The PCOS GWAS Candidate Gene ZNF217 Influences Theca Cell Expression of DENND1A.V2, CYP17A1, and Androgen Production

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Abstract
Polycystic ovary syndrome (PCOS), a common endocrine disorder of women, is characterized by increased ovarian androgen production and anovulatory infertility. Genome-wide association studies (GWAS) have identified more than 20 PCOS candidate loci. One GWAS candidate locus encompasses ZNF217, a zinc finger transcription factor. Immunohistochemical staining of ovarian tissue demonstrated significantly lower staining intensity for ZNF217 protein in PCOS theca interna compared to ovarian tissue from normal ovulatory women. Immunofluorescence staining of normal and PCOS theca cells demonstrated nuclear localization of ZNF217, with lower intensity in PCOS cells. Western blotting showed reduced ZNF217 protein in PCOS theca cells compared to normal theca cells, and that treatment with forskolin, which mimics the action of luteinizing hormone (LH), reduces ZNF217 expression. Lower ZNF217 expression in PCOS theca cells was confirmed by quantitative reverse transcription polymerase chain reaction. Notably, there was an inverse relationship between ZNF217 messenger RNA (mRNA) levels and theca cell androgen (dehydroepiandrosterone; DHEA) synthesis. The abundance of mRNA encoding a splice variant of DENND1A (DENND1A.V2), a PCOS candidate gene that positively regulates androgen biosynthesis, was also inversely related to ZNF217 mRNA levels. This relationship may be driven by increased miR-130b-3p, which targets DENND1A.V2 transcripts and is directly correlated with ZNF217 expression. Forced expression of ZNF217 in PCOS theca cells reduced androgen production, CYP17A1 and DENND1A.V2 mRNA, while increasing miR-130b-3p. Conversely, knockdown of ZNF217 in normal theca cells with short hairpin RNA-expressing lentivirus particles increased DENND1A.V2 and CYP17A1 mRNA. These observations suggest that ZNF217 is part of a network of PCOS candidate genes regulating thecal cell androgen production involving DENND1A.V2 and miR-130b-3p.

Key Words: ZNF217, DENND1A.V2, CYP17A1, miR-130b-3p, polycystic ovary syndrome, theca cells

Abbreviations: Adv, adenovirus; DHEA, dehydroepiandrosterone; ELISA, enzyme-linked immunosorbent assay; GWAS, genome-wide association study; IF, immunofluorescence; LH, luteinizing hormone; mRNA, messenger RNA; mTOR, mammalian target of rapamycin; PCOS, polycystic ovary syndrome; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ROI, region of interest; shRNA, short hairpin RNA.

Polycystic ovary syndrome (PCOS) is a common endocrinopathy affecting women of reproductive age. It is characterized by hyperandrogenemia of ovarian origin, bilateral ovarian enlargement, and follicular growth arrest leading to anovulation and infertility [1, 2]. Based on family studies, twin studies [3], genome-wide association studies (GWAS), and case-control association studies, there is compelling evidence that PCOS is a polygenic disorder [4-10] that can be modified by epigenetic and environmental factors [11, 12]. An important milestone in PCOS genetics was achieved with the publication of GWAS on Han Chinese populations that identified multiple PCOS candidate loci [6, 10], including loci encompassing DENND1A, LHCGR, ZNF217, YAP1, INSR, THADA, C90rf3, TOX3, and RAB5B. More than 20 loci associated with PCOS have now been identified from GWAS in different ethnic populations [6, 8, 9, 13].

