The molecular characteristics of polycystic ovary syndrome (PCOS) ovary defined by human ovary cDNA microarray

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Abstract

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders; it is characterized by polycystic ovaries, hyperandrogenism and chronic anovulation. To obtain a global view of those genes that might be involved in the development of this complex clinical disorder, we used recently developed cDNA microarray technology to compare differential gene expressions between normal human ovary and ovaries from PCOS patients. A total of 9216 clones randomly selected from a commercial human ovary cDNA library were screened. Among them, 290 clones showed differential expressions, including 119 known genes and 100 known or unknown expressed sequence tags (ESTs). Among 119 known genes, 88 were upregulated and 31 downregulated in the PCOS ovary, as compared with normal human ovary. These differentially expressed genes are involved in various biologic functions, such as cell division/apoptosis, regulation of gene expression and metabolism, reflecting the complexity of clinical manifestations of PCOS. The molecular characteristics established from our study will further our understanding of the pathogenesis of PCOS and help us to identify new targets for further studies and for the development of new therapeutic interventions.

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Introduction

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders, being found in 4–7% of women of reproductive age and accounting for about 75% of anovulatory infertility. It is characterized by polycystic ovaries, hyperandrogenism and chronic anovulation (Knochenhauer *et al.* 1998). PCOS is also associated with hirsutism, obesity, insulin resistance and compensatory hyperinsulinemia. Hyperinsulinemia results in an increased risk of cardiovascular disorders and a sevenfold increase in risk of non-insulin-dependent diabetes mellitus (NIDDM) (Legro *et al.* 1999). Many lipid abnormalities (most notably, low high-density lipoprotein cholesterol levels and elevated triglyceride levels) and impaired fibrinolysis are seen

in women with PCOS. Long-standing unopposed estrogen stimulation leads to an increased risk of endometrial carcinoma. PCOS is also associated with an increased rate (30–40%) of early pregnancy loss (Watson *et al.* 1993). Early diagnosis of this syndrome, close long-term follow-up and screening for diabetes and cardiovascular diseases are warranted.

Familial clustering of PCOS cases suggests that genetic factors play an important role in PCOS's etiology (Legro *et al.* 1998). Although the studies of familial cases of PCOS have produced results suggesting an autosomal dominant trait, the mode of inheritance has not been firmly established (Govind *et al.* 1999). There are several reasons for this. First, genetic heterogeneity makes the genetic studies of PCOS hard to perform. Second, several

pathways are implicated in the etiology of PCOS; therefore, several candidate genes may be responsible for this 'complex' genetic trait, making the identification of each contributing gene very difficult.

Until recently, the approach to understand the molecular basis of this complex syndrome was to study the functions of individual genes. However, since many pathways have been implicated in the etiology of PCOS, including the metabolic or regulatory pathways of steroid hormone synthesis, regulatory pathways of gonadotropin action, the insulin-signaling pathway, and pathways regulating glucose and lipid metabolism, expressions of numerous genes are coordinated both spatially and temporally during the development and progression of this disease. Earlier studies focused on the individual genes have not firmly established their roles in PCOS due to the complex nature of this disease (Jonard *et al.* 2002). In this regard, a global profiling of gene expressions will yield useful information.

The newly developed functional genomic technologies, such as cDNA microarray, have enabled researchers to study the expressions of numerous genes simultaneously. As a well-established method and a powerful tool for massively parallel analysis of gene expression, microarray analysis has been applied in various biologic studies to identify differentially expressed genes. In this study, we used cDNA microarray technology to compare the expression patterns of 1000 genes in normal human ovaries and ovaries from PCOS patients, and identified genes that are up- or downregulated in PCOS patients. These results will help us to understand the pathogenesis of this complex clinical disorder at molecular level.

Materials and methods

Amplification of the cDNA library of human ovary

A human ovary 5'-STRETCH PLUS cDNA library (Cat: HL5025t, source of insert cDNA: 25 Caucasians, aged 16–80) was purchased from Clontech (Palo Alto, CA, USA). In the supplied λ TriplEx phagemid vector, the cloning sites are located within a plasmid that is embedded in a λ phage genome and flanked by loxP sites at the λ junctions. *E. coli* BM25·8 was used to circularize a

complete plasmid from the recombinant phage at the loxP sites. To collect a set of representative clones, 12 000 positive phage plaques were randomly picked and amplified. Each λ TriplEx clone was transduced to a pTriplEx clone in *E. coli* BM 25·8, following the manufacturer's protocol (Clontech's Manual PT3003–1). Then the plasmid DNAs were extracted as previously described (Sha *et al.* 2002) and stored at -70°C until use.

Construction of the human ovary cDNA microarray

To make DNA for spotting the microarray, each gene was amplified from plasmid clone by PCR (λ TriplEx LD-insert Screening Amplimers, 5'-primer: CTCGGGAAGCGCGCCATTGTGTTGGT, 3'-primer: ATACGACTCACTATAGGGCGAATTGGCC), as described before (Sha *et al.* 2002). A total of 9216 PCR products with an average size of ~ 1.0 kb (0.5–3.0 kb) was selected and stored in a 96-well format at -20°C . Two dots for each DNA sample were spotted with a total of 18 432 dots for 9216 samples on 8×12 cm nylon membranes, using an automatic arrayer (BioRobotics, Cambridge, UK). DNA was cross-linked to the nylon membrane by UV light. Eight housekeeping genes (ribosomal protein S9, β -actin, glyceraldehyde-3-phosphate dehydrogenase, hypoxanthine phosphoribosyl transferase 1, phospholipase A₂, ubiquitin C, ubiquitin carboxy-terminal esterase L1 and *H. sapiens* mRNA for 23 kDa highly basic protein) were used as positive controls, while pTriplEx and PUC18 plasmid DNAs were used as negative controls (Zhou *et al.* 2002). Twelve spots for each control cDNA were evenly distributed in the membranes.

