

Identification of Differentially Expressed Genes in Mouse Spermatogenesis

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ABSTRACT: Complementary DNA microarray and quantitative polymerase chain reaction were used as tools for discovering genes that are differentially expressed in the mouse under normal physiological conditions at distinctive stages of male germ cell development, that is, type A spermatogonia, pachytene spermatocytes, and round spermatids. By using this strategy, we identified a set of genes exhibiting differential expression patterns in spermatogenesis, suggesting that specific functions of the encoded products occurred during the developmental process. Among them were several genes

previously not known to be active in testis, which signified undiscovered functional roles of these genes during spermatogenesis. Many of the genes identified were not previously characterized. This study highlights new targets for manipulation to unravel the molecular mechanism of spermatogenesis.

Key words: Type A spermatogonia, pachytene spermatocytes, round spermatids, spermatogenesis, complementary DNA microarray, quantitative polymerase chain reaction.

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Spermatogenesis is a tightly regulated developmental process of male germ cells. Type A spermatogonial stem cells undergo mitosis for either self-renewal or differentiation into later-stage spermatogonia that gradually become pachytene spermatocytes (PcSc) (Dym, 1994). PcSc undergo 2 meiotic divisions to give rise to haploid round spermatids (RdSd), which eventually transform into spermatozoa. The drastic change in cell morphology during germ cell differentiation and migration in seminiferous tubules suggests the presence of a highly organized network of genes, the expression of which is tightly regulated during different stages of spermatogenesis. Little is known about the molecular mechanism of this process, as signified by the presence of less than 5000 Unigene clusters assigned to male mouse germ cells, with only one half of them representing known genes (National Center for Biotechnology Information [NCBI] Mouse Unigene Cluster Build 118). This number of genes is significantly smaller than that estimated for a single cell population

(Zhang et al, 1997). Recent studies have attempted to delineate the genes involved in spermatogenesis (Wang et al, 2001; Fujii et al, 2002; Sha et al, 2002; Tanaka et al, 2002; Anway et al, 2003). However, no investigations have comprehensively compared changes in gene expression patterns at different stages of spermatogenesis. We examined the gene expression profiles of 3 distinctive stages of germ cell development in the mouse, viz. type A spermatogonia (SgA), PcSc, and RdSd, by using complementary DNA (cDNA) microarray as well as quantitative polymerase chain reaction (QPCR), and identified a set of genes exhibiting differential expression patterns in spermatogenesis.

Materials and Methods

Germ Cell Isolation and RNA Preparation

Protocols for the use of mice were approved by the Georgetown University Animal Care and Use Committee. Germ cells were isolated by the STAPUT procedure (Dym et al, 1995). Six-day-old BALB/c mouse testes were used for isolation of SgA. For PcSc and RdSd isolation, testes from 60-day-old animals were used. Purity of germ cells was routinely higher than 95% for SgA and higher than 90% for PcSc and RdSd. Total RNA was extracted from the isolated germ cells by using Trizol reagent (Invitrogen, Gaithersburg, Md) and cleaned up with RNeasy minicolumns (Qiagen, Valencia, Calif). RNA integrity was mon-

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itored by denaturing agarose gel electrophoresis. RNA content was determined by measurement of optical density at 260 nm (OD_{260}). Only RNA samples showing a $OD_{260/280}$ ratio higher than 1.8 were used for microarray hybridization and QPCR.

Hybridization of cDNA Microarray and Data Analysis

Radiolabeled cDNA probes were prepared from each type of germ cell by reverse-transcribing 1 μ g of total RNA in the presence of oligo-deoxythymidine primers and 10 μ L of [α - 33 P]deoxycytidine triphosphate (10 mCi/mL, 3000 Ci/mmol, Amersham Pharmacia, Piscataway, NJ). Mouse GeneFilters microarrays (GF400, Release I) containing 5184 mouse sequence-verified cDNA elements, each of them comprising of a \sim 1-kb fragment from the 3' end of the corresponding gene, were purchased from Research Genetics (Huntsville, Ala). Microarray hybridizations were performed according to manufacturer's instruction. Two microarrays were hybridized with probes generated from 2 separate preparations of germ cells of each stage. Two extra microarrays were hybridized in the same way with probes from a reference cell line C418. After washing, the hybridized microarrays were exposed to a phosphor screen for 5 hours and scanned for signals with a Storm 840 scanner (Molecular Dynamics, Piscataway, NJ) at a resolution of 50 μ m. Images were analyzed by IPLab/ArraySuite v2.0 (NGHRI/NIH) as described previously (Su et al, 2000). In the preliminary selection, only genes giving signal intensities less than 2-fold variation between duplicate experiments were considered to be expressed in that particular cell type. The expression level of selected genes was compared to that of C418 cells to obtain a reference ratio to eliminate experimental variation. The genes were required to exhibit comparable changes in signal ratio in the duplicate experiments. Genes showing 2-fold or greater difference in signal ratio in any 2 stages were considered to be differentially expressing. Gene identities and GenBank accession identifications were extracted from the mouse GeneFilters database (version gf400a; available at ftp://ftp.resgen.com/pub/genefilters/gf400a_final_data_070300.txt). Unigene assignment of selected genes was finalized based on Mouse Unigene cluster Build #118 (December 4, 2002) from NCBI. Biological functions of gene products were queried against LocusLink of NCBI (<http://www.ncbi.nlm.nih.gov/LocusLink>), Mouse Genome Informatics of the Jackson Laboratory (<http://www.informatics.jax.org>), and GeneCards of the Weizmann Institute of Science (<http://bioinfo.weizmann.ac.il/>).

Quantitative Polymerase Chain Reaction

Equal amounts of total RNA from different stages of germ cells were reverse transcribed to prepare the first-strand cDNA samples for QPCR analyses. Gene-specific primers (Table 1) were designed by Primer Express Version 2.0 (Applied Biosystems, Foster City, Calif) according to the sequence information provided for the cDNAs on the microarray. QPCR was carried out with the 7900 HTS Sequence Detection System (Applied Biosystems) and SYBR Green I chemistry according to manufacturer's instruction. To compare the expression level of each gene among the different cell types, a standard curve was first generated by plotting the threshold cycle (C_T) values of a series of fixed amount of RdSd cDNAs (arbitrarily assigned 0.1 \times , 1 \times , and 10 \times) against these amounts of cDNAs. The C_T values for a

gene in SgA or PcSc were fitted onto the standard curve to obtain the respective expression levels. A smaller C_T value indicates a higher expression level, and vice versa. Genes showing C_T values of 40 or higher were considered to be nonexpressing. The abundance of 18S rRNA in each cell type also was monitored and the gene expression levels were normalized to that of 18S rRNA. Genes showing 2-fold or greater difference in expression level in any 2 stages were considered to be differentially expressing.

Results and Discussion

Highly purified germ cells were used to prepare radiolabeled cDNA probes for microarray hybridization. For SgA, 190 unique Unigene clusters were identified to give consistent signals from 2 independent hybridization experiments. Among the 30 genes showing the strongest signals, two thirds (20) were expressed sequence tags (ESTs) (Table 2). For PcSc and RdSd, 272 and 245 unique Unigene clusters were recognized, respectively. The number of ESTs among the most abundant genes is 15 of 30 for PcSc (Table 3) and 14 of 30 for RdSd (Table 4). The high proportion of ESTs identified reflects the small number of characterized genes in male germ cells.

Based on the microarray signals, 79 differentially expressing genes (including known genes, uncharacterized transcripts, and ESTs) were identified, which exhibited 11 expression patterns as a function of the 3 stages of spermatogenesis (Table 5). The expression patterns of all 79 genes were verified by QPCR. When verifying gene expressions in PcSc and RdSd, only 37 genes (47%) were found to demonstrate concordant changes in expression between the microarray and QPCR experiments. Among them, 20 genes showed a 2-fold or greater difference in expression level between PcSc and RdSd. The expression levels of these genes, plus several known genes showing concordant change in expression but with less than a 2-fold difference in QPCR, in SgA, were examined. According to our selection scheme, a total of 23 genes were confirmed to be differentially expressed in the 3 stages of germ cells, with one half of them (12) representing known genes and the remaining 11 genes being ESTs or uncharacterized transcripts (Table 6). The high percentage of ESTs and uncharacterized transcripts identified corroborates the fact that little is known about gene expression in germ cells.

