3 The Genetics of Polycystic Ovary Syndrome: From Genome-Wide Association to Molecular Mechanisms

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3.1 Hyperandrogenemia and Polycystic Ovary Syndrome (PCOS)

PCOS is a common endocrine disorder reported to affect 5–7% women of reproductive age. The incidence of PCOS appears to be similar across racial/ethnic groups. Although there has been debate about the most appropriate diagnostic criteria for PCOS, hyperandrogenemia/hyperandrogenism, not explained by other causes (e.g., androgen-secreting tumors, Cushing's syndrome, late-onset congenital adrenal hyperplasia), is a hallmark of the disorder, and it is included as an essential element in all "consensus" diagnosis schemes [1, 2].

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3.2 The Phenotype of Human Theca Cells from Normal and PCOS Ovaries

Studies on freshly isolated thecal tissue from normal and PCOS ovaries or cultures of human 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 [3–9]. Our success in developing conditions to propagate human theca cells isolated from individual, size-matched follicles from ovaries of normal-cycling women and women with PCOS, provided the first evidence to show that increased *CYP17A1* (P450 17α-hydroxylase) gene expression in PCOS theca cells is associated with an excess androgen production in the PCOS ovary [10, 11]. Our previous molecular characterization of PCOS and normal theca cells from multiple individuals by microarray analysis and quantitative PCR established that normal and PCOS theca cells have distinctive molecular signatures [5–7, 12–15]. Similar results have been observed with granulosa cells collected from normal and PCOS women undergoing assisted reproduction [16].

3.3 The Genetics of PCOS

There is strong evidence for genetic predisposition to PCOS in most ethnic/racial groups studied to date [17, 18]. However, despite advances in genetic technologies, very few PCOS susceptibility genes have been validated. Numerous candidate gene association studies, based on genes selected because of their putative roles in PCOS phenotypes, have been conducted. While some of these studies yielded statistically significant associations of genetic variants with PCOS, these candidate gene studies have been uniformly conducted on small sample sizes and have had limited statistical power. Moreover, few of these studies have produced sufficiently robust results, and rarely have they been replicated by different investigators studying diverse populations of women [19, 20].

A major milestone was achieved with the publication of a genome-wide association study (GWAS) by Chen and colleagues [21], who reported their findings on a Han Chinese population. Chen and colleagues identified loci on chromosome 2 (2p16.3 and 2p21) and chromosome 9 (9q33.3) that had significant associations with PCOS, conferring protection or increased risk, at levels exceeding the threshold statistical significance for genome-wide associations. A subsequent GWAS with additional subjects identified eight new putative PCOS loci on chromosomes 2p16.3, 9q22.32, 11q22.1, 12q13.2, 12q14.3, 16q21.1, 19p13.3, and 20q13.2 [22] (Table 3.1). Replication studies conducted in populations of European ancestry confirmed a number of these associations [23, 24]. Subsequently, GWAS carried out on European populations added new putative PCOS genes, bringing the total of PCOS candidates to 22 [26, 27].

While several loci identified in the GWAS, including those in or near the *FSHB*, *FSHR*, *LHCGR*, and *INSR* genes, are plausible PCOS candidates, the pathophysiological links of other loci identified in the GWAS (e.g., *C9orf3*, *YAP1*, *RAB5B*,

Table 3.1 PCOS risk loci reported to date

Locus	Gene	GWAS index SNP	Risk/other allele	Discovery *p*-value	Discovery population	Ref.
2p16.3	FSHR	rs2268361	T/C	9.89×10^{-13}	CHN	35
		rs2349415	T/C	2.35×10^{-12}	CHN	35
2p16.3	LHCGR	rs13405728	G/A	7.55×10^{-21}	CHN	34
2p21	THADA	rs13429458	C/A	1.73×10^{-23}	CHN	34
		rs12478601	T/C	3.48×10^{-23}	CHN	34
		rs12468394	A/C	1.59×10^{-20}	CHN	34
		rs7563201	G/A	3.3×10^{-10}	EUR	36
2q34	ERBB4	rs1351592	G/C	1.2×10^{-12}	EUR	36
5q31.1	RAD50	rs13164856	T/C	3.5×10^{-9}	EUR	36
8p23.1	GATA4/NEIL2	rs804279	A/T	8×10^{-10}	EUR	37
9q22.32	C9orf3	rs4385527	A/G	5.87×10^{-9}	CHN	35
		rs3802457	A/G	5.28×10^{-14}	CHN	35
		rs10993397	C/T	4.6×10^{-13}	EUR	37
9q33.3	DENND1A	rs2479106	G/A	8.12×10^{-19}	CHN	34
		rs10818854	A/G	9.4×10^{-18}	CHN	34
		rs10986105	C/A	6.9×10^{-15}	CHN	34
		rs10760321	A/G	1.4×10^{-6}	EUR	36
11p14.1	KCNA4/FSHB	rs11031006	G/A	1.9×10^{-8}	EUR	37
11q22.1	YAP1	rs1894116	G/A	1.08×10^{-22}	CHN	35
		rs11225154	A/G	7.6×10^{-11}	EUR	36
12q13.2	RAB5B/SUOX	rs705702	G/A	8.64×10^{-26}	CHN	35
12q13.2	ERBB3	rs7312770	C/T	2.1×10^{-7}	EUR	36