Tissue collection and preparation of probes from normal and PCOS ovaries

Approval from the institutional ethics committee was granted before the initiation of this study. Samples of normal adult ovaries from women aged 30–40 years were obtained from the Body Donor Center (Nanjing Medical University). After macroscopic and histologic examinations, three ovary samples representing follicular and luteal phases respectively were selected for this study. Ovary tissues of three PCOS patients were obtained during surgical operations. These patients were

recruited by the following criteria: amenorrhea, polycystic ovaries, hyperandrogenism or hirsutism. All the samples were histologically examined and stored in liquid nitrogen. Ovarian tissues from three PCOS patients and three normal adults were pooled separately and then homogenized. Total RNA was extracted by following Trizol RNA isolation protocol (Gibco BRL, Grand Island, NY, USA). Contaminating DNA was eliminated by DNase treatment. Total RNA was quantitated by measuring A260 and A260: 280 ratio. PolyA⁺ mRNA was then purified by the Oligotex mRNA Spin-Column system (Qiagen, Hilden, Germany). Afterwards, cDNA probes were prepared by reverse-transcription reaction containing [³³P]dATP. Labeled probes were quantified according to the manufacturer's protocol (NEN Life Science, Boston, MA, USA).

Hybridization and signal analysis

After prehybridization, the nylon membranes spotted with cDNA fragments were hybridized with ³³P-labeled cDNA probes from normal and PCOS ovaries in 6 ml hybridization solution (6 × SSC, 0.5% SDS) at 65 °C overnight. The membranes were washed three times with 20 ml wash solution (10 × SSC, 0.5% SDS) at 65 °C for 20 min. Membranes were then exposed to a phosphor screen overnight and scanned with a FLA-3000A fluorescent image analyser (Fuji Photo Film, Tokyo, Japan) with a 65 536 gray grade of 50 μm pixels. The radioactive intensity of each spot was analyzed with array gauge software (Fuji Photo Film, Tokyo, Japan). After the subtraction of background chosen from an area where no cDNA was spotted, clones with signal intensities of >2 were considered as positive to ensure that they were distinguished from background, with a statistical significance of >99.9% (array gauge software). The data would be considered invalid if any of the 12 control spots for the same positive control cDNA had over a 1.0-fold difference in its intensity in one array. When the standard difference of the two dots for each DNA sample was less than 0.3, results would be accepted by the software.

Sequencing of differentially expressed clones and data analysis

A gene was considered differentially expressed if it met these two criteria: 1) beyond the 95%

predicted regression line; 2) a difference in signal intensity of >2.0-fold between normal and PCOS samples. All clones consistent with the criteria were purified with R.E.A.L. Prep 96 system (Qiagen), and the inserts were sequenced with the MegaBase1000 automatic sequencing machine (Amersham Pharmacia Biotech, Bjorkgatan, Sweden). All sequencing reactions were performed twice for confirmation. 'Good' sequences referred to those containing less than 1% ambiguous bases in sequences longer than 100 bp. All resulting sequences were analyzed by DNASTAR (<http://www.dnastar.com>) to get rid of repetitive and vector sequences. Standard BLAST searches were performed against sequences in GenBank. ESTs (expressed sequence tags) were considered as part of known genes if they shared at least 95% homology over at least 100 bp of DNA sequences. Sequences homologous to some repetitive elements were discarded. Those sequences sharing less than 95% homology with known ESTs were temporarily considered as novel ESTs. After a search against the Unigene database, all those differentially expressed ESTs were classified into seven groups based on their functional characteristics or cellular roles, which have been detailed previously (Adams *et al.* 1995).

Reverse-transcription and semiquantitative real-time PCR analysis

To validate the difference of mRNA abundance in our differential expression profiling determined by cDNA microarray analysis, we selected four genes for semiquantitative real-time PCR analysis. To rule out genomic DNA contamination, PCR primer pairs were designed to span introns. The nucleotide sequences and annealing temperatures of the primers, and the length of the amplified fragments are shown in Table 1A.

We pooled three PCOS ovary RNA samples and three normal ovary RNA samples separately for reverse transcription with the Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Production of cDNA was then added to a 20 μl reaction mixture containing 10 μl of 2 × TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 2 μl SYBR Green 1 (1:3200) (Molecular Probes, Eugene, OR, USA), and 2.5 pmol gene-specific primers according to

Table 1 Validation of four differently expressed genes by semiquantitative real-time PCR**A**

Gene name	Primers (5'–3')	Tm ^a	Size (bp)	Relative expression (P/N)	
				cDNA array	Real-time PCR
NR4A1	Forward: CATGGTGAAGGAAGTTGTC Reverse: AAAGCCAGGGATCTTCTC	55 °C	289	0.40	0.06
PDK4	Forward: CCAGACCAACCAATTCACATC Reverse: ACCAGCCAAAGGAGCATTC	55 °C	285	3.58	6.70
HIP-55	Forward: ATGTGACCATCAACGCAC Reverse: CCCAGAAGCTGTCTTTACC	55 °C	207	0.50	0.03
SET	Forward: CGAGCTACCAATGAAGGC Reverse: AAGCCTGGAAGTTCCGATAC	55 °C	199	2.01	16.70