We speculate the transition of cells throughout spermatogenesis to be the result of a programmed change in gene expression at different stages of differentiation. Up-regulation of a gene at a particular stage would suggest requirement at that period of the gene expression, as well as the inverse corollary. A comparison of the expression patterns at different stages should provide insight into the

Table 1. List of primers designed for quantitative polymerase chain reaction analyses of the 79 genes identified in microarray experiments

GenBank ID	Unigene ID	Gene Description	Forward Primer	Reverse Primer
A1449312	Mm.34012	EST* weakly similar to JC1254 Ubiquitin-protein ligase (EC 6.3.2.19) E1	agacacaaaacactcgcaggagc	aatgggtgcagttgcctcg
A1323755	Mm.196666	Hydroxysteroid 17-beta dehydrogenase 5	tcttctcagagaaaactcttggcg	aaacaatgggttgatcagagctc
A1413324	Mm.37850	EST weakly similar to T42761 rat bassoon protein	agctctgcacctctcaagtttagg	ctgaaggacattgaaagctcgg
A1449324	Mm.32145	EST	ccagggctatagttccaaaagaaga	atbtatgtgtgattgtttgcatgttag
A1465389	Mm.200636	EST weakly similar to myosin heavy chain, nonmuscle type B	acttcagcaggcgaataaag	tgctaaaactggttaatggttgagttg
A1450088	Mm.30519	EST weakly similar to zinc finger protein 111	ccaacttaattgctttaccacc	ccatatacatttgcacatcgaagg
A1414337	Mm.22902	Myosin VIIb	tcccagaacaaggccacc	tgggttgccttcgcagag
A1447686	Mm.28880	EST weakly similar to A29942 developmental control protein Krox-4 fragment	atccacataaaggcagagaaacaca	aacatggacttgggcacatagg
A1449015	Mm.206218	EST weakly similar to histone deacetylase 2	ctggagcctgaattggcactc	tcatggcctcttgcctctg
A1662233	Mm.20159	Tripartite motif protein 10 (ring finger protein 9)	cccagtgcccatcagatactc	ggcttaaaatccagcactgtttg
A1465387	Mm.87130	EST weakly similar to <i>Arabidopsis thaliana</i> expressed protein At1g58350.1	ttttacttgaaaacagtaatgcaactg	atacacaacacagactcaacaacc
A1464359	Mm.28383	EST highly similar to human downregulated in ovarian cancer 1	tgtgactatgcaggcaacacatag	tcaccccttccaaaatccctag
A1451664	Mm.17962	EST	ctagttaaacctaaagtggcctcagc	gaatgtcaattcccatccactc
A1426564	No Unigene ID	EST	gttgatcttactgagaccagatgtgtag	cctttgaactcacatgaatcctcc
A1447998	Mm.31947	EST	tgtcctggfgcagttgtgtg	tcaagatgcttcaacttggtaacctg
A1448719	Mm.210529	EST highly similar to human nucleoprotein TPR	ggatctacggaggagctegaag	gaaattgatagccagcaggaagag
A1447526	Mm.195952	Potassium large conductance calcium-activated channel, subfamily M, beta member 4	ccaagaactcactttcagtcaataa	aacccccacacagacgaaacaa
A1426019	Mm.9935	EST	cagtttccacaggcaagtcag	ggcagtttcccaactgctc
A1414204	Mm.21406	EST highly similar to human serologically defined colon cancer antigen 3	gcacagggagctgcttattgtc	gtgccttttgatagtgcaattcag
A1447598	Mm.21284	EST weakly similar to PHD finger protein 1 (T-complex testis-expressed 3)	ctgacatgaggcaaatgactgacac	gatggcgcatcacagatgaaaag
A1448283	Mm.219636	EST weakly similar to T01437 human hypothetical protein R34001_1	agtcaccccttgccttccctc	tccttacctacactttgtcctcc
A1415236	Mm.23054	EST weakly similar to platelet-activating factor acetylhydrolase IB alpha subunit	ctcaagtccccatacaaaatccc	cactaacagagaagacaggctgaagc
A1415362	Mm.4532	EST highly similar to T12543 human hypothetical protein DKFZp434M154.1 (fragment)	ccaccacaatgtatctacc	ccttacaatcacggcatttgg
A1415719	Mm.227209	Small EDRK-rich factor 2	ctcaagtagccccagctctccttac	actggatcgagcagagagccag
A1326150	Mm.24643	Centrin 2	gcatagcaaggtgaaatgaaatgg	tgactaaatcaacagggcagaaca
A1429699	Mm.12393	Downregulated by Ctnnb1, a	tagtcaagtccaccatacaaccttgc	caggtgatgtgtccgaatcgc
A1323595	Mm.17	B-cell receptor-associated protein 31	agtggggggcccaagaaatac	gctcatcttttagcttccctcaggtc
A1448833	Mm.181446	EST highly similar to alpha-amylase, salivary and hepatic precursor	cttgacacaatgttgaattacctgg	acatttgggtgatctcacattag
A1326026	Mm.6775	Ornithine decarboxylase antizyme inhibitor	aagtccactgggtcttccactcac	ccattaaactgggttcccccatc
A1448820	Mm.20904	Cartilage-associated protein	tcggatgagcacttccagc	ccgtctcaacaagctcctcc
A1415364	Mm.36746	Transducin beta-like 2 protein	ccccacacgacacacaa	ggtttctgataaagatcagcca
A1413759	Mm.22826	EST weakly similar to B56708 human extracellular signal-regulated kinase 5	caagcttcagtttctaccatcctg	tgcatacctcctagccccctc

Table 1. Continued

GenBank ID	Unigene ID	Gene Description	Forward Primer	Reverse Primer
A1426199	Mm.30222	Stromal cell-derived factor 2-like 1	tggctgagtggaacatctctg	cagcaaaaaataatccccagaccaac
A1415354	Mm.156164	EST weakly similar to B24264 proline-rich protein MP3 (fragment)	accagcacatgatgtagggagtcac	ataaatgtagaatgctgcacccccctc
A1415728	Mm.209228	EST moderately similar to human N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase	tgtggaagtgaggagcagggg	cagagaaggagcacatggagg
A1414519	Mm.27260	EST moderately similar to NEDD-4	cgattgtatgaataaaaatcctgatttg	aaggaatggcaggcagtcacaag
A1450390	Mm.138544	EST moderately similar to human HTGN29 protein	ctgcaaaagtgtgaagaagcttgg	cactgggattatcttgcgatttcc
A1448386	Mm.27288	G elongation factor	tcaagcagactccatcatccc	cagcttccccgtaaaaaaagg
A1414503	Mm.27281	EST weakly similar to I56134 human tumor necrosis factor alpha-induced protein 2	tgttattttgcctcagcccc	tcattgtctgacttgctatccccctg
A1413624	Mm.28800	SMAF1	caacacacccctgtgagttgactg	gccaaagcctcttgccaaaac
A1429624	Mm.29326	EST	cctccgagtgctggattaa	gagtgctcgaagagtcagacagatg
A1429630	Mm.19077	EST weakly similar to rat rhoB gene	ggacctgggtttgtttccaa	ggtaccagacagaagccaaatga
A1428344	Mm.23423	EST weakly similar to zinc finger protein 238	tggctgggcaaaaaaaagg	tgctagatggctccagagaagg
A1448690	Mm.142843	Hypothetical protein MNCb-2040	cctccagttctccaaaatcc	gatggagttgttgaatccgaatg
A1451014	Mm.24056	EST weakly similar to I49636 DNA-binding protein	tgctttctggtgacatagtagc	agcttgaagttcttctctgagttc
A1449806	Mm.32242	Glucokinase activity, related sequence 1	ctggacacttgacaaaatccccctag	tgaaaaccttccatggccagaaaac
A1449427	Mm.223650	EST	actgctccttaagcctgccc	tgatagccaaggatatactccagagatc
A1464441	Mm.28262	Regulator of G-protein signaling 2	gtggttctaggaaatgtggtctgatg	tgtacctgcccactgagaaatc
A1449017	Mm.46613	EST highly similar to serine/threonine protein phosphatase 2A, 56 kd regulatory subunit, epsilon isoform	agacggtcaacttcgctcacac	gaccacttcaacctatccccatcc
A1451901	Mm.1226	Complement receptor 2	tggcagcatgcagtcagac	ttaggaagcttggccttctgttg
A1428575	Mm.23465	EST moderately similar to S12207 hypothetical protein (B2 element)	ggtgagctgagccttatggc	tgggatctatcaaaagggctaactc
A1385732	Mm.30155	H ⁺ -transporting ATPase (EC 3.6.1.35), vacuolar, 16-kb chain	agcttcagcaaacaggaaccccc	agatttggacctattcatcgctctg
A1327406	Mm.29652	Glutathione-S-transferase zeta 1	acatgtaaggcagtcagtgtgc	ggaggttggccttctctgg
A1451005	Mm.30595	EST	ttattattatgaaatgatgta-tgctcgagtg	acagcctagcctatccagtgaatttc
A1426001	Mm.24669	EST	tgtctgcatttcaacactccatg	atctgtagtccagaatgcttctctc
A1448691	Mm.32059	EST	ggatctgtcaggatgcttctgttg	tgcacaggttccagcccagc
A1451316	Mm.32451	EST	atagcttctttagatgctgtgtttc	aaattcttggctactgtgtttaaactgc
A1429824	Mm.1224	T-cell receptor CD3 eta chain precursor	tctcacagtcaccacttccc	gagcactggccctgttatctg
A1413790	Mm.22848	EST	actatgagctatgcaatttaagcgatc	aagtgtggaaagctgaggcag
A1451034	Mm.34092	EST weakly similar to human hypothetical protein FLJ20721	tgagaccocagacaccactc	cttacaacttccagcctccctc
A1325471	Mm.30074	Oxoglutarate dehydrogenase (lipoamide)	tcaaaagaggagggaaggaagcag	tcccttgaacacacacagaagctg
A1429335	Mm.6735	Prolyl endopeptidase	ggggacggatcagtcagaagata	gaggtcagatcaccacagctcgg
A1666408	Mm.33584	EST highly similar to X transporter protein 3	aaattggggtccctctgcaagag	tttggggtgttctgtcccg
A1450996	No Unigene ID	EST	agtttactgaaaaatgcccccc	acggctgtctgtgtgatttg
A1323829	Mm.21758	Cytochrome P450, 2e1, ethanol inducible	aggctgaggtcgtatcccccttag	aagcgtgtgtgttggagg
A1451700	Mm.32519	EST	aactgagggcctgtgaccacttc	agtcacgccttctgttctctgtg
A1324053	Mm.18843	Transglutaminase 2, C polypeptide	acagatagcagggctcacgg	ctgggtgatgaggtcaaatcttc
A1661959	Mm.159813	EST moderately similar to I49636 DNA-binding protein	caagtgaagcaataccacatgg	tgaatgaacaaagtgaacagagcc
A1449518	Mm.44123	Differentially expressed in FDCP 8	gaccacacatcacactcaaaaggac	ggcttactcatgctgttctctctg