Although some PCOS GWAS loci encompass genes with plausible pathophysiological significance, many of the loci contain genes with no obvious link to ovarian function and androgen production [14, 15]. Moreover, the interrelationships among these putative candidate genes is not well understood [14]. We previously established that a splice variant of the PCOS GWAS candidate gene, DENND1A, named DENND1A.V2 showed increased expression in PCOS theca cells, and plays an integral role in augmented steroidogenic gene expression and excess androgen biosynthesis by PCOS theca cells [16]. Here, we report on the expression and function of another PCOS GWAS candidate gene, ZNF217, which

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encodes a zinc finger transcription factor with repressor (or corepressor) activity [17-21], and its role in mediating DENND1A.V2 and CYP17A1 expression and theca cell androgen production. Our observations strongly implicate ZNF217 in the pathogenesis of PCOS.

Materials and Methods
Normal and Polycystic Ovary Syndrome Theca Cell Cultures
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 and Virginia Commonwealth University. Ovarian tissue was obtained from women undergoing oophorectomy for treatment of endometrial hyperplasia or cancer (PCOS women) or benign gynecological conditions (normal ovulatory women). 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 previously reported in detail [22-24]. The theca cell cultures used in these studies have been described and functionally characterized previously, and the steroidogenic properties of the normal and PCOS cells have been reported to result from the inherent properties of the cells rather than the cycle phase of the ovaries from which they were isolated [25-27]. PCOS and normal ovarian tissue came from age-matched women, 38 to 40 years old. The diagnosis of PCOS was made according to National Institutes of Health consensus guidelines [28, 29], which include hyperandrogenemia, oligoovulation, polycystic ovaries, and the exclusion of 21-hydroxylase deficiency, Cushing syndrome, adrenal and ovarian tumors, and hyperprolactinemia. All of the PCOS theca cell preparations studied came from ovaries of women with fewer than 6 menses per year and elevated serum testosterone or bioavailable testosterone levels [25-27]. 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 to 35 days, and no clinical signs of hyperandrogenism. Neither PCOS nor normal individuals were receiving hormonal medications at the time of surgery. Indications for surgery were dysfunctional uterine bleeding, endometrial cancer, and/or pelvic pain. Experiments comparing PCOS and normal theca were performed using fourth-passage (31-38 population doublings) theca cells isolated from size-matched follicles obtained from age-matched participants, in the absence of in vivo stimulation. It is important to note that these fourth-passage cells are not cell lines. 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 earlier. For all studies, theca cell cultures obtained from at least 4-5 independent normal and 5 independent PCOS patients were examined. The passage conditions and split ratios for all normal and PCOS cells were identical. Methods for immunohistochemistry, immunofluorescence (IF), Western blotting, quantitative reverse transcription polymerase chain reaction (qRT-PCR) for DENND1A.V2, miR-130b-3p RNA and assay of dehydroepiandrosterone (DHEA) were performed as previously reported [16, 30-32].

Quantitation of ZNF217 Expression in Human Ovarian Tissue Sections Using Immunohistochemistry
ZNF217 immunoperoxidase staining was performed on sections of paraffin-embedded ovaries from normal cycling and PCOS women, using conditions we have previously described [16]. Anti-ZNF217 antibody (Atlas Antibodies catalog No. HPA051857, RRID:AB_2681641, https://scicrunch.org/resources-legacy/Any/search?q=AB_2681641&l=AB_2681641), which binds to both ZNF217.V1 and ZNF217.V2, and the ZNF217 recombinant protein (APREST85710) were obtained from Sigma-Aldrich. Image analysis was performed using a digital camera (Olympus QColor5), attached to an Olympus BH-2 microscope. The staining area was quantified using the CellSens imaging software (Olympus). To quantify the staining area, multiple regions of interest (ROIs) were selected in the theca cell layer, and staining area was measured within the ROIs. Each image within the same experiment contained the same number of identical ROIs. Data were reported using percentage ROI area stained.

