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
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-puberual 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/absolutevalues/stot.htm and http://www2.uniroma2.it/ricerca/ce/comparativeanalysis/stot.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 0.997 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
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

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).
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

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**Table 1**

Examples of genes selectively expressed in spermatogonia

<table>
<thead>
<tr>
<th>MG-U74Av2 target</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Notes</th>
<th>Fold difference (spermatogonia vs. spermatocytes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ott</td>
<td>Absent in spermatocytes. X-linked gene, previously defined as ‘meiosis specific’ (Kerr et al., 1996)</td>
<td>92306</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>Stra8</td>
<td>Absent in spermatocytes. Pre-meiotic germ cell-specific cytoplasmic protein encoded by Stra8, a retinoic acid-responsive gene (Oulad-Abdelghani et al., 1996)</td>
<td>101194</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>Atm</td>
<td>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)</td>
<td>101180</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Bax</td>
<td>Absent in spermatocytes. Pro-apoptotic factor (Yan et al., 2000)</td>
<td>93536</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>c-kit</td>
<td>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)</td>
<td>99956</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>E2f1</td>
<td>Absent in spermatocytes. Transcription factor crucial for mitotic cell cycle control (Dolci et al., 2001)</td>
<td>102963</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Pola1</td>
<td>Absent in spermatocytes. Dna replication (Orlando et al., 1989)</td>
<td>103207</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Bcl10</td>
<td>Present in spermatocytes. Pro-apoptotic factor</td>
<td>94448</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Ccnb1</td>
<td>Absent in spermatocytes. Subunit of cdc2/cdk1, essential for G2/M transition</td>
<td>160159</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Pold1</td>
<td>Absent in spermatocytes. Dna replication</td>
<td>103057</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Bmpr1a, ALK3</td>
<td>Absent in spermatocytes. Bone morphogenetic receptor for Bmp2 and Bmp4. Involved in spermatogonial differentiation (Pellegrini et al., 2003)</td>
<td>92767</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Prim1</td>
<td>Absent in spermatocytes. Dna replication (Orlando et al., 1989)</td>
<td>96772</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>p57</td>
<td>Absent in spermatocytes. Cdk2 inhibitor</td>
<td>95471</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>p21</td>
<td>Absent in spermatocytes. Cdk2 inhibitor</td>
<td>98067</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Chk1, Chek1, rad27</td>
<td>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</td>
<td>103064</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Cdk4</td>
<td>Present in spermatocytes. Cell cycle kinase activated and essential for G1/S transition (Dolci et al., 2001)</td>
<td>160538</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>CcnD3</td>
<td>Absent in spermatocytes. Cell cycle control in G1/S Phase (Dolci et al., 2001)</td>
<td>160545</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>Absent in spermatocytes (marginal in one sample). Apoptosis inducer and cell cycle control in G1/S Phase</td>
<td>104154</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Chk2, Chek2, rad53</td>
<td>Absent in spermatocytes (marginal in one sample). ATM-dependent. Function similar to that of Chk1</td>
<td>92481</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ccn2</td>
<td>Absent in spermatocytes. Mitotic cyclin, active in the S phase, cdk2 subunit (Dolci et al., 2001)</td>
<td>99186</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>MG-U74Av2 target</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Notes</th>
