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Analysis of the gene expression profile of mouse male meiotic germ cells ☆

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Abstract

Wide genome analysis of difference in gene expression between spermatogonial populations from 7-day-old mice and pachytene spermatocytes from 18-day-old mice was performed using Affymetrix gene chips representing ~12,500 mouse known genes or EST sequences, spanning approximately 1/3rd of the mouse genome. To delineate differences in the profile of gene expression between mitotic and meiotic stages of male germ cell differentiation, expressed genes were grouped in functional clusters. The analysis confirmed the previously described pre-meiotic or meiotic expression for several genes, in particular for those involved in the regulation of the mitotic and meiotic cell cycle, and for those whose transcripts are accumulated during the meiotic stages to be translated later in post-meiotic stages. Differential expression of several additional genes was discovered. In few cases (pro-apoptotic factors *Bak*, *Bad* and *Bax*), data were in conflict with the previously published stage-dependent expression of genes already known to be expressed in male germ cells. Northern blot analysis of selected genes confirmed the results obtained with the microarray chips. Six of these were novel genes specifically expressed in pachytene spermatocytes: a chromatin remodeling factor (*chrac1/YCL1*), a homeobox gene (*hmx1*), a novel G-coupled receptor for an unknown ligand (*Gpr19*), a glycoprotein of the intestinal epithelium (*mucin 3*), a novel RAS activator (*Ranbp9*), and the *A630056B21Rik* gene (predicted to encode a novel zinc finger protein). These studies will help to delineate the global patterns of gene expression characterizing male germ cell differentiation for a better understanding of regulation of spermatogenesis in mammals.

Keywords: DNA microarray; Transcriptome analysis; Spermatogenesis; Spermatocytes; Spermatogonia; Meiosis

1. Results and discussion

Spermatogenesis is characterized by a mitotic (spermatogonia), a meiotic (spermatocytes) and a differentiative haploid (spermatids) phase. The dissection of the mechanisms that regulate the mitotic and meiotic cell cycles in mammalian germ cells is useful for a better understanding of the molecular requirements for spermatogenesis to occur, and thus for the understanding of male sterility, which is often based on lack of spermatogonial divisions or meiotic blocks. Spermatogonia actively proliferate under the control of growth factors released by somatic cells of the seminiferous epithelium, such as Bone morphogenetic protein 4 (Bmp4), which stimulates the Alk3 receptor expressed in spermatogonial stem cells (Pellegrini et al., 2003), and Kit Ligand (also called Stem Cell Factor), which activates the c-kit receptor expressed in differentiating spermatogonia (Sorrentino et al., 1991; Yoshinaga et al., 1991; Rossi et al., 1993; Schrans-Stassen et al., 1999; Dolci et al., 2001).

Few informations are available on the control of the differentiation of spermatogonia into spermatocytes, i.e. their entrance into the meiotic cell cycle, which is characterized by two cell divisions and genetic exchange (crossing-over) between homologous chromosomes, and produce four haploid spermatids from each diploid progenitor cell (Roeder, 1997). Insights into the molecular mechanisms of the progression to the metaphase of the first meiotic division have been obtained by treatment of

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cultured spermatocytes with okadaic acid (OA), which overcomes normal checkpoints that ensure in vivo the slow progression of the meiotic prophase. OA triggers the sequential activation of ERK1, p90Rsk2 and Nek2, thus leading to chromosome condensation and progression to metaphase with the concurrent activation of the cyclin B/cdk1 complex (Sette et al., 1999; Di Agostino et al., 2002). The spermatids that result at the end of meiosis will then undergo spermiogenesis with the final production of mature spermatozoa. The aim of our work was to obtain a general profile of the expression pattern of genes specifically involved in the transition from the mitotic to the meiotic cell cycle and in the progression through the meiotic cell cycle.

DNA microarrays can be used to measure the expression patterns of thousands of genes in parallel, allowing to monitor changes in gene expression occurring during developmental events (Schena, 1996). Analysis of the results obtained with the microarray technique allows the clustering of expressed genes in functional classes. We used this approach in order to identify genes specifically involved in the meiotic program and to group these genes in clusters that should give more informations about the molecular interactions required for this peculiar type of cell cycle in mammals.

We prepared complementary RNAs from two germ cell types at different developmental stages purified from testes of pre-puberal mice, spermatogonia from 7-day-old mice and spermatocytes from 18-day-old mice. The spermatogonial population obtained from 7-day-old mice (type A and B spermatogonia) is contaminated by 10% of somatic cells, whereas germ cells in the meiotic prophase are absent (Dolci et al., 2001; Pellegrini et al., 2003; see also Section 2). Spermatocytes obtained after elutriation from 18-day-old mice are in the middle–late pachytene stage of the first meiotic prophase (85%) and in the leptotene–zygotene stage (10%). Contamination of spermatogonia and somatic cells in the meiotic cell population is less than 5%, whereas round spermatids, which are a common contaminant of pachytene fractions when using adult testis (Sette et al., 1999; Di Agostino et al., 2002), are absent, not being present in the immature testis used. The cRNAs prepared from the two different cell populations have been hybridized with commercially available MG-U74Av2 GeneChip probe arrays (Affymetrix Inc.), containing ~12,500 known mouse genes or EST sequences, and thus spanning approximately 1/3rd of the mouse genome. The analysis was performed on duplicate chip arrays, using cRNAs from two different cell preparations. The results of array data and the comparative analysis were very similar in the duplicate experiments (Fig. 1) and are available in the supplemental data. The same data are also available online at the addresses http://www2.uniroma2.it/ricerca/ce/absolutevaluestot.htm and http://www2.uniroma2.it/ricerca/ce/comparativeanalysistot.htm. The files named 'Absolute Values' contain filtered raw data of the absolute analysis. Each of the five files contains data relative to ~1/5th of the targets represented in the Affymetrix MG-U74Av2 array. In the first column, the Affymetrix identification number of the target oligonucleotide probe pairs is indicated. Signal is a numeric value measuring the abundance of a transcript revealed by the duplicate arrays (C1 and C2: spermatocytes; G1 and G2: spermatogonia). Detection indicates whether a transcript is present (P), marginal (M), or absent (A) according to statistical analysis. Detection *P*-value indicates the significance level of detection call (P: *P*-value < 0.04; M: *P*-value between 0.04 and 0.06; A: *P*-value > 0.06). Descriptions contain the Affymetrix informations about the target gene. More informations about the target genes (especially those corresponding to EST sequences) can be found with the Interacting Query online facility at www.affymetrix.com. The files named: 'Comparative Analysis' contain comparison data between paired samples of spermatocytes (C2–C1), spermatogonia (G2–G1), and between the two different cell populations in the duplicate arrays (G1–C1 and G2–C2). Signal log ratio indicates the change expression level for a transcript between the compared samples, and corresponds to the base 2 logarithm of the fold difference (for instance, a signal log ratio of 3, or of -3, indicates that the transcript corresponding to the target gene is 8-fold more abundant in the first or the second, respectively, of the two compared samples). Signal log ratio low/high represent the lower and upper limit of signal log ratio within a 95% of confidence interval. Change indicates whether the target gene expression is increased (I), marginally increased (MI), not changed (NC), marginally decreased (MD) or decreased (D) in the first vs. the second sample according to statistical analysis. Change in *P*-value indicates the significance level of change call (I: *P*-value < 0.0025; MI: *P*-value between 0.0025 and 0.003; NC: *P*-value between 0.003 and 0.997; MD: *P*-value between 0.0997 and 0.9975; D: *P*-value > 0.9975).

A positive signal (detection parameter: P in both samples) for ~3500 target sequences was obtained in spermatocytes, vs. ~5500 in spermatogonia. Comparative analysis identified ~2000 target sequences with a signal significantly higher in spermatogonia (change parameter: I in both samples), and ~700 target sequences with a signal significantly higher in spermatocytes (change parameter: D in both samples).

