BIOLOGY OF REPRODUCTION **72,** 241–255 (2005)
Published online before print 15 September 2004.
DOI 10.1095/biolreprod.104.032060

Alterations in the Ovarian Transcriptome During Primordial Follicle Assembly and Development[1]

Phillip R. Kezele, Jacquelyn M. Ague, Eric Nilsson, and Michael K. Skinner[2]

Center for Reproductive Biology, School of Molecular Biosciences, Washington State University, Pullman, Washington 99164-4231

ABSTRACT

The assembly of the developmentally arrested primordial follicle and subsequent transition to the primary follicle are poorly understood processes critical to ovarian biology. Abnormal primordial follicle development can lead to pathologies such as premature ovarian failure. The current study used a genome-wide expression profile to investigate primordial follicle assembly and development. Rat ovaries with predominantly unassembled, primordial, or primary follicles were obtained. RNA from these ovaries was hybridized to rat microarray gene chips, and the gene expression (i.e., ovarian transcriptome) was compared between the developmental stages. Analysis of the ovarian transcriptome demonstrated 148 genes up-regulated and 50 genes down-regulated between the unassembled and primordial follicle stages. Observations demonstrate 80 genes up-regulated and 44 genes down-regulated between the primordial and primary follicle stages. The analysis demonstrated 2332 genes common among the three developmental stages, 146 genes specific for the unassembled follicles, 94 genes specific for the primordial follicles, and 151 genes specific for the primary follicles. Steroidogenic genes are up-regulated between unassembled and primordial follicles, and then many are again down-regulated between primordial and primary follicles. The hormones inhibin and Müllerian inhibitory substance (MIS) display a similar pattern of expression with the highest levels of mRNA in the primordial follicles. Several novel unknown genes that had dramatic changes in expression during primordial follicle development were also identified. Gene families/clusters identified that were up-regulated from unassembled to primordial follicles include growth factors and signal transduction gene clusters, whereas a down-regulated gene family was the synaptonemal complex genes associated with meiosis. Gene families/clusters that were up-regulated between primordial and primary follicles included immune response genes, metabolic enzymes, and proteases, whereas down-regulated gene families include the globulin genes and some steroidogenic genes. The expression of several growth factors changed during primordial follicle development, including vascular endothelial growth factor and insulin-like growth factor II. Elucidation of how these changes in gene expression coordinate primordial follicle assembly and the primordial to primary follicle transition provides a better understanding of these critical biological processes and allows selection of candidate regulatory factors for further investigation.

[1]Supported by NIH grants to M.K.S. P.R.K. and J.M.A. contributed equally to the manuscript and study.
[2]Correspondence: Michael K. Skinner, Center for Reproductive Biology, School of Molecular Biosciences, Washington State University, Pullman, WA 99164-4231. FAX: 509 335 2176; e-mail: Skinner@mail.wsu.edu

Received: 12 May 2004.
First decision: 2 June 2004.
Accepted: 30 August 2004.

ISSN: 0006-3363. http://www.biolreprod.org

follicle, follicular development, gene regulation, growth factors, ovary

INTRODUCTION

The assembly and subsequent development of the developmentally arrested primordial follicle are poorly understood processes that are necessary for normal female reproduction. These processes are critical because they set the size of the primordial follicle pool. Primordial follicle numbers do not proliferate or increase once formed. The primordial follicle pool present at birth represents the total number of primordial follicles available to a female during her reproductive life [1]. Although a recent observation led to speculation that a female germ-line stem cell may exist, further research is needed to confirm this observation and identify a regenerating follicle pool [2]. Primordial follicles are the female's sole source of gametes and critical steroidogenic tissue. All of the primordial follicles will eventually be lost by ovulation or by oocyte atresia. When the primordial follicle pool is depleted, menopause occurs [3]. Abnormal primordial follicle development can cause pathological conditions such as premature ovarian failure [4]. The current study was designed to provide insight into the molecular and cellular control of primordial follicle development.

Recent studies using ovarian organ cultures or null mutant knockout mice have begun to elucidate the cell-cell interactions that coordinate primordial follicle assembly and the primordial to primary follicle transition. The process of follicular assembly begins at mid gestation in large monovulators such as humans [5] and at birth in rodents [6]. Follicular assembly is the apoptotic breakdown of groups of recently proliferating gametes called oocyte nests [7]. This apoptosis is coordinated by apoptotic factors such as tumor necrosis factor alpha (TNFα) [8]. High progesterone concentrations in utero repress oocyte apoptosis and follicular assembly in rodent pups until birth when the pups experience a dramatic decline in steroid concentrations [9].

The cell-cell interactions that occur during the primordial to primary follicle transition are better characterized than those of primordial follicle assembly. The primordial to primary follicle transition is coordinated by a combination of positive and negative regulatory factors. The epithelial granulosa cells produce kit ligand [10] and leukemia inhibitory factor [11] that act on the oocyte and the surrounding stroma to promote the primordial to primary follicle transition. Specifically, kit ligand is involved in the recruitment of mesenchymal theca cells to the follicle [12]. Basic fibroblast growth factor is produced by the oocyte and acts on the granulosa and theca to promote the primordial to primary follicle transition [13]. Nerve growth factor acts on the granulosa to promote the primordial to primary follicle transition [14]. Bone morphogenic protein

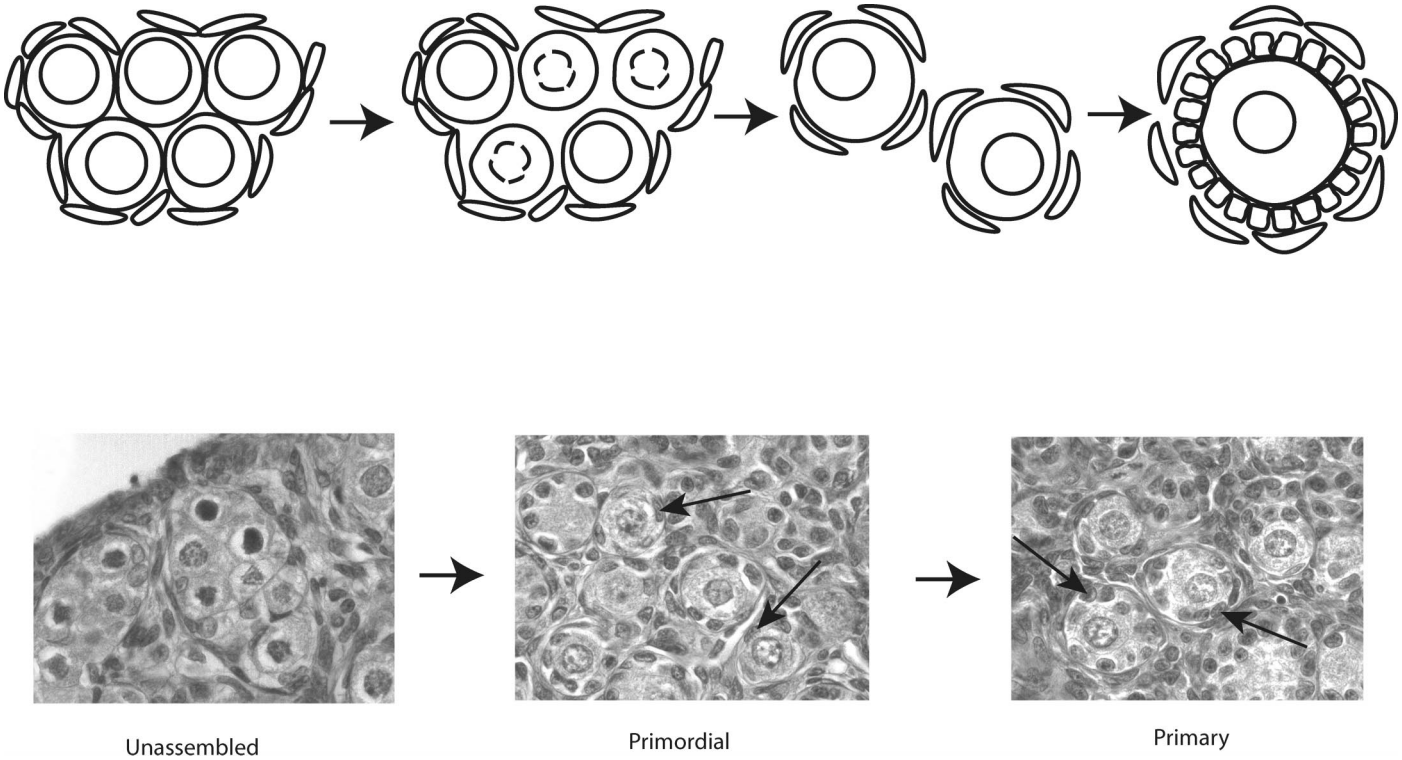

FIG. 1. Top: Schematic of follicular assembly and the primordial to primary follicle transition. The oocytes start as clusters of oocytes surrounded by bands of stromal tissue. Certain oocytes go through an apoptotic process and the ones remaining become primordial follicles that are an oocyte surrounded by a layer of squamous pregranulosa cells. At the primordial to primary follicle transition, the granulosa take on a cuboidal appearance and begin proliferating. The oocyte grows in size and theca is sequestered from the surrounding stroma. Bottom: Light micrographs of follicles at the above stages. Unassembled oocyte nests are surrounded by bands of stroma tissue. Primordial follicles, an oocyte surrounded by a layer of squamous granulosa, are indicated by arrows. Primary follicles, an oocyte surrounded by a layer of proliferating granulosa, are also indicated by arrows. Original magnification ×200.

4 produced by the theca and stroma cells acts as a follicle survival factor [15]. Insulin acts as an endocrine factor to promote the primordial to primary follicle transition [16]. Müllerian inhibitory substance (MIS) produced by larger follicles represses the primordial to primary follicle transition in adjacent follicles [17].

The current model of cell-cell interactions in primordial follicle assembly and the primordial to primary follicle transition is based primarily on the investigation of growth factors that have been shown to be important in cell-cell interactions of the better characterized large antral follicles. A number of the growth factors known to coordinate antral follicle growth have been shown to be involved in primordial follicle development. Additional factors will also likely be involved in the development of primordial follicles. The current study is a gene discovery approach to identify new factors that may coordinate primordial follicle assembly and the primordial to primary follicle transition. This gene discovery project investigates the transcriptomes of ovaries that contain predominately unassembled, primordial, or primary follicles. Primordial and primary follicles are too small to dissect or isolate from the ovary, and it is not possible to isolate individual cell types from them. The current study uses rat ovaries from different developmental stages that have predominately one follicle population. Postnatal Day 0 ovaries contain only unassembled follicles. Postnatal Day 4 ovaries contain predominately primordial follicles. Postnatal Day 0 ovaries cultured for 1 wk contain predominately primary follicles [9]. Comparing the global gene expression profiles (transcriptomes) from ovaries at these developmental stages allows a comparison between unassembled, primordial, and primary follicles.

