Modulation of the mouse testis transcriptome during postnatal development and in selected models of male infertility

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The aim of this study is to develop an overview of genetic events during spermatogenesis using a novel, specifically targeted gonadal gene set. Two subtracted cDNA libraries enriched for testis specific and germ cell specific genes were constructed, characterized and sequenced. The combined libraries contain >1905 different genes, the vast majority previously uncharacterized in testis. cDNA microarray analysis of the first wave of murine spermatogenesis and of selected germ cell-deficient models was used to correlate the expression of groups of genes with the appearance of defined germ cell types, suggesting their cellular expression patterns within the testis. Real-time RT–PCR and comparison to previously known expression patterns confirmed the array-derived transcription profiles of 65 different genes, thus establishing high confidence in the profiles of the uncharacterized genes investigated in this study. A total of 1748 out of 1905 genes showed significant change during the first spermatogenic wave, demonstrating the successful targeting of the libraries to this process. These findings highlight unknown genes likely to be important in germ cell production, and demonstrate the utility of these libraries in further studies. Transcriptional analysis of well-characterized mouse models of infertility will allow us to address the causes and progression of the pathology in related human infertility phenotypes.

Key words: development/infertility/microarray/spermatogenesis/testis

Introduction

Spermatogenesis is a highly co-ordinated process involving complex cell interactions and changing patterns of gene expression. Many of the genes involved in meiotic and postmeiotic germ cell differentiation are specific to spermatogenesis, up to 4% of the total mouse genome (Schultz et al., 2003), while other cellular processes crucial for the differentiation of multiple cell types and tissues are also important in the spermatogenic pathway. The regulation of apoptotic death, inter- and intracellular signalling pathways and complex networks of hormone action are carefully integrated to correctly orchestrate germ cell development and differentiation, the Sertoli cell being pivotal to this process. Despite detailed knowledge of testicular structure and accompanying cell biology, our understanding of how this relates to the molecular pathways that define different testicular cell types and their interactions remains rudimentary. In turn, this has hindered an understanding of the causes of male infertility at the molecular level. There are many excellent reviews available summarizing the state of current knowledge concerning the molecular regulation of male fertility (Hecht, 1998; Griswold, 1998; Okabe et al., 1998; Eddy, 2002; Matzuk and Lamb, 2002).

Microarray based approaches to mRNA expression analysis (Schena et al., 1995; Lockhart and Winzeler, 2000; Xiang et al., 2003) allow a global analysis of the testicular transcriptome that can reveal the critical genetic pathways and interactions that underlie successful germ cell production. An important caveat is that mRNA expression studies will not reveal translational controls affecting protein expression, which are of particular relevance during postmeiotic stages (Kleene, 1996; Braun, 1998; Eddy, 2002). Nevertheless, a more detailed understanding of how the testicular transcriptome evolves within the developing postnatal testis through to maturity, and how this transcriptome is affected in pathological disease states, will permit correlation with the understanding of testicular function that has emerged over a number of decades from detailed studies of testicular histology and cell biology.

The first wave of spermatogenesis in the testis of postnatal mice is a synchronized process. At defined days after birth, new germ cell types populate the testis, thus progressively more mature germ cell types appear until the entire spectrum is present (Russell et al., 1990). A successful first wave of spermatogenesis, and the establishment by apoptosis of appropriate germ cell numbers in relation to the size of the Sertoli cell population, is critical for sustained spermatogenesis in the adult (Orth et al., 1988; Russell and Griswold, 1993).

Shortly after birth, quiescent gonocytes (arrested at G0/G1) are reactivated and differentiate into self-renewing stem cells (type A0 spermatogonia) by day 5 in mice. Some of these stem cells cease division and undergo further differentiation through various intermediate spermatogonial stages to yield meiotic spermatocytes by day 10. The meiotic phase is an extended period of some 10–12 days...
resulting by day 21 in the appearance of postmeiotic round spermatids signalling the start of spermiogenesis. By around day 30 the first mature sperm are present in the testis. Thus, the first wave of spermatogenesis offers the opportunity to correlate changing patterns of gene expression with the appearance of defined populations of germ cells. This will produce an expression framework within which to investigate genetic models of infertility in the mouse.

