes two transcripts as a result of alternative splicing (31). The longer of these transcripts, DENND1A variant 1 (DENND1A.V1), encodes a 1,009-aa protein with C-terminal proline-rich domain; the other, DENND1A variant 2 (DENND1A.V2), encodes a truncated 559-aa protein that contains the DENN domain and the clathrin-binding domain, but lacks the proline-rich domain and includes a C-terminal 33-aa sequence that is not found in the larger connecdenn 1 variant. Until recently, little has been known about *DENND1A* expression in cells and tissues related to reproduction, with the exception that it is expressed in testes, theca cells, and H295 adrenal carcinoma cells: cells that make androgens (25).

Although the association of the *DENND1A* locus in PCOS has been confirmed in populations of European ancestry in multiple studies (26, 27), it is not known how *DENND1A* and the other candidate gene loci and their pathophysiological significance might contribute to the PCOS phenotype and the mechanism by which any of these genes might promote PCOS. Moreover, it is unclear how the functional variants of *DENND1A* contribute to disease risk.

We have developed conditions to propagate theca cells isolated from individual, size-matched follicles from ovaries of normal cycling and PCOS women, to our knowledge providing the first evidence that successively passaged PCOS theca cells retain the ability to produce augmented levels of androgens and progesterone compared with normal theca cells (15, 16, 35). This increased androgen and progesterone biosynthesis in PCOS theca cells have been attributed, in part, to increased *CYP17A1* and *CYP11A1* gene transcription and RNA stability (17, 20, 36). Our molecular characterization of normal and PCOS theca cells from multiple individuals by microarray analysis and quantitative PCR (qRT-PCR) also established that normal and PCOS cells have distinctive molecular signatures (16, 37, 38). These findings are consistent with the notion of an intrinsic abnormality in PCOS theca that promotes hypersecretion of androgens in response to tropic stimulation. Our theca cell-culture system provides a unique platform for identifying the biochemical and molecular mechanisms underlying genetic abnormalities identified in PCOS women.

To our knowledge, this report presents the first data to demonstrate that the alternatively spliced, truncated form of DENND1A, DENND1A.V2, is differentially expressed in normal and PCOS theca cells. We provide previously unidentified data to show that DENND1A.V2 contributes to the pathophysiological state of increased *CYP17A1* and *CYP11A1* gene expression and augmented androgen and progestin biosynthesis by PCOS theca cells, which is a hallmark of PCOS.

Results

DENND1A.V2 Protein Expression Is Increased in PCOS Theca Cells. To examine whether the alternatively spliced forms of DENND1A are differentially expressed in normal and PCOS theca cells, Western blot analysis was performed on whole-cell extracts from theca cells isolated from normal and PCOS women that were grown until subconfluent and transferred into serum-free medium treated with and without 20 μM forskolin, an activator of adenylate cyclase, for 24 h. An N-terminal DENND1A antibody (Sigma) was used, and we expected bands at ~112 kDa, corresponding to DENND1A.V1, and 62 kDa, corresponding to DENND1A.V2. As shown in Fig. 1*A*, a representative Western blot analysis demonstrated increased 62-kDa DENND1A.V2 in PCOS theca cells treated under both basal and forskolin-stimulated conditions compared with normal theca cells. However, the 112-kDa DENND1A.V1 was not significantly increased in PCOS theca cells. In Fig. 1*B*, cumulative analysis of whole-cell lysates harvested from theca cells isolated from five independent normal and five independent PCOS subjects, demonstrated that DENND1A.V2 protein is significantly increased in PCOS theca cells compared with normal theca cells under both control and forskolin-stimulated conditions. Forskolin treatment did not appear to affect DENND1A.V2 protein accumulation in normal or PCOS theca cells. In contrast, cumulative analysis of DENND1A.V1 in normal and PCOS theca cells showed that DENND1A.V1 protein level was significantly increased by forskolin treatment in normal cells (Fig. 1*C*). In addition, in forskolin-stimulated PCOS theca cells, DENND1A.V1 was significantly reduced compared normal cells. The ratio of DENND1A.V2/V1 protein was significantly increased in PCOS theca cells under control and forskolin-stimulated conditions (Fig. 1*D*).

DENND1A Immunohistochemical Staining Is Increased in the Ovarian Theca Compartment. To further examine the localization of DENND1A.V2 in the ovary, we used the paraffin-embedded blocks of ovarian tissue obtained from our normal cycling and PCOS patient populations and the N-terminal antibody for DENND1A protein used in the Western analyses presented in Fig. 1. As shown in Fig. 2*A*, immunostaining was prominent in the theca compartment of the ovary (4× magnification), and increased in PCOS theca compared with theca cells in normal ovaries (10× magnification). In Fig. 2*B*, DENND1A staining in

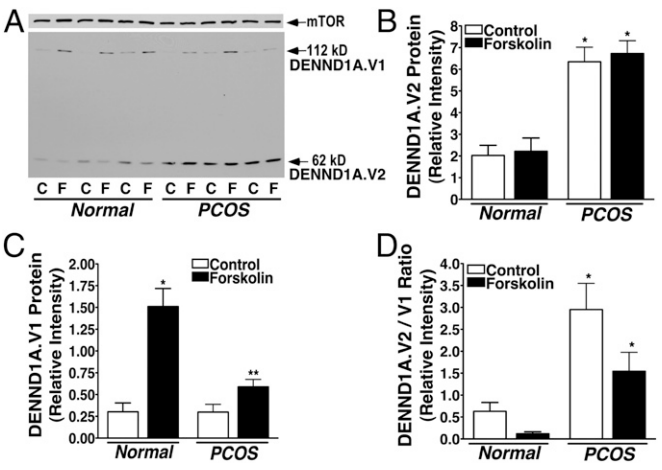

Fig. 1. Western analyses of DENND1A.V2 in normal and PCOS theca cells. (*A*) Representative Western analysis of ~62-kDa DENND1A.V2 and ~112-kDa DENND1A.V1 in whole-cell extracts from normal and PCOS theca cells treated in the absence (–) and presence (+) of 20 μM forskolin. Total mTOR was used for protein normalization. (*B*) Quantitative Western data from theca cells isolated from five normal cycling and five PCOS subjects, presented as the mean ± SEM, demonstrated that DENND1A.V2 protein was increased in both basal and forskolin-stimulated (*$P < 0.01$) PCOS theca cells compared with normal theca cells. (*C*) DENND1A.V1 was increased by forskolin treatment in normal theca cells (*$P < 0.01$). Forskolin-stimulated DENND1A.V1 was decreased in PCOS theca cells as compared normal cells (**$P < 0.01$). (*D*) The ratio of DENND1A.V2/V1 was (*$P < 0.01$) increased in PCOS theca cells under control and forskolin-stimulated conditions.

PCOS theca and granulosa cells is shown (40× magnification). Staining is primarily in the PCOS theca nuclei, cytoplasm, and cell membranes, as shown (100× oil magnification) in Fig. 2*B*. Because the N-terminal antibody predominantly reacts with DENND1A.V2 in Western blotting, and DENND1A.V2 is increased in PCOS theca cells, it is likely that the increased DENND1A immunostaining in PCOS ovaries reflects increased DENND1A.V2.

DENND1A.V2 mRNA Abundance Is Increased in PCOS Theca Cells, and Is Correlated with Increased Androgen Production. The abundance of DENND1A.V2 mRNA was examined in theca cells isolated from six normal and six PCOS patients treated with and without 20 μM forskolin (Fig. 3*A*), quantitated by qRT-PCR analysis, and normalized using TATA box binding protein (TBP) mRNA. DENND1A.V2 mRNA was significantly increased under basal and forskolin-stimulated conditions in PCOS theca cells, compared with normal cells. There was no significant increase in DENND1A.V2 mRNA accumulation in response to forskolin stimulation. To examine whether DENND1A.V2 mRNA was associated with increased androgen biosynthesis, we examined dehydroepiandrosterone (DHEA) accumulation in the matched six normal and six PCOS theca cell preparations under the same conditions. As shown in Fig. 3*B*, normal theca cells with lower DHEA synthesis have reduced DENND1A.V2 mRNA, whereas the increase in basal and forskolin-stimulated DHEA production in PCOS theca cells is associated with increased DENND1A.V2 mRNA abundance.