MATERIALS AND METHODS

Ovary Dissection, Organ Culture, and RNA Extraction

Ovaries from Postnatal Day 0 Sprague-Dawley rat pups were dissected for both immediate RNA extraction and organ culture. Postnatal Day 4 rat ovaries were dissected for immediate RNA extraction. Postnatal Day 0 rat ovaries were also cultured for 7 days. Whole ovaries were cultured as previously described [10] on floating filters (0.4 μm; Millicell-CM; Millipore, Bedford, MD) in 0.5 ml Dulbecco modified eagle medium (DMEM)–Ham F-12 medium (1:1, vol/vol) containing 0.1% bovine serum albumin (BSA; Sigma, St. Louis, MO); 0.1% albumax (Gibco BRL, Gaithersburg, MD); 2.75 μg/ml transferrin; 0.05 mg/ml L-ascorbic acid (Sigma); and 1 μg/ml insulin (bovine; Sigma) in a four-well culture plate (Nunc plate; Applied Scientific, South San Francisco, CA). Medium was supplemented with penicillin, streptomycin, and gentamicin to prevent bacterial contamination. RNA was extracted using the TRIZOL reagent (Invitrogen, Grand Island, NY) per the manufacturer's instructions. About 20 ovaries were used for each RNA extraction. All procedures were approved by the Washington State University (WSU) Animal Care and Use Committee.

Microarray Analysis

RNA was hybridized to the Affymetrix U34A 8799 gene chip (Affymetrix, Santa Clara, CA). The Genomics Core in the Center for Reproductive Biology at Washington State University performed the analysis as previously described [18, 19]. Briefly, RNA from whole ovaries was reverse transcribed into cDNA, and cDNA was transcribed into biotin-labeled RNA. Biotin-labeled RNA was then hybridized to the Affymetrix U34A 8799 gene chips. Each gene set was composed of 16 pairs of 24-oligomer oligonucleotides, with one sense strand specific for the gene and

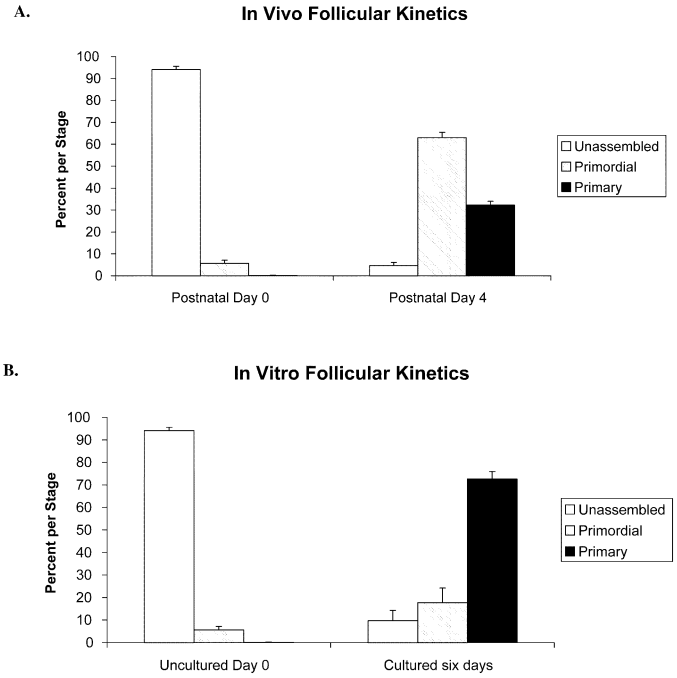

FIG. 2. **A**) Kinetics of folliculogenesis in vivo. Data displayed as percent unassembled, primordial, and primary follicles in the ovaries of the neonatal and the Postnatal Day 4 rat. At birth (Postnatal Day 0) the majority of oocytes are unassembled and are not in follicles. At Postnatal Day 4 the majority are primordial follicles. **B**) Kinetics of folliculogenesis in vitro. Data displayed as percent unassembled, primordial, and primary follicles in the neonatal ovary at 0 and 6 days of rat ovary culture. After 6 days in culture the majority of the oocytes are in primary follicles. All data are representative of a minimum of three different experiments done in replicate and presented as mean ± SEM.

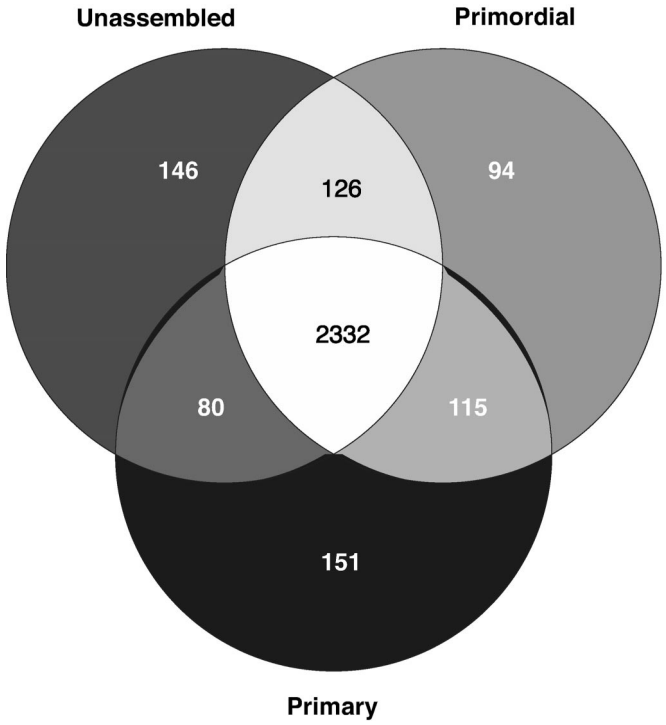

FIG. 3. Venn diagram of unassembled, primordial, and primary follicle ovary stages. Similarities and differences in genes expressed are represented. An additional 5755 genes on the microarray chip were not detected with a minimum relative signal of 100 used for each gene. Number of stage-specific genes and genes expressed in common are indicated.

one antisense strand with single point mutations for use as a comparative negative control. The oligonucleotides spanned the gene, so 5′ and 3′ regions contributed to the final signal obtained. Biotinylated RNA was then visualized by labeling with phycoerythrin-coupled avidin. The microarray was scanned on a Hewlett-Packard Gene Array Scanner (Hewlett-Packard Co., Palo Alto, CA). Two microarray chips from two different RNA samples were analyzed for each of the predominate unassembled, primordial, and primary follicle ovary preparations.

Bioinformatics and Microarray Statistics

Microarray output was examined visually for excessive background noise and physical anomalies. The default Microarray Suite (Affymetrix, Santa Clara, CA) statistical values were used for all analyses. An absolute analysis using Microarray Suite was performed to assess the relative abundance of the transcripts based on signal and detection (present, absent, or marginal) for the 16 different oligonucleotides per gene and comparison for analysis. The absolute analysis from Microarray Suite was imported into GeneSpring 5.1 software (Silicon Genetics, Redwood City, CA). The developmental time course data were normalized within GeneSpring using the default/recommended normalization methods. These included the setting of signal values below 0.01 to 0.01, total chip normalization to the 50th percentile, and normalization of each gene to the median. These normalizations allowed for the visualization of data based on relative abundance at any given time point, rather than compared with a specific control value. Data restrictions and analytical tools in GeneSpring were applied to isolate noteworthy and possibly important patterns of gene expression during the course of testicular development and spermatogenesis. Transcripts expressed differentially at a statistically significant level were determined using a one-way ANOVA parametric test with variances not assumed equal and a *P*-value cutoff of 0.05. This was applied to all three developmental stage samples and considered all transcripts represented on the arrays. Two repeats for each developmental stage was performed and allowed a 2 × 2 factorial comparison in the experiment. Subsequently, expression restrictions were applied to the transcripts expressed in a significant manner. These restrictions were designed so that the remaining transcripts met the following requirements in addition to being expressed in a significant manner: 1) each transcript must have a signal value of at least 100 in at least one of the three developmental stages, and 2) had an average fold change of two or greater in signal intensity between developmental stages. The resulting transcripts were screened using Excel (Microsoft, Redmond, WA) for redundant UniGene entries. Transcripts that passed these restrictions were considered for further analysis. Cluster analysis and patterns of gene expression were identified using unsupervised cluster analysis within the set of differentially expressed transcripts that met the requirements detailed previously. Clustering algorithms allow for the separation of distinct patterns of expression based on the similarity of expression profiles between different genes [20]. In this analysis, a hierarchical clustering algorithm using a smooth correlation with the default

TABLE 1. Summary of follicle stage transcriptome changes.