Tm^a: annealing time; P: PCOS; N: normal.**B**

Gene name	C _T P±SD	C _N ±SD	ΔΔC _T
NR4A1	30.06±0.01	27.06±0.02	–3.08
PDK4	26.91±0.07	29.68±0.04	2.69
HIP-55	29.43±0.04	25.21±0.06	–4.3
SET	23.81±0.07	27.87±0.06	3.98
β-actin	22.23±0.01	22.31±0.02	

C_TP for C_T value in PCOS ovary samples; C_TN for C_T value in normal ovary samples; SD for standard error. Note that a higher C_T is indicative of a lower mRNA content.

the manufacturer's instructions. Real-time PCR was performed with ABI Prism 7000 (Applied Biosystems). Preliminary experiments were done for each primer pairs to determine the optimal primer concentration and annealing temperature that would yield the greatest amount of specific product with melting temperature (T_m) separable from primer-primer T_m. No template control was used for each reaction to ensure the absence of carryover DNA contamination. Results were analyzed using the relative C_t method. The C_t value, which is inversely proportional to the initial template copy number, is the calculated cycle number where the fluorescence signal emitted is significantly above background levels. Each sample was assayed twice, and the intra-assay coefficient of variation was less than 3%. β-Actin from the same samples was amplified as an internal control for the normalization of starting amount of sample cDNA. The relative abundance of target gene mRNA between normal subjects and PCOS patients was then calculated.

Fold induction or repression by real-time PCR was measured and calculated after adjusting for β-actin, using $2^{-\Delta\Delta C_t}$, where ΔC_t = target gene C_t – β-actin C_t, and $\Delta\Delta C_t$ = ΔC_{tN} (in normal human ovary) – ΔC_{tP} (in PCOS ovary) ($\Delta\Delta C_t$ method, User Bulletin 2, Applied Biosystems). Statistics was done by two-sample *t*-test. Difference was considered to be statistically significant at 95% confidence level when *P* value was <0.05.

Western blot analysis

Cytosolic and nuclear proteins were extracted from two PCOS ovary samples and two normal ovary samples with the NE-PER Nuclear and Cytoplasmic Extract Kit (Pierce Biotechnology, Rockford, IL, USA). Aliquots of 50 µg protein extracts were loaded and separated by SDS–polyacrylamide gel electrophoresis. Proteins were then transferred to a nitrocellulose membrane, which was blocked for 2 h at 25 °C with 5% nonfat milk in PBS buffer (20 mM Tris, 500 mM NaCl and 0.01% Tween 20).

and incubated with polyclonal antibodies to collagen I (diluted at 1:500), PDK4 (diluted at 1:250), hnRNPA2/B1 (diluted at 1:250), NR4A1 (diluted at 1:500), TGFBR2 (diluted at 1:250) and β -actin (diluted at 1:250) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. The blot was then visualized by enhanced chemiluminescence (Amersham Biosciences). The membrane was then scanned, and the signal intensity of each band was determined with ImageMaster Elite software (Amersham Biosciences). The relative levels of proteins in each sample were then normalized to β -actin signal (signal intensity of specific protein/signal intensity of β -actin).

Results

To investigate genes that are differentially expressed between normal and PCOS ovaries, we performed the cDNA microarray hybridization with probes prepared from normal human ovaries and ovaries from PCOS patients respectively. Positive signals were obtained from 8621 clones (93.5%) hybridized with probes from normal ovaries and 8476 clones (92%) hybridized with probes from PCOS ovaries. Figure 1 shows the scatter plot of hybridization intensity of double spots on PCOS ovary cDNA array. The equation $R^2=0.9789$ indicates the reproducibility and reliability of our hybridization data.

Among the positive clones, 290 showed at least two times difference between normal and PCOS ovaries, with 200 clones expressed at a level of at least twofold higher in the PCOS ovaries and 90 clones at least twofold higher in normal ovaries. We sequenced all 290 clones and aligned consensus sequences by DNASTAR. Based on this analysis, there are 100 new or known ESTs, and 119 known genes. Among known genes, 88 expressed at a level of at least twofold higher and 31 at least twofold lower in PCOS ovaries than in normal ovaries (Tables 2 and 3).

By their biologic functions, these known genes can be grouped into seven broad categories: 1) cell division (10%); 2) cell signaling/communication (20%); 3) cell structure and motility (6%); 4) cell/organism defense (4%); 5) gene/protein expression (37%); 6) metabolism (10%); and 7) unclassified (13%) (Fig. 2). The detailed data for

Figure 1 Scatter plot of PCOS ovary cDNA hybridization. The x-axis shows the radioactive intensity of spot A in double spots, and the y-axis shows the radioactive intensity of spot B in double spots. $R^2=0.9689$.

each group are also available at www.repro-lab.net/research_date/polycystic_ovary.htm.