(continued)

Table 3.1 (continued)

Locus	Gene	GWAS index SNP	Risk/other allele	Discovery *p*-value	Discovery population	Ref.
12q14.3	HMGA2	rs2272046	C/A	1.95×10^{-21}	CHN	35
12q21.2	KRR1	rs1275468	C/T	1.9×10^{-8}	EUR	24
16q12.1	TOX3	rs4784165	G/T	3.64×10^{-11}	CHN	35
17q12	ERBB2	rs7218361	A/G	9.6×10^{-7}	EUR	36
19p13.2	INSR	rs2059807	G/A	1.09×10^{-8}	CHN	35
20q13.2	SUMO1P1/ZNF217	rs6022786	A/G	1.83×10^{-9}	CHN	35

Genetic variants associated with PCOS risk

HMGA2, *TOX3*, *SUMO1P1/ZNF217*, *THADA*, and *DENND1A*) to reproduction or ovarian function are less obvious.

The *DENND1A* locus assumed significance among these candidates as a result of several studies confirming the association of *DENND1A* SNPs with PCOS in European populations [23, 24], and its role as a guanine nucleotide exchange factor and component of clathrin-coated pits places it in pivotal position for linking signaling between plasma membrane receptors and downstream signal transduction.

3.4 *DENND1A*: A Reasonable Starting Point for Dissection of the Genetics of PCOS

The *DENND1A* gene encodes a protein named connecdenn 1, which interacts with members of the Rab family of small GTPases involved in membrane trafficking. Connecdenn 1 has a clathrin-binding domain and is thought to facilitate endocytosis (Fig. 3.1) [25].

There are at least two *DENND1A* transcripts produced as a result of alternative splicing. One of these transcripts, *DENND1A* variant 1 (V1), codes for a 1009

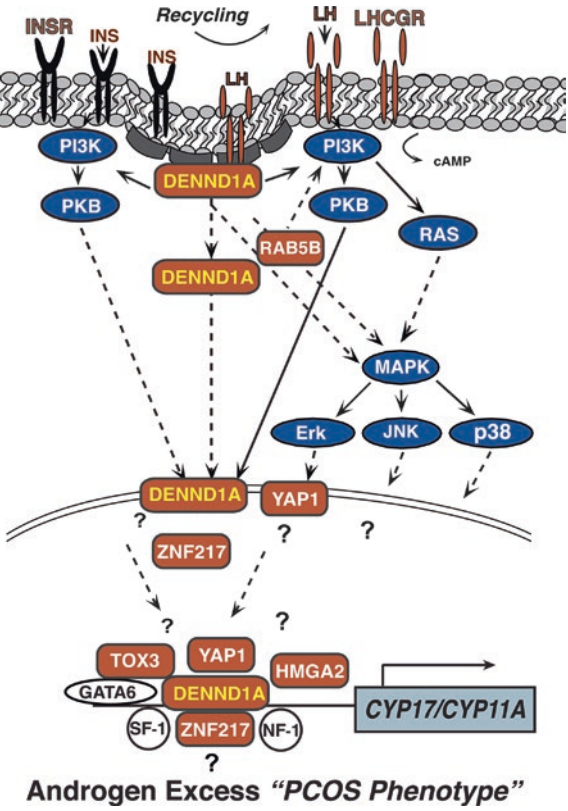

Fig. 3.1 A network of genes postulated to contribute to the hyperandrogenemia associated with PCOS based on loci identified in published GWAS and replication studies. Our preliminary observations suggest that DENND1A. V2 internalization may affect LHRH and INSR recycling and downstream signaling in conjunction with other PCOS GWAS candidates to mediate increased androgen biosynthesis as well as changes in insulin and LH-receptor sensitivity. This figure has been modified and updated from our TEM review [30]

amino acid protein with C-terminal proline-rich domain; the other, *DENND1A* variant 2 (V2), codes for a truncated 559 amino acid protein that contains the DENN domain, which serves as a guanine nucleotide exchange factor, and the clathrin-binding domain, but lacks the proline-rich domain and includes a C-terminal 33 amino acid sequence that differs from the larger V1. Interestingly, a locus encoding a Rab GTPase, RAB5B, was also identified in the GWAS noted above, suggesting a link between DENND1A and a regulator of endocytic recycling of cell surface receptors.

Up 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, ovarian theca cells, adrenocortical reticularis, brain, and H295 adrenal carcinoma cells, for the most part cells that synthesize androgens [28, 29]. Work from our laboratories has directly implicated DENND1A.V2 in the pathophysiology of PCOS: DENND1A.V2 mRNA and protein are overexpressed in PCOS theca cells compared to normal theca cells; overexpression of DENND1A.V2 in normal theca cells increased the abundance of CYP17A1 mRNA, augmented androgen (i.e., DHEA) production, and increased *CYP11A1* and *CYP17A1* promoter-reporter activity; and siRNA knockdown of V2 in PCOS theca cells reversed the PCOS phenotype. In addition, DENND1A.V2 mRNA is abundant in urinary exosomes of PCOS women, but not normal-cycling women. These results suggest the possibility of a noninvasive diagnostic for PCOS for early detection in prepubertal and adolescent females [29].