	Unassembled to primordial	Primordial to primary
Total genes (>2×)	80 genes up-regulated	148 genes up-regulated
Total genes (>2×)	44 genes down-regulated	50 genes down-regulated
Up-regulated gene clusters	5 steroidogenic genes	17 immune response genes
	3 zona pellucida genes	23 metabolism enzymes
	6 hormones and growth factors	10 proteases
	11 signal transduction genes	
Down-regulated gene clusters	2 synaptonemal complex genes	3 globin genes
		3 steroidogenic genes

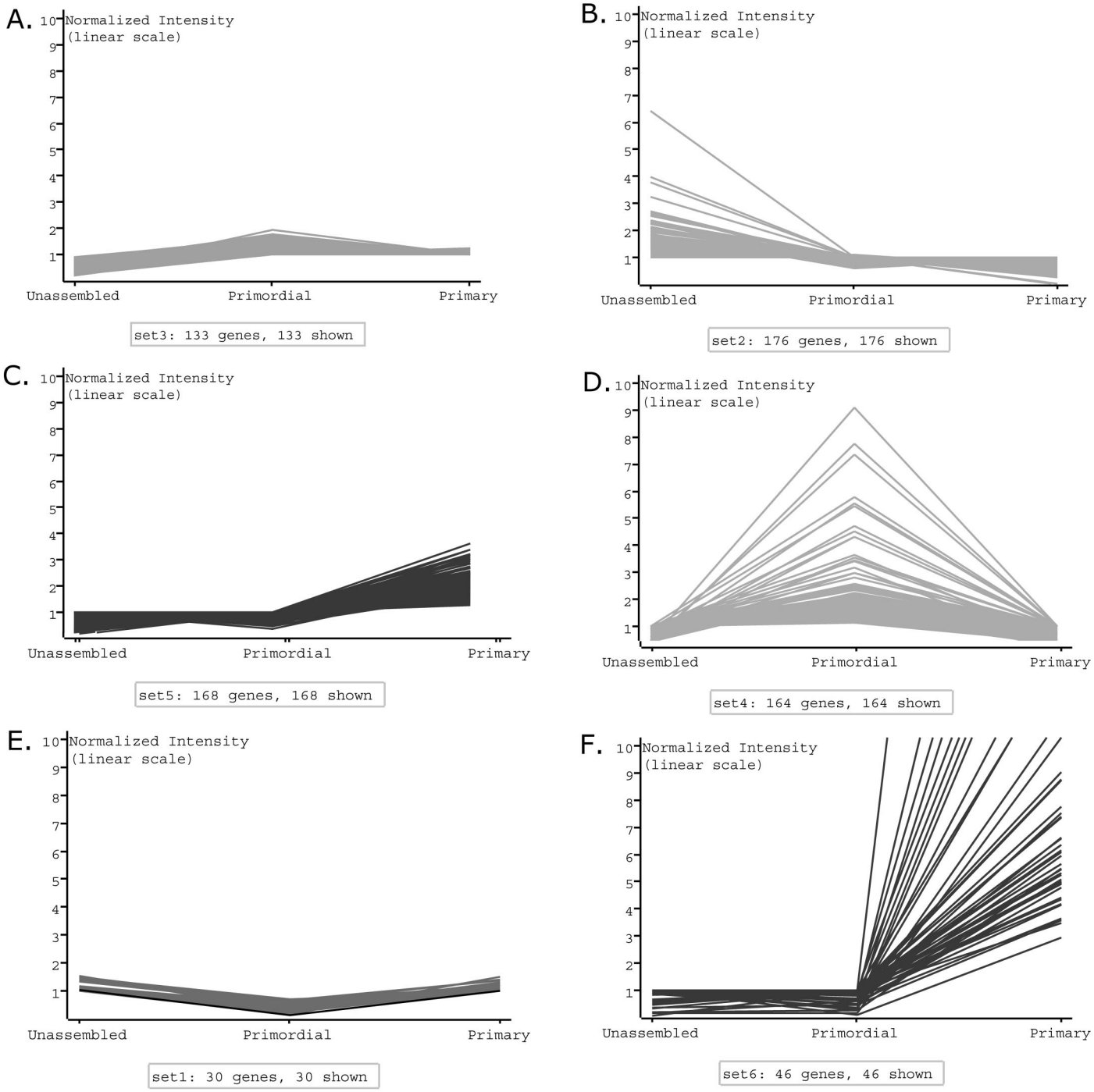

FIG. 4. Representative microarray data clustered into six distinct patterns of expression using the Genespring software. Each line represents a single gene. Data are displayed as normalized signal intensity of each gene at unassembled, primordial, and primary follicle developmental stages. Note genes with the greatest change are only expressed in one developmental stage (**B, D, F**).

parameters was used to isolate distinct, nonrepetitive patterns of expression within the time course. A nonphylogenetic gene tree that illustrates the major expressional patterns within the differentially expressed transcripts (determined through statistical analysis) in a continuous fashion was produced from this analysis. Pathway Assist (Stratagene Inc., La Jolla, CA) software was used for detailed pathway analysis and gene associations. Previous studies have shown that microarray data correlates well with real-time quantitative PCR and Northern analysis [18, 21]. Therefore, microarray data does not need to be confirmed as previously suggested [19]. However, a selected gene (i.e., vascular endothelial growth factor) was used in a real-time quantitative PCR procedure as previously described [22] to help confirm the microarray procedure.

RESULTS

The current study investigates the transcriptomes of unassembled, primordial, and primary follicles as part of a gene discovery and functional genomics analysis. RNA was taken from freshly isolated Postnatal Day 0, freshly isolated Postnatal Day 4, and Postnatal Day 0 ovaries cultured for 1 wk. The percentage of each follicle type in these ovaries has been previously determined by morphological analysis [9]. Briefly, unassembled follicles are defined as groups of oocytes adjacent to each other surrounded by a layer of

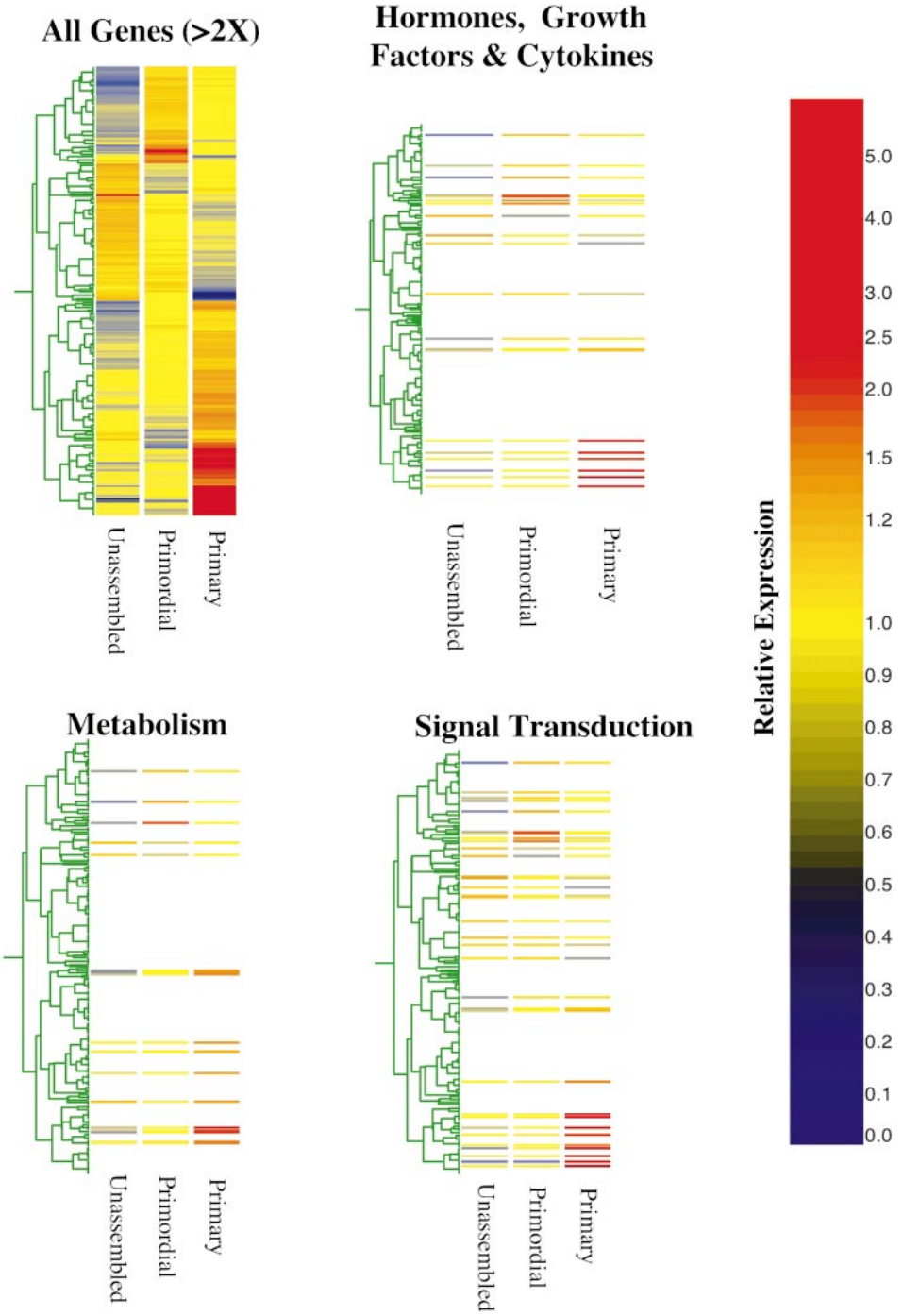

FIG. 5. Dendrogram cluster analysis of the total (all genes, >2×) ovary transcriptome in the unassembled, primordial, and primary follicle developmental states. Each line represents a single gene, and its relative expression is indicated as increased (red) or decreased (blue) according to the color key provided. Selected gene set clusters for hormones, growth factors and cytokines, metabolic genes, and signal transduction genes are shown.

stromal tissue. Primordial follicles are defined as solitary oocytes surrounded by a layer of squamous epithelial cells. Primary follicles are oocytes surrounded by a layer of proliferating cuboidal epithelial cells (Fig. 1). Postnatal Day 0 ovaries contain 95% unassembled follicles (Fig. 2). Postnatal Day 4 ovaries contain approximately 70% primordial follicles. Cultured Postnatal Day 0 ovaries contain greater than 75% primary follicles (Fig. 2). Ovaries from these three developmental stages were taken for analysis and RNA was collected.