Urine Exosomal DENND1A.V2 mRNA Is Increased in PCOS Women. Exosomes are small vesicular nucleic acid that are shed into blood and urine, and provide a stable source of RNA. Thus, to examine whether increased DENND1A.V2 in PCOS theca cells reflects corresponding increases in systemic DENND1A.V2 mRNA accumulation in PCOS women, we examined exosomal mRNA isolated from midday urine samples from multiple normal cycling and well-characterized PCOS women by qRT-PCR, as described in *SI Materials and Methods*. As shown in Fig. 4, and in agreement with our qRT-PCR in PCOS theca cells, DENND1A.V2 mRNA is

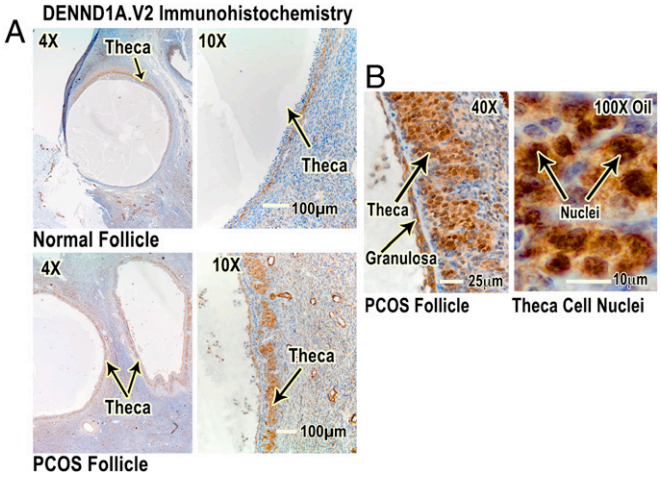

Fig. 2. Imunnohistochemical localization of DENND1A protein in normal cycling and PCOS ovary. (*A*) DENND1A protein was localized in the theca interna of the ovarian follicles, and was increased in PCOS theca (*Lower*) compared with normal theca (*Upper*). (*B*) DENND1A staining in PCOS theca and granulosa cells (*Left*, 40× magnification). Staining, primarily in the PCOS theca cell nuclei, cytoplasm, and cell membrane (*Right*, 100× magnification, under oil).

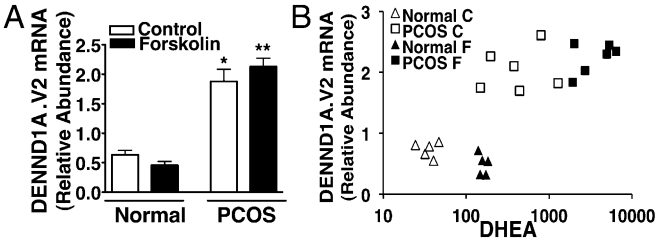

Fig. 3. DENND1A.V2 mRNA accumulation is greater in PCOS theca cells and is correlated with DHEA production. (*A*) DENND1A.V2 mRNA abundance was compared in theca cells propagated from six individual normal and six individual PCOS women that were treated in the absence (C) and presence (F) of 20 μM forskolin. DENND1A.V2 mRNA was increased under basal (*$P <$ 0.05) and forskolin (**$P <$ 0.01)-stimulated conditions in PCOS theca cells, compared with normal cells. (*B*) Control (C) and forskolin (F)-stimulated DHEA accumulation in the six normal and six PCOS women's theca cell preparations were compared with DENND1A.V2 mRNA under the same conditions.

significantly increased in urine exosomes isolated from PCOS, compared with normal cycling women.

Forced Expression of DENND1A.V2 Increases Normal Theca Cell Steroidogenesis. An adenovirus expressing human DENND1A.V2 was used to test the hypothesis that increased expression of DENND1A.V2 converts normal theca cells to a PCOS phenotype of increased androgen and progesterone production. In these experiments normal theca cells were infected with 0.3, 1.0, 3.0, and 10 pfu per cell of either empty (null-pAdenoG) or DENND1A.V2 expressing (hDENND1A.V2-pADenoG) adenovirus, treated with or without 20 μM forskolin in serum-free medium. Following 72 h of treatment, DHEA in the media was quantitated. Infection with all doses of DENND1A.V2 adenovirus significantly increased forskolin-stimulated DHEA production compared with control adenovirus (Fig. 5*A*). Western blot analysis following infection of null and DENND1A adenovirus was performed to confirm that DENND1A.V2 protein is overexpressed, consistent with our studies on cultured theca cells (Fig. 5*B*). In subsequent experiments, we investigated the effects of infection of theca cells from several individual normal women with 3 pfu of control adenovirus or adenovirus expressing DENND1A.V2 on DHEA (Fig. 5*C*), 17α-hydroxyprogesterone (17OHP4) (Fig. 5*D*), testosterone (T) (Fig. 5*E*), and progesterone (P4) (Fig. 5*F*). DENND1A.V2 adenovirus infection significantly increased basal 17OHP4, T, and P4 accumulation compared with control adenovirus. In addition, DENND1A.V2 adenovirus infection significantly increased forskolin-stimulated DHEA, 17OHP4, and P4 compared with control adenovirus. Thus, forced expression of DENND1A.V2 in normal theca cells, converted the cells to a PCOS phenotype of increased androgen and progestin biosynthesis.

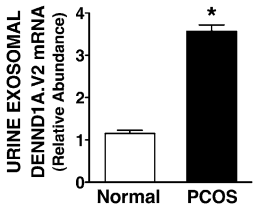

Fig. 4. Exosomal DENND1A.V2 RNA in urine from normal cycling and PCOS women. Comparison of DENND1A.V2 RNA accumulation in exosomal mRNA purified and isolated from midday urine obtained from five normal cycling and six PCOS women (*$P <$ 0.001) using qRT-PCR analysis.

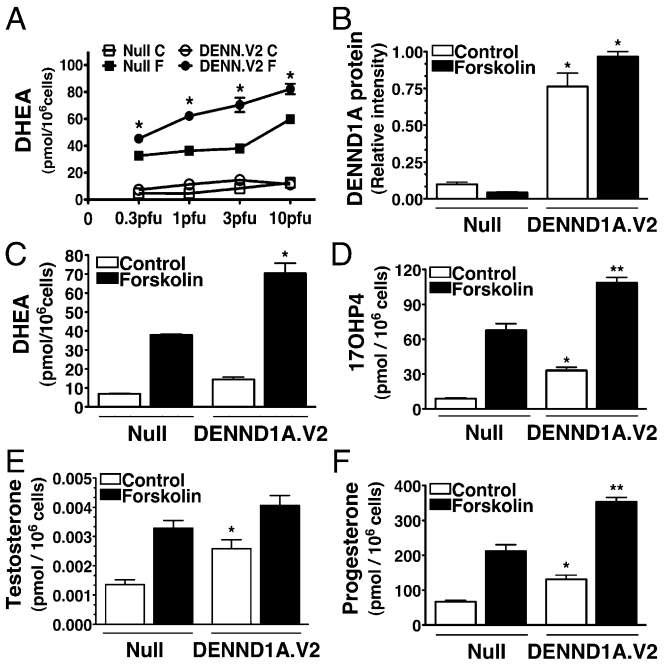

Fig. 5. Forced expression of DENND1A.V2 in normal theca cells results in augmented androgen and progestin production. (*A*) DHEA production following infection of normal theca cells, with 0.3, 1.0, 3.0, and 10 pfu per cell of either empty (Null) or DENND1A.V2 (DENN.V2) adenovirus, treated in the absence (C) or presence (F) of 20 μM forskolin for 72 h. (*B*) Quantitative Western analysis following infection of normal theca cells with 3 pfu Null or DENND1A.V2 adenovirus to confirm DENND1A.V2 protein expression. (*C*) DHEA, (*D*) 17OHP4, (*E*) T, and (*F*) Progesterone biosynthesis in normal theca cells infected with either 3 pfu per cell of DENND1A.V2 or control, Null adenovirus and treated in the absence (C) or presence of (F) of 20 μM forskolin for 72 h. DENND1A.V2 infection increased basal 17OHP4 (*$P < 0.01$), T (*$P < 0.05$), and P4 (*$P < 0.05$) accumulation compared with control Null adenovirus. DENND1A.V2 infection also increased forskolin-stimulated DHEA (*$P < 0.001$), 17OHP4 (**$P < 0.001$), and P4 (**$P < 0.001$) compared with control Null adenovirus.