Between most of the targets that gave a higher signal in spermatogonia, the selective expression in this cell population was already known from published data. A limited and representative list of such genes is shown in Table 1. We ordered these genes according to the average Signal Log Ratio parameter, which was converted in average fold difference of the signal in spermatogonia vs. spermatocytes. We also considered whether the target, besides giving a higher signal in spermatogonia, gave a positive (detection parameter: P) or negative (detection parameter: A) signal in spermatocytes. The calculation

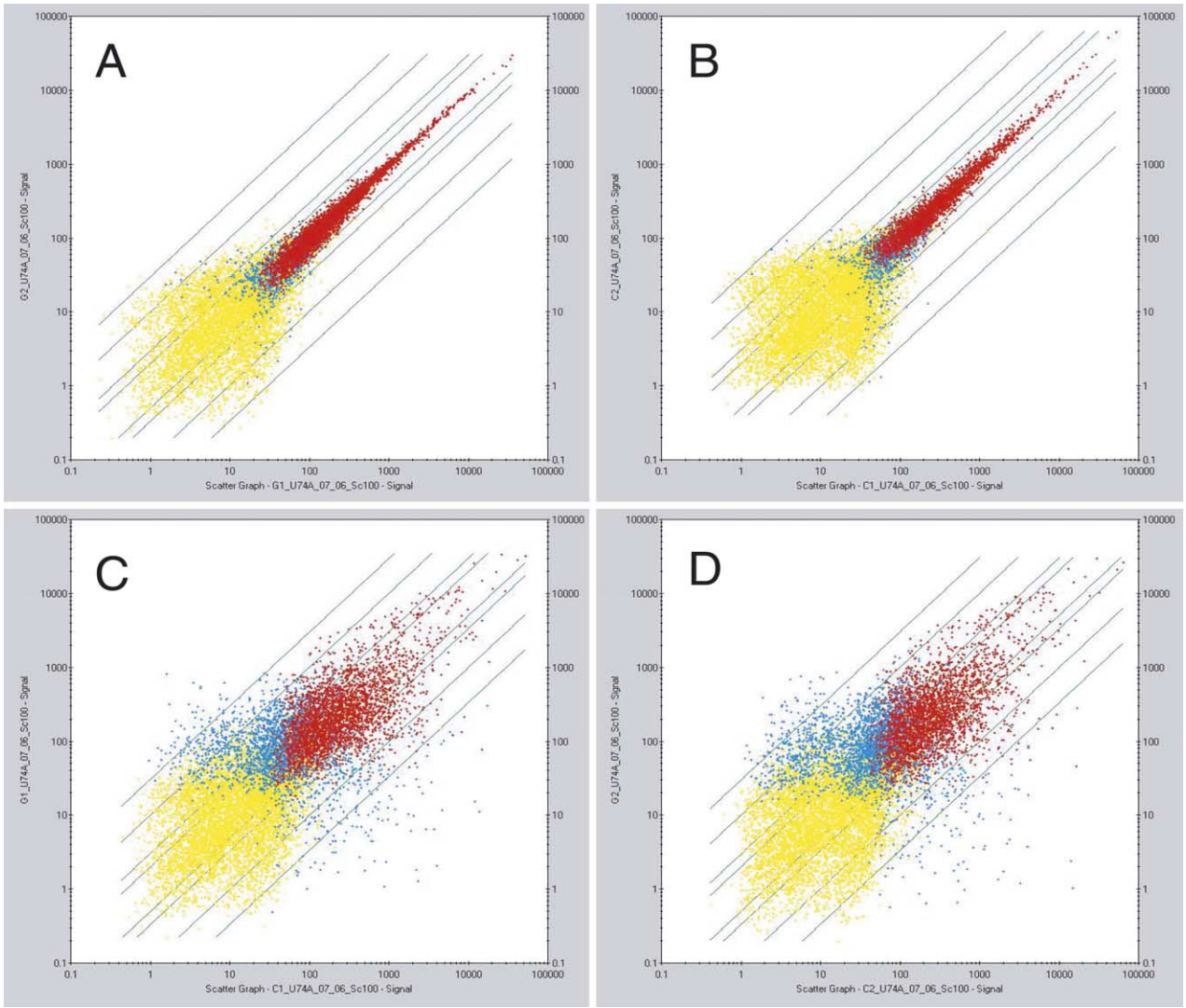

Fig. 1. Analysis of the homogeneity of microarray generated signals between duplicate samples of spermatogonia and spermatocyte cRNA probes, and comparative analysis of the divergence between spermatogonia and spermatocytes. Data represent scatter plots of (A) spermatogonia sample 1 intensities (G1) vs. spermatogonia sample 2 intensities (G2); (B) spermatocytes sample 1 (C1) vs. spermatocytes sample 2 (C2); (C) spermatogonia sample 1 (G1) vs. spermatocytes sample 1 (C1); (D) spermatogonia sample 2 (G2) vs. spermatocytes sample 2 (C2). In these scatter plots, each spot corresponds to the signal generated by a discrete Affymetrix target gene, each represented on the chip arrays by 16 specific 25mer oligonucleotide probes and by 16 one-mismatch probes. In A and in B the data fit a straight line with slope approximately equal to one and intercept near zero, demonstrating high reproducibility of the results. In C and D, the enlargement of spot distribution is very similar in both comparative analysis, and allows to define statistically significant difference in selective gene expression between the two cell populations. Red dots represent genes significantly expressed in both samples (P-value < 0.04). Blue dots genes expressed significantly only in one sample, and yellow dots genes not expressed (P-value > 0.06) in both samples. The P-values are calculated as described in the Statistical Algorithms Reference Guide by Affymetrix (see Section 2).

of the fold difference of the signal between the two cell populations does not take into account whether the target gene is significantly expressed or not in spermatocytes. It should be noticed that we found no X-linked genes whose expression was higher in spermatocytes with respect to spermatogonia, in agreement with the notion of the inactivation of the X chromosome during the first meiotic prophase (Kelly, 1987). The *Ott* (ovary–testis transcribed) gene, a member of a mouse X-linked multigene family, was found to be expressed at very high levels in spermatogonia, whereas no significant expression was detected in spermatocytes, even though, on the basis of the observation that it was not expressed in the testes of adult sex-reversed mice lacking germ cells, it was previously reported to be expressed specifically during meiosis (Kerr et al., 1996). Thus, it appears that this gene might play a role, if any, only in the pre-meiotic stages of differentiation. On the other hand, we confirmed the specific and strong pre-meiotic expression of *Stra8* (stimulated by retinoic acid gene 8) (Oulad-Abdelghani et al., 1996), and of *Atm* (ataxia