The RNA from two different preparations of each ovarian stage and follicle type were analyzed separately using the Affymetrix U34A rat gene chips. This gene chip has 14 000 genes for analysis. The global gene expression profile comparison between unassembled, primordial, and primary follicles is shown in Figure 3. The ovarian transcriptome analyzed is a combination of somatic cell and oocyte contributions. The Venn diagram demonstrates 2332 genes expressed in all three developmental stages with 146 genes specific to unassembled follicles, 94 genes specific to primordial follicles, and 151 genes specific to primary follicles (Fig. 3). Similar numbers of genes were also in common between two different developmental stages in the analysis (Fig. 3). The principle genes of interest were those that change expression levels between the developmental stages. Specific genes were categorized using a relative hybridization signal limit minimum of 100 and a minimum change greater than twofold. The cutoff 100 for signal and greater than twofold change demonstrated that over 5000 genes did not change expression levels and/or had very low levels of

TABLE 2. Unassembled to primordial up-regulated genes.*

Accession		Primordial mean signal	± SEM	Fold change
S63167	3 β-hydroxysteroid dehydrogenase (HSD)	627.35	356.55	13.20
M33986	Aromatase p450, subfamily 19	109.15	23.45	10.01
AI235576	Hydroxy-δ-5-steroid dehydrogenase	272.05	151.85	7.78
J00713	Carboxypeptidase A1	132.15	60.35	7.24
AB000778	Phospholipase D gene 1	396.40	63.50	6.03
M12492	Protein kinase, cAMP dependent regulatory, type II β	181.30	66.60	5.87
M36453	Inhibin, α subunit	1659.70	310.60	5.25
AB000929	Zona pellucida 2 glycoprotein	1624.35	157.95	5.23
S35751	3 α-HSD; 3 α-hydroxysteroid dehydrogenase	196.20	15.07	4.90
AB006007	Steroidogenic acute regulatory protein	100.65	36.75	4.89
AB012214	DNA cytosine 5 methyltransferase	835.80	211.20	4.77
AF045564	Neu development-related protein	389.45	50.55	4.46
J03190	Aminolevulinic acid synthase 1	177.60	51.10	4.24
AJ005396	Procollagen, type XI, α 1	104.50	13.80	4.12
M32754	Inhibin α-subunit gene, exon 1	1163.05	84.45	3.63
AB016800	7-Dehydrocholesterol reductase	296.75	77.35	3.52
U90887	Arginase 2	189.10	14.66	3.42
AB000928	Zona pellucida glycoprotein 1	245.15	22.75	3.38
Y10823	Zona pellucida glycoprotein 3	1774.55	307.85	3.27
U02320	Neuregulin 1	124.35	10.58	3.24
M91466	Adenosine A2B receptor	114.35	12.45	3.06
AF035951	Kinesin-related protein KRP1 (KRP1)	127.15	18.95	3.03
M64733	TRPM-2 gene	474.60	37.65	3.02
AA875411	Suppressor of K+ transport defect 3	111.05	10.17	2.99
AF054826	Vesicle-associated membrane protein 5	123.80	21.50	2.87
S53987	Nicotinic receptor α 7 subunit	173.80	14.50	2.82
U60416	Myosin 5B	275.10	26.00	2.80
AA685974	Plectin	786.35	63.48	2.72
U38253	Eukaryotic translation initiation factor 2B, subunit 3	227.80	19.42	2.68
AA998164	Cyclin B1	249.10	29.50	2.66
AI178971	Hemoglobin, α 1	151.35	42.35	2.66
X16555	Phosphoribosyl pyrophosphate synthetase 2	243.30	96.00	2.65
AA892897	Procollagen lysine, 2-oxoglutarate 5-dioxygenase 2	379.25	79.55	2.59
A03913	Glia-derived neurite-promoting factor (GdNPF)	489.65	40.77	2.59
U02315	ndf40 neu differentiation factor	116.4	3.6	2.53
AB009999	CDP-diacylglycerol synthase	156.85	20.85	2.41
S98336	MIS; anti-Müllerian hormone	487.55	98.15	2.40
D32209	Acidic nuclear phosphoprotein 32 family, member A	243.80	196.50	2.38
AA891527	4.5 LIM domains 2	197.40	17.20	2.34
AA892511	Calcium binding protein P22	114.60	20.40	2.34
AI639118	Calcium-dependent actin-binding protein	138.80	16.00	2.31
H31604	Budding uninhibited by benzimidazoles	140.30	48.30	2.24
D82363	Presenilin 1	186.60	16.73	2.23
D16237	Cell division cycle 25B	160.25	28.75	2.21
X76489	CD9 antigen (p24)	700.60	164.30	2.20
D49363	Perchrolic acid soluble protein	580.80	58.80	2.19
M64301	Mitogen-activated protein kinase 6	244.55	35.95	2.17
S72505	Glutathione S-transferase Yc1 subunit	492.75	45.21	2.14
AJ011606	DNA polymerase alpha subunit II	147.20	13.59	2.14
X73411	snRNP-associated polypeptide N	291.75	24.92	2.14
M34253	Interferon regulatory factor 1	108.30	26.20	2.13
AF016503	Procollagen C-proteinase enhancer protein	686.15	61.50	2.12
U10188	Polo-like kinase homolog (*Drosophila*)	344.45	31.48	2.12
AF076619	Growth factor receptor bound protein 14	143.45	17.95	2.12
U16359	Nitric oxide synthase gene	113.40	12.54	2.11
AI230228	Phosphoserine aminotransferase mRNA	214.60	20.11	2.08
U23769	PDZ and LIM domain 1	264.35	50.75	2.06
AF033109	Syntaxin 8	253.55	22.68	2.06
S61865	Heparan sulfate proteoglycan core protein	555.00	96.80	2.06
L10326	GNAS complex locus	136.10	25.50	2.06
AI171630	Mitogen activated protein kinase 14	281.10	26.10	2.05
AA891445	Suppressor of K+ transport defect 3	101.75	9.75	2.05
J02773	Fatty acid binding protein 3	369.05	138.55	2.03
AB017912	MAD homolog 2 (*Drosophila*)	306.25	27.40	2.03
U38253	Eukaryotic translation initiation factor 2B, subunit 3	277.05	37.55	2.03
AA848421	Uracil-DNA glycosylase	198.45	27.75	2.02
M60322	Aldose reductase gene	1141.00	113.50	2.01
AI236601	Heat-shock protein	346.80	39.60	2.01

* ESTs not included and relative minimum signal of 100 used for at least one of the developmental stages.

TABLE 3. Unassembled to primordial down-regulated genes.*

Accession		Unassembled mean signal	± SEM	Fold change
X75785	Synaptonemal complex protein 3 (SCP3)	242.30	57.70	−5.72
D83349	Cadherin 22	292.60	82.20	−4.18
Y13275	Transmembrane 4 superfamily member 3	487.65	47.35	−3.76
AI176456	Metallothionein II	991.35	361.35	−3.63
X59864	ORF; Rat ASM15	643.90	235.20	−3.62
M23566	α 2-macroglobulin gene	2328.80	866.40	−3.57
AI014091	Cbp/p300-interacting transactivator	296.55	83.55	−3.48
X17012	Insulin-like growth factor II	1633.80	373.30	−3.46
L19998	Sulfotransferase family 1A, phenol-preferring, 1	170.90	48.90	−3.19
AA800851	Carboxylesterase 3	125.60	17.10	−3.03
D28562	Solute carrier family 2, member 5	188.50	16.36	−2.93
AI029920	Insulin-like growth factor–binding protein 5	108.10	10.40	−2.61
M10934	Retinol-binding protein (RBP)	257.45	60.25	−2.51
J04488	Prostaglandin D synthase	138.55	13.26	−2.47
D12769	Kruppel-like factor 9	223.50	141.90	−2.42
X81449	Keratin complex 1, acidic, gene 19	1117.20	102.43	−2.40
D84336	δ-like homolog (*Drosophila*)	582.25	173.85	−2.36
AI103238	Protein phosphatase 2	107.30	10.71	−2.36
M83143	Sialyltransferase 1	146.45	13.65	−2.35
X81449	Keratin complex 1, acidic, gene 19	586.05	54.43	−2.29
AA894092	Osteoblast-specific factor 2	1489.00	127.29	−2.29
AA852004	Glutamine synthease 1	100.75	45.15	−2.28
U67908	Chymase 1	343.55	135.55	−2.24
D00036	Phospholipase A2, group 1B	293.85	28.95	−2.19
AB010999	Peptidyl arginine deiminase, type 4	185.70	17.79	−2.18
Z46614	Caveolin	195.65	50.85	−2.15
M83680	GTPase Rab14	117.25	63.95	−2.15
D89730	T16 mRNA	115.95	21.65	−2.14
U67911	Mast cell protease 8	141.30	14.29	−2.11
M77694	Fumarylacetoacetate hydrolase	106.85	10.72	−2.09
AA965147	Heterogeneous nuclear ribonucleoprotein A1	483.65	71.75	−2.04
Z35138	Fibroblast growth factor receptor 2b	126.20	12.99	−2.02
H31813	Hypothetical protein DKFZp586B1621.1	117.25	13.85	−2.01

* ESTs not included and relative minimum signal of 100 used for at least one of the developmental stages.

expression. Analysis of primordial follicle assembly demonstrated that 80 genes are up-regulated and 44 genes are down-regulated between unassembled and primordial follicles. Analysis of the primordial to primary follicle transition demonstrated that 148 genes are up-regulated and 50 genes are down-regulated between primordial and primary follicles (Table 1).

Genes were grouped by expression pattern into six gene clusters (Fig. 4). Half of these clusters displayed dramatic changes in gene expression (Fig. 4, B, D, and F). The transcriptomes of each developmental point were unique. The other three clusters displayed smaller changes in gene expression (Fig. 4, A, C, and E). The first displayed up-regulation at primordial and primary stage follicles (Fig. 4A). The second displayed up-regulation at the primary follicle stage (Fig. 4C). The third displayed up-regulation at the unassembled and primary follicle stage (Fig. 4E). A dendrogram cluster analysis for all of the genes changing greater than twofold between the developmental stages is shown in Figure 5. The genes with increased (red) and decreased (blue) expression between the stages are grouped and show unique clusters at each developmental stage. Specific examples of functional gene clusters of metabolic, signal transduction, and hormones/growth factors/cytokines are also shown in Figure 5. Each developmental stage has gene clusters increasing and decreasing between the developmental stages. The specific genes examined are listed by change in signal in Tables 2–5.

Of the 80 genes up-regulated during primordial follicle assembly (Tables 1, 2, and 6), a number were steroidogenetic enzymes. Also up-regulated was the hormonal factor inhibin and the known repressor of the primordial to primary follicle transition MIS. All three zona pellucida genes, which code for the thick protein coat of the oocyte, were up-regulated. Members of the neu differentiation factor family were also up-regulated (Tables 2 and 6).

Of the 44 genes down-regulated during follicular assembly (Tables 3 and 6), two were synaptonemal complex family genes involved in meiosis. A growth factor strongly down-regulated between the unassembled and primordial follicle stage was insulin-like growth factor II (IGFII), as was an IGF binding protein–2 (IGFBP2).