Table 1. Continued

GenBank ID	Unigene ID	Gene Description	Forward Primer	Reverse Primer
A1447538	Mm.31862	EST	actagaccacatagcccagcg	gagacctatggaggactactgggttc
A1452049	Mm.31742	EST weakly similar to rat Ubiquitin-specific protease 2	cagctctgtaactccttgagctgccc	tctgtttcgaagctgctgtc
A1427661	Mm.104771	Vascular Rab-GAP/TBC-containing; BUB2-like protein 1	aaccttgaggacaaacattaaaggc	caagagatcccagaaccattgc
A1428089	Mm.129498	EST weakly similar to T50638 human synaptic glycoprotein SC2 (imported)	tgacgttttccatgcatcaccac	actgttgggtttccaaggagcctc
A1450803	Mm.32360	EST	ttcctagttctttgtgaacctacctg	aactcccacagacaccatgg
A1413123	Mm.179409	EST weakly similar to T08696 human hypothetical protein DKFZp564A043.1 (fragment)	tggtattgttacacttccttaaaagc	cgggaaacttgttttctccagc
A1426683	No Unigene ID	EST	ccaccttaggaggtgggtatg	ccatgtataacctcagattggtccttac
A1428924	Mm.23498	RIKEN cDNA 1110051B16	gacggaccggaggatata	cactgttactctggctgcttga
A1428846	Mm.29310	EST weakly similar to rat O-GlcNAc transferase p110 subunit	ataagaacagcacacaatccagagc	aaagtgggtttcccatctccatg
A1448817	Mm.231899	EST	ggacagctcctccatccactgag	tgtagtgtttgaaagagagtggg

* EST indicates expressed sequence tag.

potential roles these genes would play during spermatogenesis. The expression of the 23 genes could be clustered into 5 changing patterns (group I through V; Figure). Group I genes (n = 7) displayed very low or no expression in SgA, a gradual increase in PcSc, and a maximal increase in RdSd. These genes may be specific for meiotic or postmeiotic functions or cellular activities in a more differentiated state. Four of the genes in this group are known genes, and the remaining 3 are ESTs and a gene encoding a hypothetical protein (Table 6). In the mouse, glucokinase (glycerol kinase) activity-related sequence 1 (*Gk-rs1*) is an autosomal intronless retrotransposed element from the X-linked glycerol kinase and expressed only in the testis (Pan et al, 1999). Nothing is known about its biological function because it lacks glycerol kinase activity. From our data, the total absence in SgA suggests that *Gk-rs1* is involved in activities of postmitotic germ cells. Within this group there is a novel gene *Smaf1* that has uncharacterized biological function.

Two immunocyte-specific genes, namely complement receptor 2 (*Cr2/CD21*) and T-cell receptor CD3 eta precursor (*CD3η*), also were identified. Cr2 is a receptor for C3d,g complement fragment-tagged immune complexes. The receptor is expressed mainly on follicular dendritic and B cell surfaces and was found to enhance B-cell activation and differentiation by lowering the signal threshold for activation (Prechl and Erdei, 2000). *CD3η* is one of the noncovalently associated subunits of the T-cell receptor (TCR) complexes. It has been shown to participate in the assembly and cell surface expression of TCR complexes and transduction of signal from TCR that leads to intrathymic T-cell differentiation (Bauer et al, 1991; Malissen et al, 1993). Because cross-contamination by B or T cells is very unlikely in our germ cell isolation procedure, the expression of *Cr2* and *CD3η* in male germ cells strongly suggests their involvement in spermatogenesis and the immunocyte-specific expression is the result of exclusion of testis tissues in previous studies. As both gene products were expressed on cell surface and involved in signaling processes, we speculate a similar mode of action for Cr2 and CD3η in germ cells by regulating or transducing signals for cellular differentiation from the extracellular environment.

Group II genes (n = 8) expressed at a lower level in SgA; after reaching a maximum in PcSc, the expression level declined in RdSd. Such expression pattern implies that the gene activities are more important to meiotic germ cells, as the expression level dropped beyond this stage, possibly involved in meiosis or the maintenance of the tetraploid state. Three known genes are in this group.

Mitochondrial elongation factor G (*Gfm*) catalyzes the A-to-P site translocation of peptidyl-tRNA after peptide bond formation in protein biosynthesis (Gao et al, 2001). Its preferential expression in PcSc suggests a more de-

Table 2. List of top 30 genes identified in type A spermatogonia. Raw signal data from the duplicate microarray hybridizations are shown