Table 1
Examples of genes selectively expressed in spermatogonia

MG-U74Av2 target Affymetrix	Gene name	Gene symbol	Notes	Fold difference (spermatogonia vs. spermatocytes)
92306	Ovary–testis transcribed	Ott	Absent in spermatocytes. X-linked gene, previously defined as 'meiosis specific' (Kerr et al., 1996)	104
101194	Stimulated by retinoic acid gene 8	Stra8	Absent in spermatocytes. Pre-meiotic germ cell-specific cytoplasmic protein encoded by Stra8, a retinoic acid-responsive gene (Oulad-Abdelghani et al., 1996)	50
101180	Ataxia telangiectasia mutated homolog	Atm	Absent in spermatocytes. Protein kinase involved in DNA repair and DNA damage response (induction of apoptosis by DNA damage). In knock-out mice gametogenesis is severely disrupted as early as leptonema of prophase I (Barlow et al., 1998)	15
93536	Bcl2-associated X protein	Bax	Absent in spermatocytes. Pro-apoptotic factor (Yan et al., 2000)	9
99956	Stem cell factor receptor (KL receptor)	c-kit	Absent in spermatocytes. Transmembrane tyrosine kinase receptor (Sorrentino et al., 1991; Yoshinaga et al., 1991; Schrans-Stassen et al., 1999). Essential for pre-meiotic spermatogenesis (Rossi et al., 1993; Kissel et al., 2000; Blume-Jensen et al., 2000; Dolci et al., 2001)	7
102963	E2F transcription factor 1	E2f1	Absent in spermatocytes. Transcription factor crucial for mitotic cell cycle control (Dolci et al., 2001)	6
103207	DNA polymerase α 1, 180 kDa	Pola1	Absent in spermatocytes. Dna replication (Orlando et al., 1989)	6
94448	B-cell leukemia/lymphoma 10	Bcl10	Present in spermatocytes. Pro-apoptotic factor	6
160159	Cyclin B1	Ccnb1	Absent in spermatocytes. Subunit of cdc2/cdk1, essential for G2/M transition	5
103057	DNA polymerase δ 1, catalytic domain	Pold1	Absent in spermatocytes. Dna replication	4
92767	Bone morphogenetic protein receptor, type 1A	Bmpr1a, ALK3	Absent in spermatocytes. Bone morphogenetic receptor for Bmp2 and Bmp4. Involved in spermatogonial differentiation (Pellegrini et al., 2003)	4
96772	DNA primase, p49 subunit	Prim1	Absent in spermatocytes. Dna replication (Orlando et al., 1989)	4
95471	Cyclin-dependent kinase inhibitor 1C (P57)	p57	Absent in spermatocytes. Cdk2 inhibitor	4
98067	Cyclin-dependent kinase inhibitor 1A (P21)	p21	Absent in spermatocytes. Cdk2 inhibitor	3
103064	Checkpoint kinase 1 homolog	Chk1, Chek1, rad27	Absent in spermatocytes. Protein kinase, which is required for the DNA damage checkpoint. In response to DNA damage, Chk1 phosphorylates and inhibits Cdc25C, thus preventing activation of the Cdc2–cyclin B complex and mitotic entry	3
160538	D-type G1 cyclin catalytic subunit (PSK-J3/CDK4)	Cdk4	Present in spermatocytes. Cell cycle kinase activated and essential for G1/S transition (Dolci et al., 2001)	2
160545	Cyclin D3	Ccnd3	Absent in spermatocytes. Cell cycle control in G1/S Phase (Dolci et al., 2001)	2
104154	Transformation related protein 53	p53	Absent in spermatocytes (marginal in one sample). Apoptosis inducer and cell cycle control in G1/S Phase	2
92481	Checkpoint kinase 2 homolog	Chk2, Chek2, rad53	Absent in spermatocytes (marginal in one sample). ATM-dependent. Function similar to that of Chk1	2
99186	Cyclin A2	Ccna2	Absent in spermatocytes. Mitotic cyclin, active in the S phase, cdk2 subunit (Dolci et al., 2001)	2

teleangectasia mutated homolog) (Barlow et al., 1998). Between growth factor receptors, *c-kit* (encoding the KL receptor) and *Alk3* (encoding the Bmp4 receptor) were confirmed to be expressed in pre-meiotic stages, and not in spermatocytes (Sorrentino et al., 1991; Yoshinaga et al., 1991; Schrans-Stassen et al., 1999; Pellegrini et al., 2003).

As expected, many of the genes selectively expressed in spermatogonia and not in spermatocytes encode proteins involved in the regulation of the mitotic cell cycle (transcription factor E2f1, cyclin-dependent-kinase-inhibitors p57 and p21, cyclin D3, cyclin B1, cyclin A2, cyclin-dependent-kinase 4), and replicative DNA synthesis (DNA

polymerase α, DNA polymerase δ, DNA primase), generally confirming previously published data (Dolci et al., 2001; Orlando et al., 1989). Between genes encoding proteins involved in pro-apoptotic programs, *p53* and *Bax* were found to be selectively expressed in spermatogonia. The lack of *Bax* expression in spermatocytes, according to the microarray statistical analysis, is in partial conflict with previously published data (Yan et al., 2000, see below). *Bcl10* was found to be expressed also in spermatocytes, even though at a much lower level.

As for the targets that gave a higher signal in spermatocytes, we tried to classify them in a series of functional clusters: apoptosis/cell-cycle, chromatin/transcription, cytoskeleton/traffic, meiosis/spermatogenesis,

Table 2
Genes expressed in spermatocytes: apoptosis/cell cycle

MG-U74Av2 target Affymetrix	Gene name	Gene symbol	Notes	Fold difference (spermatocytes vs. spermatogonia)
160644	BCL2-antagonist/killer 1	Bak1	Absent in spermatogonia. Involved in apoptosis; caspase activation via cytochrome c (Yan et al., 2000)	45
92911	Cyclin A1	Ccna1	Absent in spermatogonia. In knock-out mice, meiotic arrest during meiotic divisions (Liu et al., 1998)	23
103094	Small EDRK-rich factor 1	Serf1	Present in spermatogonia. (Survival of Motoneuron in SMA1)	18
99522	Germ cell-specific gene 2	Gsg2	Absent in spermatogonia (marginal in one sample). Atypical serine–threonine kinase, named Haspin (for haploid germ cell-specific nuclear protein kinase)	17
160761	Upregulated during skeletal muscle growth 4	Usmg4	Absent in spermatogonia	11
100054	DNA segment Chr2	D2Wsu81e	Present in spermatogonia. Endonuclease G: a mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation	10
92929	Cytochrome c, testis	Cyct	Present in spermatogonia. Null mice produce functional sperm but undergo early testicular atrophy (Narisawa et al., 2002)	10
94971	KAP1, Cdk inhibiting phosphatase	Cdkn3	Present in spermatogonia. cdk2-associated dual specificity phosphatase	9
94521	Cyclin-dependent kinase inhibitor 2D	Cdkn2d, Ink4d, p19	Absent in spermatogonia. Selective cdk4/6 inhibitor. Double p19 and p18 (Ink4c) knock-out provokes sterility due to a delayed exit of spermatogonia from the mitotic cell cycle (Zindy et al., 2001)	9
101885	Growth arrest specific 5	Gas5	Absent in spermatogonia. Preferentially expressed in the growth phase arrest of the cell cycle	6
160638	Cyclin-dependent kinase inhibitor 2C	Cdkn2c, Ink4c, p18	Absent in spermatogonia. Selective cdk4/6 inhibitor. Double p19 (Ink4d) and p18 knock-out provokes sterility due to a delayed exit of spermatogonia from the mitotic cell cycle (Zindy et al., 2001)	6
99670	Bcl-associated death promoter	Bad	Absent in spermatogonia. Pro-apoptotic factor (Yan et al., 2000)	5
92902	Myeloblastosis oncogene-like 1	Mybl1, A-Myb	Present in one sample in spermatogonia. Transcription factor. Knockout male mice are sterile due to arrest in pachytene (Toscani et al., 1997)	5
98945	SH3-domain GRB2-like B1 (endophilin)	Sh3glb1	Present in spermatogonia. Synaptically enriched protein implicated in synaptic vesicle endocytosis. Might be involved in apoptotic programs since it interacts with Bax	4
92879	Protein phosphatase 1G, γ isoform	Ppm1g, PP2C-γ	Present in spermatogonia. Formerly called protein phosphatase 2C. Magnesium-dependent serine–threonine phosphatase, known to be expressed in the testis and skeletal muscle	4
94294	Cyclin B2	Ccnb2	Present in spermatogonia. Interacts with cdc2 (cdk1) as a subunit. Component of MPF (Dolci et al., 2001)	4
104738	Zuotin related factor 2	Zrf2	Present in spermatogonia. A ribosome-associated DnaJ molecular chaperone. Also called MIDA-1, associates with Id HLH transcription factors	3
102734	Baculoviral IAP repeat-containing 3	Birc3, mIAP-2	Present in spermatogonia. Also called Apoptosis inhibitor 2. Caspase inhibitor	3
104476	Retinoblastoma-like 1 (p107)	Rbl1	Absent in spermatogonia (marginal in one sample). Homolog of pRb, involved in negative regulation of the cell cycle	2
101521	Baculoviral IAP repeat-containing 5	Birc5, TIAP	Present in spermatogonia. Homologous to human survivin. Caspase inhibitor	2

membrane-bound-proteins/receptors, metabolism, RNA binding proteins, signal-transduction/protein-kinases (Tables 2–9). Also in this case, we ordered these genes according to the average Signal Log Ratio parameter, which was converted in average fold difference of the signal in spermatocytes vs. spermatogonia. We also considered whether the target, beside giving a higher signal in spermatocytes, gave a positive (detection parameter: P) or negative (detection parameter: A) signal in spermatogonia, and the calculation of the fold difference of the signal between the two cell populations does not take into account whether the target gene is significantly expressed or not in spermatogonia.