Naturally occurring and experimentally produced (for example knock-out and ENU mutagenesis) models of infertility in mouse provide a means of studying, *in vivo*, the complex interactions within the testis that govern the formation of functioning germ cells. It is unlikely that such studies could be accomplished using *in vitro* cell culture systems as these will be unable to model the full complexity of the spermatogenic process.

This paper describes a rigorously controlled analysis of gene expression patterns using cDNA microarrays, during the first wave of spermatogenesis in normal fertile mice, and compares these in detail to other spermatogenic models of infertility. The four models are: (i) XXY*sr* (formerly termed XXY*sr*) in which few if any germ cells survive beyond the immediate postnatal period (McLaren and Monk, 1981; McLaren *et al.*, 1984; Mroz *et al.*, 1999; Mazeyrat *et al.*, 2001); (ii) *mshi* homozygotes in which there are reduced numbers of spermatogonia and those that are present do not progress beyond meiotic stages (Ward-Bailey *et al.*, 1996); (iii) *Bax* −/− males that are homozygous for a targeted mutation of the pro-apoptotic *Bax* gene, and accumulate atypical premeiotic cells including multinucleate giant cells and have spermatids. Two further models, namely *azh* and *unt* with teratozoospermia and subfertility (Hugenholtz, 1984) and a model bearing a targeted mutation of the anti-apoptotic *Bcl-w* gene (Print *et al.*, 1998), were also examined and showed few transcriptional changes when compared to normal controls at the ages analysed.

### Materials and methods

#### Sample collection

All animals were housed and killed in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986. Paired testes were collected from multiple C57BL6J animals (Harlan Ltd, UK) at the following ages: 1 dpp (days post partum) (*n* = 75), 5 dpp (*n* = 50), 10 dpp (*n* = 35), 15 dpp (*n* = 30), 23 dpp (*n* = 10), 35 dpp (*n* = 7) and 56 dpp (8 weeks, *n* = 15), taking the day of birth as 1 dpp. Animals were killed via intraperitoneal injection of 50 μl Euthatal, testes dissected free from all surrounding tissue including epididymal tissue, snap-frozen in isopentane on dry ice and stored at −80°C. Supplementary Figure 1 shows the histological composition of representative testes from each age used in this study. All supplementary material is located at http://www-dpms.path.cam.ac.uk/~pjell2/data/Supplementary/.

Paired testes were also obtained from adult male XXY*sr* mice (*n* = 13) on the random-bred albino MF1 (NIMR colony) background and from three normal adult males from the same strain background. For each of the homozygous *mshi*, *Bax*, *bs* and *azh* mutants, paired testes were obtained from 8 week adult males (*n* = 6 for *mshi*, *Bax* and *azh*, *n* = 4 for *bs*), together with unaffected control males (homozygous normal for *Bax* and either homozygous normal or heterozygous unaffected for *mshi*, *bs* and *azh*) from the same strains, purchased from the Jackson Laboratories (Bar Harbor, USA). Males were killed with carbon dioxide, and the testes dissected and cryopreserved. For the *Bcl-w* knockout, paired testes were obtained from homozygous null, heterozygous and homozygous normal animals (*n* = 2 in each case), preserved in RNALater (Ambion, USA) and stored at −20°C.

#### RNA preparation

The samples for each age/mutant were pooled at the RNA preparation stage to reduce sample numbers to manageable levels, thus constituting a pre-averaging of biological replicates reducing the impact of individual variation. Cryopreserved testis tissue was homogenized using either a rotor-stator homogenizer or a mortar and pestle, and total RNA extracted using TRI reagent (Sigma, UK) according to the manufacturer’s protocol. Total RNA was then further purified using the RNaseasy filter-binding system (Qiagen, UK). The XXY*sr* poly-A*+* mRNA used for the subtracted library production was isolated using the Oligotex affinity purification kit (Qiagen).

#### Subtracted library production and arraying

Two separate subtracted cDNA libraries were constructed using the Clontech PCR-Select system. For the first subtraction, normal adult Balb/c testis was used as the tester cDNA and a pool of six different Balb/c somatic cDNA (see below) as the driver. This subtraction selected for genes specifically expressed in the testis, and is hereafter referred to as the somatic subtracted library. For the second subtraction, normal adult Balb/c testis cDNA was used as the tester, and germ cell deficient XXY*sr* testis cDNA as the driver. This subtraction selected for genes associated with the presence of germ cells in the testis, and is hereafter referred to as the XXY*sr* subtracted library.