First Experiment	Second Experiment	GenBank ID	Gene Description
819.9288	1621.9736	AI464583	ESTs,* weakly similar to podocalyxin (<i>Rattus norvegicus</i>)
661.26186	1297.5354	AI325463	ESTs, highly similar to NADH-ubiquinone oxidoreductase PDSW subunit (<i>Bos taurus</i>)
345.88605	675.84675	AI413169	Ferritin heavy chain
873.81201	1692.67821	AI327250	ESTs, highly similar to fibrinogen beta chain precursor (<i>Homo sapiens</i>)
277.89312	537.14944	AI327126	ESTs, moderately similar to probable peptidyl-prolyl <i>cis-trans</i> isomerase C21E11.05C (<i>Schizosaccharomyces pombe</i>)
436.89368	836.5056	AI324036	ESTs, highly similar to alpha-1-antiproteinase f precursor (<i>Oryctolagus cuniculus</i>)
355.12456	679.59642	AI327195	ESTs, highly similar to hypothetical 66.5-kd protein in ADE12-RAP1 intergenic region (<i>Saccharomyces cerevisiae</i>)
352.29766	670.39518	AI573377	Peroxisomal membrane protein 3, 35 kDa
637.33303	1210.04042	AI449200	ESTs, weakly similar to survival motor neuron protein 1 (<i>Mus musculus</i>)
447.06816	836.14464	AI448856	ESTs, weakly similar to (define not available 5931573) (<i>M. musculus</i>)
761.8534	1404.02724	AI448901	ESTs, highly similar to KIAA0738 protein (<i>H. sapiens</i>)
567.6756	1020.81784	AI662203	ESTs, moderately similar to phosphatidylinositol (<i>H. sapiens</i>)
428.0736	760.64936	AI427137	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 13
585.09297	1036.85184	AI427147	DNA segment, Chr 18, Wayne State University 70, expressed
320.90373	568.12147	AI449974	ESTs, highly similar to KIAA0476 protein (<i>H. sapiens</i>)
714.64624	1264.79684	AI327236	<i>Mus musculus</i> myotubularin related protein 7 mRNA, partial cds
509.81061	902.09591	AI326004	ESTs, highly similar to T-cell surface protein tactile precursor (<i>H. sapiens</i>)
1076.80761	1891.41795	AI385678	Even skipped homeotic gene 2 homolog
494.2896	857.092	AI429293	ESTs, highly similar to ubiquitin-conjugating enzyme E2-21 kd UBCH6 (<i>M. musculus</i>)
472.2036	790.38504	AI415195	Demethylarginine dimethylaminohydrolase 2
439.25025	716.17032	AI662233	Ring finger protein 9
494.60676	800.15812	AI447686	ESTs, weakly similar to zinc finger protein ZFP-37 (<i>M. musculus</i>)
366.6106	581.8431	AI661959	ESTs, weakly similar to DNA-binding protein (<i>M. musculus</i>)
336.85208	533.94272	AI426508	ESTs, highly similar to cytochrome P450 L1 (<i>S. cerevisiae</i>)
621.63515	924.97285	AI325930	ESTs, highly similar to cell division control protein 23 (<i>S. cerevisiae</i>)
409.51328	586.37425	AI414965	Glutamine synthetase
486.34593	675.37809	AI451489	ESTs, highly similar to KIAA0652 protein (<i>H. sapiens</i>)
399.81781	533.37109	AI449154	Phosphatase and tensin homolog
734.0025	951.95265	AI414785	ESTs, weakly similar to DAP-kinase related protein 1 (<i>M. musculus</i>)
421.85715	546.7	AI413310	ESTs, weakly similar to type VI collagen alpha 3 subunit (<i>M. musculus</i>)

* EST indicates expressed sequence tag.

manding need of the germ cell for protein synthesis at this stage, presumably to support cellular events at the tetraploid state or the 2 rounds of meiosis.

Regulator of G-protein signaling 2 (*Rgs2*) belongs to a family of proteins that regulate G-protein signaling by accelerating hydrolysis of guanosine triphosphate bound to activated G α subunits, thus limiting the duration of signaling (Kehrl and Sinnarajah, 2002). *Rgs2* also was involved in cellular differentiation (Imagawa et al, 1999). Specifically it was up-regulated during early stages of differentiation but down-regulated thereafter. We did not observe a concordant expression pattern of *Rgs2* in germ cells; this may be attributed to the difference in cellular contexts. The increased expression of *Rgs2* in PcSc suggests the occurrence of more active transmembrane signaling events during this stage.

The large conductance calcium-activated potassium channel (BK or MaxiK) is a member of the Shaker-related 6 transmembrane domain potassium channel family that is activated by voltage and calcium. BK channel is composed of a pore-forming α subunit and a modulatory

transmembrane β subunit. The tissue specificity of β subunits confers different physiological properties to the channels, for example, β_4 subunit (encoded by *KCNMB4*), which is highly expressed in brain and testis, could enhance the opening of BK channel at high [Ca²⁺] (Brenner et al, 2000). Despite the α subunit gene being absent on the microarray, the detection of *Kcnmb4* in germ cells indicates the presence of functional α and β_4 BK channels. In fact, both α - and β_4 -subunits were found to be active in human testes (Behrens et al, 2000; Brenner et al, 2000). The augmented expression of *Kcnmb4* in PcSc suggests more active modulation of the BK channel during this stage. In neurons, BK channels were associated with calcium channels (Marrion and Tavalin, 1998). We postulate in male germ cells, the Ca²⁺ influx resulting from signaling events would activate BK channels to open to allow entry of K⁺ that triggers downstream biochemical responses. Interestingly, heterologously expressed α and β_4 BK channels could be activated by 17 β -estradiol (Behrens et al, 2000). This finding suggests that sex steroids may act on α and β_4 BK channels in germ

Table 3. List of top 30 genes identified in pachytene spermatocytes. Raw signal data from the duplicate microarray hybridizations are shown

First Experiment	Second Experiment	GenBank ID	Gene Description
439.91906	564.09682	AI451433	ATP-binding cassette, sub-family A (ABC1), member 2
392.2892	497.1226	AI415313	Calcium channel beta 3 subunit
370.0781	455.90226	AI666534	CD4 antigen
403.722	496.2672	AI451039	ESTs,* highly similar to mitochondrial 60S ribosomal protein L3 (<i>Rattus norvegicus</i>)
407.12595	499.91667	AI448900	Potassium inwardly rectifying channel, subfamily J, member 8
548.37475	655.88908	AI448901	ESTs, highly similar to KIAA0738 protein (<i>Homo sapiens</i>)
488.02971	571.36558	AI415629	Calumenin
404.1804	460.7616	AI413758	Potassium channel, subfamily K, member 1
509.70815	566.69305	AI324156	ESTs, highly similar to NPL4 protein (<i>Saccharomyces cerevisiae</i>)
539.11363	593.47948	AI323810	Nucleophosmin 1
467.3853	514.35498	AI324267	Deiodinase, iodothyronine, type II
438.3236	480.55384	AI324113	Noncatalytic region of tyrosine kinase adaptor protein 2
632.1731	688.72336	AI666635	Gene trap locus 3
425.58195	463.34723	AI427864	ESTs, highly similar to transcription factor-like 5 (<i>H. sapiens</i>)
451.24736	482.5746	AI448065	Aryl-hydrocarbon receptor-interacting protein
454.073	485.0515	AI413710	ESTs, weakly similar to ZnT4 (<i>Mus musculus</i>)
766.26333	814.74408	AI385678	Even skipped homeotic gene 2 homolog
506.85768	537.67098	AI325463	ESTs, highly similar to NADH-ubiquinone oxidoreductase PDSW subunit (<i>Bos taurus</i>)
582.1242	611.6328	AI464583	ESTs, weakly similar to podocalyxin (<i>R. norvegicus</i>)
452.0926	472.80295	AI661337	ESTs, weakly similar to retinoblastoma-associated protein HEC (<i>H. sapiens</i>)
461.08251	476.91683	AI325930	ESTs, highly similar to cell division control protein 23 (<i>S. cerevisiae</i>)
572.85956	590.77785	AI465327	ESTs, moderately similar to hypothetical protein (<i>H. sapiens</i>)
643.05081	661.36608	AI327250	ESTs, highly similar to fibrinogen beta chain precursor (<i>H. sapiens</i>)
444.10926	454.75855	AI413153	ESTs, weakly similar to C11H1.7 (<i>Caenorhabditis elegans</i>)
458.79372	460.50444	AI430998	Vav2 oncogene
460.04493	461.18492	AI449200	ESTs, weakly similar to survival motor neuron protein 1 (<i>M. musculus</i>)
535.31302	526.88674	AI327236	<i>Mus musculus</i> myotubularin related protein 7 mRNA, partial cds
509.91435	458.15055	AI414785	ESTs, weakly similar to DAP-kinase related protein 1 (<i>M. musculus</i>)
597.42995	518.11004	AI449123	ESTs, moderately similar to tumor suppressor (<i>H. sapiens</i>)
690.6384	548.1351	AI666733	Calcium/calmodulin-dependent protein kinase IV

* EST indicates expressed sequence tag.

cells. It would be tempting to investigate the interplay between sex steroids and α and β BK channels in modulating germ cell physiology in vivo.

The expression level of group III genes ($n = 2$) was maintained in SgA and PcSc but dropped in RdSd, suggesting the encoded functions were less essential to the postmeiotic germ cells. Only 1 known gene is present in this group. *Trim10* (also known as *Herf1*) was reported to be involved in erythroid differentiation (Harada et al, 1999). The higher expression of *Trim10* in SgA and PcSc suggests its involvement in differentiation in the earlier stages of spermatogenesis. Similar to *Cr2* and *CD3 η* , *Trim10* was previously not known to be active in testis.