For the large majority of these targets, detection of a high signal in spermatocytes by the microarray analysis confirmed data that are available in published literature or in

Table 3
Genes expressed in spermatocytes: chromatin/transcription

MG-U74Av2 target Affymetrix	Gene name	Gene symbol	Notes	Fold difference (spermatocytes vs. spermatogonia)
160599	Testis-specific gene A2	Tsga2	Absent in spermatogonia. Male meiotic metaphase chromosome-associated acidic protein	56
102795	Mesoderm posterior 1	Mesp1	Absent in spermatogonia. HLH protein	42
161064	PHD finger protein 7	Phf7	Present in spermatogonia. Isolated from a mouse testis cDNA library	42
102079	Mus musculus Aip1	Aip1, Aym1	Absent in spermatogonia. IME-1 functional homolog (Personal communication from Jeremy Don, Bar-Ilan University, Ramat Gan, Israel)	40
104622	Transcription elongation factor A (SII), 2	Tcea2	Absent in spermatogonia	34
97745	Homeo box A4	Hoxa4	Absent in spermatogonia	29
95755	Cold shock domain protein A	Csda	Present in spermatogonia	25
92190	Nuclear receptor subfamily 2, group C, member 1	Nr2c1	Absent in spermatogonia	22
93182	Glial and testis-specific homeobox gene	Nkx6-2, Gtx	Absent in spermatogonia. Murine homeobox-containing gene, expressed specifically in glial cells of the brain and germ cells of testis. Knock-out mice are viable and fertile (Cai et al., 2001)	17
102219	Regulatory factor X, 2	Rfx2	Absent in spermatogonia. Influences HLA class II expression	16
99987	RIKEN cDNA A630056B21 gene	A630056B21Rik	Absent in spermatogonia. Weakly similar to zinc finger protein 2 (Zfp2) (mKR2 protein)	15
98414	Zinc finger protein 42	Zfp42	Present in one sample in spermatogonia. Expressed also in embryonic stem cells	14
160204	RIKEN cDNA 3110013H01 gene	3110013H01Rik	Present in spermatogonia. Nuclear protein p30, a protein of the nuclear pore complex	11
93221	RIKEN cDNA 4921540P06 gene (Homeo box D8)	4921540P06Rik	Absent in spermatogonia. Other names: Hox-4.3 140, HOXD8, Hox5.4	11
100126	Chromatin accessibility complex 1	Chrac1	Present in spermatogonia. NF-YC-like protein. Also called YCL1 (Bolognese et al., 2000)	11
160068	Sin3 associated polypeptide, 30 kDa	Sap30	Present in spermatogonia. Component of a histone deacetylase complex	10
97893	TATA box binding protein-like protein	Tlp	Present in spermatogonia. Also named TLF, TRF2 or TBPL1. Knockout mice arrest at spermiogenesis (Martianov et al., 2001)	9
92432	Zinc finger protein 93	Zfp93	Absent in spermatogonia	9
104604	Zinc finger protein 96	Zfp96	Present in one sample in spermatogonia	6
103629	Lymphoid enhancer binding factor 1	Lef1	Absent in spermatogonia	6
96144	Inhibitor of DNA binding 4	Idb4	Absent in spermatogonia. Id4, dominant negative helix-loop-helix protein	6
92195	CCAAT/enhancer binding protein (C/EBP), γ	Cebpg	Present in spermatogonia	6
94406	Putative homeodomain transcription factor	Phtf	Present in spermatogonia	5
98032	Zinc finger protein 35	Zfp35	Present in spermatogonia	5
160220	Zinc finger protein 110	Zfp110	Absent in spermatogonia	4
94102	H6 homeo box 1	Hmx1	Absent in spermatogonia (Yoshiura et al., 1998)	4

Table 4
Genes expressed in spermatocytes: cytoskeleton/traffic

MG-U74Av2 target Affymetrix	Gene name	Gene symbol	Notes	Fold difference (spermatocytes vs.spermatogonia)
99995	Centrin 1	Cetn1	Absent in spermatogonia. Also called caltractin. Testis-specific centrosomal protein encoded by an intronless retroposon	588
101864	Actin-like 7b	Actl7b	Absent in spermatogonia. Testis-specific actin isoform, encoded by an intronless gene	76
161035	Kinesin family member 9	Kif9	Absent in spermatogonia. Microtubule motor associated protein abundantly expressed in the testis	58
160631	Sarcoglycan, α (50 kDa dystrophin-associated glycoprotein)	Sgca, adhalin	Absent in spermatogonia. Integral plasma membrane protein considered specifically expressed in striated muscle	36
99531	Synaptogyrin 4	Syngr4	Absent in spermatogonia. Integral membrane protein present in synaptic vesicles	36
101195	Myosin light chain 2	Mylc2pl	Absent in spermatogonia. Considered specifically expressed in precursor B and T lymphocytes	17
92496	Vesicle-associated membrane protein 5	Vamp5	Absent in spermatogonia. Also called synaptobrevin. Expressed during myogenesis in striated muscles	15
101520	RIKEN cDNA 1700062C23 gene	1700062C23Rik	Absent in spermatogonia. Kinesin-related protein HASH. Rat homolog known to be expressed during spermatogenesis in meiotic cells	10
160487	Myosin light chain, alkali, cardiac atria	myla	Absent in spermatogonia. Expressed during striated muscle development	9
103684	Tektin-2	Tekt2	Absent in spermatogonia. A sperm flagellar protein also called tektin-t and different from tektin-1	8
94321	Keratin complex 1, acidic, gene 10	Krt1-10	Absent in spermatogonia (marginal in one sample). Protein of intermediate filaments	4
95097	ARP10 actin-related protein 10 homolog	Actr10	Present in spermatogonia. Protein of the dynactin complex	4
93567	Profilin 2	Pfn2	Present in spermatogonia. Actin binding ubiquitous protein	4
102732	Talin	tln	Present in spermatogonia. Integrin and actin binding protein	4
93499	Capping protein α 1	cappa1	Present in spermatogonia. Actin binding protein	4
94248	Adaptor-related protein complex AP-1, μ subunit 1	Ap1m1	Present in spermatogonia. Adaptor protein of clathrin-coated vesicles involved in intracellular protein transport and endocytosis	4
104565	Adaptor-related protein complex AP-4, sigma 1	Ap4s1	Present in spermatogonia. Adaptor protein of clathrin-coated vesicles involved in intracellular protein transport and endocytosis	3
92643	Neurofibromatosis 2	Nf2	Present in spermatogonia. Tumor suppressor protein involved in mediating interactions between the plasma membrane and the cytoskeleton	3
93333	Tubulin cofactor a	Tbca	Present in spermatogonia. Molecular chaperonin involved in tubulin folding	2
103878	Adaptor-related protein complex AP-3, β 1 subunit	Ap3b1	Present in spermatogonia. Adaptor protein of clathrin-coated vesicles involved in intracellular protein transport and endocytosis	2

expression databases, indicating that our analysis faithfully reflected the actual differences in the pattern of gene expression between male mitotic and meiotic germ cells. Many of these genes encode proteins specifically involved in the control of the meiotic cell cycle, such as cyclin A1 (Table 2) (Liu et al., 1998), cdk4-inhibitors p18 and p19 (Table 2) (Zindy et al., 2001), A-myb (Table 2) (Toscani et al., 1997), Nek2 (Table 9) (Di Agostino et al., 2002), but in many cases their expression reflects meiotic accumulation of transcripts destined to be translated later during spermiogenesis, such as testis-specific lactate dehydrogenase (Table 7) (Li et al., 1998), testis-specific poly(A) polymerase β (Table 8) Kashiwabara et al., 2002), calmegin (Table 9) (Ikawa et al., 1997), preproacrosin (Table 5) (Kremling et al., 1991), fertilin β (Table 5) (Cho et al., 1998), Trf2 (Table 3) (Martianov et al., 2001), MSJ-1 (Table 5) (Berruti and Martegani, 2001), Tpx1 (Table 5) (Kasahara et al., 1989), Tekt1 (Table 5) (Larsson et al., 2000), Tesp1 (Table 5) (Kohno et al., 1998) and so on. It is noteworthy that the spermatocyte-specific expression of a large number of genes encoding enzymes is involved in glycolysis and gluconeogenesis, beside that of *Pgk2*, encoding a well known meiotic isoform of phosphoglycerate kinase (Boer et al., 1987) (Table 7). Thus, metabolic pathways distinct