To validate our cDNA microarray data, we selected four genes (NR4A1, PDK4, HIP-55 and SET) for semiquantitative real-time PCR analysis. These genes are differentially expressed in PCOS ovaries (Tables 2 and 3) and involved in various biologic functions that might have a role in the pathogenesis of PCOS. The sequences of primers used and relative changes in expression levels of these genes as determined by real-time RT-PCR are listed in Table 1A. A paired *t*-test showed that the differences in the levels of expression of these genes between normal subjects and PCOS patients are statistically significant (Table 1B). The patterns of these gene expressions obtained from cDNA microarray and real-time RT-PCR analyses were similar, although the absolute ratios were different due to the potential difference in assay sensitivity and dynamics of these two assays (Fig. 3).

To determine whether the differential gene expressions at mRNA levels seen between normal and PCOS ovaries are also reflected in their protein products, we did Western blot analysis to determine the protein levels of five genes (PDK4, hnRNPA2/B1, TGFBR2, collagen I and NR4A1), using protein samples from the ovaries of two normal subjects and two PCOS patients (Fig. 4A). Levels of β -actin in these samples were also measured and used to normalize sample

Table 2 Genes expressed at higher levels in PCOS ovary

	GenBank ID	Gene name	Abbreviation	Unigene	P/N
Classification					
Cell division	NM_014335	CREBBP/EP300 inhibitory protein 1	CR11	Hs. 381137	3:12
	NM_144624.1	Kinase interacting with leukemia-associated (stathmin)	KIS	Hs. 127310	2:97
	NM_002107.2	H3 histone, family 3A	H3F3A	Hs. 181307	2:40
	NM_004862	Lipopolysaccharide-induced TNF-alpha factor	LITAF	Hs. 76507	2:32
	NM_014886	TGF beta inducible nuclear protein TINP1	TINP1	Hs. 8170	2:31
	NM_005324.2	H3 histone, family 3B	H3F3B	Hs. 393660	2:28
	NM_002305	Lectin, galactoside-binding, soluble, 1	Galectin 1	Hs. 382367	2:21
	NM_021962	Active BCR-related gene	ABR	Hs. 118021	2:15
Cell signal communication	NM_003292	Nuclear pore complex-associated protein TPR (tpr)	TPR	Hs. 169750	2:13
	M33132	Proliferating cell nucleolar protein P120	CHD4/P120	Hs. 74441	2:01
	NM_003270	Transmembrane 4 superfamily member 6	TM4SF6	Hs. 121068	3:24
	NM_001892	Casein kinase 1, alpha 1	CSNK1A1	Hs. 283738	3:15
	NM_030571.1	Nedd4 family interacting protein 1	NDFIP1	Hs. 9788	2:90
	XM_291015	Homolog of rat kinase D-interacting substance	KIDINS220	Hs. 9873	2:89
	NM_021135	Ribosomal protein S6 kinase, 90 kDa polypeptide 2	RPS6KA2	Hs. 301664	2:87
	XM_113275.1	Reticulocalbin 2 precursor (EF-hand calcium-binding protein ERC-55) (E6-binding protein) (E6BP)	RCN2	Hs. 79088	2:76
	NM_133376.1	Integrin beta-1; fibronectin receptor, beta subunit-like	ITGB1	Hs. 287797	2:58
	NM_020824	Rho-GTPase activating protein 10	ARHGAP10	Hs. 11611	2:36
	X75252	Phosphatidylethanolamine-binding protein	PBP	Hs. 80423	2:28
	NM_152828	Sorting nexin 3	SNX3	Hs. 12102	2:25
	XM_003118	Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC	Hs. 111779	2:23
	NM_003242	TGF-beta1R alpha	TGFB2	Hs. 82028	2:22
	NM_004342	Caldesmon	CALD1	Hs. 325474	2:21
	NM_004844	SH3-domain binding protein 5 (BTK-associated)	SH3BP5	Hs. 109150	2:11
	NM_004684	High endothelial venule SPARC-like 1 (mast9, hevin)	SPARCL1	Hs. 75445	2:10
	NM_005216	Dolichyl-diphosphooligosaccharide-protein glycosyltransferase	DDOST	Hs. 34789	2:07
	AF536326	<i>H. sapiens</i> adenovirus E1B19k-binding protein B5	BNIP3L	Hs. 132955	2:01
Cell structure/mobility	NM_000089	Collagen, type I, alpha 2	COL1A2	Hs. 179573	3:15
	NM_000090.2	Collagen, type III, alpha 1	COL3A1	Hs. 119571	2:76
	NM_001920.2	Decorin, transcript variant A1	DCN	Hs. 433989	2:72
	NM_006087	Tubulin, beta 5	TUBB5	Hs. 110837	2:17
	XM_008854.7	Myosin IXB	MYO9B	Hs. 159629	2:01
Cell/organism defense	NM_005545	Immunoglobulin superfamily containing leucine-rich repeat	ISLR	Hs. 102171	2:59
	NM_005348.1	Heat-shock 90 kDa protein 1, alpha (HSPCA)	HSPCA	Hs. 356531	2:12
	NM_000062	Serine (or cysteine) proteinase inhibitor, clade G (C1) inhibitor, member 1	SERPING1	Hs. 151242	2:12
Gene/protein expression	NM_022365.2	DnaJ (Hsp40) homolog, subfamily C, member 1	DNAJL1	Hs. 13015	2:03
	NM_001967	Eukaryotic translation initiation factor 4A, isoform 2	EIF4A2	Hs. 173912	6:18
	BC026276	Ribosomal protein L10	RPL10	Hs. 458148	5:20
	NM_001417.1	Eukaryotic translation initiation factor 4B	EIF4B	Hs. 93379	3:55
	NM_003750	Eukaryotic translation initiation factor 3, subunit 10 theta	EIF3S10	Hs. 154796	3:43
	HSA011779	SEC63 protein	SEC63	Hs. 31575	3:33
	NM_021005	Nuclear receptor subfamily 2, group F, member 2	NR2F2	Hs. 347991	3:13
	NM_000506	Prothrombin (F2) gene, exon XIV and partial cds	F2	Hs. 76530	2:87
	NM_001961	Eukaryotic translation elongation factor 2	EEF2	Hs. 75309	2:77