Group IV genes ($n = 5$) had the highest expression level in SgA, the lowest in PcSc, and more elevated in RdSd, but less than in SgA. These genes are likely to mediate functions specific to spermatogonia. The up-regulation in RdSd may reflect the requirement of the gene products at this stage (eg, for subsequent sperm maturation) or result from relaxation of restraints imposed by X-chromosome inactivation process (McCarrey et al, 2002).

Four known genes and 1 uncharacterized transcript are in this group.

Cartilage-associated protein (*Crtap*) was first identified to exhibit a developmentally regulated expression pattern in chick chondrocytes (Castagnola et al, 1997). *Crtap* was implied to function in the differentiation process, because its expression was up-regulated when chondrocytes differentiated. Examination of our data demonstrated a similar expression pattern of *Crtap*, in which the more differentiated RdSd showed a higher expression than PcSc. However, the less differentiated SgA showed the highest expression level. This observation suggests an alternate function for *Crtap* in SgA or *Crtap* may play a totally different role in germ cells than in chick chondrocytes. Because little is known about *Crtap*, the significance of this gene in germ cell development will be revealed by direct manipulation of its expression.

Prolyl oligopeptidase (*Pop*) is a widely distributed serine endopeptidase catalyzing the hydrolysis of the carboxyl side of proline residues in peptides. *Pop* was found to involve in peptide hormone maturation and degrada-

Table 4. List of top 30 genes identified in RdSd. Raw signal data from the duplicate microarray hybridizations are shown

First Experiment	Second Experiment	GenBank ID	Gene Description
641.50305	766.96656	AI413324	ESTs,* moderately similar to HH0712 cDNA clone for KIAA0442 has a 574-bp insertion at position 1474
552.27326	511.73815	AI448901	ESTs, highly similar to KIAA0738 protein (<i>Homo sapiens</i>)
577.02315	502.75335	AI414785	ESTs, weakly similar to DAP-kinase-related protein 1 (<i>Mus musculus</i>)
580.65013	497.9895	AI415629	Calumenin
667.86324	565.42095	AI327250	ESTs, highly similar to fibrinogen beta chain precursor (<i>H. sapiens</i>)
676.96453	565.5837	AI413315	Glutamine synthetase
859.9482	716.77953	AI385678	Even skipped homeotic gene 2 homolog
571.5958	473.80374	AI449164	Immunoglobulin-associated beta
604.773	474.201	AI450790	ESTs, highly similar to hypothetical 36.7-kd protein AH6.2 in chromosome II (<i>Caenorhabditis elegans</i>)
582.53664	447.0034	AI449362	ESTs, weakly similar to testis-specific protein A (<i>Rattus norvegicus</i>)
647.35812	491.47194	AI327236	<i>Mus musculus</i> myotubularin-related protein 7 mRNA, partial cds
590.77785	443.66387	AI465327	ESTs, moderately similar to hypothetical protein (<i>H. sapiens</i>)
649.8204	483.1836	AI464583	ESTs, weakly similar to podocalyxin (<i>R. norvegicus</i>)
631.00937	464.61794	AI325160	MARCKS-like protein
596.44431	436.49766	AI666733	Calcium/calmodulin-dependent protein kinase IV
671.15772	486.66149	AI428004	DNA segment, Chr 9, University of California at Los Angeles 2
645.23664	467.5776	AI893883	Cardiotrophin 1
825.2827	590.39695	AI413310	ESTs, weakly similar to type VI collagen alpha 3 subunit (<i>M. musculus</i>)
653.23197	462.7485	AI427478	ESTs, highly similar to diphosphomevalonate decarboxylase (<i>R. norvegicus</i>)
642.19656	453.73482	AI385562	Cytochrome P450, 2b9, phenobarbital inducible, type a
659.72767	456.73985	AI323314	Neuropeptide nociceptin 1
639.6042	441.10254	AI894225	ESTs, highly similar to drebrins E1 and E2 (<i>Gallus gallus</i>)
656.49264	441.85944	AI528643	Adaptor-related protein complex AP-1, mu subunit 1
755.43444	501.06412	AI894196	ESTs, weakly similar to ubiquitin carboxyl-terminal hydrolase 13 (<i>Saccharomyces cerevisiae</i>)
853.24248	561.93552	AI413305	ESTs, highly similar to neural F box protein NFB42 (<i>R. norvegicus</i>)
680.79528	443.75364	AI430998	Vav2 oncogene
696.9402	447.9948	AI894211	Otoconin 90
723.36218	459.1604	AI323310	Fos-like antigen 2
695.79203	433.60411	AI414297	Phosphodiesterase 6D, cGMP-specific, rod, delta
862.92892	485.37042	AI451489	ESTs, highly similar to KIAA0652 protein (<i>H. sapiens</i>)

* EST indicates expressed sequence tag.

tion, cellular differentiation (Kimura et al, 1999), and recently meiosis of spermatocytes and differentiation of spermatids in mice (Kimura et al, 2002). In the latter study, *Pop* showed the highest expression level in 2-week-old mouse testes that contained differentiated cell species from SgA to PcSc; the expression decreased successively until 8 weeks of age when all stages of spermatogenesis were present. In situ hybridization revealed *Pop* mRNA in all germ cells in 2-week-old testes, but only in RdSd in 8-week-old testes. Because the PcSc and RdSd used in our study were isolated from animals at a similar age (60 days) as in study of Kimura et al (2002) (8 weeks), we expected a similar result. Indeed, we observed a higher expression of *Pop* in RdSd. In contrast, we did not see a total absence of *Pop* expression in PcSc. We also found that SgA displayed the highest level of *Pop* expression, which was not addressed by Kimura et al (2002). Thus, rather than being involved in meiosis, we believe *Pop* plays a more important role in SgA mitotic functions.

Among the genes in this group are two X-linked genes, namely B-cell receptor-associated protein 31 (*Bap31*) and centrin 2 (*Cetn2*). *Bap31* associates with membrane immunoglobulin D on mature B cells and is highly enriched in endoplasmic reticulum (Ng et al, 1997). It also regulates apoptosis by processing procaspase-8L to modulate caspase activation (Breckenridge et al, 2002). In fact, *Bap31* is a preferred substrate of caspase-8, and its cleavage product p20 contributes directly to apoptosis progression (Nguyen et al, 2000). *Cetn2* is a centriole protein within the centrosome. In addition to its involvement in cytokinesis and cell cycle progression, *Cetn2* is essential to centriole duplication and the proper progression of mitosis; its loss leads to aberrant mitosis and cell death (Salisbury et al, 2002). Both genes were preferentially expressed in SgA, suggestive of a role for *Bap31* and *Cetn2* in regulating spermatogonial apoptosis and proper maintenance of mitosis, respectively. As germ cells differentiate, the expression of *Bap31* and *Cetn2* dropped dramatically in PcSc, a phenomenon attributable to X-chro-

Table 5. List of 79 genes showing differential expression pattern in microarray analyses. Red color denotes a higher gene expression level in the respective type of germ cells than in reference cells, whereas green color shows the opposite phenomenon