Of the 148 genes up-regulated during the primordial to primary follicle transition (Tables 1, 4, and 7), 17 were immune or inflammatory response–related genes such as cytokines. In addition, 10 proteases (e.g., cathepsins) were up-regulated. Twenty-three of the genes were metabolic enzymes. Also up-regulated were the IGFII growth factor and the IGFBP2 (Fig. 6C). Interestingly, vascular endothelial growth factor (VEGF) was also found to be up-regulated (Fig. 6C). To confirm the microarray analysis, the VEGF gene expression change was also assessed with a real-time quantitative PCR procedure and found to give the same relative increase in expression (data not shown). Absent from this list are growth factors known to promote the primordial to primary follicle transition—such as kit ligand (data not shown)—primarily due to low levels (i.e., <100 signal) of expression. In addition to the known genes, two genes of unknown function were dramatically up-regulated during the primordial to primary follicle transition. The kid-

TABLE 4. Primordial to primary up-regulated genes.*

Accession		Primary mean signal	±SEM	Fold change
X52477	Complement component 3	428.35	187.25	133.86
AF022147	Integral membrane–associated protein 1	1262.60	184.70	55.26
AF022147	UOTP	375.8	104.2	42.95
J02585	Stearoyl-coenzyme A desaturase 1	957.30	622.80	41.00
M14656	Secreted phosphoprotein 1 (osteopontin)	1193.05	289.15	37.58
L24374	Matrix metalloproteinase 7 (matrilysin)	462.45	73.55	35.99
X98517	Macrophage metalloproteinase	208.65	17.75	32.56
NM052802	KAP	84.65	18.25	28.21
U23055	C-CAM4 gene	254.60	15.60	26.94
M92059	Adipsin	231.85	15.34	23.30
X71127	Complement component c1q β-chain	282.95	42.95	18.99
AA892775	Lysozyme	1611.35	655.65	16.80
X73371	Immunoglobin γ FC region	117.05	50.55	13.85
M32062	Fc receptor, IgG, low affinity III	334.90	152.70	13.64
X51529	Platelet phospholipase A2	1164.00	746.60	11.87
U10894	Allograft inflammatory factor 1	207.10	92.90	10.87
AA945737	Chemokine receptor (LCR1)	142.75	66.15	10.65
J02962	Lectin, galactose binding, soluble 3	736.35	95.35	10.56
X17053	Immediate early serum–responsive JE gene	456.60	89.10	10.44
AA946503	Lipocalin 2	951.55	156.45	10.05
M57276	CD53 antigen	153.10	66.20	9.78
AI639117	Complement factor B precursor	276.10	87.60	9.42
S53527	S-100 β subunit	158.40	35.20	9.37
AI169612	Fatty acid binding protein 4	647.80	135.50	9.16
M64795	MHC class I antigen gene (RT1-u haplotype)	224.55	66.35	9.11
AA800587	Glutathione peroxidase (GSHPX-1)	198.20	13.43	8.66
AA799861	Interferon regulatory factor 7 (IRF-7)	103.60	27.00	7.85
M98820	Interleukin 1 β	152.25	34.55	7.56
L14004	Polymeric immunoglobulin receptor	122.40	30.90	7.46
U17919	Allograft inflammatory factor 1	140.20	55.00	7.38
X17053	Immediate early serum–responsive JE gene	257.00	63.00	7.26
L03201	Cathepsin S	315.10	104.70	7.08
M23566	α-2-macroglobulin gene	377.20	72.80	6.81
K00994	Intestinal calcium binding protein	142.55	40.95	6.80
AI237731	Lipoprotein lipase	347.25	138.25	6.45
AI072634	Keratin complex 1, acidic, gene 18	362.90	26.85	6.24
U90448	CXC chemokine LIX	207.60	29.20	5.65
U42719	Complement component, C4 complement protein	351.05	78.85	5.05
L33869	Ceruloplasmin	126.40	10.40	4.95
AA800750	Retrovirus-related POL polyprotein	1648.30	116.15	4.67
U49062	CD24 antigen	526.45	38.88	4.64
S74141	Tyrosine kinase	140.10	23.50	4.39
U95368	GABA-A receptor π subunit	123.70	10.81	4.35
U13275	Transmembrane 4 superfamily member 3	556.30	66.70	4.29
M63656	Aldolase C	164.60	15.70	4.23
D10354	Glutamic-pyruvate transaminase	111.45	10.29	4.07
J02612	UDP glycosyltransferase 1 family, polypeptide A6	200.70	34.80	3.91
U18729	Cytochrome b558 α-subunit	658.90	160.50	3.84
M15880	Neuropeptide Y	194.80	34.10	3.76
S57478	Lipocortin I	423.00	78.30	3.73
AI169104	Platelet factor 4 precursor (PF-4) (CXCL4)	215.45	100.35	3.60
AI170268	Beta-2-microglobulin	1694.10	126.12	3.58
X07729	Neuron-specific enolase	110.95	10.17	3.46
AA800318	Protein C inhibitor	392.30	138.40	3.40
AI171962	Annexin 1	335.30	27.49	3.39
AA894004	Pervin	296.50	121.50	3.32
X13044	CD74 antigen	113.10	39.20	3.27
AF037072	Carbonic anhydrase 3	261.00	22.00	3.26
S56937	3-methylcholanthrene-inducible	112.45	10.07	3.25
L40362	RT1 class Ib gene	488.75	63.45	3.23
M88469	f-spondin	165.25	56.15	3.22
AA874848	Thymus cell surface antigen	191.40	48.00	3.11
D90404	Cathepsin C	325.60	26.41	3.05
AF065438	Peptidylprolyl isomerase C-associated protein	461.20	34.41	3.04
AB003042	Complement component 5, receptor 1	134.70	29.60	3.01
AA852004	Glutamine synthetase 1	132.65	68.05	3.00
D89730	T16 mRNA	159.90	29.80	2.95
S76054	Cytokeratin-8	267.45	22.02	2.91
X62322	Granulin	1523.60	329.70	2.90
D90109	Fatty acid coenzyme A ligase, long chain 2	260.80	62.40	2.88
AI179610	Heme oxygenase	240.15	50.85	2.86
X06916	Protein p9Ka homologous to calcium-binding protein	152.95	51.35	2.79

TABLE 4. Continued.

Accession		Primary mean signal	±SEM	Fold change
AA799560	N-myc downstream-regulated gene 2	266.10	20.86	2.71
AA891690	Tumor necrosis factor (ligand) member 13	245.25	19.99	2.68
AF083269	Actin-related protein 2/3 complex, subunit 1B	491.80	39.47	2.68
M10934	Retinol-binding protein (RBP)	271.05	108.25	2.64
M15562	MHC class II RT1.u-D-α chain	105.60	20.00	2.61
AI639535	Transporter-like protein	172.70	31.80	2.61
AI176456	METALLOTHIONEIN-II (MT-II)	714.40	113.10	2.61
X60661	Ligand-binding protein	141.45	44.15	2.59
AI014091	Cbp/p300-interacting transactivator	220.80	55.30	2.59
AI235585	Cathepsin D	655.55	142.35	2.56
J02722	Heme oxygenase	103.95	33.45	2.55
AI008888	Cystatin B	222.05	27.65	2.55
AI231213	Kangai 1	591.15	96.25	2.49
AF087943	CD14 antigen	191.85	16.86	2.48
D10729	Proteasome subunit RC1	268.20	22.77	2.42
J02791	Acetyl-coenzyme A dehydrogenase	289.10	24.33	2.42
S68135	Glucose transporter 1	561.70	202.20	2.42
AA850734	Vascular endothelial growth factor	108.90	19.60	2.41
AF003835	Isopentenyl-diphosphate delta isomerase	183.65	29.15	2.41
X82396	Cathepsin B	1046.25	245.55	2.38
AI232783	Glutamine synthetase 1	384.60	139.70	2.37
AF025308	MHC class Ib antigen (RT1.C1)	475.25	75.25	2.34
AF087944	Monocyte differentiation antigen CD14	156.45	14.91	2.31
X96437	PRG1 gene	254.65	23.58	2.29
AI009405	Insulin-like growth factor binding protein 3	183.35	78.25	2.28
X76985	Latexin	181.25	67.75	2.27
U90610	Chemokine receptor (LCR1)	275.85	97.25	2.27
AI169327	Tissue inhibitor of metalloproteinase 1	424.30	38.61	2.26
AA892797	Phosphoglycerate kinase 1	1400.80	340.80	2.26
AA926129	Schlafen 4	136.30	23.80	2.24
M83143	Sialyltransferase 1	139.55	32.45	2.24
U09870	Major vault protein	134.25	14.45	2.22
J05122	Benzodiazepin receptor (peripheral)	353.40	31.32	2.21
J03752	Microsomal glutathione S-transferase 1	1576.20	186.50	2.19
M93257	Catechol-O-methyltransferase	107.15	13.25	2.18
AA893235	Putative lymphocyte G0/G1 switch protein 2	207.60	20.70	2.18
H32189	Glutathione S-transferase, μ 1	531.95	73.25	2.18
AA925752	cd36 antigen	137.75	12.98	2.16
M31788	Phosphoglycerate kinase 1	760.05	142.85	2.16
D30649	Alkaline phosphodiesterase	126.00	11.80	2.13
L40364	MHC class I RT1.O type-149	854.90	109.90	2.04
C07012	Peptidylprolyl isomerase C-associated protein	331.15	28.68	2.03
X02610	Enolase, 1, α	1217.10	113.05	2.03
U27562	SPARC-like 1	470.35	269.95	2.01

* ESTs not included and relative minimum signal of 100 used for at least one of the developmental stages.

ney-specific androgen-regulated protein (KAP) and the uterus ovary–specific transmembrane protein (UOSTP) were both up-regulated (28- and 42-fold, respectively; Fig. 6A).

Of the 50 genes down-regulated during the primordial to primary follicle transition (Tables 1, 5, and 7), three where steroidogenic enzymes. The endocrine factors inhibin and MIS were also down-regulated in a situation reciprocal to the change between the unassembled and primordial follicles (Fig. 6B). Three globin genes were strongly down-regulated between the primordial and primary follicle stages (Tables 5 and 7).

Further analysis of the data with Pathway Assist software identified groups of genes in specific cellular pathways. The relationship of the steroidogenic genes and other interacting proteins is shown in Figure 7. Critical steroidogenic genes such as CYP19A1 and STAR have interactions with a large number of proteins. Some regulate activity (Fig. 7, dashed lines) and some regulate expression (Fig. 7, solid lines). All of the genes identified as changing during primordial follicle development are linked except Scd1 and DCHR7 (Fig. 7).