Western Blot Analysis of ZNF217 and DENND1A.V2 Protein
Whole-cell protein extracts were prepared from normal and PCOS theca cells treated with and without 20 μM forskolin for 48 hours, and Western blot analyses were performed using 30 μg protein per lane as we have previously described [16, 31]. A rabbit polyclonal anti-ZNF217 antibody recognizing ZNF217.V1 and V2 was obtained from Sigma-Aldrich, and a rabbit polyclonal antibody specific for the unique 33 amino acid C-terminal sequence of DENND1A.V2 (Jan M. McAllister, Penn State College of Medicine catalog No. Bunny-226n-V2, RRID:AB_2800456, https://scicrunch.org/resources-legacy/Any/search?q=AB_2800456&l=AB_2800456) generated in our laboratory, were used as primary antibodies [16, 31]. ZNF217 and DENND1A.V2 protein was visualized using electrochemiluminescence (Rockland Immunocchemicals) and quantitated using a ProteinSimple FluorChem R. Protein expression was normalized to total mammalian target of rapamycin (mTOR), which is not significantly different in normal and PCOS theca cells, nor regulated by forskolin treatment [16]. The data presented are representative of multiple experiments repeated 3 times.

Quantitation of Quantitative Reverse Transcription Polymerase Chain Reaction for ZNF217, CYP17A1, and DENND1A.V2
Quantification of ZNF217 mRNA accumulation was performed using the Single Step Brilliant III Ultra-Fast qRT-PCR Reagent (Agilent) with a final concentration of 200 nM for
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Statistical Methods

Relative differences in staining at the ROI were measured by highlighting specific stained areas with red overlay. Data were quantified as area stained (μm²). Where indicated, statistical analyses were performed with Prism 8 by GraphPad Software. The t test was used for comparison of 2 groups of data, and 1-way analysis of variance with Bonferroni corrections when multiple groups of data are compared. Data were presented as means ± SE. Normality of data was assessed by D’Agostino and Pearson omnibus normality test. Samples were considered statistically significantly different when the P value was less than .05.

Correlations were determined in experiments where ZNF217, CYP17A1, DENND1A.V2 mRNA, miR-130b-3p, and steroid production (DHEA) were examined from multiple individual patients treated with or without forskolin. Spearman nonparametric correlation coefficient (ρ) was calculated to estimate the degree of correlation between all pairwise comparisons (ρ was considered statistically significant when P < .05), using JMP/SAAS.

Expression of ZNF217 Protein in Human Ovaries and Cultured Theca Cells

To examine the localization of ZNF217, we used paraffin-embedded blocks of ovarian tissue obtained from normal cycling women and our PCOS patient population. Immunohistochemical staining revealed lower staining intensity for ZNF217 in PCOS theca cells (Fig. 1A). Specificity of the anti-ZNF217 antibody was confirmed by neutralization with recombinant ZNF217 peptide (see Fig. 1A, lower panels). IF images of cultured normal and PCOS theca cells established nuclear localization of ZNF217 and revealed lower signal intensity in PCOS theca cells and in normal theca cells treated with 20 μM forskolin (Fig. 2A). Quantification of Western blot data from 5 normal and 5 PCOS theca cell preparations demonstrated that ZNF217 protein (normalized by mTOR) was significantly reduced in normal cells treated with forskolin (Fig. 2B and 2C). In addition, ZNF217 protein in PCOS theca cells was significantly reduced under control culture conditions compared to normal theca cells (see Fig. 2C). Although it did not reach statistical significance, ZNF217 in forskolin-stimulated PCOS theca cells trended toward lower expression as compared to normal theca cells (see Fig. 2C). In contrast, DENND1A.V2 expression was significantly increased in PCOS theca cells compared to normal theca cells (Fig. 2D), in agreement with our prior observations [16].