Double-stranded cDNA samples were prepared from 2 μg each of normal adult mouse testis, brain, liver, lung, kidney, heart and skeletal muscle poly-A*+* mRNA (Clontech, USA), and from 2 μg of XXY*sr* poly-A*+* mRNA (prepared in-house, see above), using the Promega Universal RiboClone system. The double stranded cDNA was digested to completion with 50 IU of Roche Rnasefree HaeIII enzyme for 2 h at 37°C, and used to perform suppression subtractive hybridization (SSH) procedure according to the Clontech PCR-Select protocol, using a 16 h incubation for the first hybridization and 8 h for the second. The efficiency of the SSH was tested by semi-quantitative PCR of selected housekeeping genes. The degree of subtraction of these genes varied between ~20-fold (~5 cycles of PCR) for beta actin and 2000-fold (~11 cycles of PCR) for G3PDH. The final subtracted PCR product populations were ligated into the pGem-T Easy plasmid vector (Promega, UK), subcloned into *E. coli* XL-1 Blue strain supercompetent cells (Stratagene, USA), and colonies picked into 96-well microtitre plates using a BioPICK colony picking robot (BioRobotics, UK). In all, 5145 colonies were picked from the somatic subtracted library, and 1920 colonies from the XXY*sr* subtracted library.

Library inserts were amplified via PCR and deposited in duplicate with negative and positive controls (Table I) to form cDNA microarrays. The panel of control genes chosen covers several different functional categories of ‘housekeeping’ gene, with a slight bias towards ribosomal components. Use of

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Gene name</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3I3a</td>
<td>H3 histone, family 3A</td>
<td>Chromatin</td>
</tr>
<tr>
<td>HsrpF</td>
<td>Heterogeneous ribonucleoprotein F</td>
<td>RNA processing</td>
</tr>
<tr>
<td>Acta2</td>
<td>Alpha 2 actin</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Actb</td>
<td>Beta actin</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Krt18</td>
<td>Keratin complex 1, acidic, gene 18</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Vim</td>
<td>Vimentin</td>
<td>Cytoskeleton</td>
</tr>
<tr>
<td>Cox4a</td>
<td>Cytochrome C oxidase, subunit Iva</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
<td>Metabolism</td>
</tr>
<tr>
<td>PpiA</td>
<td>Cyclophilin A</td>
<td>Metabolism</td>
</tr>
<tr>
<td>Hprt</td>
<td>Hypoxanthine guanine phosphoribosyl transferase</td>
<td>Metabolism</td>
</tr>
<tr>
<td>UbB</td>
<td>Ubiquitin B</td>
<td>Metabolism</td>
</tr>
<tr>
<td>RplP7</td>
<td>Ribosomal protein L7</td>
<td>Ribosomal</td>
</tr>
<tr>
<td>RplA4I</td>
<td>Ribosomal protein L4I</td>
<td>Ribosomal</td>
</tr>
<tr>
<td>Rps7</td>
<td>Ribosomal protein S7</td>
<td>Ribosomal</td>
</tr>
<tr>
<td>Rps13</td>
<td>Ribosomal protein S13 (Rps13)</td>
<td>Ribosomal</td>
</tr>
<tr>
<td>Rps20</td>
<td>Ribosomal protein S20</td>
<td>Ribosomal</td>
</tr>
<tr>
<td>Rps21</td>
<td>Ribosomal protein S21</td>
<td>Ribosomal</td>
</tr>
<tr>
<td>Rps22</td>
<td>Ribosomal protein, mitochondrial, S22</td>
<td>Ribosomal</td>
</tr>
<tr>
<td>Rps25</td>
<td>Ribosomal protein S25</td>
<td>Ribosomal</td>
</tr>
<tr>
<td>Rps29</td>
<td>Ribosomal protein S29</td>
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<table>
<thead>
<tr>
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<th>Function</th>
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</thead>
<tbody>
<tr>
<td>Rps29</td>
<td>Ribosomal protein S29</td>
<td>Ribosomal</td>
</tr>
</tbody>
</table>