<th>Fold difference (spermatocytes vs. spermatogonia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160644</td>
<td>BCL2-antagonist/killer 1</td>
<td>Bak1</td>
<td>Absent in spermatogonia. Involved in apoptosis; caspase activation via cytochrome c (Yan et al., 2000)</td>
<td>45</td>
</tr>
<tr>
<td>92911</td>
<td>Cyclin A1</td>
<td>Ccna1</td>
<td>Absent in spermatogonia. In knock-out mice, meiotic arrest during meiotic divisions (Liu et al., 1998)</td>
<td>23</td>
</tr>
<tr>
<td>103094</td>
<td>Small EDRK-rich factor 1</td>
<td>Serf1</td>
<td>Present in spermatogonia. (Survival of Motoneuron in SMA1)</td>
<td>18</td>
</tr>
<tr>
<td>99522</td>
<td>Germ cell-specific gene 2</td>
<td>Gsg2</td>
<td>Absent in spermatogonia (marginal in one sample). Atypical serine–threonine kinase, named Haspin (for haploid germ cell-specific nuclear protein kinase)</td>
<td>17</td>
</tr>
<tr>
<td>160761</td>
<td>Upregulated during skeletal muscle growth 4</td>
<td>Usmg4</td>
<td>Absent in spermatogonia</td>
<td>11</td>
</tr>
<tr>
<td>100054</td>
<td>DNA segment Chr2</td>
<td>D2Wsu81e</td>
<td>Protein released in apoptosis and involved in caspase-independent DNA degradation</td>
<td>10</td>
</tr>
<tr>
<td>92929</td>
<td>Cytochrome c, testis</td>
<td>Cyt</td>
<td>Present in spermatogonia. Null mice produce functional sperm but undergo early testicular atrophy (Narisawa et al., 2002)</td>
<td>10</td>
</tr>
<tr>
<td>94971</td>
<td>KAP1, Cdk inhibiting phosphatase</td>
<td>Cdkna3</td>
<td>Present in spermatogonia. cdk2-associated dual specificity phosphatase</td>
<td>9</td>
</tr>
<tr>
<td>94521</td>
<td>Cyclin-dependent kinase inhibitor 2D</td>
<td>Cdkn2d, Ink4d, p19</td>
<td>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)</td>
<td>9</td>
</tr>
<tr>
<td>101885</td>
<td>Growth arrest specific 5</td>
<td>Gas5</td>
<td>Absent in spermatogonia. Preferentially expressed in the growth phase arrest of the cell cycle</td>
<td>6</td>
</tr>
<tr>
<td>160638</td>
<td>Cyclin-dependent kinase inhibitor 2C</td>
<td>Cdkn2c, Ink4c, p18</td>
<td>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)</td>
<td>6</td>
</tr>
<tr>
<td>99670</td>
<td>Bcl-associated death promoter</td>
<td>Bad</td>
<td>Absent in spermatogonia. Pro-apoptotic factor (Yan et al., 2000)</td>
<td>5</td>
</tr>
<tr>
<td>92902</td>
<td>Myeloblastosis oncogene-like 1</td>
<td>Myb11, A-Myb</td>
<td>Present in one sample in spermatogonia. Transcription factor. Knockout male mice are sterile due to arrest in pachytenne</td>
<td>5</td>
</tr>
<tr>
<td>98945</td>
<td>SH3-domain GRB2-like B1 (endophilin)</td>
<td>Sh3glb1</td>
<td>Present in spermatogonia. Synaptically enriched protein implicated in synaptic vesicle endocytosis. Might be involved in apoptotic programs since it interacts with Bax</td>
<td>4</td>
</tr>
<tr>
<td>92879</td>
<td>Protein phosphatase 1G, γ isofrom</td>
<td>Ppm1g, PP2C-γ</td>
<td>Present in spermatogonia. Formerly called protein phosphatase 2C. Magnesium-dependent serine–threonine phosphatase, known to be expressed in the testis and skeletal muscle</td>
<td>4</td>
</tr>
<tr>
<td>94294</td>
<td>Cyclin B2</td>
<td>Cenb2</td>
<td>Present in spermatogonia. Interacts with cdc2 (cdk1) as a subunit. Component of MPF (Dolci et al., 2001)</td>
<td>4</td>
</tr>
<tr>
<td>104738</td>
<td>Zuo1in related factor 2</td>
<td>Zof2</td>
<td>Present in spermatogonia. A ribosome-associated DnaJ molecular chaperone. Also called MIDA-1, associates with Id HLH transcription factors</td>
<td>3</td>
</tr>
<tr>
<td>102734</td>
<td>Baculoviral IAP repeat-containing 3</td>
<td>Birc3, mIAP-2</td>
<td>Present in spermatogonia. Also called Apoptosis inhibitor 2. Caspase inhibitor</td>
<td>3</td>
</tr>
<tr>
<td>104476</td>
<td>Retinoblastoma-like 1 (p107)</td>
<td>Rbl1</td>
<td>Absent in spermatogonia (marginal in one sample). Homolog of pRb, involved in negative regulation of the cell cycle</td>
<td>2</td>
</tr>
<tr>
<td>101521</td>
<td>Baculoviral IAP repeat-containing 5</td>
<td>Birc5, TIAP</td>
<td>Present in spermatogonia. Homologous to human survivin. Caspase inhibitor</td>
<td>2</td>
</tr>
</tbody>
</table>
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

<table>
<thead>
<tr>