ZNF217 Gene Expression in Normal and Polycystic Ovary Syndrome Theca Cells and Relationships to Dehydroepiandrosterone Production, CYP17A1, DENND1A.V2, and miR-130b-3p expression

Comparison of ZNF217 expression in normal and PCOS theca cell preparations (Fig. 3A) demonstrated a significantly lower level of ZNF217 mRNA compared to normal theca cells under both forskolin-treated and untreated conditions. Moreover, forskolin treatment of normal theca cells significantly reduced ZNF217 expression. ZNF217 expression, DHEA production (Fig. 3B), CYP17A1 (Fig. 3C), and DENND1A.V2 mRNA expression (Fig. 3D), and miR-130b-3p abundance (Fig. 3E) were correlated among normal and PCOS theca cells. There was a significant negative relationship between ZNF217 mRNA levels and DHEA production (see Fig. 3B). Decreased ZNF217 expression in PCOS theca cells was negatively correlated with CYP17A1 gene expression, which encodes P450 17α-hydroxylase, the key enzyme in androgen biosynthesis (see Fig. 3C) [24, 33, 34]. Reduced ZNF217 expression in PCOS theca cells was associated with increased expression of CYP17A1 (see Fig. 3C).
of ZNF217 mRNA expression with DENND1A.V2 mRNA abundance in PCOS and normal theca cells treated without or with forskolin revealed a negative inverse correlation (see Fig. 3D). miR-130b-3p, which targets DENND1A.V2 mRNA, was positively correlated with ZNF217 expression (see Fig. 3E).

ZNF217 Overexpression in Polycystic Ovary Syndrome Theca Cells Reduces DENND1A.V2 and CYP17A1 Expression and Androgen Production, While Increasing miR-130b-3p Abundance

A ZNF217 Adv was generated to examine the effects of forced overexpression of ZNF217 in PCOS theca cells that have reduced ZNF217 expression (see Fig. 3A). PCOS theca cells were grown until 85% confluent and infected with ZNF217 Adv (3 pfu/cell) or empty vector (Null Adv) at 3 pfu/cell in serum-free medium for 16 hours, then cultured in the absence (control) or presence of 20 μM forskolin. After 36 hours, the infected cells were harvested, and RNA was prepared. ZNF217, DENND1A.V2, CYP17A1, and miR-130b-3p expression was quantified by qRT-PCR, as previously described [16, 31]. In parallel studies, 72 hours following treatment media were collected for DHEA measurements and cells were counted. As shown in Fig. 4A, ZNF217 mRNA was significantly increased in ZNF217 Adv-infected cells compared to Null Adv–infected cells under both basal (control) and forskolin-stimulated conditions. Of note, forskolin treatment reduced ZNF217 mRNA both in Null and ZNF217 Adv–infected cells, in agreement with data presented in Fig. 3. ZNF217 Adv infection significantly inhibited both basal and forskolin-stimulated DENND1A.V2 expression, as compared to Null Adv infection, and converted the PCOS cells to a normal phenotype of diminished DENND1A.V2 mRNA levels (Fig. 4B). CYP17A1 mRNA accumulation was significantly increased in Null Adv–infected cells following forskolin treatment (Fig. 4C). In contrast, forced ZNF217 overexpression dramatically decreased CYP17A1 mRNA accumulation under both control and forskolin-stimulated culture conditions (see Fig. 4C). In parallel studies, DHEA accumulation was also observed to be increased by forskolin treatment in Null Adv–infected cells (Fig. 4D), whereas ZNF217 Adv infection reduced DHEA biosynthesis both in control and forskolin-treated cells. ZNF217 Adv infection significantly augmented both basal and forskolin-treated miR-130b-3p accumulation, as compared to Null Adv–infected cells (Fig. 4E). Forkolin treatment significantly reduced miR-130b-3p in ZNF217 Adv–infected cells. These data are consistent with the notion that relatively high ZNF217 expression in normal theca cells is linked to lower levels of DENND1A.V2 and CYP17A1 gene expression.