To further define the role of DENND1A.V2 in the pathophysiology of PCOS, we created a transgenic mouse that expresses human DENND1A.V2 under control of the CMV promoter. To our advantage, rodents do not express truncated DENND1A.V2. Although characterization of these animals is still under way, Cyp17A1 and Cyp11A1 mRNA were observed to be overexpressed in ovaries, testes, and adrenals of the DENND1A.V2 transgenic mouse (V2-Tg) compared to wild-type (WT) littermates. Plasma androstenedione, DHEA, testosterone, and progesterone were also observed to be elevated in the female V2-Tg compared to the WT mouse. One of the most striking findings of these studies was the discovery of *Cyp17a1* expression in the V2-Tg mouse adrenal cortex, and cortisol production by V2-Tg mouse adrenocortical cells in culture. *Cyp17a1* is not normally expressed in the mouse adrenal gland, and we could not detect *Cyp17a1* mRNA in wild-type adrenals, only in the V2-Tg mice. These findings implicate DENND1A.V2 in control of the pathways that govern androgen biosynthesis.

3.5 What Is the Mechanism for DENND1A.V2 Overexpression in PCOS Theca Cells?

The exact mechanism through which V2 is overexpressed in PCOS theca cells has not yet been determined. GWAS results indicate that genetic variation contributes to some extent. However, the *DENND1A* GWAS SNPs associated with PCOS in all populations are located in introns, and none are near the alternative splice sites, so on the surface it is not evident that they have functional roles in controlling or

transcript splicing or gene expression, although they could be embedded in intronic enhancer elements with the SNP alleles affecting transcription factor binding. This possibility has not been formally excluded.

We have yet to identify genetic variants in coding sequences from whole exome sequencing (WES) studies that can account for overexpression in PCOS. Also, copy number variants do not appear to be a common mechanism. A detailed examination of splicing mechanisms that generate DENND1A.V2 failed to disclose variants in the intron involved in V2 splicing (lying between exons 20 and 20A, which encodes the C-terminal V2 sequence) [28]. The SNPs in *DENND1A* identified by GWAS are located in introns and lack apparent functions. As suggested previously, overexpression of DENND1A.V2 in PCOS suggests the contribution of gain-of-function variation [29]. Thus, we sought to determine the mechanisms leading to production of DENND1A.V2 and its overexpression in PCOS by testing several possible mechanisms including examination of coding or splicing variants in the *DENND1A* gene via available WES data, investigation of copy number variations in the *DENND1A* gene, and sequencing of the intronic region between exons 20 and exon 20A [28] (see below) for identification and characterization of variations affecting alternative splicing of DENND1A.V1 and DENND1A.V2.

We have recently examined the possibility that epigenetic mechanisms contribute to the overexpression of V2 in human PCOS theca cells. Three lines of evidence suggest that these mechanisms are operative:

First, treatment of normal human theca cells with 5-azacytosine, which results in genomic DNA demethylation and increased DENND1A.V2 expression.

Second, treatment of normal theca cells with the histone deactylase inhibitor valproic acid augmented DENND1A.V2 mRNA accumulation.

Third, an examination of differentially expressed microRNAs in normal and PCOS theca cells identified that miR-125a-3p was downregulated in PCOS theca cells.

Our studies have shown that miR-125a-3p targets *DENND1A*; miR-125a-3p mimic reduces DENND1A.V2 but not DENND1A.V1 mRNA accumulation in human adrenal 295R cells, implicating that changes in abundance of this microRNA directly affect DENND1A.V2 mRNA. Collectively, these observations suggest that both genetic and epigenetic mechanisms contribute to DENND1A.V2 overexpression in PCOS.

3.6 A PCOS Genetic Network Incorporating *DENND1A*

Among the loci associated with PCOS in Han Chinese, several reside in or near genes that potentially define a network, including the *FSHR*, *LHCGR*, and *INSR*, which encode receptors that reside on the plasma membrane, and which are internalized by coated pits, where DENND1A protein is located (Fig. 3.1) [19, 22]. RAB5B is thought to be involved in endocytosis and could, therefore, be a molecule interacting with the DENN domain. YAP1, TOX3, HMGA2, and ZNF217 are all

involved in transcriptional regulation, although none of them have been specifically implicated in the expression of genes involved in steroidogenesis. However, TOX3 (transcriptional coactivator of the p300/CBP-mediated transcription complex) transactivates through cAMP response element (CRE) sites, which are present in gene-encoding steroidogenic proteins. These genes can be assembled into a signaling network beginning at the receptor level, receptor coupling, or recycling and downstream molecules that ultimately regulate gene transcription, either of steroidogenic genes directly or possibly through the upregulation of other transcription factors that directly influence steroidogenic gene promoter function (Fig. 3.1). This framework provides a road map for the identification of genetic variation/mutations that predispose to PCOS and the molecular basis for the action of the identified risk alleles [30].

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