<th>MG-U74Av2 target Affymetrix</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Notes</th>
<th>Fold difference (spermatocytes vs. spermatogonia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>160599</td>
<td>Testis-specific gene A2</td>
<td>Tsga2</td>
<td>Absent in spermatogonia. Male meiotic metaphase chromosome-associated acidic protein</td>
<td>56</td>
</tr>
<tr>
<td>102795</td>
<td>Mesoderm posterior 1</td>
<td>Mesp1</td>
<td>Absent in spermatogonia. HLH protein</td>
<td>42</td>
</tr>
<tr>
<td>161064</td>
<td>PHD finger protein 7</td>
<td>Pfl7</td>
<td>Present in spermatogonia. Isolated from a mouse testis cDNA library</td>
<td>42</td>
</tr>
<tr>
<td>102079</td>
<td>Mus musculus Aip1</td>
<td>Aip1, Aym1</td>
<td>Absent in spermatogonia. IME-1 functional homolog (Personal communication from Jeremy Don, Bar-Ilan University, Ramat Gan, Israel)</td>
<td>40</td>
</tr>
<tr>
<td>104622</td>
<td>Transcription elongation factor A (SII), 2</td>
<td>Tcea2</td>
<td>Absent in spermatogonia</td>
<td>34</td>
</tr>
<tr>
<td>97745</td>
<td>Homeo box A4</td>
<td>Hoxa4</td>
<td>Absent in spermatogonia</td>
<td>29</td>
</tr>
<tr>
<td>95755</td>
<td>Cold shock domain protein A</td>
<td>Csda</td>
<td>Present in spermatogonia</td>
<td>25</td>
</tr>
<tr>
<td>92190</td>
<td>Nuclear receptor subfamily 2, group C, member 1</td>
<td>Nr2c1</td>
<td>Absent in spermatogonia</td>
<td>22</td>
</tr>
<tr>
<td>93182</td>
<td>Glial and testis-specific homeobox gene</td>
<td>Nkx6-2, Gtx</td>
<td>Absent in spermatogonia. Murine homeobox-containing gene, expressed specifically in glial cells of the brain and germ cells of tests. Knock-out mice are viable and fertile (Cai et al., 2001)</td>
<td>17</td>
</tr>
<tr>
<td>102219</td>
<td>Regulatory factor X, 2</td>
<td>Rfx2</td>
<td>Absent in spermatogonia. Influences HLA class II expression</td>
<td>16</td>
</tr>
<tr>
<td>99987</td>
<td>RIKEN cDNA A630056B21 gene</td>
<td>A630056B21Rik</td>
<td>Absent in spermatogonia. Weakly similar to zinc finger protein 2 (Zfp2) (mKR2 protein)</td>
<td>15</td>
</tr>
<tr>
<td>98414</td>
<td>Zinc finger protein 42</td>
<td>Zfp42</td>
<td>Present in one sample in spermatogonia. Expressed also in embryonic stem cells</td>
<td>14</td>
</tr>
<tr>
<td>160204</td>
<td>RIKEN cDNA 3110013H01 gene</td>
<td>3110013H01Rik</td>
<td>Present in spermatogonia. Nuclear protein p30, a protein of the nuclear pore complex</td>
<td>11</td>
</tr>
<tr>
<td>93221</td>
<td>RIKEN cDNA 4921540P06 gene (Homeo box D8)</td>
<td>4921540P06Rik</td>
<td>Present in spermatogonia. Other names: Hox-4.3 140, HOXD8, Hox5.4</td>
<td>11</td>
</tr>
<tr>
<td>100126</td>
<td>Chromatin accessibility complex 1</td>
<td>Chrac1</td>
<td>Present in spermatogonia. NF-YC-like protein. Also called YCL1 (Bolognese et al., 2000)</td>
<td>11</td>
</tr>
<tr>
<td>160068</td>
<td>Sin3 associated polypeptide, 30 kDa</td>
<td>Sap30</td>
<td>Present in spermatogonia. Component of a histone deacetylase complex</td>
<td>10</td>
</tr>
<tr>
<td>97893</td>
<td>TATA box binding protein-like protein</td>
<td>Tlp</td>
<td>Present in spermatogonia. Also named TLF, TRF2 or TBPL1. Knockout mice arrest at spermiogenesis (Martianov et al., 2001)</td>
<td>9</td>
</tr>
<tr>
<td>92342</td>
<td>Zinc finger protein 93</td>
<td>Zfp93</td>
<td>Absent in spermatogonia</td>
<td>9</td>
</tr>
<tr>
<td>104604</td>
<td>Zinc finger protein 96</td>
<td>Zfp96</td>
<td>Present in one sample in spermatogonia</td>
<td>6</td>
</tr>
<tr>
<td>103629</td>
<td>Lymphoid enhancer binding factor 1</td>
<td>Lef1</td>
<td>Absent in spermatogonia</td>
<td>6</td>
</tr>
<tr>
<td>96144</td>
<td>Inhibitor of DNA binding 4</td>
<td>Id4</td>
<td>Absent in spermatogonia. Id4, dominant negative helix-loop-helix protein</td>
<td>6</td>
</tr>
<tr>
<td>92195</td>
<td>CCAAT/enhancer binding protein (C/EBP), γ</td>
<td>Cebpg</td>
<td>Present in spermatogonia</td>
<td>6</td>
</tr>
<tr>
<td>94406</td>
<td>Putative homeodomain transcription factor</td>
<td>Phtf</td>
<td>Present in spermatogonia</td>
<td>5</td>
</tr>
<tr>
<td>98032</td>
<td>Zinc finger protein 35</td>
<td>Zfp35</td>
<td>Present in spermatogonia</td>
<td>5</td>
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<tr>
<td>160220</td>
<td>Zinc finger protein 110</td>