Forced Expression of DENND1A.V2 Increases *CYP17A1* and *CYP11A1* Gene Expression in Normal Theca Cells. To examine the effects of DENND1A.V2 expression on CYP17 and CYP11A1 mRNA accumulation, normal theca cells were infected with 3 pfu DENND1A.V2 adenovirus or null adenovirus, and treated with or without 20 μM forskolin for 16 h. Following treatment, RNA was harvested, and CYP17 and CYP11A1 mRNA abundance was quantitated and normalized by TBP abundance. Both CYP17 mRNA (Fig. 6*A*) and CYP11A1 mRNA (Fig. 6*B*) accumulation were significantly increased following 3 pfu per cell DENND1A.V2 infection under forskolin-stimulated conditions, compared with infection with control adenovirus.

To examine the effects of DENND1A.V2 on *CYP17A1* transcription, normal theca cells were transfected with pGl3 luciferase reporter plasmid containing −770/+44 of the 5′-flanking regions of the human *CYP17A1* gene (*−770 CYP17A1/LUC*) using the calcium phosphate method (17, 36). Following transfection the cells were infected with the DENND1A.V2 adenovirus or control adenovirus for 1 h, and treated with serum-free medium with and without 20 μM forskolin, an activator of adenylate cyclase. Twenty-four hours later, luciferase activity was determined. Transfections were performed in triplicate and normalized for transfection efficiency using β-galactosidase. Fig. 6*C* shows that 3 pfu per cell DENND1A.V2 adenovirus infection increases both basal and forskolin-stimulated *−770 CYP17A1/LUC* promoter activity compared with control adenovirus. The *−770 CYP17A1/LUC* was used based on our prior reports demonstrating that the 5′ −770/+44 bp flanking sequence of the *CYP17A1* gene confers increased transcriptional regulation in PCOS theca cells (17, 36). The results of the above studies showed that DENND1A.V2 significantly increased *−770 CYP17A1/LUC* promoter function in normal theca cells, converting the cells to a PCOS phenotype. Similar results were observed with a reporter construct containing −235/+44 of the 5′ flanking sequence of the *CYP17A1* gene (*−235 CYP17A1/LUC*), which also confers increased transcriptional regulation in PCOS theca cells (17, 36).

We used a *CYP11A1* pGl3 reporter construct containing the −160/−90 bp element of the proximal *CYP11A1* promoter (*−160/−90 CYP11A1/LUC*), which confers increased *CYP11A1* expression in PCOS theca cells (20) to examine the effects of DENND1A.V2 on *CYP11A1* transcription in theca cells. Normal theca cells were transfected with the *−160/−90 CYP11A1/LUC* plasmid with a DENND1A.V2/pCMV-XL4 or empty pCMV-XL4. Forty-eight hours following transfection the cells were harvested and luciferase was measured. As shown in Fig. 6*D*, DENND1A.V2 increases forskolin-stimulated *−160/−90 CYP11A1/LUC* promoter activity compared with empty plasmid. Collectively, these data suggest that the increased theca cell steroidogenesis resulting from augmented DENND1A.V2 expression is at least in part because of transcriptional activation of the *CYP17A1* and *CYP11A1* genes.

Knock-Down of DENND1A.V2 mRNA in PCOS Theca Cells Reduces *CYP17A1* and *CYP11A1* Expression, Androgen, and Progesterone Production. To determine the effect of knock-down of endogenous DENND1A.V2 mRNA on CYP17 and CYP11A1 mRNA

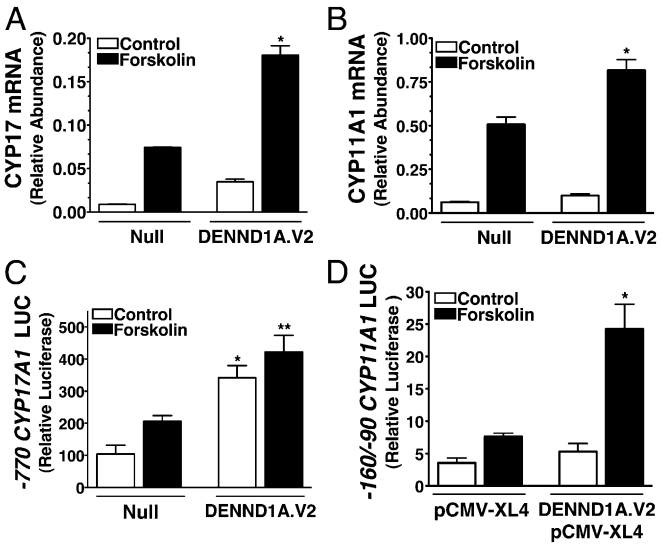

Fig. 6. Forced expression of DENND1A.V2 in normal theca cells results in augmented CYP17A1 and CYP11A1 expression. To examine the effects of DENND1A.V2 on (*A*) CYP17 and (*B*) CYP11A1 mRNA accumulation, normal theca cells were infected either 3 pfu per cell of DENND1A.V2, or control, Null adenovirus and treated in the absence (C) or presence of (F) of 20 μM forskolin. DENND1A.V2 infection increased forskolin-stimulated (*A*) CYP17 mRNA (*$P < 0.01$) and (*B*) CYP11A1 mRNA (*$P < 0.05$) accumulation. (*C*) To examine the effects of DENND1A.V2 on *CYP17A1* transcription, normal theca cells were transfected with a *CYP17A1* promoter gene plasmid *(−770 CYP17A1/LUC)*, and infected with DENND1A V2 or Null adenovirus. DENND1A.V2 infection increased both basal (*$P < 0.05$) and forskolin-stimulated (**$P < 0.05$) *−770 CYP17A1* promoter activity, compared with Null control adenovirus. (*D*) The *CYP11A1* promoter construct (*−160/−90 CYP11A1/LUC*) was used to examine the effects of DENND1A.V2 on *CYP11A1* transcription in theca cells. Normal theca cells were transfected with the *−160/−90 CYP11A1/LUC* plasmid and a DENND1A.V2/pCMV-XL4 or control pCMV-XL4 plasmid. DENND1A.V2 increased forskolin-stimulated (*$P < 0.001$) *−160/−90 CYP11A1/LUC* promoter activity compared with empty plasmid.