Table 5
Genes expressed in spermatocytes: meiosis/spermatogenesis

MG-U74Av2 target Affymetrix	Gene name	Gene symbol	Notes	Fold difference (spermatocytes vs. spermatogonia)
160219	Meiosis expressed gene 1	Meg1	Absent in spermatogonia (Don and Wolgemuth, 1992)	653
94927	Fatty acid binding protein 9	Fabp9, Perf 15	Absent in spermatogonia. Perforatorial protein in the perinuclear theca of spermatozoa	388
92825	Testis-specific gene 1	Tpx1	Absent in spermatogonia (Kasahara et al., 1989)	349
100526	A disintegrin and metalloprotease domain 3	Adam3	Absent in spermatogonia. Also known as cyritestin	326
92732	A disintegrin and metalloprotease domain 2	Adam2	Absent in spermatogonia. Also called Fertilin β. Knock-out mice are sterile for failure of sperm–egg or sperm–oviduct interactions (Cho et al., 1998)	256
103058	T-complex protein 10b	Tcp10b	Absent in spermatogonia	238
99545	Tektin 1	Tekt1	Absent in spermatogonia. Sperm axonemal protein (Larsson et al., 2000)	187
100359	T-complex protein-10	Tcp10	Absent in spermatogonia	168
99134	T-complex-associated testis expressed 3	Tcte3	Present in spermatogonia	163
93955	Zona-pellucida-binding protein	Zpbp, sp38	Absent in spermatogonia	157
99474	A disintegrin and metalloprotease domain 5	Adam5	Absent in spermatogonia	147
160506	A kinase anchoring protein-associated sperm protein	Akapasp	Absent in spermatogonia	133
100358	T-complex protein 10a	Tcp10a	Absent in spermatogonia	128
97381	T-complex protein 11	Tcp11	Absent in spermatogonia	79
93207	Preproacrosin	Acr	Absent in spermatogonia (Kremling et al., 1991)	52
160122	RIKEN cDNA 2410004D18 gene	2410004D18Rik	Absent in spermatogonia. Asparaginase-like sperm autoantigen	50
97481	DnaJ (Hsp40) homolog, subfamily B, member 3	Dnajb3, MSJ-1	Absent in spermatogonia. Member of the DNAj co-chaperon family (Berruti and Martegani, 2001)	42
99456	Proacrosin binding protein	Acrbp, sp32	Absent in spermatogonia	39
95299	Dynein, axonemal, heavy chain 8	Dnahc8	Absent in spermatogonia. Absent also in one sample of spermatocytes	39
99816	Heat shock protein 2	Hspa2, Hsp70-2	Present in spermatogonia. Knock-out mice arrest in pachytene (Dix et al., 1996)	37
102244	Testicular serine protease 1	Tesp1	Absent in spermatogonia. Sperm acrosomal protein (Kohno et al., 1998)	36
97785	DNA segment, human D6S2654E	D0H6S2654E	Present in one sample of spermatogonia. Also called X5L protein (XAP5 like protein, retroposon-encoded copy of an X-linked gene)	30
100626	Outer dense fiber 2	Odf2	Present in spermatogonia	16
103541	T-complex-associated testis expressed 2	Tcte2	Absent in spermatogonia	16
103468	Meiosis-specific nuclear structural protein 1	Mns1	Present in spermatogonia	12
102747	T-complex-associated testis expressed 1	Tcte1	Absent in spermatogonia	11
103956	5-azacytidine induced gene 1	Azi1	Absent in spermatogonia. Pre-acrosomal protein	10
102818	Xlr-related, meiosis regulated	Xmr	Absent in spermatogonia	5
94891	Male enhanced antigen 1	Mea1	Present in spermatogonia	3
92888	Phosphoserine/threonine/tyrosine interaction protein	Styx	Present in spermatogonia. Complexes with a testicular phosphorylated RNA-binding protein and is essential for normal spermiogenesis (Wishart and Dixon, 2002)	2
92692	Synaptonemal complex protein 1	Sycp1	Present in spermatogonia	2

from those operating in mitotic germ cells and somatic cells might drive carbohydrate utilization in meiotic and/or post-meiotic germ cells, even though this hypothesis needs to be substantiated by more specific studies. We also noticed several targets whose relative gene expression in spermatogonia or in spermatocytes was either not known, or controversial, or conflicting with data available in the literature. The pattern of expression in spermatocytes vs.

Table 6
Genes expressed in spermatocytes: membrane-bound proteins/receptors

MG-U74Av2 target Affymetrix	Gene name	Gene symbol	Notes	Fold difference (spermatocytes vs. spermatogonia)
101390	Mucin 3, intestinal	Muc3	Absent in spermatogonia. Glycoprotein of the colon epithelium	62
92198	Decay accelerating factor 2	Daf2	Absent in spermatogonia. Integral membrane protein involved in complement activation	31
93390	Prominin 1	Prom1	Absent in spermatogonia. A microvilli-specific polytopic membrane protein of the apical surface of epithelial cells targeted to plasmalemmal protrusions of non-epithelial cells	11
103289	Low density lipoprotein receptor-related protein 4	Lrp4, corin	Absent in spermatogonia. Atrial natriuteric peptide-converting enzyme (pro-ANP-converting enzyme). Serine protease of the trypsin family	10
103656	LanC (bacterial lantibiotic synthetase component C)-like	Lancl1, p40GPRT, p40/GPR69A	Present in spermatogonia. Originally proposed as a G-protein coupled receptor, was then characterized as a loosely membrane-associated protein related to the LanC family of bacterial proteins involved in the biosynthesis of antimicrobial peptides	8
160876	B-cell receptor-associated protein 29	Bcap29	Present in spermatogonia. Associated with the membrane IgD and IgM receptors in B lymphocytes	8
100438	G protein coupled receptor 19	Gpr19	Absent in spermatogonia. G-protein coupled receptor for an unknown ligand (O'Dowd et al., 1996)	4
99160 and 99161	RIKEN cDNA 1110025J15 gene	1110025J15Rik	Present in spermatogonia. Similar to membrane proteins related to a glutamate binding protein (NMDA receptor)	4
102343	Hypothetical protein 425O18-1	425O18-1	Present in spermatogonia. Contains a low density lipoprotein-receptor class A domain	4
103726	RIKEN cDNA 2610311I19 gene	2610311I19Rik	Absent in spermatogonia. Similar to Golgi membrane protein SB140. I	3
161046	Cytokine receptor-like factor 1	Crlf1	Present in spermatogonia. Soluble cytokine receptor subunit or part of a cytokine responsive complex, possibly playing a regulatory role in the immune system and during fetal development	2

spermatogonia for a selection of these genes is shown in Fig. 2. Northern blot analysis confirmed both qualitatively and quantitatively the data obtained by the microarray experiments.