DISCUSSION

The current study analyzed the transcriptomes of ovaries with predominately unassembled follicles, primordial follicles, and primary follicles as part of a functional genomics investigation. A similar approach has recently been used to characterize the transcriptome of ovarian cell types to assess disease states (e.g., polycystic ovarian syndrome) and folliculogenesis characteristics [23–25]. The genes identified whose transcriptional regulation was correlated with primordial follicle assembly, and the primordial to primary follicle transition are candidate regulators of these developmental processes. A number of candidate genes were identified, as well as genes that confirmed and supported the experimental approach and methodology. Those genes that were strongly up-regulated were expressed during one developmental stage exclusively, and each follicle stage be-

TABLE 5. Primordial to primary down-regulated genes.*

Accession		Primary mean signal	± SEM	Fold change
M94918	β-globin gene	1298.80	186.60	−71.05
X56325	2-α-1 globin gene	3661.20	371.40	−26.17
J02773	Fatty acid binding protein 3	369.05	138.55	−7.88
M33986	Cytochrome P450, subfamily 19	109.15	23.45	−6.76
M23721	Carboxypeptidase (CA2)	194.80	64.50	−5.89
S63167	3 β-hydroxysteroid dehydrogenase isomerase type II	196.20	16.78	−5.29
D32209	Acidic nuclear phosphoprotein 32 family, member A	243.80	196.50	−4.40
AI235576	Hydroxy-δ-5-steroid dehydrogenase	272.05	151.85	−4.19
M36453	Inhibin, α	1163.05	84.45	−4.03
AI178971	Hemoglobin, α 1	151.35	42.35	−3.95
S98336	MIS; Müllerian inhibiting substance	487.55	98.15	−3.54
U25264	Selenoprotein W muscle 1	326.65	26.52	−3.18
X03347	Polyprotein gag-fos-p75; FBR murine osteosarcoma	116.55	78.85	−3.16
AA875033	Fibulin 5	169.60	15.03	−2.94
AI639448	A5D3 protein	896.15	73.50	−2.84
J00713	Carboxypeptidase A1	132.15	60.35	−2.66
M12492	Protein kinase, cAMP dependent regulatory, type II β	181.30	66.60	−2.61
AF021936	Cdc42-binding protein kinase β	110.70	26.20	−2.58
M22400	Glypican 3	937.40	84.40	−2.50
D00575	Glycoprotein hormones, α subunit	206.25	50.75	−2.42
AA891527	4.5 LIM domains 2	197.40	17.32	−2.37
X07365	Glutathione peroxidase	1171.95	106.83	−2.35
U16359	Nitric oxide synthase gene	113.40	12.18	−2.25
AI102839	Calbindin 1	102.85	16.65	−2.24
J04035	Elastin	737.35	180.05	−2.21
D14441	Brain acidic membrane protein	154.70	15.35	−2.19
X06769	c-fox protein (AA 1-380)	279.90	181.70	−2.17
L22761	GATA-binding protein 4	417.45	66.85	−2.13
X74565	Polypyrimidine tract binding protein	350.25	46.25	−2.12
AA866454	Procollagen, type I, α 2	135.65	28.15	−2.07
Z78279	Collagen, type 1, α 1	2479.55	664.55	−2.07
L22760	GATA-binding protein 6	349.75	30.24	−2.06
AI012275	Developmentally regulated protein TPO1	983.60	141.30	−2.04
M55017	Nucleolin gene	128.15	12.61	−2.03
AB011532	MEGF6	125.00	27.60	−2.01
D49785	Mitogen-activated protein kinase kinase kinase 12	250.60	111.10	−2.01

* ESTs not included and relative minimum signal of 100 used for at least one of the developmental stages.

TABLE 6. Unassembled to primordial follicles genes.

Groups/clusters		Fold change
Steroid synthesis	3 β-hydroxysteroid dehydrogenase	13
	Aromatase P450, subfamily 19	10
	Steroidogenic acute regulatory protein	4.89
	Steroid 3-alpha-dehydrogenase	4.90
	7-Dehydrocholesterol reductase	3.52
Zona pellucida	Zona pellucida 2	5.23
	Zona pellucida glycoprotein 3	3.38
	Zona pellucida 1	3.27
Synaptonemal complex proteins	SCP3	−5.72
	SCP1	−3.99
Hormones and growth factors	Müllerian inhibiting substance	2.41
	Insulin-like growth factor II	−3.46
	inhibin α-subunit	5.25
	ndf40 neu differentiation factor	2.53
	Neu development–related protein	4.46
	Neuregulin	3.24
Signal transduction	Phospholipase D	6.0
	cAMP-protein kinase A	3.5
	Nicotinic receptor	2.82
	CDP-diacylglycerol synthase	2.41
	Adenosine A2B receptor	3.06
	Cadherin 22	−4.18
	Mitogen-activated protein kinase 14	2.05

TABLE 7. Primordial to primary follicles genes.

Groups/clusters		Fold change
Immune response	Complement component C3	101.88
	Fc Γ receptor	13.4
	IgE binding protein	10.87
	Osteopontin	37.58
	Complement protein C1q β chain	18.99
	C4 complement protein	6.46
	CXC chemokine LIX	5.65
	MHC class I RT1.Aa α-chain	2.04
	Fc receptor, IgG low-affinity III	13.64
	Complement factor B	9.42
	Compliment component 4a	5.05
	Chemokine receptor (LCR1)	2.81
	Allograft inflammation factor I	10.87
Proteases	Matrillysin (MMP-7)	36.00
	Macrophage metalloelastase	32.60
	Serine protease	5.85
	Proteosome subunit RC1	2.42
	Cathepsin S	7.08
	Cathepsin C	3.05
Globin	β-globin gene	−71.41
	2-α-1 globin	−26.17
	EST222653 hemoglobin	−4.19
Steroid metabolism	Stearyl CoA desaturase	23.77
	3 β-hydroxysteroid dehydrogenase isomerase type II.2	−2.08
	Aromatase cytochrome P450	−6.76
	Steroid 3-α-dehydrogenase	−2.64
Hormones and growth factors	Inhibin α-subunit gene	−3.66
	Müllerian inhibiting substance	−3.54
	VEGF	2.41
	Insulin-like growth factor II	1.89
	Interleukin 1 β	7.55
	Neuropeptide Y	3.76
	TNF-13	2.68
Unknown function	Kidney-specific androgen-regulated protein (KAP)	28
	Uterus-ovary–specific putative transmembrane protein	42

ing examined displayed a very unique transcriptome. This is consistent with the dramatic change in morphology and cell function during primordial follicle assembly and the primordial to primary follicle transition (Fig. 1).

Changes in gene expression are anticipated as the primordial follicles leave developmental arrest and the somatic cells proliferate. The gene expression changes reflect contributions of both the somatic cells and developing oocyte. Of the 148 genes that are up-regulated, 23 are metabolic enzymes suggesting the follicles are generally more metabolically active. This increase in metabolic state in the primary stage follicles is anticipated [26]. The increase in expression of the zona pellucida genes during follicular assembly validated the experimental methodology and design. The zona pellucida is the protein coat of the oocyte that is produced as the follicle assembles [27]. The rise in zona pellucida gene expression was anticipated as the primordial follicles assembled [27, 28].

The transcriptional profile of MIS is shown in Figure 6B. MIS is known to inhibit the primordial to primary follicle transition [17]. MIS was up-regulated during primordial follicle assembly and down-regulated during the primordial to primary follicle transition, consistent with the inhibitory role of MIS. The accompanying change in inhibin expression was unexpected (Fig. 6B) [29]. Although MIS and inhibin production are known to be stimulated by estrogen in the antral follicle [30], these early stages of folliculogenesis have negligible steroidogenesis. In addition, the pituitary/gonadal axis is not active at this stage of development, and these follicle stages are hormone-independent. These data suggest that low levels of inhibin operating within the ovary may have a function in maintaining the primordial follicles in their developmentally arrested state. Characterization of this potential unique function for inhibin requires further investigation.

A number of steroidogenic enzymes were up-regulated in primordial follicles and then down-regulated in primary follicles. Although a number of steroidogenic genes have increased expression levels (Fig. 7), critical genes in the steroidogenic pathway (e.g., CYP11A) were not changed. Therefore, steroid production may be minimal. Two important genes, STAR and CYP19A1, were altered (Fig. 7). Previous studies have suggested steroids may be produced and be involved in primordial follicle development [31]. This unique role of steroids in early primordial follicle development requires further investigation.

The transcriptional profile of IGFII and IGFBP2 displayed a pattern inverse to that of MIS and inhibin (Fig. 6C). They were down-regulated during primordial follicle assembly and up-regulated at the primordial to primary follicle transition. Although IGFII is known to have important functions in the antral follicle and corpus luteum [32], it has no known function in the early developing primordial follicle. The microarray data suggests a potential role for IGFII at this stage of development.

Three neu differentiation factors were up-regulated at the time of follicular assembly. This large gene family has been shown to be involved in the differentiation and growth of mesenchymal and neuronal cells [33]. This may be related to the innervation of the ovary by neural cells. Previous