<td>Zfp110</td>
<td>Absent in spermatogonia</td>
<td>4</td>
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<tr>
<td>94102</td>
<td>H6 homeo box 1</td>
<td>Hmx1</td>
<td>Absent in spermatogonia (Yoshiura et al., 1998)</td>
<td>4</td>
</tr>
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</table>
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), 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), calmodulin (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 Pgd2, encoding a well-known meiotic isoform of phosphoglycerate kinase (Boer et al., 1987) (Table 7). Thus, metabolic pathways distinct

<table>
<thead>
<tr>
<th>MG-U74Av2 target Affymetrix Gene name</th>
<th>Gene symbol</th>
<th>Notes</th>
<th>Fold difference (spermatocytes vs.spermatogonia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>99995 Centrin 1</td>
<td>Cetn1</td>
<td>Absent in spermatogonia. Also called caltractin. Testis-specific centrosomal protein encoded by an intronless retroposon</td>
<td>588</td>
</tr>
<tr>
<td>101864 Actin-like 7b</td>
<td>Actl7b</td>
<td>Absent in spermatogonia. Testis-specific actin isoform, encoded by an intronless gene</td>
<td>76</td>
</tr>
<tr>
<td>161035 Kinesin family member 9</td>
<td>Kif9</td>
<td>Absent in spermatogonia. Microtubule motor associated protein abundantly expressed in the testis</td>
<td>58</td>
</tr>
<tr>
<td>160631 Sarcoglycan, 50 kDa dystrophin-associated glycoprotein</td>
<td>Sgca, adhalin</td>
<td>Absent in spermatogonia. Integral plasma membrane protein considered specifically expressed in striated muscle</td>
<td>36</td>
</tr>
<tr>
<td>99531 Synaptogyrin 4</td>
<td>Syng4</td>
<td>Absent in spermatogonia. Integral membrane protein present in synaptic vesicles</td>
<td>36</td>
</tr>
<tr>
<td>101195 Myosin light chain 2</td>
<td>Mylc2pl</td>
<td>Absent in spermatogonia. Considered specifically expressed in precursor B and T lymphocytes</td>
<td>17</td>
</tr>
<tr>
<td>92496 Vesicle-associated membrane protein 5</td>
<td>Vamp5</td>
<td>Absent in spermatogonia. Also called synaptobrevin. Expressed during myogenesis in striated muscles</td>
<td>15</td>
</tr>
<tr>
<td>101520 RIKEN cDNA 1700062C23 gene</td>
<td>1700062C23Rik</td>
<td>Absent in spermatogonia. Kinesin-related protein HASH. Rat homolog known to be expressed during spermatogenesis in meiotic cells</td>
<td>10</td>
</tr>
<tr>
<td>160487 Myosin light chain, alkali, cardiac atria</td>
<td>myla</td>
<td>Absent in spermatogonia. Expressed during striated muscle development</td>
<td>9</td>
</tr>
<tr>
<td>103684 Tektin-2</td>
<td>Tekt2</td>
<td>Absent in spermatogonia. A sperm flagellar protein also called tekin-1 and different from tekin-1</td>
<td>8</td>
</tr>
<tr>
<td>94321 Keratin complex 1, acidic, gene 10</td>
<td>Krt1-10</td>
<td>Absent in spermatogonia (marginal in one sample). Protein of intermediate filaments</td>
<td>4</td>
</tr>
<tr>
<td>95097 ARP10 actin-related protein 10 homolog</td>
<td>Actr10</td>
<td>Present in spermatogonia. Protein of the dynactin complex</td>
<td>4</td>
</tr>
<tr>
<td>93567 Profilin 2</td>
<td>Pfn2</td>
<td>Present in spermatogonia. Actin binding ubiquitous protein</td>
<td>4</td>
</tr>
<tr>
<td>102732 Talin</td>
<td>Tln</td>
<td>Present in spermatogonia. Integrin and actin binding protein</td>
<td>4</td>
</tr>
<tr>
<td>93499 Capping protein α 1</td>
<td>Cappt1</td>
<td>Present in spermatogonia. Actin binding protein</td>
<td>4</td>
</tr>
<tr>
<td>94248 Adaptor-related protein complex AP-1, μ subunit 1</td>
<td>Ap1m1</td>
<td>Present in spermatogonia. Adaptor protein of clathrin-coated vesicles involved in intracellular protein transport and endocytosis</td>
<td>4</td>
</tr>
<tr>
<td>104565 Adaptor-related protein complex AP-4, sigma 1</td>
<td>Ap4s1</td>
<td>Present in spermatogonia. Adaptor protein of clathrin-coated vesicles involved in intracellular protein transport and endocytosis</td>
<td>3</td>
</tr>
<tr>
<td>92643 Neurofibromatosis 2</td>
<td>Nf2</td>
<td>Present in spermatogonia. Tumor suppressor protein involved in mediating interactions between the plasma membrane and the cytoskeleton</td>
<td>3</td>
</tr>
<tr>
<td>93333 Tubulin cofactor a</td>
<td>Tbca</td>