ZNF217 knockdown in normal theca cells with ZNF217 short hairpin RNA lentiviral particles

To determine whether decreased expression of ZNF217 was associated with increased DENND1A.V2 and CYP17A1 mRNA abundance and androgen production, PCOS theca cells and normal theca cells were infected with either control shRNA or ZNF217 shRNA lentiviral particles for 36 hours. As shown in Fig. 5A, ZNF217 expression was significantly suppressed following ZNF217 shRNA lentivirus infection. In agreement with results presented in Fig. 3A, forskolin treatment significantly inhibited ZNF217 expression (see Fig. 5A). As shown in Fig. 5B, DENND1A.V2 expression was significantly increased following treatment with ZNF217 shRNA, as compared to control shRNA. ZNF217 knockdown significantly increased both basal and forskolin-stimulated CYP17A1 mRNA abundance (Fig. 5C). Combined, these results provide further evidence that the decrease in ZNF217 expression in PCOS is associated with augmented CYP17A1 expression, as well as androgen biosynthesis, possibly through the intermediacy of DENND1A.V2 and a microRNA that targets the DENND1A.V2 transcript.

Discussion

PCOS is currently thought to be a polygenic disorder [14]. The first PCOS candidate loci [6, 10] were identified by GWAS performed in Han Chinese populations. These loci included DENND1A, LHCGR, ZNF217, YAP1, INSR,
THADA, C9orf3, TOX3, and RAB5B [10]. Subsequent GWAS, performed in various populations, revealed more than 20 PCOS candidate loci [35-37]. Despite substantial research on the genetic origins of PCOS, including follow-up association studies examining the correlation between PCOS candidate genes and various PCOS phenotypes, the mechanism(s) by which specific genes disrupt ovarian function and produce other features characteristic of PCOS is currently unknown [14, 15]. Moreover, the genetic variants that have a causal role in PCOS in different populations remain to be identified.

The functional importance of DENND1A.V2 in the PCOS phenotype of human theca cells was established by our studies demonstrating that increased DENND1A.V2 stimulates androgen synthesis in normal theca cells, whereas knockdown of DENND1A.V2 diminishes androgen production in PCOS theca cells [16, 21]. Although our prior studies provided evidence of a direct role for DENND1A.V2 in the production of the ovarian PCOS phenotype, the mechanisms of DENND1A.V2 action and the relationship of DENND1A.V2 to other PCOS candidate genes was unknown. We have expanded our studies of the functional genomics of PCOS and the interrelationship of another PCOS GWAS candidate gene, ZNF217, with respect to the genesis of hyperandrogenemia of ovarian origin, widely considered to be one of the most common characteristics of women with PCOS.

ZNF217 encodes a zinc finger DNA-binding transcription factor that usually acts as a corepressor [17]. Work by others showed that ZNF217 is expressed in granulosa cells and theca cells [19, 20]. ZNF217 has also been shown to increase estradiol synthesis in granulosa cells from normal cycling women, and to be reduced in granulosa cells collected from women with PCOS [19]. These studies agree with our immunostaining of ovarian tissue sections from normal cycling and PCOS women (see Fig. 1) that revealed lower staining intensity in PCOS theca.