Table 2 Continued

	GenBank ID	Gene name	Abbreviation	Unigene	P/N
Classification Gene/protein expression <i>continued</i>	NM_000982	Ribosomal protein L21	RPL21	Hs. 431927	2:65
	NM_001402	Eukaryotic translation elongation factor 1, alpha 1	EEF1A1	Hs. 422118	2:61
	NM_000999.2	Ribosomal protein L38	RPL38	Hs. 425668	2:61
	NM_002137.2	Heterogenous nuclear ribonucleoprotein A2/B1	HNRPA2B1	Hs. 232400	2:56
	NM_006431.1	Chaperonin containing TCP1, subunit 2 (beta)	CCT2	Hs. 432970	2:54
	AB002330	U2-associated SR140 protein	SR140	Hs. 7976	2:42
	NM_004768	Splicing factor, arginine/serine-rich 11	SFRS11	Hs. 433581	2:35
	BC002327	Ribosomal protein L30	RPL30	Hs. 334807	2:22
	NM_001002	Ribosomal protein, large P0 (RPLP0)	RPLP0	Hs. 406511	2:21
	BC000287	General transcription factor IIA, 2	GTF2A2	Hs. 76362	2:20
	NM_000987.2	Ribosomal protein L26	RPL26	Hs. 406682	2:18
	AF100620	Transcription factor-like protein MRGX	MRGX	Hs. 173714	2:17
	BC007603	Ribosomal protein S11	RPS11	Hs. 182740	2:17
	NM_006937	SMT3 suppressor of mif two 3 homolog 2	SMT3H2	Hs. 180139	2:17
	BC017343	Ribosomal protein L31	RPL31	Hs. 184014	2:13
	NM_031157	Heterogenous nuclear ribonucleoprotein A1	HNRPA1	Hs. 376844	2:09
	AF419331	TLS-associated protein TASR-1	TASR-1	Hs. 3530	2:08
	NM_133476.1	Zinc finger protein 384	ZNF384	Hs. 103315	2:06
	NM_003134	Signal recognition particle 14 kDa	SRP14	Hs. 180394	2:03
	NM_022551	Ribosomal protein S18/S6-like mRNA	RPS18	Hs. 275865	2:02
	NM_003011	SET translocation (myeloid leukemia-associated)	SET	Hs. 145279	2:01
	X63237	Uba80 mRNA for ubiquitin	Uba80	Hs. 311640	2:01
	NM_002948	Ribosomal protein L15	RPL15	Hs. 74267	2:01
	NM_016333.2	Splicing coactivator subunit SRn300/serine/arginine repetitive matrix 2 (SRRM2)	SRm300	Hs. 197114	2:00
Metabolism	NM_002612	Pyruvate dehydrogenase kinase 4 mRNA	PDK4	Hs. 8364	3:58
	NM_006895	Histamine N-methyltransferase	HNMT	Hs. 81182	2:82
	NM_001159.2	Aldehyde oxidase 1	AOX1	Hs. 406238	2:60
	NM_001863	Cytochrome c oxidase subunit VIb	COX6B	Hs. 431668	2:26
	NM_000365.2	Triosephosphate isomerase 1	TP11	Hs. 83848	2:24
	XM_051100.4	Ceroid-lipofuscinosis, neuronal 3, juvenile	CLN3	Hs. 194660	2:10
	NM_003115	UDP-N-acetylglucosamine pyrophosphorylase 1	UAP1	Hs. 21293	2:08
Unclassified	NM_000087	Cyclic nucleotide gated channel alpha 1, cGMP gated	CNGA1	Hs. 1323	2:05
	M97168	X (inactive)-specific transcript	XIST	Hs. 352403	3:53
	AF203815	<i>H. sapiens</i> alpha gene	PRO1073	Hs. 356442	2:90
	XM_291015	Homolog of rat kinase D-interacting substance of 220 kDa	KIDINS220	Hs. 9873	2:89
	XM_003952.5	HTGN29 protein	HTGN29	Hs. 283437	2:84
	NM_018507	Hypothetical protein PRO1843 (PRO1843)	PRO1843	Hs. 283330	2:50
	XM_086515	Hypothetical protein LOC200030 (LOC200030)	LOC200030	Hs. 446489	2:40
	NM_173638	Hypothetical protein MGC8902	MGC8902	Hs. 323463	2:20
	NM_018084	Hypothetical protein FLJ10392	FLJ10392	Hs. 20887	2:18
	BC022218.1	Hypothetical protein SP192	SP192	Hs. 169854	2:17
	NM_021825	Hypothetical protein MDS025	MDS025	Hs. 154938	2:10
	AF314542	B lymphocyte activation-related protein mRNA	FLJ21174	Hs. 194329	2:06
	NM_024334	Hypothetical protein MGC3222	MGC3222	Hs. 323193	2:05

loading. Afterwards, the ratio of relative levels of specific proteins in normal and PCOS ovaries was calculated (Fig. 4B). As shown in Fig. 4B, the differential protein expression patterns for these

genes are similar to their relating mRNA expression patterns. Thus, our data from Western blotting analysis further validated our findings from cDNA microarray analysis.