<td>Present in spermatogonia. Molecular chaperonin involved in tubulin folding</td>
<td>2</td>
</tr>
<tr>
<td>103878 Adaptor-related protein complex AP-3, β 1 subunit</td>
<td>Ap3b1</td>
<td>Present in spermatogonia. Adaptor protein of clathrin-coated vesicles involved in intracellular protein transport and endocytosis</td>
<td>2</td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>Table 5</th>
<th>Genes expressed in spermatocytes: meiosis/spermatogenesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>MG-U74Av2 target</td>
<td>Affymetrix</td>
</tr>
<tr>
<td>160219</td>
<td>94927</td>
</tr>
<tr>
<td>92825</td>
<td>100526</td>
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<td>92732</td>
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<td>103058</td>
<td>99545</td>
</tr>
<tr>
<td>100359</td>
<td>99134</td>
</tr>
<tr>
<td>93955</td>
<td>99474</td>
</tr>
<tr>
<td>160506</td>
<td></td>
</tr>
<tr>
<td>100358</td>
<td>97381</td>
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<tr>
<td>99456</td>
<td>95299</td>
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<tr>
<td>99816</td>
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</tr>
<tr>
<td>102244</td>
<td>97785</td>
</tr>
<tr>
<td>100626</td>
<td>103468</td>
</tr>
<tr>
<td>103541</td>
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</tr>
<tr>
<td>103468</td>
<td></td>
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<tr>
<td>102747</td>
<td></td>
</tr>
<tr>
<td>103956</td>
<td>102818</td>
</tr>
<tr>
<td>104691</td>
<td></td>
</tr>
<tr>
<td>92888</td>
<td></td>
</tr>
<tr>
<td>92692</td>
<td></td>
</tr>
</tbody>
</table>
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 (Arana 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>
<thead>
<tr>
<th>MG-U74Av2 target Affymetrix</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Notes</th>
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</tr>
</thead>
<tbody>
<tr>
<td>101390</td>
<td>Mucin 3, intestinal</td>
<td>Muc3</td>
<td>Absent in spermatogonia. Glycoprotein of the colon epithelium</td>
<td>62</td>
</tr>
<tr>
<td>92198</td>
<td>Decay accelerating factor 2</td>
<td>Daf2</td>
<td>Absent in spermatogonia. Integral membrane protein involved in complement activation</td>
<td>31</td>
</tr>
<tr>
<td>93390</td>
<td>Prominin 1</td>
<td>Prom1</td>
<td>Absent in spermatogonia. A microvilli-specific polypeptide membrane protein of the apical surface of epithelial cells targeted to plasmalemmal protrusions of non-epithelial cells</td>
<td>11</td>
</tr>
<tr>
<td>103289</td>
<td>Low density lipoprotein receptor-related protein 4</td>
<td>Lp4, corin</td>
<td>Absent in spermatogonia. Atrial natriuretic peptide-converting enzyme (pro-ANP-converting enzyme). Serine protease of the trypsin family</td>
<td>10</td>
</tr>
<tr>
<td>103656</td>
<td>LanC (bacterial lantibiotic synthetase component C)-like</td>
<td>Lnc1l, p40GPRPT, p40/GPR69A</td>
<td>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</td>
<td>8</td>
</tr>
<tr>
<td>160876</td>
<td>B-cell receptor-associated protein 29</td>
<td>Bcap29</td>
<td>Present in spermatogonia. Associated with the membrane IgD and IgM receptors in B lymphocytes</td>
<td>8</td>
</tr>
<tr>
<td>100438</td>
<td>G protein coupled receptor 19</td>
<td>Gpr19</td>
<td>Absent in spermatogonia. G-protein coupled receptor for an unknown ligand (O’Dowd et al., 1996)</td>
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<tr>
<td>99160 and 99161</td>
<td>RIKEN cDNA 1110025J15 gene</td>
<td>1110025J15Rik</td>
<td>Present in spermatogonia. Similar to membrane proteins related to a glutamate binding protein (NMDA receptor)</td>
<td>4</td>
</tr>
<tr>
<td>102343</td>
<td>Hypothetical protein 425018-1</td>
<td>425018-1</td>
<td>Present in spermatogonia. Contains a low density lipoprotein-receptor class A domain</td>
<td>4</td>
</tr>
<tr>
<td>103726</td>
<td>RIKEN cDNA 261031119 gene</td>
<td>2610311119Rik</td>
<td>Absent in spermatogonia. Similar to Golgi membrane protein SB140.1</td>
<td>3</td>
</tr>
<tr>
<td>161046</td>
<td>Cytokine receptor-like factor 1</td>
<td>Crlf1</td>
<td>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</td>
<td>2</td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>MG-U74Av2 target Affymetrix</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Notes</th>