An expression pattern in partial conflict with previously published data was particularly evident for several pro-apoptotic members of the Bcl2 family. Bad was previously reported to be expressed in spermatogonia and in Sertoli cells, but not in spermatocytes, nor in spermatids (Yan et al., 2000), while both microarray and Northern blot analysis showed that *Bad* mRNA is expressed in spermatocytes, but not in spermatogonia, nor in Sertoli cells (Table 2 and Fig. 2). Moreover, its expression was very strong in spermatids, in which a slower migrating transcript was observed. Bak was reported to be expressed in Sertoli cells, in spermatogonia and in spermatocytes, but not in spermatids (Yan et al., 2000), but we found a very abundant transcript only in meiotic and post-meiotic cells, and no expression in spermatogonia, nor in Sertoli cells (Table 2 and Fig. 2). Interestingly it has been recently reported that apoptosis-like mechanisms are required for spermatid differentiation in *Drosophila* (Arama et al., 2003). An analogy between cytoplasmic apoptotic events and the formation of residual bodies has been also noticed in mammalian spermiogenesis (Blanco-Rodriguez and Martinez-Garcia, 1999). On the other hand, Bax was reported to be expressed, besides in spermatogonia and Sertoli cells, also in spermatocytes (Yan et al., 2000), but we found an abundant transcript in mitotic germ cells and in Sertoli cells, with the highest level of expression at 7 dpn, whereas only a very faint signal was detectable in spermatocytes (Table 1 and Fig. 2).

Chrac1 (chromatin accessibility complex 1, also named Ycl1) is a histone-fold protein that interacts with other histone-fold proteins to bind DNA in a sequence-independent manner. These histone-fold protein dimers combine within larger enzymatic complexes for DNA transcription, replication, and packaging (Bolognese et al., 2000). *Chrac1* mRNA was found to be very abundant in spermatocytes (Table 3 and Fig. 2), suggesting that it might be involved in chromatin remodeling during the first meiotic prophase. This might help to regulate changes in gene expression patterns that characterize specific developmental events during spermatogenesis.

In the cluster of membrane-bound proteins and receptors, microarray analysis revealed the unexpected expression of

Table 7
Genes expressed in spermatocytes: metabolism

MG-U74Av2 target Affymetrix	Gene name	Gene symbol	Notes	Fold difference (spermatocytes vs. spermatogonia)
93103	Lactate dehydrogenase 3, C chain, sperm specific	Ldh3	Absent in spermatogonia. Glycolysis and gluconeogenesis (Li et al., 1998)	3565
92599	Phosphoglycerate mutase 2	Pgam2	Absent in spermatogonia. Glycolysis and gluconeogenesis	1260
96918	Fructose bisphosphatase 1	Fbp1	Absent in spermatogonia. Glycolysis and gluconeogenesis	401
100931	Arylsulfatase A	Arsa	Absent in spermatogonia. Sulfuric ester hydrolase	194
92292	Solute carrier family 2 (facilitated glucose transporter), member 3	Slc2a3	Absent in spermatogonia	132
95060	Solute carrier family 16 (monocarboxylic acid transporters), member 7	Slc16a7	Absent in spermatogonia	68
93560	RIKEN cDNA 1110039O14 gene	1110039O14Rik	Present in spermatogonia. Similar to human acylphosphatase	59
103982	Alcohol dehydrogenase 4 (class II), pi polypeptide	Adh4	Absent in spermatogonia. Glycolysis and gluconeogenesis	43
104328	Aquaporin 9	Aqp9	Absent in spermatogonia. Water transport	43
104372	RIKEN cDNA 0910001L24 gene	0910001L24Rik	Absent in spermatogonia. Xenobiotic metabolism	39
99011	UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyl-transferase 3	Galnt3	Absent in spermatogonia	39
103646	Carnitine acetyltransferase	Crat	Present in one sample in spermatogonia. Fatty acid metabolism	33
101388	Phosphoglycerate kinase 2	Pgk2	Absent in spermatogonia. Spermatocyte-specific PGK isoform encoded by an intronless retroposon (Boer et al., 1987). Glycolysis and gluconeogenesis	33
99542	Pyruvate dehydrogenase E1 α 2	Pdha2	Present in spermatogonia. Glycolysis and gluconeogenesis	30
94540	RIKEN cDNA 1300006E06 gene	1300006E06Rik	Absent in spermatogonia. Cytochrome C P-450-16α. Electron transport	22
103689	ATP-binding cassette, sub-family C (CFTR/MRP), member 3	Abcc3	Absent in spermatogonia. Similar to human multidrug resistance associated protein	19
161243	RIKEN cDNA 0910001L24 gene	0910001L24Rik	Absent in spermatogonia. Xenobiotic metabolism	17
103531	RIKEN cDNA 1300013B24 gene	1300013B24Rik	Absent in spermatogonia. Low similarity to endoplasmic oxidoreductase 1 β	16
92841	Chromogranin B	Chgb	Absent in spermatogonia	16
103068	Aldo-keto reductase family 1, member E1	Akr1e1	Absent in spermatogonia. Aldehyde reductase	15
99591	Retinol dehydrogenase 11	Rdh11	Absent in spermatogonia. Similar to human androgen-regulated prostate short-chain dehydrogenase/reductase 1	15
93557	Selenophosphate synthetase 2	Sps2	Absent in spermatogonia	13
97511	Monoglyceride lipase	Mgll	Absent in spermatogonia	13
97834	Phosphofructokinase-1 C	Pfkp	Absent in spermatogonia. Glycolysis and gluconeogenesis	12
160839	Solute carrier family 2 (facilitated glucose transporter), member 5	Slc2a5	Absent in spermatogonia	11
96072	Lactate dehydrogenase 1, A chain	Ldh1e	Present in spermatogonia. Glycolysis and gluconeogenesis	11

the transcript encoding mucin3, a protein known to be specifically expressed in the colon epithelium (Table 6). Northern blot analysis confirmed high levels of expression of mucin3 mRNA in spermatocytes, and, at a lesser extent, in spermatids (Fig. 2). Interestingly, another component of the mucosal glycocalyx, contributing to anti-adhesive and protective cell functions, mucin1, has been reported to be expressed in maturing germ cells of the human testis (Franke et al., 2001), and a mucin glycoprotein was found to be an universal constituent of stable intercellular bridges in the *Drosophila melanogaster* germ line (Kramerova and Kramerov, 1999).

Table 8
Genes expressed in spermatocytes: RNA binding proteins

MG-U74Av2 target Affymetrix	Gene name	Gene symbol	Notes	Fold difference (spermatocytes vs. spermatogonia)
101938	Poly(A) binding protein, cytoplasmic 2	Pabpc2	Absent in spermatogonia. Encoded by an intronless retroposon during spermatogenesis	94
104440	Y box protein 2	Ybx2	Absent in spermatogonia. RNA-binding protein which might delay polysomal association of transcripts during spermiogenesis	90
161033	Poly(A) polymerase β (testis-specific)	Papolb	Absent in spermatogonia. Responsible for cytoplasmic polyadenylation of pre-existing mRNAs in male haploid germ cells. Knock-out results in the arrest of spermiogenesis (Kashiwabara et al., 2002)	20
97661	Testis nuclear RNA binding protein	Tenr	Present in spermatogonia. Expressed in meiotic and haploid male germ cells	11
161041 and 92678	DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 25	Ddx25	Present in spermatogonia. Gonadotropin regulated RNA helicase also expressed in Leydig cells	11
96850	Hypothetical protein 4833436O05	4833436O05	Present in spermatogonia. Similar to eukaryotic translation initiation factors	10
160429	NTF2-related export protein 1	Nxt1	Present in spermatogonia. RAN-binding protein involved in nuclear RNA export from the nucleus	7
100720	Poly(A) binding protein, cytoplasmic 1	Pabpc1	Present in spermatogonia	4
101579	Signal recognition particle 9 kDa	Srp9	Present in spermatogonia. Cytoplasmic ribonucleoprotein targeting nascent polypeptide chains to the endoplasmic reticulum	4
103101	TAR (HIV) RNA binding protein 2	Tarbp2, Prbp	Present in spermatogonia. Interacts with the 3′ untranslated region of the Protamine-1 RNA	4
101519	Signal recognition particle 14 kDa (homologous Alu RNA binding protein)	Srp14	Present in spermatogonia. Cytoplasmic ribonucleoprotein targeting nascent polypeptide chains to the endoplasmic reticulum	3
94552	Poly(rC) binding protein 1	Pcbp1	Present in spermatogonia. Implicated in mRNA stabilization	2
103330	Spermatid perinuclear RNA binding protein	Spnr	Present in spermatogonia. Binds to the to the 3′ UTR of Protamine-1 mRNA. Microtubule-associated RNA-binding protein that localizes to the manchette in developing spermatids	2

Few receptors for potential growth factors were found to be expressed in spermatocytes through the microarray analysis. One of these was Gpr19 (O'Dowd et al., 1996), a seven transmembrane G-coupled receptor for an unknown ligand (Table 6). Northern blot analysis showed an high abundance of the *Gpr19* transcript in spermatocytes, a lower level of expression in spermatids, while a faint band was observed in spermatogonia from 7-day, but not 4-day-old mice (Fig. 2). This receptor might thus play a role in the regulation of meiotic entry and/or meiotic progression. The gene encoding Ranbp9 (Ran binding protein 9), a protein shown to be a positive regulator of Ras function (Wang et al., 2002), was found to be highly expressed in spermatids, with a complex migratory pattern, but the signals were evident also in spermatocytes, implying its possible involvement in the regulation of the Ras/MEK/ERK cascade during the transition through the meiotic divisions and/or the morphogenetic events of spermiogenesis (Table 9 and Fig. 2).