Table 3 Genes expressed at lower levels in PCOS ovary

	GenBank ID	Gene name	Abbreviation	Unigene	P/N
Classification					
Cell division	NM_002128	High-mobility group (nonhistone chromosomal) protein 1	HMGB1	Hs. 427696	0.48
Cell signal communication	NM_005487	High-mobility group protein 2-like 1	HMG2L1	Hs. 92260	0.40
	NM_014394	Growth hormone-inducible transmembrane protein	GHITM	Hs. 433957	0.49
	U59305	Ser-Thr protein kinase PK428	PK428	Hs. 18586	0.48
	XM_037447.4	SET binding factor 1	SBF1	Hs. 112049	0.48
	NM_078474	BBP-like protein 2 (BLP2), transcript variant 1	BLP2	Hs. 288912	0.44
	NM_022036	G protein-coupled receptor, family C, group 5, member C	GPRC5C	Hs. 58014	0.43
Cell structure/mobility	XM_083884	Nuclear receptor subfamily 4, group A, member 1	NR4A1	Hs. 1119	0.40
	NM_014063.3	src homology 3 domain-containing protein HIP-55	HIP-55	Hs. 183373	0.39
	NM_015180	Spectrin repeat containing, nuclear envelope 2	SYNE-2	Hs. 57749	0.43
	NM_004261	15 kDa selenoprotein	SEP15	Hs. 90606	0.43
Cell/organism defense	NM_014311	Single-strand selective monofunctional uracil DNA glycosylase	DGU	Hs. 5212	0.22
Gene/protein expression	NM_020414	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 24	DDX24	Hs. 155986	0.50
	NM_145011	Zinc finger protein 25 (KOX 19)	ZNF25	Hs. 5856	0.49
	AF132969	CGI-35 protein	CGI-35	Hs. 343173	0.48
	NM_001029	Ribosomal protein S26	RPS26	Hs. 299465	0.48
Metabolism	NM_005461	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B	MAFB	Hs. 169487	0.48
	NM_000971	Ribosomal protein L7	RPL7	Hs. 153	0.47
	BC003373	Prefoldin 5	PFDN5	Hs. 288856	0.39
	BC004971	Ribosomal protein L36	RPL36	Hs. 433411	0.37
	NM_001514	General transcription factor IIB	GTF2B	Hs. 258561	0.27
	NM_001019	Ribosomal protein S15a	RPS15a	Hs. 433406	0.27
	NM_002142	Homeo box A9 (HOXA9), transcript variant 2	Hox-A9	Hs. 127428	0.24
	NM_004569.1	Phosphatidylinositol glycan, class H (GPI-H)	PIGH	Hs. 177	0.49
	NM_001866	Cytochrome c oxidase subunit VIIb	COX7B	Hs. 432170	0.47
	NM_015523	Small fragment nuclease	SFN	Hs. 7527	0.46
Unclassified	NM_004273.2	Carbohydrate (chondroitin 6) sulfotransferase 3	CHST3	Hs. 158304	0.45
	NM_017627.1	Hypothetical protein FLJ20030	FLJ20030	Hs. 433489	0.45
	NM_152415	Hypothetical protein FLJ32642	FLJ32642	Hs. 101617	0.43
	NM_139030.1	CD151 antigen	CD151	Hs. 75564	0.41
	NM_004404.1	Neural precursor cell expressed, developmentally downregulated 5	NEDD5	Hs. 155595	0.34

Discussion

PCOS was firstly reported as Stein–Leventhal syndrome in 1935, and since then has attracted more and more attention due to its genetic heterogenesis and diversity of clinical manifestations. It has been used as an important clinical model to investigate the relationships among endocrine, reproductive activity and energy metabolism.

In this study, we used recently developed cDNA microarray technology to examine the differential gene expression patterns between normal and PCOS ovaries, and were able to identify several

hundred genes expressed at changed levels in PCOS patients compared with normal subjects. These genes are involved in a wide range of biologic functions, including gene/protein expression, cell signaling/cell communication and metabolism. This picture is consistent with the complex nature of PCOS. Many genes identified in this study were found to be associated with hormone production, metabolism and apoptosis in other tissues, but their roles in human ovaries and PCOS have not been documented before. Furthermore, we found that different genes with similar functions changed their expression patterns as a group and might contribute to some aspects of PCOS

Figure 2 The functional classification of genes differentially expressed between normal and PCOS ovaries. The 119 known genes identified by cDNA microarray analysis are divided into seven groups by their biologic functions. Percentage of genes in each group is indicated.

development, such as impaired apoptosis, abnormal insulin-stimulated pathways, insulin resistance and anovulation.