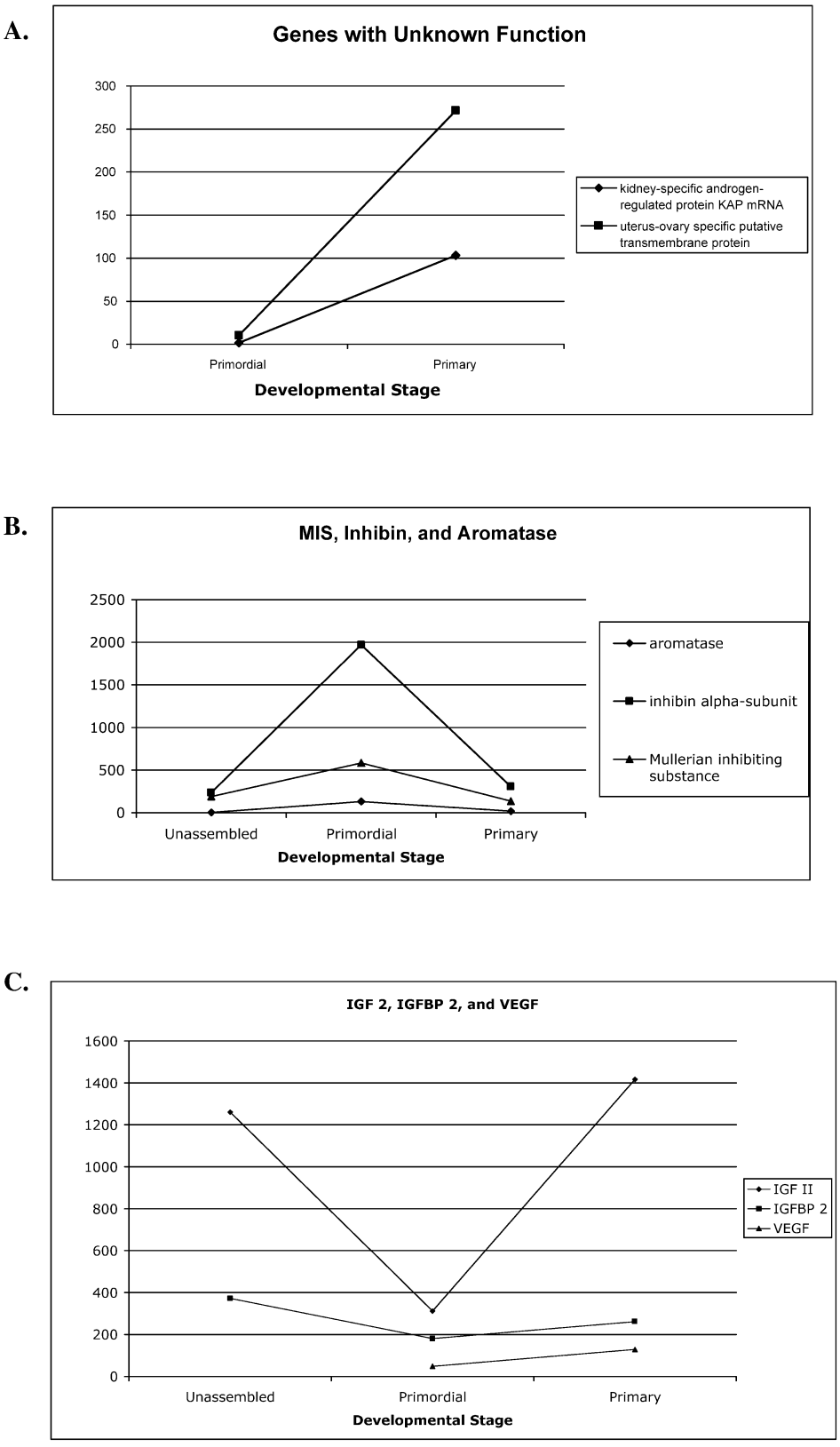

FIG. 6. **A**) Signal intensity change of two genes of unknown function (KAP and the UOSTP) between the primordial and primary follicle stages. **B**) Representative change of MIS, inhibin, and aromatase signal between unassembled, primordial, and primary follicle stages. **C**) Representative change of IGFII, IGFBP2, and VEGF signals between unassembled, primordial, and primary follicle stages. Data are displayed as a representative normalized microarray signal for the different probe sets in unassembled, primordial, and primary follicle stages.

studies have suggested ovarian innervation may be critical for the normal timing of early folliculogenesis [34]. Because the neu differentiation factor family is such a large and diverse group of factors, it is possible that these factors may act directly on primordial follicles themselves. Therefore, the neu differentiation factors are candidates for potential coordinators of primordial follicle assembly.

Interestingly, several growth factors known to promote the primordial to primary follicle transition such as kit ligand, leukemia inhibitory factor, and basic fibroblast growth factor [10–12] had no change in gene expression between the developmental stages (data not shown). This was in large part due to the low levels of gene expression of these factors in the whole ovary. These known facilita-

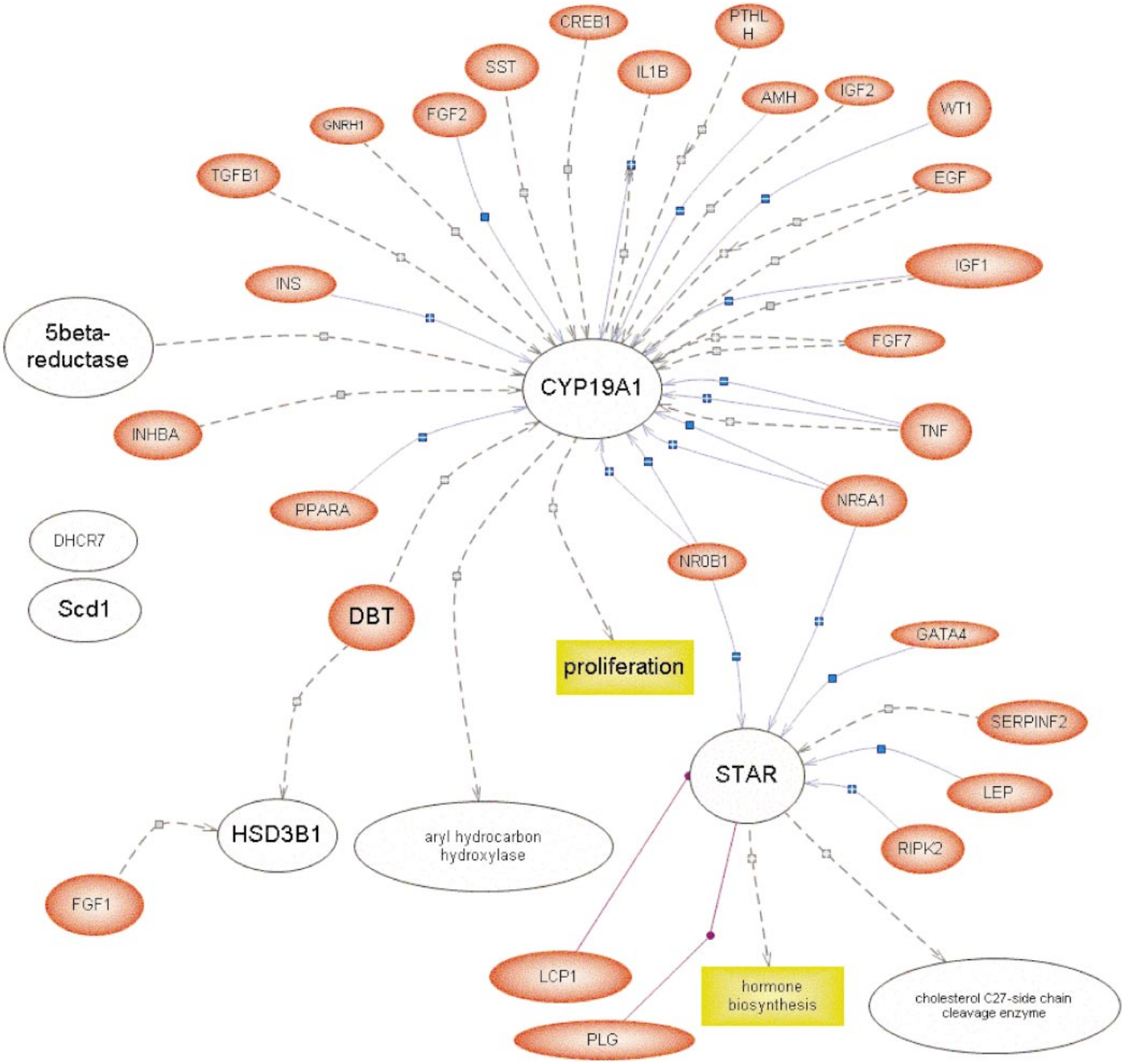

FIG. 7. A Pathway Assist and gene interaction diagram. Open circles represent steroidogenic genes identified in the microarray analysis. Shaded circles identify interacting proteins with hatched lines for regulation of activity and solid lines for regulation of gene expression. The interrelationship of the genes identified is shown with other regulatory factors. Positive (+) and negative (−) regulation of the interaction pathways is also indicated.

tors of the primordial to primary follicle transition may be expected to be up-regulated during the primordial to primary follicle transition. Alteration of gene expression may not be required if translational control and/or the cells responsiveness to the growth factors (e.g., receptor expression) are more critical. Although the experimental approach identifies potential candidate regulatory factors, it is important to note that gene expression changes are not always essential. Further analysis at an individual gene level is required to determine the specific function of a given gene product.

VEGF (Fig. 6C) gene expression is significantly up-regulated during the primordial to primary follicle transition. This observation was confirmed with a quantitative polymerase chain reaction (PCR) procedure that helped validate the microarray procedure. VEGF effects endothelial cell migration and proliferation to influence angiogenesis of the ovary [35–37]. No function of VEGF that affects early stages of folliculogenesis has been identified, but VEGF does influence preantral follicle development [38]. The microarray data associates this growth factor with the primordial to primary follicle transition. An obvious function of VEGF in the primordial to primary follicle transition would be in the angiogenesis of the ovary and follicles. The actual role of VEGF in the primordial to primary follicle transition will require further characterization.

Immune response genes, such as cytokines, were also found to be up-regulated during the primordial to primary

follicle transition. Interleukin 1-β, for example, was up-regulated sevenfold. Interleukins have been shown to have activities in the ovary, but not at this early stage of development [39]. The relationship and functions of these genes in primordial follicle development remains to be elucidated. Microarray observations suggest these factors might also play a role in the coordination of the primordial to primary follicle transition.

In addition to genes with a characterized known function, the microarray analysis identified two genes of unknown function that are up-regulated during the primordial to primary follicle transition (Fig. 6A). These are the kidney-specific androgen-regulated protein KAP (Genebank reference NM_052802) [40] and the uterus ovary–specific transmembrane protein (Genebank reference AF022147) [41]. Both of these genes are known only by screening of cDNA libraries from androgen-treated kidney from the first case and from estrogen-treated uterus in the second. Nothing can be deduced about the function or localization of these proteins except that they are highly expressed at the time of primordial to primary follicle transition. These two genes are good candidates for regulators of the primordial to primary follicle transition and require further characterization of their localization and function.

In conclusion, a gene discovery project was undertaken to identify new coordinators of primordial follicle assembly and the primordial to primary follicle transition. The Affymetrix rat U34A 8799 gene chip was used to analyze the transcriptomes of unassembled, primordial, and primary follicles. This gene chip contains 14 000 genes, and therefore does not reflect the entire genome. The majority of these genes did not change expression during primordial follicle development or were expressed at low levels below the cutoff used in the current study. Further investigations are needed to assess a genome-wide transcriptome of the ovary. The unassembled and primordial follicle stages used in vivo tissue, whereas the primary stage used cultured ovaries. Although we have shown no effect of culture on organ or follicle viability [10–12], changes potentially induced during culture need to be considered in any data interpretation. A summary of the potential factors involved in the coordination of primordial follicle development are summarized below. The neu differentiation factors are among the genes identified as being up-regulated at the time of follicular assembly. The neu factors are candidates for promoters of follicular differentiation. MIS was found to be highly expressed at the primordial follicle, consistent with its proposed function as an inhibitor of the primordial to primary follicle transition. Its site of synthesis and action remain to be determined. Unexpectedly, the steroidogenic apparatus and inhibin were also highly expressed at the primordial follicle stage. This suggests that potentially both inhibin and steroids may have novel functions in maintaining the primordial follicle in its developmentally arrested state. Estrogen receptor activation has been shown to regulate MIS expression [42]. The speculation is that steroids may be performing a similar function in the neonatal ovary. Further studies are needed to characterize the role of MIS, steroids, and inhibin in the coordination of early folliculogenesis. IGFII was down-regulated in the primordial follicle stage, and therefore may influence follicle assembly. The growth factor VEGF was up-regulated in the primary follicle, making the factor a candidate for a facilitator of the primordial to primary follicle transition. Many cytokines such as interleukin 1-β were also up-regulated in the primary follicle. These factors are also candidates for coordinators of the primordial to primary follicle transition. In addition, two new genes of unknown function, KAP and the UOSTP, were up-regulated and are also candidates for coordinators of the primordial to primary follicle transition. Therefore, the microarray approach identified a number of factors potentially involved in primordial follicle development.