Finally, in the cluster of transcription factors (Table 3) we confirmed through Northern blot analysis the selective germ cell expression starting from the meiotic stage of *Hmx1*, a homeodomain gene previously not known to be expressed during spermatogenesis (Yoshiura et al., 1998), and of a transcript corresponding to RIKEN cDNA A630056B21Rik (Affymetrix target 99987_at in the MG-U74Av2 array) predicted to encode a novel zinc finger protein (Fig. 2). These transcription factors might play an important role in driving the spermatogenic program. As in the case of Ranbp9, the signal generated by the 99987 target in Northern blots was rather complex: this might be due to either the presence of multiple alternative transcripts, or to cross-hybridization with closely related RNAs.

Even though, recently, an initial microarray screen of spermatogenic cells at different developmental stages has been reported (Yu et al., 2003), only 1176 mouse target genes were represented in these arrays. We noticed partial overlapping of our data with the ones published by Yu et al. (2003), but also some discrepancies were evident: for instance, cyclin D3 was reported to be not expressed in spermatogonia, but present in spermatocytes. One should note that we used oligonucleotide based DNA chips, in which each gene is represented by 16 couples of probes and mismatch probes, whereas in the gene arrays used by Yu et al. each target gene is represented by a single longer

Table 9
Genes expressed in spermatocytes: signal transduction/protein kinases

MG-U74Av2 target Affymetrix	Gene name	Gene symbol	Notes	Fold difference (spermatocytes vs. spermatogonia)
104029	Calmegin	Clgn	Absent in spermatogonia. Knockout male mice are sterile for defective sperm function (Ikawa et al., 1997)	52
101850	Sperm autoantigenic protein 17	Spa17	Present in spermatogonia. A calmodulin-binding protein enriched in the sperm acrosome and interacting with the zona pellucida	31
161839	RAS-like, family 2, locus 9	Rasl2-9	Absent in spermatogonia. Strongly related to Ran GTPase, probably a testis-specific isoform	30
100972	Chemokine (C-C motif) ligand 27	Ccl27	Absent in spermatogonia. Also called ALP, CTAK, mILC, CTACK, PESKY, ESkine, skinkine	29
102948	hematopoietic cell transcript 1	Hemt1	Absent in spermatogonia. Contains a calcium-activated BK potassium channel α subunit signature	26
99869	Hepatoma-derived growth factor	HRP1, Pwwp1	Absent in spermatogonia. Also called PWWP domain containing 1. Present in the nucleus of spermatocytes and spermatids	23
98614	Nephrocystin	Nphp1	Present in spermatogonia. SH3 containing protein which forms protein complexes with p130(Cas), proline-rich tyrosine kinase 2 (Pyk2), and tensin	23
103489	Socius	Soc	Absent in spermatogonia. A Rho-related GTPase-interacting protein involved in disassembly of actin stress fibers	22
93210	NIMA-related-kinase 4	Nek4	Absent in spermatogonia	18
93658	Protein tyrosine phosphatase, non-receptor type 20	Ptpn20	Absent in spermatogonia	16
100287	Immunoglobulin (CD79A) binding protein 1b	Igbp1b	Absent in spermatogonia. Binds to protein phosphatase 2A. Also called α4-b	16
160623	Cyclin-dependent kinase-like 2	Cdkl2	Absent in spermatogonia. CDC2-related kinase also called KKIAMRE	13
104166	Renal tumor antigen	Rage, MOK	Absent in spermatogonia. Protein kinase with homologies with members of the MAPK family	13
104135	ADP-ribosylation-like 3	Arl3	Present in spermatogonia. Small monomeric GTPase of the Ras superfamily	12
160948	Testis-specific calcineurin isoform	Ppp3cc	Present in spermatogonia. Calmodulin-dependent protein phosphatase	11
92805	ADP-ribosylation-like 4	Arl4	Present in spermatogonia. Knock-out mice show a significant reduction of testis weight and sperm count (Schurmann et al., 2002)	10
100562	Ran. guanine nucleotide release factor	Rangnrf	Present in one sample in spermatogonia. Also called MOG1	9
100291	Casitas B-lineage lymphoma	Cbl	Absent in spermatogonia. Adaptor protein	8
102033	Testis-specific protein kinase 1	Tesk1	Absent in spermatogonia	8
92639	Serine/threonine kinase 6	Stk6, Ayk1	Present in spermatogonia. Also called Aurora/IPL1-related kinase 1. Specifically expressed in meiotic cells just before the first meiotic division	7
100885	NIMA-related kinase 2	Nek2	Present in spermatogonia. Involved in chromosome condensation during meiotic divisions (Di Agostino et al., 2002). Controls splitting of duplicated centrosomes	7
161575	Mitogen activated protein kinase 10	Mapk10,	Absent in spermatogonia. Also called SAPK (β), JNK3, SERK2, p54bSAPK, p439F12	4
97812	RAN binding protein 9	Ranbp9	Present in spermatogonia. Stimulates Ras activation by recruiting Sos. Also called RanbpM (Wang et al., 2002)	4

oligonucleotide, making the possibility of cross-hybridization easier and hindering the statistical evaluation of the generated signals (see also Section 2). In conclusion, our results represent a first extensive attempt to delineate the global patterns of gene expression characterizing male germ cell differentiation, and should be extended to other germ cell types, namely spermatogonial stem cells and spermatids.

2. Experimental procedures

2.1. Cell preparations

Germ cell populations highly enriched in mitotic spermatogonia were obtained as previously described from testes of 4–7-day-old mice (Rossi et al., 1993; Pellegrini et al., 2003; Dolci et al., 2001). Briefly, germ