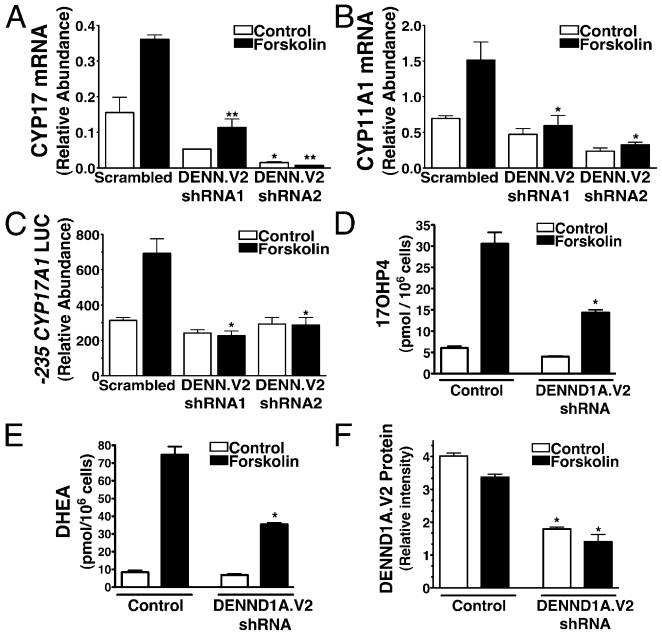

Fig. 7. Knock-down of DENND1A.V2 in PCOS theca cells results in a reduction in CYP17A1 and CYP11A1 expression, and decreased androgen and progestin biosynthesis. (*A*) Transfection of PCOS theca cells with silencing DENND1A.V2 (DENN.V2) shRNA1 and shRNA2 plasmids inhibited both basal ($P < 0.05$) and 20 μM forskolin (*$P < 0.01$) -stimulated CYP17 mRNA accumulation, compared with scrambled plasmid. (*B*) The DENND1A.V2 shRNA plasmids also inhibited forskolin-stimulated (*$P < 0.05$) CYP11A1 mRNA accumulation in PCOS theca cells. (*C*) Cotransfection of −235/+44 of the *CYP17A1* promoter fused to the luciferase gene in a pGL3 plasmid (*−235 CYP17A1*/LUC) with DENND1A.V2 shRNA1 and shRNA2 plasmids resulted in an inhibition of forskolin-dependent *CYP17A1* reporter activity in PCOS theca cells, compared with scrambled shRNA (*$P < 0.05$). (*D–F*) Infection with silencing shRNA DENND1A.V2 lentivirus particles inhibited forskolin-stimulated (*D*) 17OHP4 (*$P < 0.001$), (*E*) DHEA (*$P < 0.001$), and (*F*) Progesterone biosynthesis (*$P < 0.001$), compared with control nonsilencing lentivirus.

levels, silencing DENND1A.V2 shRNA plasmids were transfected into PCOS theca cells, and basal and forskolin-stimulated CYP17 mRNA was assessed using qRT-PCR. In these experiments PCOS theca cells were transfected with pRSV-scrambled plasmid or plasmids specific to DENND1A.V2 (pSV-shRNA1 or pRSV-shRNA2). Six hours following transfection, the cells were treated with and without 20 μM forskolin and, 24 h later, total RNA was harvested, and CYP17, CYP11A1, and TBP mRNA abundance was measured. As shown in Fig. 7*A*, DENND1A.V2 shRNA1 and shRNA2 retrovirus plasmid significantly inhibited both basal and forskolin-stimulated CYP17 mRNA accumulation in PCOS theca cells. Both of the DENND1A.V2 shRNA plasmids also significantly inhibited forskolin-stimulated CYP11A1 mRNA (Fig. 7*B*) in PCOS theca cells.

Parallel studies were performed to evaluate whether silencing shRNAs specific to DENND1A.V2 would inhibit *CYP17A1* promoter function (i.e., transcription) in PCOS theca cells. PCOS theca cells were transfected with a *CYP17A1* luciferase reporter plasmid (*−235 CYP17A1*/LUC) containing −235/+44 of the *CYP17A1* promoter fused to the luciferase gene in pGL3. Scrambled pRV expression vector or plasmid encoding the silencing DENND1A.V2 pRV-shRNA1 or pRV-shRNA2 was also added to the transfection mixture. Six hours following transfection, the cells were rinsed with PBS, and treated in serum-free medium with and without 20 μM forskolin. Twenty-four hours later luciferase activity was determined. The results from these experiments showed that transfection of DENND1A.V2 shRNA1 inhibited basal and cAMP-dependent *CYP17A1* reporter activity in PCOS theca cells compared with scrambled shRNA. As we previously reported, we observed an increase in both basal and forskolin-stimulated *−235 CYP17A1*/LUC promoter regulation in PCOS theca cells (36). As shown in Fig. 7*C*, cotransfection of *−235 CYP17A1*/LUC with a silencing DENND1A.V2 shRNA1 or shRNA2 resulted in a significant reduction of forskolin-dependent *CYP17A1* reporter activity in PCOS theca cells, compared with scrambled shRNA.

Silencing of DENND1A.V2 Expression by shRNA Lentivirus Particles Inhibits PCOS Theca Cell Steroidogenesis. To evaluate the effect of knockdown of DENND1A.V2 on steroid biosynthesis, we used custom Thermo/Dharmacon GIPZ DENND1A.V2 shRNA particles. PCOS theca cells were infected with 300,000 particles per well of silencing shRNA DENND1A.V2 lentivirus or a control nonsilencing lentivirus in serum-free medium. Six hours later the lentivirus mixture was removed and the cells were transferred into serum-free medium in the presence or absence of forskolin for 72 h. Infection with silencing shRNA DENND1A.V2 lentivirus significantly inhibited forskolin-stimulated 17OHP4 (Fig. 7*D*), DHEA biosynthesis (Fig. 7*E*), and P4 (Fig. 7*F*). Although

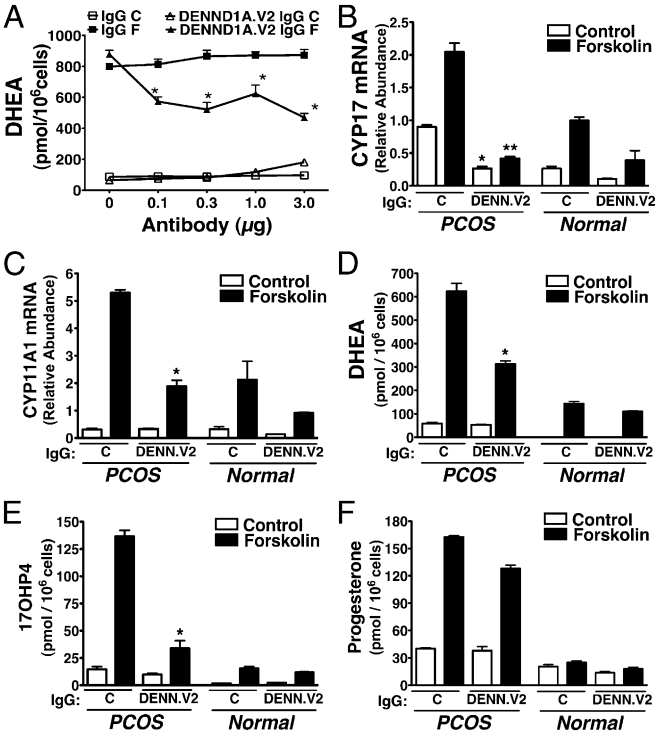

Fig. 8. Anti-DENND1A.V2 IgG reduces androgen biosynthesis and CYP17 and CYP11A1 mRNA PCOS theca cells. (*A*) PCOS theca cells were treated with increasing concentrations (0.1–3.0 μg/mL) of anti-DENND1A.V2 IgG or nonspecific IgG, in the absence (C) or presence of 20 μM forskolin (F). Anti-DENND1A.V2 IgG inhibited forskolin-stimulated DHEA (*$P < 0.01$) with an approximate ID_{50} of 0.25 μg/mL, compared with nonspecific IgG. (*B* and *C*) Experiments were performed to examine the effects of 0.5 μg/mL of anti-DENND1A.V2 IgG (DENNN.V2) or 0.5 μg/mL nonspecific IgG on (*B*) CYP17 and (*C*) CYP11A1 mRNA (Fig. 7*C*) accumulation in the absence (C) or presence of 20 μM forskolin (F), demonstrated that anti-DENND1A.V2 IgG inhibits CYP17 mRNA accumulation under control (*$P < 0.01$) and CYP17 mRNA and CYP11A1 mRNA under forskolin-stimulated (**$P < 0.01$) conditions in PCOS theca cells, but had no effect in normal theca cells. (*D–F*) Parallel experiments to examine the effects of 0.5 μg/mL of anti-DENND1A.V2 IgG or control IgG on basal and forskolin-stimulated (*D*) DHEA (*E*) 17OHP4, and (*F*) P4 biosynthesis similarly demonstrated that anti-DENND1A.V2 IgG-inhibited forskolin-stimulated DHEA (*$P < 0.001$) and 17OHP4 (*$P < 0.001$), in PCOS theca cells by 50% compared with control IgG, without affecting normal theca cells.

there was a trend toward inhibition in basal steroidogenesis following DENND1A.V2 lentivirus infection, the design of lentiviral infection experiments precluded the accurate assessment of basal steroid concentrations.