<th>Fold difference (spermatocytes vs. spermatogonia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>93103</td>
<td>Lactate dehydrogenase 3, C chain, sperm specific</td>
<td>Ldh3</td>
<td>Absent in spermatogonia. Glycolysis and gluconeogenesis (Li et al., 1998)</td>
<td>3565</td>
</tr>
<tr>
<td>92599</td>
<td>Phosphoglycerate mutase 2</td>
<td>Pgam2</td>
<td>Absent in spermatogonia. Glycolysis and gluconeogenesis</td>
<td>1260</td>
</tr>
<tr>
<td>96918</td>
<td>Fructose bisphosphatase 1</td>
<td>Fbp1</td>
<td>Absent in spermatogonia. Glycolysis and gluconeogenesis</td>
<td>401</td>
</tr>
<tr>
<td>100931</td>
<td>Arylsulfatase A</td>
<td>ArsA</td>
<td>Absent in spermatogonia. Sulfuric ester hydrolase</td>
<td>194</td>
</tr>
<tr>
<td>92292</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 3</td>
<td>Slc2a3</td>
<td>Absent in spermatogonia</td>
<td>132</td>
</tr>
<tr>
<td>95060</td>
<td>Solute carrier family 16 (monocarboxyl acid transporters), member 7</td>
<td>Slc16a7</td>
<td>Absent in spermatogonia</td>
<td>68</td>
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<tr>
<td>93560</td>
<td>RIKEN cDNA 1110039O14 gene</td>
<td>1110039O14Rik</td>
<td>Present in spermatogonia. Similar to human acylphosphatase</td>
<td>59</td>
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<tr>
<td>103982</td>
<td>Alcohol dehydrogenase 4 (class II), pi polypeptide</td>
<td>Adh4</td>
<td>Absent in spermatogonia. Glycolysis and gluconeogenesis</td>
<td>43</td>
</tr>
<tr>
<td>104328</td>
<td>Aquaporin 9</td>
<td>Aqp9</td>
<td>Absent in spermatogonia. Water transport</td>
<td>43</td>
</tr>
<tr>
<td>104372</td>
<td>RIKEN cDNA 0910001L24 gene</td>
<td>0910001L24Rik</td>
<td>Absent in spermatogonia. Xenobiotic metabolism</td>
<td>39</td>
</tr>
<tr>
<td>99011</td>
<td>UDP-N-acetyl-α-D-galactosamine-polypeptide N-acetylgalactosaminyl-transferase 3</td>
<td>Galnt3</td>
<td>Absent in spermatogonia</td>
<td>39</td>
</tr>
<tr>
<td>103646</td>
<td>Carnitine acetyltransferase</td>
<td>Crat</td>
<td>Present in one sample in spermatogonia. Fatty acid metabolism</td>
<td>33</td>
</tr>
<tr>
<td>101388</td>
<td>Phosphoglycerate kinase 2</td>
<td>Pgi2</td>
<td>Absent in spermatogonia. Spermatocyte-specific PGK isoform encoded by an intronless retropon (Boer et al., 1987). Glycolysis and gluconeogenesis</td>
<td>33</td>
</tr>
<tr>
<td>99542</td>
<td>Pyruvate dehydrogenase E1 α 2</td>
<td>Pdhα2</td>
<td>Present in spermatogonia. Glycolysis and gluconeogenesis</td>
<td>30</td>
</tr>
<tr>
<td>94540</td>
<td>RIKEN cDNA 1300006E06 gene</td>
<td>1300006E06Rik</td>
<td>Absent in spermatogonia. Cytochrome C P-450-16α. Electron transport</td>
<td>22</td>
</tr>
<tr>
<td>103689</td>
<td>ATP-binding cassette, sub-family C (CFTR/MRP), member 3</td>
<td>Abcc3</td>
<td>Absent in spermatogonia. Similar to human multidrug resistance associated protein</td>
<td>19</td>
</tr>
<tr>
<td>161243</td>
<td>RIKEN cDNA 0910001L24 gene</td>
<td>0910001L24Rik</td>
<td>Absent in spermatogonia. Xenobiotic metabolism</td>
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</tr>
<tr>
<td>103531</td>
<td>RIKEN cDNA 1300013B24 gene</td>
<td>1300013B24Rik</td>
<td>Absent in spermatogonia. Low similarity to endoplasmic oxidoreductase 1 B</td>
<td>16</td>
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<tr>
<td>92841</td>
<td>Chromogranin B</td>
<td>Chgb</td>
<td>Absent in spermatogonia</td>
<td>16</td>
</tr>
<tr>
<td>103068</td>
<td>Aldo-keto reductase family 1, member E1</td>
<td>Akr1e1</td>
<td>Absent in spermatogonia. Aldehyde reductase</td>
<td>15</td>
</tr>
<tr>
<td>99591</td>
<td>Retinol dehydrogenase 11</td>
<td>Rdh11</td>
<td>Absent in spermatogonia. Similar to human androgen-regulated prostate short-chain dehydrogenase/reductase 1</td>
<td>15</td>
</tr>
<tr>
<td>93557</td>
<td>Selenophosphate synthetase 2</td>
<td>Spds2</td>
<td>Absent in spermatogonia</td>
<td>13</td>
</tr>
<tr>
<td>97511</td>
<td>Monoglyceride lipase</td>
<td>MglL</td>
<td>Absent in spermatogonia</td>
<td>13</td>
</tr>
<tr>
<td>97834</td>
<td>Phosphofructokinase-1 C</td>
<td>Pfkp</td>
<td>Absent in spermatogonia. Glycolysis and gluconeogenesis</td>
<td>12</td>
</tr>
<tr>