Pattern	SgA	PcSc	RdSd	GenBank ID	Unigene ID	Gene Description
1	Red	Red	Red	AI449312	Mm.34012	EST weakly similar to JCI254 Ubiquitin-protein ligase (EC 6.3.2.19) E1
				AI323755	Mm.196666	Hydroxysteroid 17-beta dehydrogenase 5
				AI413324	Mm.37850	EST weakly similar to T42761 rat Bassoon protein
2	Red	White	White	AI449324	Mm.32145	EST
				AI465389	Mm.200636	EST weakly similar to Myosin heavy chain, nonmuscle type B
				AI450088	Mm.30519	EST weakly similar to Zinc Finger Protein 111
3	Red	White	White	AI414337	Mm.22902	Myosin VIIb
				AI447686	Mm.28880	EST weakly similar to A29942 developmental control protein Krox-4 fragment
				AI449015	Mm.206218	EST weakly similar to Histone Deacetylase 2
				AI662233	Mm.20159	Tripartite motif protein 10 (Ring finger protein 9)
				AI465387	Mm.87130	EST weakly similar to <i>A. thaliana</i> expressed protein At1g58350.1
				AI464359	Mm.28383	EST highly similar to human Downregulated in ovarian cancer 1
4	White	Red	White	AI451664	Mm.17962	EST
				AI426564	No Unigene ID	EST
				AI447998	Mm.31947	EST
				AI448719	Mm.210529	EST highly similar to human Nucleoprotein TPR
				AI447526	Mm.195952	Potassium large conductance calcium-activated channel, subfamily M, beta member 4
				AI426019	Mm.9935	EST
				AI414204	Mm.21406	EST highly similar to human Serologically defined colon cancer antigen 3
				AI447598	Mm.21284	EST weakly similar to PHD finger protein 1 (T-complex testis-expressed 3)
				AI448283	Mm.219636	EST weakly similar T01437 human Hypothetical protein R34001_1
				AI415236	Mm.23054	EST weakly similar to Platelet-activating factor acetylhydrolase IB alpha subunit
5	White	Red	White	AI415362	Mm.4532	EST highly similar to T12543 human Hypothetical protein DKFZp434M154.1 (fragment)
				AI415719	Mm.227209	Small EDRK-rich factor 2
				AI326150	Mm.24643	Centrin 2
				AI429699	Mm.12393	Down-regulated by Ctnnb1, a
				AI323595	Mm.17	B-cell receptor-associated protein 31
				AI448833	Mm.181446	EST highly similar to Alpha-amylase, salivary and hepatic precursor
6	White	Red	White	AI326026	Mm.6775	Ornithine decarboxylase antizyme inhibitor
				AI448820	Mm.20904	Cartilage-associated protein
				AI415364	Mm.36746	Transducin beta-like 2 protein
				AI413759	Mm.22826	EST weakly similar to B56708 human Extracellular signal-regulated kinase 5
				AI426199	Mm.30222	Stromal cell-derived factor 2-like 1
				AI415354	Mm.156164	EST weakly similar to B24264 Proline-rich protein MP3 (fragment)
7	White	Red	Green	AI415728	Mm.209228	EST moderately similar to human N-Acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase
				AI414519	Mm.27260	EST moderately similar to NEDD-4
				AI450390	Mm.138544	EST moderately similar to human HTGN29 protein
				AI448386	Mm.27288	G elongation factor
				AI414503	Mm.27281	EST weakly similar to I56134 human Tumor necrosis factor alpha-induced protein 2
				AI413624	Mm.28800	SMAF1
8	White	Red	Green	AI429624	Mm.29326	EST
				AI429630	Mm.19077	EST weakly similar to rat rhoB gene
				AI428344	Mm.23423	EST weakly similar to Zinc finger protein 238
				AI448690	Mm.142843	Hypothetical protein MNCb-2040
				AI451014	Mm.24056	EST weakly similar to I49636 DNA-binding protein
				AI449806	Mm.32242	Glucokinase activity, related sequence 1
				AI449427	Mm.223650	EST
				AI464441	Mm.28262	Regulator of G-protein signaling 2
				AI449017	Mm.46613	EST highly similar to Serine/threonine protein phosphatase 2A, 56 kDa regulatory subunit, epsilon isoform
				AI451901	Mm.1226	Complement receptor 2
				AI428575	Mm.23465	EST moderately similar to S12207 Hypothetical protein (B2 element)
				AI385732	Mm.30155	H+-transporting ATPase (EC 3.6.1.35), vacuolar, 16K chain
				AI327406	Mm.29652	Glutathione-S-transferase zeta 1
				AI451005	Mm.30595	EST
AI426001	Mm.24669	EST				
9	White	Red	Green	AI448691	Mm.32059	EST
				AI451316	Mm.32451	EST
				AI429824	Mm.1224	T-cell receptor CD3 eta chain precursor
				AI413790	Mm.22848	EST
				AI451034	Mm.34092	EST weakly similar to human Hypothetical protein FLJ20721
				AI325471	Mm.30074	Oxoglutarate dehydrogenase (lipoamide)
				AI429335	Mm.6735	Prolyl endopeptidase
				AI666408	Mm.33584	EST highly similar to X transporter protein 3
				AI450996	No Unigene ID	EST
				AI323829	Mm.21758	Cytochrome P450, 2e1, ethanol inducible
10	White	Red	Green	AI451700	Mm.32519	EST
				AI324053	Mm.18843	Transglutaminase 2, C polypeptide
				AI661959	Mm.159813	EST moderately similar to I49636 DNA-binding protein
				AI449518	Mm.44123	Differentially expressed in FDCC 8
				AI447538	Mm.31862	EST
				AI452049	Mm.31742	EST weakly similar to rat Ubiquitin specific protease 2
11	White	Red	Green	AI427661	Mm.104771	Vascular Rab-GAP/TBC-containing; BUB2-like protein 1
				AI428089	Mm.129498	EST weakly similar to T50638 human Synaptic glycoprotein SC2 (imported)
				AI450803	Mm.32360	EST
				AI413123	Mm.179409	EST weakly similar to T08696 human Hypothetical protein DKFZp564A043.1 (fragment)
	White	Red	Green	AI426683	No Unigene ID	EST
				AI428924	Mm.23498	RIKEN cDNA 1110051B16
				AI428846	Mm.29310	EST weakly similar to rat O-GlcNAc transferase p110 subunit
				AI448817	Mm.231899	EST

Table 6. List of 23 genes showing differential expression pattern as identified by microarray analysis and quantitative polymerase chain reaction*