Anti-DENND1A.V2–Specific IgG Reduces DHEA Secretion and CYP17 mRNA in PCOS Theca Cells. DENND1A is known to be associated with the cell membrane and clathrin-coated pits (31, 33), potentially making DENND1A epitopes available to antibodies added to the cell exterior. We generated a rabbit polyclonal antibody against a 21-aa peptide ([C]-QKSITHFAAKFPTRGWTSSSH) that is specific to DENND1A.V2. Western blot analysis demonstrating the specificity of this polypeptide antibody to the 62-kDa form of DENND1A.V2, and increased expression in PCOS theca cells, is provided in Fig. S1. This DENND1A.V2-specific IgG was added to the culture medium of normal and PCOS theca cells to determine whether it could be used to block or neutralize DENND1A.V2 function, and thus alter steroid biosynthesis in normal and PCOS theca cells. In these experiments, PCOS theca cells were treated with increasing concentrations of a DENND1A.V2 isoform-specific IgG or nonspecific IgG, in the presence or absence of 20 μM forskolin. As shown in Fig. 8*A*, DENND1A.V2 IgG significantly inhibits forskolin-stimulated DHEA biosynthesis, with an approximate ID_{50} of 0.25 μg/mL In contrast, nonspecific IgG had no effect on DHEA biosynthesis.

Subsequent experiments were performed to examine the effects of 0.5 μg/mL of DENND1A.V2-specific IgG or 0.5 μg/mL nonspecific IgG on CYP17 mRNA (Fig. 8*B*) and CYP11A1 mRNA (Fig. 8*C*) accumulation following 16-h treatment in the presence and absence of 20 μM forskolin in normal and PCOS theca cells from various individual patients. The data in Fig. 8*B* demonstrate that 0.5 μg/mL DENND1A.V2-specific IgG significantly inhibits CYP17 mRNA accumulation in PCOS theca cells, under control and forskolin-stimulated conditions following 16 h of treatment. In contrast, in normal theca cells, DENND1A.V2 IgG had no effect on basal or forskolin-stimulated CYP17 mRNA (Fig. 8*B*). As shown in Fig. 8*C*, DENND1A.V2-specific IgG also significantly inhibited forskolin-stimulated CYP11A1 mRNA accumulation in PCOS theca cells, but had no effect on CYP11A1 mRNA accumulation in normal theca cells.

In parallel experiments, normal and PCOS theca cells from individual patients were treated with 0.5 μg/mL of DENND1A.V2-specific IgG or 0.5 μg/mL or nonspecific IgG and basal and forskolin-stimulated absence DHEA (Fig. 8*D*), 17OHP4 (Fig. 8*E*), and P4 (Fig. 8*F*) biosynthesis on a per cell basis was measured following 72-h treatment. The results of these experiments showed that DENND1A.V2-specific IgG significantly inhibited forskolin-stimulated DHEA (Fig. 8*D*) and 17OHP4 (Fig. 8*E*), in PCOS theca cells by 50% compared with control IgG, without affecting normal theca cells.

Discussion

Studies conducted over the past decade have built a convincing argument that genetic factors contribute to PCOS. Despite advances in genetic technologies, very few PCOS susceptibility genes have been validated. Numerous candidate gene-association studies had been conducted, and a number of these have yielded statistically significant associations of variants with PCOS (25). These include studies using family-based methods, like the transmission-disequilibrium test, which test for both association and linkage (39). However, the candidate gene studies have been uniformly conducted on small sample populations and have had limited statistical power. Additionally, few of these studies have yielded sufficiently robust results that have been consistently replicated by different investigators studying different populations.

Important milestones in PCOS genetics were achieved with the publication of GWAS on Han Chinese populations that identified PCOS candidate loci: *DENND1A*, *INSR*, *YAP1*, *C9orf3*, *RAB5B*, *HMGA2*, *TOX3*, *SUMO1P1*/*ZNF217*, *THADA*, and *FSHR*/*LHCGR* (23, 24). The initial study encompassed a total of 4,082 cases and 6,678 controls, with cases defined according to the "Rotterdam Criteria." The replication of the association of SNPs in the *DENND1A* locus with PCOS in different populations by different investigators provides strong support for *DENND1A* as a PCOS candidate gene (26–29). However, before our studies there was no pathophysiological link between *DENND1A* and the other GWAS loci to reproduction or ovarian function. In addition, the specific variants encoded by *DENND1A* loci that contribute to PCOS phenotype of increased androgen production and disease risk are unknown.

We have, to our knowledge, carried out the first studies to examine the expression of *DENND1A* in well-characterized theca cells from normal cycling and PCOS women. The finding that increased expression of a splice variant of DENND1A RNA, DENND1A.V2, is characteristic of PCOS theca cells provided a basis for pursuing studies on the functional consequences of this molecule in terms of theca cell function. Forced expression of DENND1A.V2 in normal theca cells increases *CYP17A1* and *CYP11A1* gene expression and converts the cells to a PCOS phenotype of augmented androgen and progestin biosynthesis. In contrast, knock-down of DENND1A.V2 with silencing shRNA plasmids or lentivirus in PCOS theca cells reverts the cells to a normal phenotype of reduced *CYP17A1* and *CYP11A1* gene expression and androgen and progestin biosynthesis. These observations suggest that DENND1A is involved in a signaling cascade that augments transcription of steroidogenic genes that subsequently results in increased androgen production.

What are the possible mechanisms of DENND1A.V2 action? DENND1A.V2 is a truncated connecdenn 1, which has a clathrin-binding domain and is thought to facilitate endocytosis. Among the loci associated with PCOS in Han Chinese, several reside in or near genes that potentially define a network, including *FSHR, LHCGR,* and *INSR*, which encode receptors that reside on the plasma membrane and which are internalized by clathrin-coated pits, where the DENND1A protein is located (24, 25, 33). The DENN domains of DENND1A function as Rab-specific guanine nucleotide-exchange factors. Ras related protein 5B (RAB5B), another PCOS GWAS candidate, is a Rab-GTPase, also thought to be involved in endocytosis and receptor recycling and could, therefore, be a molecule interacting with the DENN domain (40, 41). RAB5B signaling has also been reported to involve PI3K, PKB, and MAPK/ERK components (33, 40–42). Therefore, the truncated form of DENND1A.V2 could potentially affect insulin or luteinizing hormone (LH)-receptor turnover and sensitivity in theca and granulosa cells, further affecting ovarian function and steroid biosynthesis in PCOS women. DENND1A has also been shown to be associated with lipids, particularly phosphoinsitol-3-phosphate, and other endocytosis/endosome proteins (31, 34), and could potentially be involved in insulin and LH-receptor signaling. Alternatively, DENND1A.V2 may have a more direct role in controlling gene expression. The observation of DENND1A.V2 localization in the nucleus of theca cells suggests that DENND1A.V2 translocates into the nucleus, possibly by a process that is facilitated by RAB5B, another GWAS-discovered PCOS candidate, where it may be involved in transcriptional activation of *CYP17A1* and *CYP11A1*.