**Table I.** Control genes used in the normalization panel for all array experiments
a panel of multiple genes was necessary owing to the existence of testis specific, differentially regulated homologues of many metabolic genes, which makes the testis an exceptionally awkward tissue with regard to control genes. The total number of different clones spotted to form the arrays used in this study was 7749. This value includes both of the subtracted libraries (7065 clones), all positive and negative control clones, and a panel of IMAGE clones selected to represent as many members as possible of the integrin/ADAM/ADAM-TS families of cell–cell and cell–matrix interaction molecules (Wolfsberg et al., 1995). The chip designs used in this experiment have been made public through the ArrayExpress database at http://www.ebi.ac.uk/arrayexpress with accession numbers A-MEXP-31 and A-MEXP-33.

**Microarray probe preparation and hybridization**

These arrays were used to investigate the first wave of spermatogenesis in prepubertal mice, and the steady state adult phenotype of several different germ cell depleted models of infertility, using a common control strategy. For the first wave study, cDNA produced from testes at 1, 5, 10, 15, 23 and 35 dpp were compared to adult (8 week) testis cDNA via competitive hybridization to the arrays. A self–self hybridization of the adult material was used as the endpoint of this time-course. For the germ cell depleted models, cDNA from adult (8 week) mutant mice were compared to age-matched unaffected controls from the same colonies. Four technical replicates (repeat hybridizations on separate slides using independent labellings of the same starting RNA preparations) were obtained for all experiments except the ayz model, for which two technical replicates were obtained.

Cy3 was used to label the test sample and Cy5 the control sample. In a common reference design, dye reversal comparisons (Tseng et al., 2001) are unnecessary, since each different age stage/mutant is labelled with the same dye, in this case Cy3. Nevertheless, the comparison of 15 dpp testis to adult was also performed with the dyes reversed to check for dye bias. The topic of dye reversal in dual dye experiments has recently been discussed in detail (Dobbin et al., 2003).

The complete data set generated in this experiment has been submitted to the public ArrayExpress database, accession number E-MEXP-35. The ArrayExpress submission also contains full details of labelling, hybridization and scanning protocols. A second archive of these data, including the original tiff image files, is also publicly accessible, linked from http://www-dpms.path.cam.ac.uk/~pjie2/data/.

Briefly, 10 μg of total RNA from test and control samples was fluorescently labelled using an amino-allyl indirect labelling protocol, and dual-colour hybridizations performed. Slides were scanned using an ArrayWorx CCD-based scanner (Applied Precision, UK), and spotfinding and quantification of fluorescence data were performed using GenePix 3.1 software (Axon, USA). Manual curation was used to exclude unreliable data. Microsoft Excel was then used to exclude data from spots flagged ‘bad’ or ‘not found’, also data with a background subtracted fluorescence value of <100 in the control channel.

On import to Genesis, the ratio between the background subtracted values for the test and control samples was calculated for each spot, and intra-slide replicate spots were averaged. The ratio values for all spots on each slide were then normalized by dividing by the median ratio value for the positive control spots on the slide. Following normalization, the data from technical replicate slides were averaged. Thus, in total, data from up to 8 measurements (four technical replicate slides × duplicate spotting on each slide) were integrated for every library clone.

**RT–PCR validation of selected profiles**

Real-time semi-quantitative RT–PCR was performed using the Quantitect SYBR Green RT–PCR kit (Qiagen) according to the manufacturer’s protocols, quantifying the resulting fluorescence using an iCycler system (BioRad, UK). Fifty nanograms of total RNA was used for each RT–PCR reaction. To control for genomic DNA contamination, primer pairs (see Supplementary Table I) were designed to span an intron boundary and to include at least one intron. Duplicate RT–PCR reactions for each gene were performed for each time-point. A ‘crossing-point’ C₀ was obtained for each gene, being the fractional cycle number at which the measured fluorescence crossed a threshold value, which was set such that all reactions were in log phase. Starting concentration for each gene in the sample was estimated semi-quantitatively as 2−C₀, thus assuming full efficiency of the PCR.