Insulin has numerous biologic functions in target tissues, such as glucogen synthesis, steroidogenesis, DNA synthesis and lipogenesis. There is increasing evidence that PCOS is associated with hyperinsulinemia, insulin-resistance and dyslipidemia. Insulin resistance has been observed in cultured skin fibroblasts derived from PCOS patients (Book & Dunaif 1999). But peripheral insulin-resistance cannot fully explain the abnormal insulin action in PCOS ovary. In our study, we found that some genes involved in insulin functions changed their expression levels in PCOS ovary, including PDK4 (pyruvate dehydrogenase kinase 4) (Rosa *et al.* 2003, Sugden *et al.* 2003), PIGH (phosphatidylinositol glycan, class H) and UDP-GalNAcPP (UDP-GalNAc pyrophosphorylase) (Muller *et al.* 1998, Pouwels *et al.* 2002). Pyruvate dehydrogenase complex (PDHC) regulates the oxidative metabolism of glucose, which can be inhibited by isoforms of PDK. Recently, increased PDK4 activity has been implicated in the pathogenesis of insulin resistance and non-insulin-dependent diabetes mellitus (NIDDM). In the muscle cell of NIDDM patients, PDK4 mRNA expression

correlates negatively with glucose uptake rate, and decreases following the improvement of insulin sensitivity and the reduction of weight (Muller *et al.* 1998, Rosa *et al.* 2003). We found that PDK4 mRNA was upregulated in PCOS ovary, although we need to investigate whether this increased expression of PDK4 mRNA means the existence of insulin resistance in PCOS ovary. Some genes involved in insulin mitogenic pathway were also upregulated in PCOS ovary, mainly those in the MAPKs (mitogen-activated protein kinases) signaling pathway, such as RPS6KA2 (Dufresne *et al.* 2001). Intrinsic selective insulin-resistance in PCOS ovary could be important to the development of metabolic and reproductive disturbance in this syndrome.

Apoptosis, a form of programmed cell death, is recognized as the mechanism of germ cell death and follicle atresia at all stages of ovary development and in the each cycle of folliculogenesis. The imbalance between apoptosis and proliferation affects the stages of follicle development, such as primary follicle pool formation, follicle recruitment and dominant follicle selection. PCOS patients have an obvious disturbance in their follicle development, since, in their ovaries, there are twice the number of primary and

Figure 3 Comparison of mRNA expression patterns of four genes analyzed by quantitative real-time RT-PCR and cDNA microarray. Ratio of gene expression levels between normal and PCOS ovaries (N/P) or between PCOS and normal ovaries (P/N) is presented. Gray columns represent data from cDNA microarray analysis, while black columns represent data from real-time PCR.

secondary follicles (Franks *et al.* 1998), a subcortical necklaced array containing ≥ 10 follicles of 3–10 mm in diameter which are arrested at the small antral follicle stage and without dominant follicle, and thickened and hyperechogenic stroma and hyperproliferated theca-interstitial cells.

Previously, researchers suggested that the increased apoptosis of follicle cells contribute significantly to the premature arrest of follicle growth, but this alone cannot account for all three characteristics of disturbed follicle development in PCOS mentioned above. Furthermore, *in vitro* studies revealed that these arrested follicles are not atretic and show increased metabolic activity under normal culture condition (Nelson *et al.* 1999). Our study indicates that there is a group of genes up- or downregulated in PCOS ovary that might result in reduced apoptosis. These genes include NR4A1 (nuclear receptor subfamily 4, group A, member 1)

(Li *et al.* 2000), HIP-55 (src homology 3 domain-containing protein) (Chen *et al.* 2001) and HMGB1 (high-mobility group protein 1) (Stros *et al.* 2002). They regulate apoptosis via different pathways such as the c-Jun N-terminal kinase signaling cascade (JNK)/NF- κ B, p53/BCL2/BAX and p73/c-myc (Watanabe *et al.* 2002, Zhang *et al.* 2002). Most of them finally influence apoptosis through the mitochondrial death signaling pathway, resulting in the defect in the release of cytochrome C (Tournier *et al.* 2000, De Smaele *et al.* 2001, Anning 2003).

These data suggest that the overexpression of survival or antiapoptotic factors and downregulation of apoptosis inducers led to the blocking of follicle apoptosis and atresia in PCOS ovary. We hypothesize that the blocking of apoptosis and atresia affects follicle development at both gonadotropin-independent and -dependent stages, and contributes to the excessive recruitment of

(A)

(B)

Figure 4 Western blot analysis of proteins encoded by genes differentially expressed between normal and PCOS ovaries. (A) Western blotting for six gene products was carried out using specific antibodies, as indicated in Materials and methods. There are two samples from PCOS patients (P1 and P2) and two samples from normal subjects (N1 and N2). (B) The relative levels of gene and protein expressions between normal and PCOS ovaries determined by cDNA microarray and Western blotting. The y-axis represents the ratio of gene expressions at mRNA and protein levels between PCOS and normal ovaries. The signal intensity of β -actin was used for the normalization of sample loading in Western blot analysis, as indicated in Materials and methods. Black columns represent data from Western blotting and white columns represent data from cDNA microarray.

preantral follicles and accumulation of multiple small antral follicles as well as hyperproliferation of theca-interstitial cells.