Our studies with a specific anti-DENND1A.V2 IgG suggest that DENND1A.V2 epitopes are available to extracellular antibodies. The observation that augmented CYP17 and CYP11A mRNA and androgen biosynthesis in PCOS theca cells can be reduced using DENND1A.V2-specific IgG raises the possibility that humanized monoclonal antibodies against DENND1A.V2 may be a useful biologic therapeutic agents for the hyperandrogenemia associated with PCOS, and possibly other phenotypes related to insulin action.

We demonstrated that DENND1A.V2 mRNA was significantly increased in urine exosomal mRNA from PCOS women, compared with normal cycling women. Exosomes are small vesicles of about 40–100 nm that are secreted from cells into extracellular fluid, and are found in different bodily fluids, such blood, blood derivatives, urine, and amniotic fluid. Exosomes are produced by all cell types and have a molecular phenotype, which largely reflects that of a parent cell (43, 44) The contents of exosomes reflect the origin and the physiological status of the source cells, and thus exosomal RNAs, such as DENND1A.V2, can serve as biomarkers for various diseases. These data further confirm our findings in that DENND1A.V2 mRNA is increased in PCOS, and provide the basis for a potential noninvasive diagnostic for PCOS. The source of the urine exosomal DENND1A mRNA is not known. The kidney expresses the DENND1A gene, and it is a likely source (26, 33). If this is indeed the case, the finding of increased DENND1A.V2 mRNA in urine speaks to a generalized alteration in expression of this variant in PCOS.

What is the genetic mechanism resulting in increased DENND1A.V2 expression in PCOS? The *DENND1A* SNPs reported to be associated with PCOS in the GWAS, and those from replication studies, lie in intronic regions and are not associated with canonical sites for DENND1A expression or splicing. The fact that DENND1A.V2 expression is increased in PCOS suggests that there must be a gain-of-function variation, which could be related to copy-number variants, variation in the promoter region that increases promoter activity, variants that affect splicing, or mRNA stability. Moreover, because the odds ratio for *DENND1A* association with PCOS is modest (<2.0), it is likely that variation in other genes other than the *DENND1A* locus accounts for the consistent elevation of DENND1A.V2 expression (e.g., transcription factors controlling *DENND1A* promoter function). In an initial attempt to identify functional genetic variants in the *DENND1A* locus, we performed whole-exome sequencing (Table S1) as presented in Fig. S1. We found only synonymous coding-sequence variants, and no missense or nonsense variants. This result is consistent with a recent whole-exome sequencing study reporting no changes in *DENND1A* exons or splice junctions in PCOS patients (45). Intronic sequences captured in the whole-exome sequencing also contained variants, some of which were only found in PCOS genomic DNA. None of these were in introns, where it was likely that splicing to generate DENND1A.V2 could be affected. However, we cannot exclude at this time the possibility that these variants contribute to increased DENND1A.V2 expression because they lie in enhancer elements or have long-range effects on promoter function or splicing. The DENND1A.V2 locus also encodes a microRNA, miR601. We have characterized the microRNA profiles of normal and PCOS theca cells and found no differences in miR601 expression. Moreover, none of the SNPs identified in whole-exome sequencing appear to affect miR601 structure. Another possibility that remains to be explored is whether genetic variation in the *DENND1A* promoter contributes to increased *DENND1A* expression. The promoter is GC-rich, and therefore variation could also affect epigenetic control through altered DNA methylation. Alternatively, copy-number variation could result in increased *DENND1A* expression. Thus, at this juncture the genetic mechanism underlying the consistent overexpression of *DENND1A* in PCOS remains elusive.

In conclusion, we have demonstrated that a splice variant (DENND1A.V2) derived from the *DENND1A* gene, a gene implicated in the pathogenesis of PCOS from GWAS and replication studies, has a functional role in controlling theca cell steroidogenesis. We have established that overexpression of DENND1A.V2 is sufficient to convert normal theca cells into a PCOS biochemical phenotype characterized by increased *CYP17A1* and *CYP11A1* gene expression and augmented androgen and progestin production, and that suppression of DENND1A.V2 function pushes PCOS theca cells toward normal phenotype in terms of steroidogenic enzyme gene expression and steroid production. The fact that DENND1A.V2 mRNA is elevated in urine of women with PCOS and that a DENND1A.V2-specific IgG can transform the biochemical characteristics of PCOS theca cells, reducing steroidogenesis, opens up new possibilities for noninvasive detection of PCOS and biological therapy.

Materials and Methods

Normal and PCOS Theca Cells. Human theca interna tissue was obtained from follicles of women undergoing hysterectomy, following informed consent under a protocol approved by the Institutional Review Board of The Pennsylvania State University College of Medicine. As a standard of care, oophorectomies were performed during the luteal phase of the cycle. Theca cells from normal cycling and PCOS follicles were isolated and grown as we have as previously reported in detail (20, 46, 47). The theca cell preparations used in these studies have been described and characterized previously (15–17, 20, 35–38, 47). The steroidogenic phenotypes of the normal and PCOS theca cells have been reported to result from the inherent properties of the cells, rather than the cycle phase at the time that they were isolated (15, 17, 48). PCOS and normal ovarian tissue came from age-matched women, 38–40 y old. The diagnosis of PCOS was made according to National Institutes of Health (NIH) consensus guidelines (10, 49), which include hyperandrogenemia, oligoovulation, polycystic ovaries, and the exclusion of 21-hydroxylase deficiency, Cushing's syndrome, and hyperprolactinemia. All of the PCOS theca cell preparations studied came from ovaries of women with fewer than six menses per year and elevated serum total testosterone or bioavailable testosterone levels (15, 17, 48). Each of the PCOS ovaries contained multiple subcortical follicles of less than 10 mm in diameter. The control (normal) theca cell preparations came from ovaries of fertile women with normal menstrual histories, menstrual cycles of 21–35 d, and no clinical signs of hyperandrogenism. Neither PCOS nor normal subjects were receiving hormonal medications at the time of surgery. Indications for surgery were dysfunctional uterine bleeding, endometrial cancer, and pelvic pain. Experiments comparing PCOS and normal theca were performed using fourth-passage (31–38 population doublings) theca cells isolated from individual size-matched follicles obtained from age-matched subjects, in the absence of in vivo stimulation. The use of fourth-passage cells allowed us to perform multiple experiments from the same patient population, and were propagated from frozen stocks of second passage cells in the media described above. For all studies, theca cell cultures obtained from at least five independent normal and five independent PCOS patients were examined. The passage conditions and split ratios for all normal and PCOS cells were identical.

Quantitative DENND1A.V1, DENND1A.V2, CYP17, and CYP11A1 qRT-PCR. Quantitation of DENND1A.V1 and V.2, CYP17, and CYP11A1 mRNA abundance was determined using the Single Step Brilliant III Ultra Fast qRT-PCR Reagents (Agilent) using 50–100 ng total RNA/tube, and 200-nM final concentration of each forward and reverse primers and 100-nM probe. The specific primer and probe sequences used are provided in detail in *SI Materials and Methods*. DENND1A.V2 data were verified with the second confirmatory DENND1A.V2 primer probe set provided. The gene-specific one-step PCR was carried out in duplicate for each mRNA sample and for a series of dilutions in an Mx3000p Thermocycler system (Stratagene) according to the manufacturer's instructions for this instrument, as previously described (20). The same process was carried out for TBP to use TBP values for normalization of each reaction. The mean target value for each unknown was divided by the mean TBP value to normalize each sample.