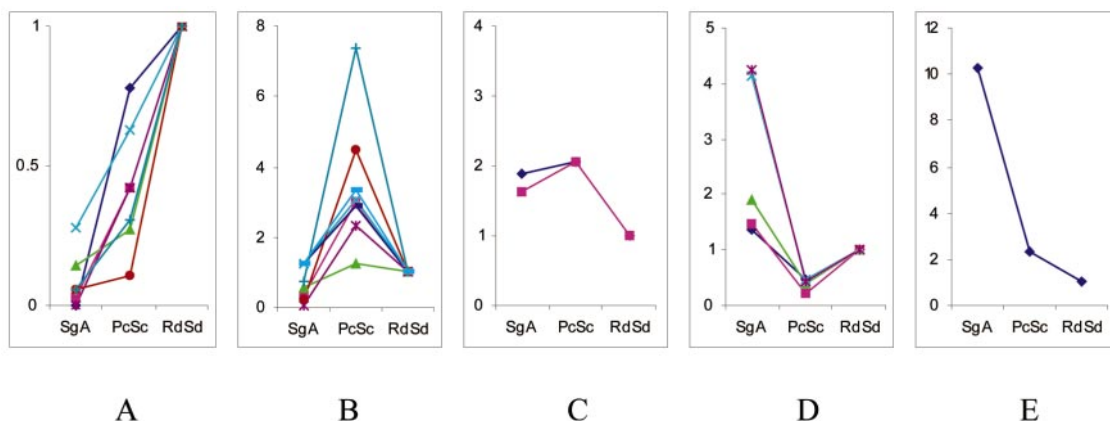
Group I	Gene Description	GenBank ID	Unigene ID	Reported Testis Expression	Biological Function	Fold Change in QPCR			
						SgA	PcSc	RdSd	C _T in RdSd†
Group I	Glucokinase activity, related sequence 1 (<i>Gk-rs1</i>)	A1449806	Mm.32242	Yes‡	Unknown	0	0.78 ± 0.05	1§	24.62 ± 0.50
	<i>Smaf1</i>	A1413624	Mm.28800	Yes	Unknown	0.03 ± 0.004	0.42 ± 0.02	1	27.84 ± 0.11
	Complement receptor 2 (<i>Cr2</i>)	A1451901	Mm.1226	No	Signaling	0.14 ± 0.02	0.27 ± 0.05	1	33.61 ± 0.08
	T-cell receptor CD3 eta chain precursor (<i>Cd3η</i>)	A1429824	Mm.1224	No	Signaling	0.28 ± 0.01	0.63 ± 0.01	1	28.41 ± 0.05
Group II	EST weakly similar to B24264 proline-rich protein MP3 (fragment)	A1415354	Mm.156164	Yes	Unknown	0	0.42 ± 0.01	1	32.55 ± 0.14
	Hypothetical protein MNCb-2040	A1448690	Mm.142843	Yes	Unknown	0.06 ± 0.01	0.11 ± 0.002	1	22.30 ± 0.02
	EST weakly similar to human hypothetical protein FLJ20721	A1451034	Mm.34092	Yes	Unknown	0.06 ± 0.01	0.31 ± 0.01	1	25.68 ± 0.04
	G elongation factor (<i>Gfm</i>)	A1448386	Mm.27288	Yes	Protein biosynthesis	0.33 ± 0.05	3.03 ± 0.07	1	30.99 ± 0.22
	Regulator of G-protein signaling 2 (<i>Rgs2</i>)	A1464441	Mm.28262	Yes	Signaling	0.55 ± 0.05	1.27 ± 0.14	1	35.27 ± 0.28
	Potassium large conductance calcium-activated channel, subfamily M, beta member 4 (<i>Kcrrmb4</i>)	A1447526	Mm.195952	Yes	Signaling	1.22 ± 0.06	3.09 ± 0.07	1	31.97 ± 0.04
	EST weakly similar to 156134 human tumor necrosis factor alpha-induced protein 2	A1414503	Mm.27281	Yes	Unknown	0.05 ± 0.004	2.33 ± 0.05	1	26.91 ± 0.04
	EST weakly similar to myosin heavy chain, nonmuscle type B	A1465389	Mm.200636	Yes	Unknown	0.21 ± 0.02	4.50 ± 0.21	1	31.43 ± 0.12
	EST	A1449427	Mm.223650	No	Unknown	0.76 ± 0.04	7.36 ± 0.21	1	33.84 ± 0.55
	ESTs weakly similar to zinc finger protein 111	A1450088	Mm.30519	No	Unknown	1.25 ± 0.11	2.88 ± 0.14	1	33.13 ± 0.10
Group III	EST highly similar to human nucleoprotein TPR	A1448719	Mm.210529	Yes	Nuclear protein import (?)	1.25 ± 0.09	3.32 ± 0.06	1	27.89 ± 0.15
	Tripartite motif protein 10 (<i>Trim10</i>)	A1662233	Mm.20159	No	Cell differentiation	1.89 ± 0.32	2.07 ± 0.49	1	34.61 ± 0.35
Group IV	EST weakly similar to PHD finger protein 1 (T-complex testis-expressed 3)	A1447598	Mm.21284	Yes	Unknown	1.64 ± 0.21	2.06 ± 0.20	1	31.20 ± 0.09
	Cartilage-associated protein (<i>Crtp</i>)	A1448820	Mm.20904	Yes	Cell differentiation	1.38 ± 0.02	0.48 ± 0.02	1	26.20 ± 0.04
Group V	B-cell receptor-associated protein 31 (<i>Bap31</i>)	A1323595	Mm.17	Yes	Apoptosis	1.49 ± 0.05	0.22 ± 0.01	1	26.72 ± 0.02
	Centrin 2 (<i>Cent2</i>)	A1326150	Mm.24643	Yes	Centriole duplication cytokinesis	1.89 ± 0.18	0.38 ± 0.01	1	31.49 ± 0.06
	Prolyl oligopeptidase (<i>Pop</i>)	A1429335	Mm.6735	Yes	Cell differentiation sperm motility	4.14 ± 0.28	0.47 ± 0.02	1	27.39 ± 0.05
	RIKEN cDNA 1110051B16 EST	A1428924	Mm.23498	No	Unknown	4.24 ± 0.12	0.43 ± 0.07	1	36.99 ± 0.25
		A1426564	No Unigene ID	No	Unknown	10.28 ± 0.89	2.34 ± 0.36	1	36.18 ± 0.57

* QPCR indicates quantitative polymerase chain reaction; SgA, type A spermatogonia; PcSc, pachytene spermatocytes; RdSd, round spermatids; C_T, threshold cycle; and EST, expressed sequence tag.

† C_T values of RdSd when expression level of RdSd is considered to be 1.

‡ Shown to be present in both spermatocytes and spermatids.

§ Expression level in RdSd is set to be 1 for comparison.



The expression patterns of the 23 differentially expressing genes in type A spermatogonia (SgA), pachytene spermatocytes (PcSc), and round spermatids (RdSd). The fold differences in expression level of each gene at the 3 stages of germ cells were compared. The genes were classified into group I through V (A through E, accordingly) as described in the text.

mosome inactivation. This functionally elusive inactivation process takes place in PcSc, which results in a transient repression of gene transcription from the single X chromosome in male germ cells (McCarrey et al, 2002). The down-regulation of *Cetn2* coincides with an up-regulation of its autosomal retrotransposon *Cetn1* that may compensate for its repression and allow completion of meiosis (Hart et al, 1999). This mechanism implies an indispensable role of the *Cetn* family in spermatogenesis. To date no retrotransposon of *Bap31* has been identified. The repression likely reflects a cessation of the need for apoptosis in PcSc. For some X-linked genes the inactivation process persists beyond the pachytene stage, but other X-linked genes would be reactivated in post-meiotic RdSd, for example, *Ube1x* (Odorisio et al, 1996) and *Pgk-1* (Kumari et al, 1996), or activated for the first time at this stage, for example, *Mage* (McCarrey et al, 2002). From our data, both *Bap31* and *Cetn2* became reactivated in postmeiotic RdSd, indicating that their functions were required in this stage.

Only 1 EST was classified into group V. The limited sequence information made us unable to speculate on the function of the encoded protein. This EST reached the highest expression level in SgA but it dropped drastically in PcSc and RdSd, implying that the translated product is important to cellular activities in spermatogonia, possibly related to mitosis, and is less important in the following stages. The full characterization of the ESTs and uncharacterized transcripts identified in the 5 groups should permit speculation about their roles in spermatogenesis.

A search of the GenBank cDNA database identified 7 of the 23 genes not known to be expressed in the testis (Table 6). In addition to the discovery of novel testicular transcripts, the identification of 3 known genes (*Cr2*, *CD3 η* , and *Trim10*) suggests a more general biological function of them in diverse cellular contexts than previ-

ously postulated. Of the known genes identified, a high proportion are implicated to be functional in the process of signaling (4 of 12) and cellular differentiation (3 of 12; Table 6), suggesting these as the major biological activities in spermatogenesis. Nevertheless, the small number of genes identified limits the generality of this speculation. A more detailed study is required to elucidate the biochemistry of male germ cells.

Extensive analysis of gene expression in germ cells is limited by the small number of germ cells at earlier stages of development and the availability of efficient germ cell isolation method. Nevertheless, recent advances in molecular biotechnology have led to multiple studies of testicular gene expression on a larger scale, for example, identification of novel genes at particular stages of spermatogenesis by differential display (Anway et al, 2003), cDNA subtraction (Wang et al, 2001; Fujii et al, 2002), and microarray analysis of testes in mutant animals enriched for specific types of germ cells (Tanaka et al, 2002). Gene profiling experiments were reported on the spermatozoal mRNA profiles of healthy fertile men (Ostermeier et al, 2002) and the pattern of testicular gene expression in neonatal and mature animals (Sha et al, 2002). However, the conclusions that may be drawn from these experiments are limited to single stages of germ cells only. We adopted the use of 3 different developmental stages of germ cells isolated from mice to examine the changes in gene expression patterns during spermatogenesis. Although one may challenge the potential risk of altering the properties, and consequently the gene expression, of germ cells upon isolation because the cells are not maintained in their natural microenvironment, the use of whole testes from animals of different ages would yield results that may be difficult to interpret because the identified transcripts can be contributed by a single or multiple cell types. Also, one cannot eliminate the interference

from other testicular somatic cells whose gene expression patterns can be changing over time.

Just before submission of this manuscript, a report on gene expression profiling of various stages of mouse germ cells with a 1176 cDNA microarray was published (Yu et al, 2003). In that report, radioactive signals of the genes from one stage were compared with those of the neighboring stage to identify differentially expressed genes. From our experience and that of others (Piétu et al, 1996; Eickhoff et al, 1999), radioactive signals generated from membrane-based microarrays are not totally reliable because of various experimental variances. This is illustrated by the low level of concordance between the membrane hybridization and QPCR results in this report. Conclusions based solely on radioactive signals are highly prone to errors. In contrast, we used microarray analysis as a tool to screen for differentially expressed genes. The leads identified were confirmed by QPCR. Only genes showing consistent changes in both experiments were considered to be truly differentially expressed during spermatogenesis. To our knowledge, our report is the most comprehensive comparison of the changes in gene expression patterns during spermatogenesis. In our study, the transcriptomes of germ cells were partially characterized because of the incomplete representation of genes on the microarray. A more detailed analysis by more powerful tools such as SAGE is required to identify the molecular signature of male germ cells. We reported the differential expression patterns of a set of genes and transcripts at different stages of spermatogenesis. The specific gene/transcript expression patterns strongly suggest specialized functions for the encoded products during male germ cell development, and identify targets for manipulation to unravel the molecular mechanism of spermatogenesis.