The most interesting gene among this group is NR4A1, which is dramatically upregulated in normal adult ovary compared with fetal ovary

(adult/fetal=7.1) (Xu & Diao *et al.*, unpublished data), but downregulated in PCOS ovary. NR4A1, also known as TR3, Nur77 or NGFI-B, has been confirmed as an inducer of apoptosis. It causes mitochondria to release cytochrome C (Li *et al.* 2000). Considering that the major cell types

expressing NR4A1 are thecal cells of follicles in different sizes, we suggest that the downregulation of NR4A1 might contribute to the hyperproliferation of theca cells from small antral follicles in PCOS ovary (Park *et al.* 2001). As a nuclear receptor, NR4A1 mRNA expresses rapidly and transiently in granulosa cells of preovulatory follicles after the preovulatory luteinizing hormone (LH) surge in adult cycling rats (Park *et al.* 2001), suggesting that NR4A1 may play a role in ovulation by initiating a cascade of expression of ovulation-specific genes in ovulatory follicles in response to LH surge. Considering that NR4A1 might play a protective role in atherogenesis, which is one of the long-term sequelae of PCOS, the expression pattern of NR4A1 in the PCOS patient's circulation system needs further investigation (Arkenbout *et al.* 2002). These findings suggest that the downregulation of NR4A1 may affect multiple signal pathways and contribute to the development of various abnormalities in PCOS ovary simultaneously.

Theca cells are the major androgen-secreting cells in the human ovarian follicle. The enzymes involved in the synthesis of ovarian androgens, such as P450c17 and 3 β -HSD, were observed in follicular theca cells, but not granulosa cells, in PCOS ovary (Kaaikj *et al.* 2000). The hyperproliferation of theca cells, induced by blocked apoptosis and increased mitogenic actions, might contribute to the development of hyperandrogenism in PCOS.

Our cDNA microarray analysis also discovered a gene named SET (SET translocation) that can regulate androgen production by P450c17. Cytochrome P450c17 catalyzes 17 α -hydroxylation during cortisol synthesis and 17,20-lyase activity during sex steroid production. 17,2-lyase activity, but not 17 α -hydroxylation activity, can be inhibited by PP2A (protein phosphatase 2A). The serine phosphoprotein SET inhibits PP2A specifically and fosters 17,20-lyase activity (Pandey & Synthia 2003). Since SET and PP2A are the post-translational regulators of androgen biosynthesis, changes in their expression might contribute to the development of hyperandrogenism in PCOS.

Our study also revealed some other genes, such as C1 inhibitor (C1 inh) (Falus *et al.* 1990, Han *et al.* 2002), HNMT (histamine *N*-methyltransferase), prothrombin (Roach *et al.* 2002, Yildiz *et al.* 2002) and TGFBR2 (TGF-betaIIIR alpha) (Qi *et al.* 1999), which are differentially expressed in PCOS

ovaries. The changed levels of expression of these genes might contribute to the impaired vascular permeability, fibrinolysis, and increased fibrogenesis in PCOS, as consistent with previous reports.

The differentially expressed genes we identified in this study also include some encoding initiation factors, elongation factors, general transcription factors and splicing factors. These factors control gene expression at levels of transcription, post-transcription and translation respectively. The upregulation of translation factors in PCOS ovary is consistent with the decreased apoptosis and increased mitogenesis (Wang *et al.* 2001). Alternative splicing is another important mechanism for gene expression regulation. Alternative splicing can expand the coding capacity of limited number of genes to achieve the high molecular complexity needed to sustain normal body functions and facilitate responses to altered conditions (Hastings & Krainer 2001). In our expression profiling, there are two members and one cooperator of serine-arginine-rich (SR) proteins (SFRS11 (arginine/serine-rich 11), TASR-1 (TLS-associated serine-arginine-1) and SRRM2 (serine/arginine repetitive matrix 2/SRM300)) upregulated in PCOS ovary. Their precise functions in PCOS ovary are not yet known. But it has been reported recently that SR proteins can regulate the alternative splicing of members of the insulin signal pathway, such as IGF-1 and protein kinase C beta II, in response to metabolic and hormonal changes (Patel *et al.* 2001, Smith *et al.* 2002). In addition to SR proteins, we also found genes of some other splicing factors (hnRNP A1 and hnRNP A2) upregulated in PCOS ovary. It has been reported that hnRNP A2 expression is increased during human endometrial hyperplasia or adenocarcinoma (Byrjalsen *et al.* 1999). Since endometrial hyperplasia is one of the long-term sequelae of PCOS, the potential association of the upregulation of hnRNP A2 expression we found in PCOS ovaries with the increased risk of endometrial carcinoma in these patients warrants further study.

During the preparation of this paper, Wood *et al.* published their research on the gene expression profile in PCOS theca cells using Affymetrix GeneChip (Wood *et al.* 2003). Our results are consistent with theirs on some genes, such as SH3-domain binding protein 5, C1-inhibitor/SERP1 and DnaJ (HSP40) homolog, but differed in results on some other genes, such as

SPARC-like 1 (mast9, hev1) and collagen I. We found these genes were upregulated in PCOS ovaries, while they found that the mRNA levels of these genes decreased in PCOS theca cells. Such a discrepancy could be due to the fact that the whole PCOS ovaries used in our study contain other types of cells, in addition to theca cells, which might have different levels of gene expressions. Therefore, future studies should take these factors into consideration.

In conclusion, we identified numerous genes that are differentially expressed in normal and PCOS ovaries. These genes are involved in various biologic functions, and their changed levels of expression might contribute to the pathogenesis of PCOS. Due to the complex nature of PCOS, no single factor can account for all aspects of this disorder. The global profiling of gene expressions will provide us with some insight into the molecular mechanism underlying clinical manifestations of PCOS.

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