Exosomal RNA Extraction and Purification. Midday urine samples were obtained from five normal women and six women with PCOS, using the same clinical criteria described above for normal and PCOS theca cells, according to NIH consensus guidelines (10, 49). Following informed consent under an Institutional Review Board protocol approved by the Institutional Review Board of The Pennsylvania State University College of Medicine. The urine samples were collected and placed at 4 °C until processed. Cleared urine was frozen at −80 °C and extracted using a modified protocol of the "Urine Exosome RNA Isolation Kit" from Norgen. The resulting RNA was quantified using a Nano-drop. DENND1A.V2 mRNA was then quantitated by qRT-PCR, and normalized using 5S mRNA abundance.

Quantitation of Steroid Biosynthesis. ELISAs for DHEA, 17OHP4, T, and P4 were performed without organic solvent extraction using kits from DRG International, as described by the manufacturer's protocol, and normalized by

cell count. Information pertaining to the specificity and cross-reactivity of the specific ELISAs for the DHEA, 17OHP4, T, and P4 is provided in *SI Materials and Methods*.

Western Blot Analysis. Western blot analysis was performed on 35 μg/ln whole-cell lysates harvested from fourth-passage normal and PCOS theca cells were grown until subconfluent and transferred into serum-free medium with and without forskolin for 24 h, as we have previously described (48). Similar results were obtained in Western analyses performed using Abcam antibody specific for the N-terminal and Sigma antibody for intrapeptide sequence of DENND1A, which were visualized using ECL (Pierce). DENND1A Western data were quantitated and normalized by total mammalian target of rapamycin (mTOR), which is not significantly different in normal and PCOS theca cells, nor regulated by forskolin treatment. Actin and GAPDH cannot be used to normalize Westerns as they are regulated by forskolin in theca cells and are differentially expressed in normal and PCOS cells.

Immunohistochemical Localization of DENND1A. Immunohistochemical (IHC) analysis for DENND1A was performed on 4- to 5-μm–thick sections of formalin-fixed, paraffin-embedded tissue on a Ventana Discovery XT stainer (Ventana Medical Systems), using a the Sigma DENND1A antibody that we found to react predominantly with DENND1A.V2 in the Western blotting, as shown in Fig. 1*A*. The staining protocol consisted of incubation in the primary antibody for 30 min at 1:300 dilution, followed by detection and visualization with Ventana OmniMap DAB anti-Rabbit detection kit (Ventana Medical Systems). FFPE samples of adrenal cortex were used as a positive tissue control. After using various antigen retrieval methods and examining several serial dilutions of the DENND1A.V2 polypeptide antibody (made against the DENND1A.V2 21 amino acid sequence [C]-QKSITHFAAKFPTRGWTSSSH), we could not obtain specific immunostaining.

Replication-Deficient Adenovirus Infections. DENND1A.V2 adenovirus (hDENND1A.V2-pADenoG) was constructed by Applied Biological Materials by cloning DENND1A.V2 from pCMV6-XL4 plasmid encoding the DENND1A.V2 into pADenoG, from Origene. Control empty null nonexpressing adenovirus (pAdenoG-null) was also obtained from Applied Biological Materials. Recombinant adenoviruses were propagated and expanded in HEK293T cells, purified using a Virabind Adenovirus Miniprep Kit, Cell Biolabs, and tittered by QuickTiter Adenovirus Titer Elisa Kit, Cell Biolabs. Both the DENND1A.V2 or Control empty null nonexpressing adenovirus (pAdenoG-null) were used to infect in normal theca cells as we previously described (47).

Transient Transfection of Normal and PCOS Theca Cells. Human theca cells isolated from normal cycling women and women with PCOS were transfected using the calcium phosphate precipitation method as we have previously described (17, 36, 50). The calcium phosphate precipitate contained 2 μg per dish of luciferase plasmid, and 0.1 μg per dish of an expression vector for β-galactosidase, pSVβ-gal (Promega) for each 30-mm well. Luciferase activity was quantitated and normalized by β-Galactosidase activity as we have previously described (20, 36). Transfections were performed in triplicate in theca cells isolated from four or more independent normal, and four or more independent PCOS patients.

Statistical Analysis. Data are presented and described in the text as the mean ± SEM performed in triplicate. The results from qRT-PCR, steroid, and transfection analyses were collected from individual patients and ANOVA was performed using Prism 5.0c (GraphPad Software). *P* values were determined by the Boniferri method for multiple comparisons when significant differences were indicated by ANOVA.

ACKNOWLEDGMENTS. We thank Dr. Walter Miller for his scientific and editorial insight; Barbara Sheetz and Dr. Nazia Raja-Khan, for enrolling normal and polycystic ovary syndrome women for our urine exosomal studies; Kira Oshaben and Lauren Sanders-Miller for their technical assistance; Charity Pavlesich for her administrative assistance; and the Morphologic and Molecular Pathology Core Research Laboratory, Department of Pathology, Pennsylvania State Hershey College of Medicine, for assistance with the ovarian immunohistochemistry. This research was funded by National Institutes of Health Grants U54HD344449 (to J.F.S., J.M.M., and R.S.L.), R01HD058300 (to J.M.M.), and R01HD33852 (to J.M.M. and R.S.L.); and the cooperative agreement between Pennsylvania State University and Virginia Commonwealth University Clinical and Translational Science Awards, UL1T000058 (Virginia Commonwealth University) and UL1T0000127 (Pennsylvania State University).