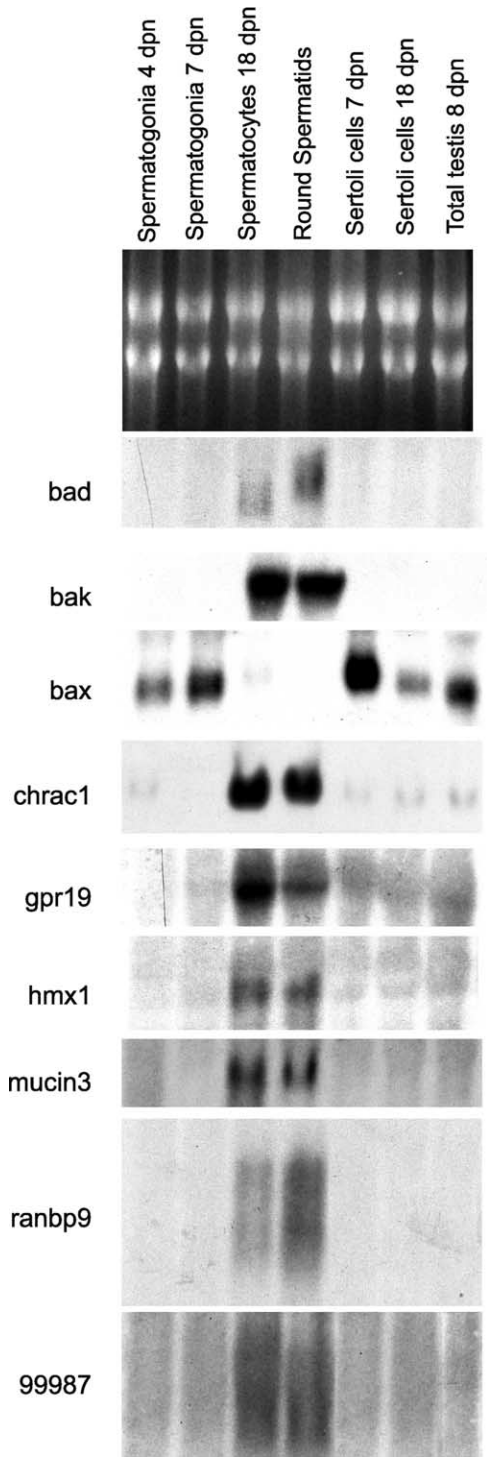

Fig. 2. Germ cell-type-specific expression of a selection of genes identified by Affymetrix microarray hybridizations was verified by Northern blot analysis, using 10 μg of total RNA for each indicated testicular cell type. The representative top panel shows ethidium bromide staining, indicating that RNA loading was qualitatively and quantitatively comparable for each sample. Specific labeled gene probes for hybridization were obtained by nick translation of RT-PCR amplified cDNAs, as indicated in Section 2. These Northern blots are representative of at least two experiments, which gave similar results.

cell suspensions were obtained by sequential collagenase–hyaluronidase–trypsin digestions of freshly withdrawn testes. A 3 h period of culture in E-MEM additioned with 10% FCS was performed to facilitate adhesion of contaminating somatic cells to the plastic dishes. At the end of this pre-plating treatment, enriched mitotic germ cell suspensions were rinsed from FCS. Purity of 7 dpn spermatogonia was about 90% after the pre-plating treatment, whereas a 50% enrichment was obtained for 4 dpn spermatogonia. The homogeneity of the cell populations was assessed through both morphological criteria and by specific immunostaining with antibodies directed against three specific markers of mitotic germ cells, which are not expressed in testicular somatic cells (Smad5, Alk3 and c-kit). Homogeneous populations (purity $>90\%$) of spermatocytes and round spermatids were obtained from testes of either 18-day-old or 36-day-old mice, respectively, by differential elutriation as previously described (Sette et al., 1999; Di Agostino et al., 2002). Spermatocyte populations from 18-day-old mice (10% at the leptotene–zygotene and 85% at the middle–late pachytene stage of the meiotic prophase) are devoid of round spermatids, which contaminate elutriation fractions from adult animals, and their purity was assessed through morphological criteria (namely, cell size and the characteristic aspect of partially condensed meiotic chromatin). Sertoli cell monolayers from 7 to 17-day-old mice, devoid of contaminating germ cells, were prepared as previously described (Grimaldi et al., 1993).

2.2. RNA extraction, cDNA and cRNA preparation

RNA was purified by adding cold Trizol reagent (Invitrogen) to freshly prepared cell samples and extracted according to the manufacturer's instructions.

Total cellular RNA (25 μg) was used to synthesize cDNA using the cDNA Synthesis Kit (Life Technologies BRL 11917-010) and T7-$(dT)_{24}$ oligonucleotide (5′-GGCCAGTGAATTGTAATACGACTCACTATAGG-GAGGCGG-$(dT)_{24}$-3′) according to manufacturer's instructions. Second strand cDNA was synthesized by adding 10 U of DNA ligase, 40 U of DNA polymerase and 2 U of RNaseH and incubating at 16 °C for additional 2 h. At the end of the incubation, 20 U of T4 DNA polymerase were added to the reaction and incubated for 5 min at the same temperature. Reactions were stopped by adding EDTA (30 mM final concentration). Double stranded cDNA was purified by phenol/chloroform extraction followed by precipitation with 0.5 volumes of 7.5 M ammonium acetate and 2.5 volumes of ethanol and its concentration measured by optical densitometry.

Complementary RNA (cRNA) synthesis was performed using the Essential ENZO kit (Bioarray High Yield TNA transcription kit 900182) and following manufacturer's instructions. The resulting cRNA was purified using QIAGEN Rneasy spin columns (74103) and the standard procedure. RNA was then precipitated as described above

for cDNA, resuspended in 15 μl of RNase-free H_2O and quantified by optical densitometry. cRNA was then fragmented in a Tris-acetate buffer (200 mM, pH 8.1) containing 500 mM KOAc and 150 mM MgOAc by incubation at 94 °C for 35 min. At the end of the incubation, fragmented cRNA was stored at −80 °C until hybridization.

2.3. DNA microarray analysis

cRNA samples from two independent cell preparations were used for hybridization to duplicate mouse MG-U74Av2 microarray sets from Affymetrix. This array represents approximately 12,500 murine genes or EST sequences. In each array, target genes are represented by 16 pairs (exact match and single base mismatch) of 25-mer oligonucleotides for each gene. The signals of the pairs are compared to assess specificity of hybridization, thus, beside the intensity of the signal, its statistical significance can be estimated. Biotinylated cRNA (15 μg) was hybridized to the array and then processed following the standard Affymetrix protocol. Phycoerythrin-coupled avidin bound microarrays were scanned with a Hewlett-Packard Gene Array Scanner (Hewlett-Packard Co., Palo Alto, CA), and the results were analyzed using the Affymetrix MAS5 statistical algorithm. For more informations about the statistical analysis, see the Affymetrix Statistical Algorithms Reference Guide at http://www.affymetrix.com/support/technical/technotes/statistical_reference_guide.pdf.

Target genes represented in the MG-U74Av2 Affymetrix chips were grouped in several functional clusters by using specific keywords with the Interacting Query online facility at www.affymetrix.com.

2.4. RT-PCR preparation of probes and Northern blot analysis

cDNA probes for Northern blot hybridization of total RNAs were prepared by RT-PCR amplification of selected mRNAs, by using specific oligonucleotide primers designed on the basis of the sequence of the corresponding Affymetrix target genes. Specificity of the primers was previously controlled through BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/). The couples of primers used were: TAGCCCTTTTCGAGGACGCTCG and TGGAGCCTCCTTTGCCCAAGTT (for *Bad*, amplification product 220 bp); AGTTGGCTCTCAAGGATGGCTT and TCGTTGCACTGACAGAATCTTC (for *Bak1*, 229 bp); ACCAAGAAGCTGAGCGAGTGT and TCCAGCCCATGATGGTTCTGAT (for *Bax*, 253 bp); ATCTGGAGAATAGGCACGGACG and CCGAAATGCCCACATAGTTTCT (for *gpr19*, 378 bp); TGCTCTACAGTGTACCGGACAG and CAGCACTCTGTACTGTCCCTTG (for *hmx1*, 291 bp); GACTCTGTGTACAACACCTTCC and GCCCTTGTAAAGACAGATGGTC (for *mucin3*, 524 bp); CAAATTGGGAGCTGTTCCGACC and CTACAACAGAAGTCATCTGTAG (for *Ranbp9*, 267 bp); TGTGTAGCCG GGAGTTTGGTA and TGAAAACGGACTCCGCACTCCT (for *A630056B21Rik*, 357 bp). The cDNA probe for *Chrac1* was kindly provided by Prof. Roberto Mantovani (University of Milan).

cDNAs were labeled by random priming with α^{32}PdNTPs and hybridized using standard conditions to blotted total RNA samples. After stringency washes, blots were exposed overnight at −80 °C with intensifier screens for autoradiography.

Acknowledgements

Due to space restrictions, we apologize for not being able to cite all the relevant papers describing germ cell-specific-expression of several genes that we have confirmed through microarray analysis and included in our tables. We thank Prof. Roberto Mantovani (University of Milan) for supplying a *Chrac1* cDNA probe. This work has been supported by MIUR CoFin 2002, by a grant of 'Centro di Eccellenza per lo Studio del Rischio Genomico in Patologie Complesse Multifattoriali' and by Agenzia Spaziale Italiana.

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