Primordial follicle assembly and the rate of primordial to primary follicle transition influences the size of the primordial follicle pool [43]. The size of the primordial follicle pool sets the number and availability of follicles in the female. Although it has recently been speculated that new follicles can be recruited into the primordial follicle pool in adult rodents from germ-line stem cells [2], further investigation is required to confirm this possibility. When the primordial follicle pool is depleted, reproduction and steroidogenesis ends and menopause begins. Dysfunction in primordial follicle assembly and the primordial to primary follicle transition compromises the primordial follicle pool and may lead to an early menopause and/or the condition of premature ovarian failure. Further analysis of the factors involved in these processes of primordial follicle assembly and the primordial to primary follicle transition will improve our understanding of such pathological conditions as premature ovarian failure. The genes identified in the current study provide candidates for further analysis and will help elucidate this critical biological process.

ACKNOWLEDGMENTS

We would like to thank Dr. Ingrid Sadler-Riggleman for invaluable technical assistance. Thanks also to Jill Griffin and Mary Francis for assistance in preparing the manuscript. We acknowledge the use of the Center for Reproductive Biology, Genomics Core Laboratory, and Bioinformatics Core Laboratory, and the technical assistance of Derek Pouchnik and Jim Shima. Electronic access to the microarray database for this study can be obtained at www.skinner.wsu.edu.

REFERENCES

1. Kezele P, Nilsson E, Skinner MK. Cell-cell interactions in primordial follicle assembly and development. Front Biosci 2002; 7:d1990–d1996.
2. Johnson J, Canning J, Kaneko T, Pru JK, Tilly JL. Germline stem cells and follicular renewal in the postnatal mammalian ovary. Nature 2004; 428:145–150.
3. Richardson SJ, Senikas V, Nelson JF. Follicular depletion during the menopausal transition: evidence for accelerated loss and ultimate exhaustion. J Clin Endocrinol Metab 1987; 65:1231–1237.
4. Santoro N. Research on the mechanisms of premature ovarian failure. J Soc Gynecol Investig 2001; 8:S10–S12.
5. Fortune JE, Cushman RA, Wahl CM, Kito S. The primordial to primary follicle transition. Mol Cell Endocrinol 2000; 163:53–60.
6. Rajah R, Glaser EM, Hirshfield AN. The changing architecture of the neonatal rat ovary during histogenesis. Dev Dyn 1992; 194:177–192.
7. Pepling ME, Spradling AC. Mouse ovarian germ cell cysts undergo programmed breakdown to form primordial follicles. Dev Biol 2001; 234:339–351.
8. Morrison LJ, Marcinkiewicz JL. Tumor necrosis factor alpha enhances oocyte/follicle apoptosis in the neonatal rat ovary. Biol Reprod 2002; 66:450–457.
9. Kezele P, Skinner MK. Regulation of ovarian primordial follicle assembly and development by estrogen and progesterone: endocrine model of follicle assembly. Endocrinology 2003; 144:3329–3337.
10. Parrott JA, Skinner MK. Direct actions of kit-ligand on theca cell growth and differentiation during follicle development. Endocrinology 1997; 138:3819–3827.
11. Nilsson EE, Kezele P, Skinner MK. Leukemia inhibitory factor (LIF) promotes the primordial to primary follicle transition in rat ovaries. Mol Cell Endocrinol 2002; 188:65–73.
12. Parrott JA, Skinner MK. Kit ligand actions on ovarian stromal cells:

effects on theca cell recruitment and steroid production. Mol Reprod Dev 2000; 55:55–64.
13. Nilsson E, Parrott JA, Skinner MK. Basic fibroblast growth factor induces primordial follicle development and initiates folliculogenesis. Mol Cell Endocrinol 2001; 175:123–130.
14. Dissen GA, Romero C, Hirshfield AN, Ojeda SR. Nerve growth factor is required for early follicular development in the mammalian ovary. Endocrinology 2001; 142:2078–2086.
15. Nilsson EE, Skinner MK. Bone morphogenetic protein-4 acts as an ovarian follicle survival factor and promotes primordial follicle development. Biol Reprod 2003; 69:1265–1272.
16. Kezele PR, Nilsson EE, Skinner MK. Insulin but not insulin-like growth factor-1 promotes the primordial to primary follicle transition. Mol Cell Endocrinol 2002; 192:37–43.
17. Durlinger AL, Gruijters MJ, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JT, Grootegoed JA, Themmen AP. Anti-Mullerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 2002; 143:1076–1084.
18. McLean DJ, Friel PJ, Pouchnik D, Griswold MD. Oligonucleotide microarray analysis of gene expression in follicle-stimulating hormone-treated rat Sertoli cells. Mol Endocrinol 2002; 16:2780–2792.
19. Shima JE, McLean DJ, McCarrey JR, Griswold MD. The Murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod 2004; 71: 560–569.
20. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. Proc Natl Acad Sci USA 1998; 95:14863–14868.
21. Sadate-Ngatchou PI, Pouchnik DJ, Griswold MD. Identification of testosterone-regulated genes in testes of hypogonadal mice using oligonucleotide microarray. Mol Endocrinol 2004; 18:422–433.
22. McChlery SM, Clarke SC. The use of hydrolysis and hairpin probes in real-time PCR. Mol Biotechnol 2003; 25:267–274.
23. Wood JR, Nelson VL, Ho C, Jansen E, Wang CY, Urbanek M, McAllister JM, Mosselman S, Strauss JF 3rd. The molecular phenotype of polycystic ovary syndrome (PCOS) theca cells and new candidate PCOS genes defined by microarray analysis. J Biol Chem 2003; 278: 26380–26390.
24. Chin KV, Seifer DB, Feng B, Lin Y, Shih WC. DNA microarray analysis of the expression profiles of luteinized granulosa cells as a function of ovarian reserve. Fertil Steril 2002; 77:1214–1218.
25. Liu HC, He Z, Rosenwaks Z. Application of complementary DNA microarray (DNA chip) technology in the study of gene expression profiles during folliculogenesis. Fertil Steril 2001; 75:947–955.
26. Mihm M, Bleach EC. Endocrine regulation of ovarian antral follicle development in cattle. Anim Reprod Sci 2003; 78:217–237.
27. Zhao M, Dean J. The zona pellucida in folliculogenesis, fertilization and early development. Rev Endocr Metab Disord 2002; 3:19–26.
28. Dean J. Oocyte-specific genes regulate follicle formation, fertility and early mouse development. J Reprod Immunol 2002; 53:171–180.
29. Kang JS, Lee CJ, Lee JM, Rha JY, Song KW, Park MH. Follicular expression of c-Kit/SCF and inhibin-alpha in mouse ovary during development. J Histochem Cytochem 2003; 51:1447–1458.
30. Ikeda Y, Nagai A, Ikeda MA, Hayashi S. Increased expression of Mullerian-inhibiting substance correlates with inhibition of follicular growth in the developing ovary of rats treated with E2 benzoate. Endocrinology 2002; 143:304–312.
31. Juengel JL, Sawyer HR, Smith PR, Quirke LD, Heath DA, Lun S, Wakefield SJ, McNatty KP. Origins of follicular cells and ontogeny of steroidogenesis in ovine fetal ovaries. Mol Cell Endocrinol 2002; 191:1–10.
32. Yoshinaga K, Nishikawa S, Ogawa M, Hayashi S, Kunisada T, Fujimoto T. Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. Development 1991; 113:689–699.
33. Ben-Baruch N, Yarden Y. Neu differentiation factors: a family of alternatively spliced neuronal and mesenchymal factors. Proc Soc Exp Biol Med 1994; 206:221–227.
34. Ojeda SR, Romero C, Tapia V, Dissen GA. Neurotrophic and cell-cell dependent control of early follicular development. Mol Cell Endocrinol 2000; 163:67–71.
35. Fraser HM, Wulff C. Angiogenesis in the primate ovary. Reprod Fertil Dev 2001; 13:557–566.
36. Reynolds LP, Grazul-Bilska AT, Redmer DA. Angiogenesis in the corpus luteum. Endocrine 2000; 12:1–9.
37. Lebovic DI, Mueller MD, Taylor RN. Vascular endothelial growth factor in reproductive biology. Curr Opin Obstet Gynecol 1999; 11: 255–260.
38. Danforth DR, Arbogast LK, Ghosh S, Dickerman A, Rofagha R, Friedman CI. Vascular endothelial growth factor stimulates preantral follicle growth in the rat ovary. Biol Reprod 2003; 68:1736–1741.
39. Wang LJ, Brannstrom M, Cui KH, Simula AP, Hart RP, Maddocks S, Norman RJ. Localisation of mRNA for interleukin-1 receptor and interleukin-1 receptor antagonist in the rat ovary. J Endocrinol 1997; 152:11–17.
40. Malstrom SE, Tornavaca O, Meseguer A, Purchio AF, West DB. The characterization and hormonal regulation of kidney androgen-regulated protein (Kap)-luciferase transgenic mice. Toxicol Sci 2004; 79: 266–277.
41. Huynh H, Ng CY, Lim KB, Ong CK, Ong CS, Tran E, Tuyen Nguyen TT, Chan TW. Induction of UO-44 gene expression by tamoxifen in the rat uterus and ovary. Endocrinology 2001; 142:2985–2995.
42. Chen G, Shinka T, Kinoshita K, Yan HT, Iwamoto T, Nakahori Y. Roles of estrogen receptor alpha (ER alpha) in the regulation of the human Mullerian inhibitory substance (MIS) promoter. J Med Invest 2003; 50:192–198.
43. Fortune JE. The early stages of follicular development: activation of primordial follicles and growth of preantral follicles. Anim Reprod Sci 2003; 78:135–163.