1. Dunaif A (2012) Polycystic ovary syndrome in 2011: Genes, aging and sleep apnea in polycystic ovary syndrome. *Nat Rev Endocrinol* 8(2):72–74.
2. Balen A, Homburg R, Franks S (2009) Defining polycystic ovary syndrome. *BMJ* 338: a2968.
3. Franks S, et al. (2008) Ovarian morphology is a marker of heritable biochemical traits in sisters with polycystic ovaries. *J Clin Endocrinol Metab* 93(9):3396–3402.
4. Franks S, Stark J, Hardy K (2008) Follicle dynamics and anovulation in polycystic ovary syndrome. *Hum Reprod Update* 14(4):367–378.
5. Dunaif A, et al., eds (2008) *Polycystic Ovary Syndrome: Current Controversies, from the Ovary to the Pancreas* (Humana Press, Totowa, NJ).
6. Azziz R, et al.; Androgen Excess Society (2006) Positions statement: Criteria for defining polycystic ovary syndrome as a predominantly hyperandrogenic syndrome: An Androgen Excess Society guideline. *J Clin Endocrinol Metab* 91(11):4237–4245.
7. Rotteredam E, et al.; Rotterdam ESHRE/ASRM-Sponsored PCOS consensus workshop group (2004) Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). *Hum Reprod* 19(1):41–47.
8. Franks S, Gilling-Smith C, Gharani N, McCarthy M (2000) Pathogenesis of polycystic ovary syndrome: Evidence for a genetically determined disorder of ovarian androgen production. *Hum Fertil (Camb)* 3(2):77–79.
9. Goodarzi MO, Dumesic DA, Chazenbalk G, Azziz R (2011) Polycystic ovary syndrome: Etiology, pathogenesis and diagnosis. *Nat Rev Endocrinol* 7(4):219–231.
10. Zawadzki J, Dunaif A (1992) *Diagnostic Criteria for Polycystic Ovary Syndrome: Towards a Rational Approach* (Blackwell Scientific, Boston), pp 377–384.
11. Jakubowicz DJ, Nestler JE (1997) 17 α-Hydroxyprogesterone responses to leuprolide and serum androgens in obese women with and without polycystic ovary syndrome offer dietary weight loss. *J Clin Endocrinol Metab* 82(2):556–560.
12. Gilling-Smith C, Storey H, Rogers V, Franks S (1997) Evidence for a primary abnormality in theca cell steroidogenesis in the polycystic ovarian syndrome. *Clin Endocrinol (Oxf)* 47(93–99):1158–1165.
13. Nestler JE, et al. (1998) Insulin stimulates testosterone biosynthesis by human thecal cells from women with polycystic ovary syndrome by activating its own receptor and using inositolglycan mediators as the signal transduction system. *J Clin Endocrinol Metab* 83(6):2001–2005.
14. Gilling-Smith C, Willis DS, Beard RW, Franks S (1994) Hypersecretion of androstenedione by isolated thecal cells from polycystic ovaries. *J Clin Endocrinol Metab* 79(4):1158–1165.
15. Nelson VL, Legro RS, Strauss JF, 3rd, McAllister JM (1999) Augmented androgen production is a stable steroidogenic phenotype of propagated theca cells from polycystic ovaries. *Mol Endocrinol* 13(6):946–957.
16. Nelson VL, et al. (2001) The biochemical basis for increased testosterone production in theca cells propagated from patients with polycystic ovary syndrome. *J Clin Endocrinol Metab* 86(12):5925–5933.
17. Wickenheisser JK, et al. (2000) Differential activity of the cytochrome P450 17alpha-hydroxylase and steroidogenic acute regulatory protein gene promoters in normal and polycystic ovary syndrome theca cells. *J Clin Endocrinol Metab* 85(6):2304–2311.
18. Magoffin DA (2006) Ovarian enzyme activities in women with polycystic ovary syndrome. *Fertil Steril* 86(Suppl 1):S9–S11.
19. Jakimiuk AJ, Weitsman SR, Navab A, Magoffin DA (2001) Luteinizing hormone receptor, steroidogenesis acute regulatory protein, and steroidogenic enzyme messenger ribonucleic acids are overexpressed in thecal and granulosa cells from polycystic ovaries. *J Clin Endocrinol Metab* 86(3):1318–1323.
20. Wickenheisser JK, et al. (2012) Cholesterol side-chain cleavage gene expression in theca cells: Augmented transcriptional regulation and mRNA stability in polycystic ovary syndrome. *PLoS ONE* 7(11):e48963.
21. Legro RS, Driscoll D, Strauss JF, 3rd, Fox J, Dunaif A (1998) Evidence for a genetic basis for hyperandrogenemia in polycystic ovary syndrome. *Proc Natl Acad Sci USA* 95(25): 14956–14960.
22. Legro RS, Strauss JF (2002) Molecular progress in infertility: Polycystic ovary syndrome. *Fertil Steril* 78(3):569–576.
23. Chen ZJ, et al. (2011) Genome-wide association study identifies susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21 and 9q33.3. *Nat Genet* 43(1): 55–59.
24. Shi Y, et al. (2012) Genome-wide association study identifies eight new risk loci for polycystic ovary syndrome. *Nat Genet* 44(9):1020–1025.
25. Strauss JF, 3rd, McAllister JM, Urbanek M (2012) Persistence pays off for PCOS gene prospectors. *J Clin Endocrinol Metab* 97(7):2286–2288.
26. Goodarzi MO, et al. (2012) Replication of association of DENND1A and THADA variants with polycystic ovary syndrome in European cohorts. *J Med Genet* 49(2):90–95.
27. Welt CK, et al. (2012) Variants in DENND1A are associated with polycystic ovary syndrome in women of European ancestry. *J Clin Endocrinol Metab* 97(7):E1342–E1347.
28. Lerchbaum E, et al. (2011) Susceptibility loci for polycystic ovary syndrome on chromosome 2p16.3, 2p21, and 9q33.3 in a cohort of Caucasian women. *Horm Metab Res* 43(11):743–747.
29. Eriksen MB, et al. (2012) Association of polycystic ovary syndrome susceptibility single nucleotide polymorphism rs2479106 and PCOS in Caucasian patients with PCOS or hirsutism as referral diagnosis. *Eur J Obstet Gynecol Reprod Biol* 163(1):39–42.
30. Kosova G, Urbanek M (2013) Genetics of the polycystic ovary syndrome. *Mol Cell Endocrinol* 373(1–2):29–38.
31. Marat AL, Dokainish H, McPherson PS (2011) DENN domain proteins: regulators of Rab GTPases. *J Biol Chem* 286(16):13791–13800.

32. Chaineau M, Ioannou MS, McPherson PS (2013) Rab35: GEFs, GAPs and effectors. *Traffic* 14(11):1109–1117.
33. Allaire PD, et al. (2010) The Connecdenn DENN domain: A GEF for Rab35 mediating cargo-specific exit from early endosomes. *Mol Cell* 37(3):370–382.
34. Allaire PD, et al. (2006) Connecdenn, a novel DENN domain-containing protein of neuronal clathrin-coated vesicles functioning in synaptic vesicle endocytosis. *J Neurosci* 26(51):13202–13212.
35. Strauss JF, 3rd (2003) Some new thoughts on the pathophysiology and genetics of polycystic ovary syndrome. *Ann N Y Acad Sci* 997:42–48.
36. Wickenheisser JK, Nelson-DeGrave VL, Quinn PG, McAllister JM (2004) Increased cytochrome P450 17alpha-hydroxylase promoter function in theca cells isolated from patients with polycystic ovary syndrome involves nuclear factor-1. *Mol Endocrinol* 18(3):588–605.
37. Wood JR, Ho CK, Nelson-Degrave VL, McAllister JM, Strauss JF, 3rd (2004) The molecular signature of polycystic ovary syndrome (PCOS) theca cells defined by gene expression profiling. *J Reprod Immunol* 63(1):51–60.
38. Wood JR, et al. (2003) The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis. *J Biol Chem* 278(29):26380–26390.
39. Ewens WJ, Spielman RS (1995) The transmission/disequilibrium test: History, subdivision, and admixture. *Am J Hum Genet* 57(2):455–464.
40. Stenmark H (2009) Rab GTPases as coordinators of vesicle traffic. *Nat Rev Mol Cell Biol* 10(8):513–525.
41. Stenmark H, Olkkonen VM (2001) The Rab GTPase family. *Genome Biol* 2(5):S3007.
42. Chiariello M, Bruni CB, Bucci C (1999) The small GTPases Rab5a, Rab5b and Rab5c are differentially phosphorylated in vitro. *FEBS Lett* 453(1-2):20–24.
43. Lv LL, et al. (2013) Isolation and quantification of microRNAs from urinary exosomes/microvesicles for biomarker discovery. *Int J Biol Sci* 9(10):1021–1031.
44. Alvarez ML, Khosroheidari M, Kanchi Ravi R, DiStefano JK (2012) Comparison of protein, microRNA, and mRNA yields using different methods of urinary exosome isolation for the discovery of kidney disease biomarkers. *Kidney Int* 82(9):1024–1032.
45. Eriksen MB, et al. (2013) Genetic alterations within the DENND1A gene in patients with polycystic ovary syndrome (PCOS). *PLoS ONE* 8(9):e77186.
46. McAllister J, Simpson E (1993) Human theca interna cells in culture. *Methods in Toxicology*, eds Heindel JJ, Chapin RE (Academic, San Diego), pp 330–339.
47. Nelson-Degrave VL, et al. (2005) Alterations in mitogen-activated protein kinase kinase and extracellular regulated kinase signaling in theca cells contribute to excessive androgen production in polycystic ovary syndrome. *Mol Endocrinol* 19(2):379–390.
48. Nelson-DeGrave VL, et al. (2004) Valproate potentiates androgen biosynthesis in human ovarian theca cells. *Endocrinology* 145(2):799–808.
49. Legro RS, et al. (2013) Diagnosis and treatment of polycystic ovary syndrome: An endocrine society clinical practice guideline. *J Clin Endocrinol Metab* 98(12):4565–4592.
50. Graham FL, van der Eb AJ (1973) A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52(2):456–467.