Our findings suggest that decreased expression of ZNF217 in PCOS theca cells allows for the upregulation of DENND1A.V2 and CYP17A1 mRNA, resulting in enhanced...
androgen biosynthesis. In contrast, miR-130b-3p, which targets DENND1A.V2, was positively correlated with ZNF217 expression (see Fig. 3E). This finding raises the possibility that ZNF217 directly influences DENND1A.V2 abundance, and consequently CYP17A1 expression and androgen biosynthesis (Fig. 6). Alternatively, ZNF217 could indirectly
Forced expression of ZNF217 in polycystic ovary syndrome (PCOS) theca cells converts PCOS theca cells to a normal phenotype of reduced DENND1A.V2, CYP17A1, and dehydroepiandrosterone (DHEA) biosynthesis using a ZNF217 adenovirus (Adv). PCOS theca cells (N=5) were infected with either 3 pfu/cell of Null (empty) vector adenovirus (Null Adv) or ZNF217 adenovirus (ZNF217 Adv) overnight, then treated in the absence (Control) and presence of forskolin (20 μM) for 36 hours for RNA studies or 72 hours for DHEA studies. A, ZNF217 Adv infection augmented ZNF217 messenger RNA (mRNA) expression under unstimulated (control, ***P < .0001) and forskolin-stimulated conditions (**P < .001). ZNF217 Adv infection following forskolin stimulation resulted in decreased ZNF217 mRNA compared to control untreated cells (αP < .01). B, Following ZNF217 Adv infection, PCOS theca cells were converted to a normal phenotype of diminished DENND1A.V2 mRNA accumulation, under both control (**P < .001) and forskolin-stimulated conditions (**P < .001), compared to Null Adv (control) infected cells. C, CYP17A1 mRNA accumulation was increased following forskolin-treatment (**P < .01) in Null Adv–infected cells. In addition, ZNF217 Adv infection dramatically decreased CYP17A1 mRNA accumulation under both control (**P < .001) and forskolin-stimulated conditions (**P < .001). D, At 72 hours following infection with Null Adv, DHEA accumulation was observed to be increased by forskolin treatment (**P < .01). ZNF217 Adv infection resulted in decreased DHEA biosynthesis under both control (**P < .001) and forskolin-stimulated conditions (**P < .001). E, Forced ZNF217 overexpression with ZNF217 Adv, augmented miR-130b-3p expression under unstimulated (control, **P < .001) and forskolin-stimulated conditions (**P < .01). ZNF217 Adv infection following forskolin stimulation resulted in decreased ZNF217 mRNA compared to control untreated cells (**P < .01).
influence DENND1A.V2 and CYP17A1 expression through the intermediacy of miR-130b-3p (see Fig. 6).

In addition to ZNF217, DENND1A, and CYP17A1, 2 other GWAS candidate genes, LHCGR and RAB5B, should be incorporated into the network of interacting genes regulating thecal androgen production. We have previously shown that RAB5B protein colocalizes with DENND1A.V2. We propose that LHCGR, an activator of adenylate cyclase, is an upstream regulator of DENND1A.V2, which has a clathrin-binding domain and, therefore, has potential interactions with LHCGR localized in plasma membrane coated pits and endocytic vesicles [30]. It is notable that databases, including TargetMiner (https://www.isical.ac.in/~bioinfo_miu/targetminer20.htm) and miRBase (https://www.mirbase.org), list ZNF217 and RAB5B as miR-130b-3p targets. Although we have no direct experimental evidence that miR-130b-3p regulates the expression of these genes in human theca cells, the potential role of miR-130b-3p in controlling expression of ZNF217 and RAB5B adds greater complexity to the proposed gene network (see Fig. 6, dashed lines), with the potential for autoregulation of ZNF217 expression and control of another gene, RAB5B, in the proposed cascade involving LHCGR and DENND1A.

Figure 6. A polycystic ovary syndrome (PCOS) genome-wide association study (GWAS) candidate gene network encompassing ZNF217 and DENND1A.V2. PCOS is a manifestation of altered expression of a network of PCOS GWAS candidate genes including LHCGR, ZNF217, DENND1A, and RAB5B. Decreased ZNF217 expression in PCOS theca cells is associated with increased DENND1A.V2 and CYP17A1 expression, and downstream, androgen biosynthesis. Alternatively, ZNF217 could indirectly influence DENND1A.V2, RAB5B, and subsequently CYP17A1 expression, through the intermediacy of miR-130b-3p.

In summary, studies on normal and PCOS theca and granulosa cells provide a molecular framework for understanding the hormonal imbalance characteristic of PCOS, increased androgens of thecal origin, and reduced estradiol production by granulosa cells.

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Disclosures
The authors have nothing to disclose.

Data Availability
Original data generated and or analyzed during the present study are not available but are available from the corresponding author on reasonable request.

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