<td>160839</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 5</td>
<td>Slc2a5</td>
<td>Absent in spermatogonia</td>
<td>11</td>
</tr>
<tr>
<td>96072</td>
<td>Lactate dehydrogenase 1, A chain</td>
<td>Ldh1e</td>
<td>Present in spermatogonia. Glycolysis and gluconeogenesis</td>
<td>11</td>
</tr>
</tbody>
</table>
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 a 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>
<thead>
<tr>
<th>MG-U74Av2</th>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Notes</th>
<th>Fold Difference (Spermatocytes vs. Spermatogonia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>101938</td>
<td>Poly(A) binding protein, cytoplasmic 2</td>
<td>Pabpc2</td>
<td>Absent in spermatogonia. Encoded by an intronless retroposon during spermatogenesis</td>
<td>94</td>
</tr>
<tr>
<td>104440</td>
<td>Y box protein 2</td>
<td>Ybx2</td>
<td>Absent in spermatogonia. RNA-binding protein which might delay polysomal association of transcripts during spermiogenesis</td>
<td>90</td>
</tr>
<tr>
<td>161033</td>
<td>Poly(A) polymerase β (testis-specific)</td>
<td>Papob</td>
<td>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)</td>
<td>20</td>
</tr>
<tr>
<td>97661</td>
<td>Testis nuclear RNA binding protein</td>
<td>Tenr</td>
<td>Present in spermatogonia. Expressed in meiotic and haploid male germ cells</td>
<td>11</td>
</tr>
<tr>
<td>161041 and 92678</td>
<td>DEAD/H (Asp-Glu-Ala-Asp/His) box poly peptide 25</td>
<td>Ddx25</td>
<td>Present in spermatogonia. Gonadotropin regulated RNA helicase also expressed in Leydig cells</td>
<td>11</td>
</tr>
<tr>
<td>96850</td>
<td>Hypothetical protein 4833436005</td>
<td>4833436005</td>
<td>Present in spermatogonia. Similar to eukaryotic translation initiation factors</td>
<td>10</td>
</tr>
<tr>
<td>160429</td>
<td>NTF2-related export protein 1 Nxt1</td>
<td>4833436005</td>
<td>Present in spermatogonia. RAN-binding protein involved in nuclear RNA export from the nucleus</td>
<td>7</td>
</tr>
<tr>
<td>100720</td>
<td>Poly(A) binding protein, cytoplasmic 1</td>
<td>Pabpc1</td>
<td>Present in spermatogonia</td>
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</tr>
<tr>
<td>101579</td>
<td>Signal recognition particle 9 kDa</td>
<td>Srp9</td>
<td>Present in spermatogonia. Cytoplasmic ribonucleoprotein targeting nascent polypeptide chains to the endoplasmic reticulum</td>
<td>4</td>
</tr>
<tr>
<td>103101</td>
<td>TAR (HSV) RNA binding protein 2</td>
<td>Tarbp2, Prbp</td>
<td>Present in spermatogonia. Interacts with the 3' untranslated region of the Protamine-1 RNA</td>
<td>4</td>
</tr>
<tr>
<td>101519</td>
<td>Signal recognition particle 14 kDa (homologous Alu RNA binding protein)</td>
<td>Srp14</td>
<td>Present in spermatogonia. Cytoplasmic ribonucleoprotein targeting nascent polypeptide chains to the endoplasmic reticulum</td>
<td>3</td>
</tr>
<tr>
<td>94552</td>
<td>Poly(C) binding protein 1</td>
<td>Pcbp1</td>
<td>Present in spermatogonia. Implicated in mRNA stabilization</td>
<td>2</td>
</tr>
<tr>
<td>103330</td>
<td>Spermatid perinuclear RNA binding protein</td>
<td>Spnr</td>
<td>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</td>
<td>2</td>
</tr>
</tbody>
</table>
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.

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

<table>
<thead>
<tr>
<th>MG-U74Av2 target Affymetrix</th>
<th>Gene name</th>
<th>Gene symbol</th>
<th>Notes</th>
<th>Fold difference (spermatocytes vs. spermatogonia)</th>
</tr>
</thead>
<tbody>
<tr>
<td>104029</td>
<td>Calmegin</td>
<td>Clgn</td>
<td>Absent in spermatogonia. Knockout male mice are sterile for defective sperm function (Ikawa et al., 1997)</td>
<td>52</td>
</tr>
<tr>
<td>101850</td>
<td>Sperm autoantigen protein 17</td>
<td>Spa17</td>
<td>Present in spermatogonia. A calmodulin-binding protein enriched in the sperm acrosome and interacting with the zona pellucida</td>
<td>31</td>
</tr>
<tr>
<td>161839</td>
<td>RAS-like, family 2, locus 9</td>
<td>Rasl2-9</td>
<td>Absent in spermatogonia. Strongly related to Ran GTPase, probably a testis-specific isoform</td>
<td>30</td>
</tr>
<tr>
<td>100972</td>
<td>Chemokine (C-C motif) ligand 27</td>
<td>Ccl27</td>