References

- Anway MD, Li Y, Ravindranath N, Dym M, Griswold MD. Expression of testicular germ cell genes identified by differential display analysis. *J Androl.* 2003;24:173–184.
- Bauer A, McConkey DJ, Howard FD, Clayton LK, Novick D, Koyasu S, Reinherz EL. Differential signal transduction via T-cell receptor CD3 ζ_2 , CD3 ζ - η , and CD3 η_2 isoforms. *Proc Natl Acad Sci USA.* 1991; 88:3842–3846.
- Behrens R, Nolting A, Reimann F, Schwarz M, Waldschütz R, Pongs O. hKCNMB3 and hKCNMB4, cloning and characterization of two members of the large-conductance calcium-activated potassium channel β subunit family. *FEBS Lett.* 2000;474:99–106.
- Breckenridge DG, Nguyen M, Kuppig S, Reth M, Shore GC. The procaspase-8 isoform, procaspase-8L, recruited to the BAP31 complex at the endoplasmic reticulum. *Proc Natl Acad Sci USA.* 2002;99: 4331–4336.
- Brenner R, Jegla TJ, Wickenden A, Liu Y, Aldrich RW. Cloning and functional characterization of novel large conductance calcium-activated potassium channel β subunits, hKCNMB3 and hKCNMB4. *J Biol Chem.* 2000;275:6453–6461.
- Castagnola P, Gennari M, Morello R, Tonachini L, Marin O, Gaggero A, Cancedda R. Cartilage associated protein (CASP) is a novel developmentally regulated chick embryo protein. *J Cell Sci.* 1997;110: 1351–1359.
- Dym M. Spermatogonial stem cells of the testis. *Proc Natl Acad Sci USA.* 1994;91:11287–11289.
- Dym M, Jia M-C, Dirami G, Price M, Rabin SJ, Mocchetti I, Ravindranath N. Expression of *c-kit* receptor and its autophosphorylation in immature rat type A spermatogonia. *Biol Reprod.* 1995;52:8–19.
- Eickhoff B, Korn B, Schick M, Poustka A, van der Bosch J. Normalization of array hybridization experiments in differential gene expression analysis. *Nucleic Acids Res.* 1999;27:e33.
- Fujii T, Tamura K, Masai K, Tanaka H, Nishimune Y, Nojima H. Use of stepwise subtraction to comprehensively isolate mouse genes whose transcription is up-regulated during spermiogenesis. *EMBO Rep.* 2002;3:367–372.
- Gao J, Yu L, Zhang P, Jiang J, Chen J, Peng J, Wei Y, Zhao S. Cloning and characterization of human and mouse mitochondrial elongation factor G, *GFM* and *Gfm*, and mapping of *GFM* to human chromosome 3q25.1-q26.2. *Genomics.* 2001;74:109–114.
- Harada H, Harada Y, O'Brien DP, Rice DS, Naeve CW, Downing JR. HERF1, a novel hematopoiesis-specific RING finger protein, is required for terminal differentiation of erythroid cells. *Mol Cell Biol.* 1999;19:3808–3815.
- Hart PE, Glantz JN, Orth JD, Poynter GM, Salisbury JL. Testis-specific murine centrin, *Cetn1*: genomic characterization and evidence for repositioning of a gene encoding a centrosome protein. *Genomics.* 1999; 60:111–120.
- Imagawa M, Tsuchiya T, Nishihara T. Identification of inducible genes at the early stage of adipocyte differentiation of 3T3-L1 cells. *Biochem Biophys Res Commun.* 1999;254:299–305.
- Kehrl JH, Sinnarajah S. RGS2: a multifunctional regulator of G-protein signaling. *Int J Biochem Cell Biol.* 2002;34:432–438.
- Kimura A, Matsui H, Takahashi T. Expression and localization of prolyl oligopeptidase in mouse testis and its possible involvement in sperm motility. *Zool Sci.* 2002;19:93–102.
- Kimura A, Yoshida I, Takagi N, Takahashi T. Structure and localization of the mouse prolyl oligopeptidase gene. *J Biol Chem.* 1999;274: 24047–24053.
- Kumari M, Stroud JC, Anji A, McCarrey JR. Differential appearance of DNase I-hypersensitive sites correlates with differential transcription of *Pgk* genes during spermatogenesis in the mouse. *J Biol Chem.* 1996;271:14390–14397.
- Malissen M, Gillet A, Rocha B, et al. T cell development in mice lacking the CD3- ζ/η gene. *EMBO J.* 1993;12:4347–4355.
- Marrion NV, Tavalin SJ. Selective activation of Ca²⁺-activated K⁺ channels by co-localized Ca²⁺ channels in hippocampal neurons. *Nature.* 1998;395:900–905.
- McCarrey JR, Watson C, Atencio J, Ostermeier GC, Marahrens Y, Jaenisch R, Krawetz SA. X-chromosome inactivation during spermatogenesis is regulated by an *Xist/Tsix*-independent mechanism in the mouse. *Genesis.* 2002;34:257–266.
- Ng FWH, Nguyen M, Kwan T, Branton PE, Nicholson DW, Cromlish JA, Shore GC. p28 Bap31, a Bcl-2/Bcl-X_L- and procaspase-8-associated protein in the endoplasmic reticulum. *J Cell Biol.* 1997;139: 327–338.
- Nguyen M, Breckenridge DG, Ducret A, Shore GC. Caspase-resistant BAP31 inhibits Fas-mediated apoptotic membrane fragmentation and release of cytochrome *c* from mitochondria. *Mol Cell Biol.* 2000;20: 6731–6740.
- Odoriso T, Mahadevaiah SK, McCarrey JR, Burgoyne PS. Transcriptional analysis of the candidate spermatogenesis gene *Ube1y* and of the closely related *Ube1x* shows that they are coexpressed in spermatogenesis.

- gonia and spermatids but are repressed in pachytene spermatocytes. *Dev Biol.* 1996;180:336–343.
- Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA. Spermatozoal RNA profiles of normal fertile men. *Lancet.* 2002;360:772–777.
- Pan Y, Decker WK, Huq AHHM, Craigen WJ. Retrotransposition of glycerol kinase-related genes from the chromosome to autosomes: functional and evolutionary aspects. *Genomics.* 1999;59:282–290.
- Piétu G, Alibert O, Guichard V, et al. Novel gene transcripts preferentially expressed in human muscles revealed by quantitative hybridization of a high density cDNA array. *Genome Res.* 1996;6:492–503.
- Prechl J and Erdei A. Immunomodulatory functions of murine CR1/2. *Immunopharmacology.* 2000;49:117–124.
- Salisbury JL, Suino KM, Busby R, Springett M. Centrin-2 is required for centriole duplication in mammalian cells. *Curr Biol.* 2002;12:1287–1292.
- Sha J, Zhou Z, Li J, et al. Identification of testis development and spermatogenesis-related genes in human and mouse testes using cDNA arrays. *Mol Hum Reprod.* 2002;8:511–517.
- Su YA, Bittner ML, Chen Y, Tao L, Jiang Y, Zhang Y, Stephan DA, Trent JM. Identification of tumor-suppressor genes using human melanoma cell lines UACC903, UACC903(+6), and SRS3 by comparison of expression profiles. *Mol Carcinog.* 2000;28:119–127.
- Tanaka K, Tamura H, Tanaka H, Katoh M, Futamata Y, Seki N, Nishimune Y, Hara T. Spermatogonia-dependent expression of testicular genes in mice. *Dev Biol.* 2002;246:466–479.
- Wang PJ, McCarrey JR, Yang F, Page DC. An abundance of X-linked genes expressed in spermatogonia. *Nat Genet.* 2001;27:422–426.
- Yu Z, Guo R, Ge Y, et al. Gene expression profiles in different stages of mouse spermatogenic cells during spermatogenesis. *Biol. Reprod.* 2003;69:37–47.
- Zhang L, Zhou W, Velculescu VE, Kern SE, Hruban RH, Hamilton SR, Vogelstein B, Kinzler KW. Gene expression profiles in normal and cancer cells. *Science.* 1997;276:1268–1272.