<td>Absent in spermatogonia. Also called ALP, CTAK, mILC, CTACK, PESKY, ESkine, skinkine</td>
<td>29</td>
</tr>
<tr>
<td>102948</td>
<td>Hematopoietic cell transcript 1</td>
<td>Hmt1</td>
<td>Absent in spermatogonia. Contains a calcium-activated BK potassium channel α subunit signature</td>
<td>26</td>
</tr>
<tr>
<td>99869</td>
<td>Hepatoma-derived growth factor</td>
<td>Hrpl, Pwwp1</td>
<td>Absent in spermatogonia. Also called PWWP domain containing 1. Present in the nucleus of spermatocytes and spermatids</td>
<td>23</td>
</tr>
<tr>
<td>98614</td>
<td>Nephrocystin</td>
<td>Nphp1</td>
<td>Present in spermatogonia. SH3 containing protein which forms protein complexes with p130(Cas), proline-rich tyrosine kinase 2 (Pyk2), and tensin</td>
<td>23</td>
</tr>
<tr>
<td>103489</td>
<td>Socius</td>
<td>Soc</td>
<td>Absent in spermatogonia. A Rho-related GTPase-interacting protein involved in disassembly of actin stress fibers</td>
<td>22</td>
</tr>
<tr>
<td>93210</td>
<td>NIMA-related-kinase 4</td>
<td>Nek4</td>
<td>Absent in spermatogonia</td>
<td>18</td>
</tr>
<tr>
<td>93658</td>
<td>Protein tyrosine phosphatase, non-receptor type 20</td>
<td>Ptnp20</td>
<td>Absent in spermatogonia</td>
<td>16</td>
</tr>
<tr>
<td>100287</td>
<td>Immunoglobulin (CD79A) binding protein 1b</td>
<td>Igblb</td>
<td>Absent in spermatogonia. Binds to protein phosphatase 2A. Also called α-β</td>
<td>16</td>
</tr>
<tr>
<td>160623</td>
<td>Cyclin-dependent kinase-like 2</td>
<td>Cdkl2</td>
<td>Absent in spermatogonia. CDC2-related kinase also called KIAMRE</td>
<td>13</td>
</tr>
<tr>
<td>104166</td>
<td>Renal tumor antigen</td>
<td>Ral, MOK</td>
<td>Present in spermatogonia. Protein kinase with homologies with members of the MAPK family</td>
<td>13</td>
</tr>
<tr>
<td>104135</td>
<td>ADP-ribosylation-like 3</td>
<td>Arl3</td>
<td>Present in spermatogonia. Small monomeric GTPase of the Ras superfamily</td>
<td>12</td>
</tr>
<tr>
<td>160948</td>
<td>Testis-specific calcineurin isoform</td>
<td>Ppp3cc</td>
<td>Present in spermatogonia. Calmodulin-dependent protein phosphatase</td>
<td>11</td>
</tr>
<tr>
<td>92805</td>
<td>ADP-ribosylation-like 4</td>
<td>ARL4</td>
<td>Present in spermatogonia. Knock-out mice show a significant reduction of testis weight and sperm count (Schramm et al., 2002)</td>
<td>10</td>
</tr>
<tr>
<td>100562</td>
<td>Ran guanine nucleotide release factor</td>
<td>Rangnrf</td>
<td>Present in one sample in spermatogonia. Also called MOG1</td>
<td>9</td>
</tr>
<tr>
<td>100291</td>
<td>Casitas B-lineage lymphoma</td>
<td>Cbl</td>
<td>Absent in spermatogonia. Adaptor protein</td>
<td>8</td>
</tr>
<tr>
<td>102033</td>
<td>Testis-specific protein kinase 1</td>
<td>Tesk1</td>
<td>Absent in spermatogonia</td>
<td>8</td>
</tr>
<tr>
<td>92639</td>
<td>Serine/threonine kinase 6</td>
<td>Stk6, Ayk1</td>
<td>Present in spermatogonia. Also called Aurora/IPL1-related kinase 1. Specifically expressed in meiotic cells just before the first meiotic division</td>
<td>7</td>
</tr>
<tr>
<td>100885</td>
<td>NIMA-related kinase 2</td>
<td>Nek2</td>
<td>Present in spermatogonia. Involved in chromosome condensation during meiotic divisions (Di Agostino et al., 2002). Controls splitting of duplicated centrosomes</td>
<td>7</td>
</tr>
<tr>
<td>161575</td>
<td>Mitogen activated protein kinase 10</td>
<td>Mapk10,</td>
<td>Absent in spermatogonia. Also called SAPK (β), JNK3, SERK2, p54SAPK, p439F12</td>
<td>4</td>
</tr>
<tr>
<td>97812</td>
<td>RAN binding protein 9</td>
<td>Ranbp9</td>
<td>Present in spermatogonia. Stimulates Ras activation by recruiting Sos. Also called RanbpM (Wang et al., 2002)</td>
<td>4</td>
</tr>
</tbody>
</table>

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
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'-GGCCGATGAAATTGAAATCGACTCATATAGG-GAGGCCG-(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 H2O 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 couple of primers 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: TAGGCCCTTTTCGAGGACGCTCG and TGGAGCTCTGTTCCGGACC and CTACAACAGATTGCTTAG (for Ranbp9, 267 bp); TGTGTAGCCGGAGGTTTTGTTGTA and TGAAACGGACTCCGGACTCCTC (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 α32PdNTPs 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.

References