**Results**

**Library characterization**

Single-pass sequencing was performed on all clones from both libraries using a vector-directed primer. In all, 3797 successful sequences were obtained from the somatic subtracted library and 1454 sequences from the XXSxsubtracted library, giving a total of 5251. These sequences were searched against the nr database of full-length gene sequences at http://www.ncbi.nlm.nih.org/blast/ using default parameters. A ‘hit’ was defined as a match better than 1e−40. Any sequence with no hit or a genomic hit only was further searched against the dbEST database of EST. Of the 5251 sequences searched, 100 (1.9%) were chimeric and 310 (5.9%) were uninserted clones.

The GenBank ID assigned to each clone was used to interrogate the LocusLink database of genes and EST clusters, obtaining a single unique identifier for each gene present in the libraries. The successful sequences represented 1498 different genes for the somatic subtracted library and 778 for the XXSxlibrary. The total non-redundant set for both libraries combined was 1905 genes, indicating that the two subtractions have enriched for overlapping but distinct subsections of the testicular transcriptome. The total is expected to be >2000 once all remaining sequencing is successful. Since the sequencing was single-pass only, full length sequence was not obtained in all cases, thus there may be a small number of remaining undetected chimeric clones. For this reason, and because clones from different sections of a gene may show different profiles if the gene is differentially spliced, results from different library clones representing the same gene were not averaged together during data analysis.

**Validation of microarrays and array hybridizations**

Supplementary Figure 2 illustrates the typical quality of raw data from a single slide. The self–self hybridizations from the first wave time-course were used to assess experimental noise in the system. This comparison yielded a very high correlation (r² = 0.992), with no clone showing change of more than a few percentage points between the fluorescence channels, demonstrating the low noise and high quality of data obtained. High correlation was also observed between successive technical replicate ratio measurements (r² = 0.9561), and between the ratios obtained in the 15 dpp/adult dye-reversal test (r² = 0.9245). Finally, a lack of correlation between successive self–self hybridizations (r² = 0.1322) demonstrates that there are no systematic sources of error in the system (e.g. dye bias, scan field non-uniformity), since these would lead to a positive trend in this scatter plot. These regression analyses are shown in Supplementary Figure 3.

We tested the applicability of LOESS normalization (Yang et al., 2002) to our data set by comparing the fluorescence ratios detected by multiple clones representing the Odf2 gene, of differing insert size and PCR product concentration. These constitute an internal dilution series allowing confirmation of the linearity of ratio measurements across the range of fluorescence intensities. Before application of LOESS, the measured fluorescence values for the clones fall on a straight line, indicating a lack of intensity-dependent bias in the data. After LOESS, this good arrangement is distorted, demonstrating the inapplicability of this technique to our data set (Supplementary Figure 4).

**Analysis of the first wave of spermatogenesis**

The data set for the first wave hybridizations was filtered to select clones for which data was available from at least two separate technical replicates at each age stage, ensuring that a mean and SD could be calculated for each data point. A total of 5867 of 7749 clones passed this filtering step, showing that the vast majority of library clones are reliably detected by the array process.
The expression profiles for these 5867 clones were then subjected to k-means clustering to detect which clones are co-expressed and thus potentially co-regulated. A series of clusterings was performed with increasing k in order to determine the optimal value for this parameter. In general, larger values of k yielded a higher explained diversity, but values of ≥25 consistently generated empty clusters. Thus, a 24-cluster partitioning of the gene set was chosen as the basis for further analysis, with an explained diversity of 81.27%. Where multiple clones representing the same gene were present on the array, they were always grouped into the same or closely related clusters, thus constituting a useful internal check on the performance of the clustering algorithm. The complete clustering data are contained in Supplementary Table II.

The 24 clusters can be arranged into several broader ‘supergroups’ based on the overall shape of the expression profiles across the first wave. These can then be assigned to germ cell stages based upon the germ cell proportions shown in Table II, which shows the proportions of different cell types present in the seminiferous epithelium at different ages. This table is based on the data of Sutcliffe and Burgoyne (1989) and Sutcliffe et al. (1991), and a fuller version is shown in Supplementary Table III.

This study uses day 35 rather than day 31 (the latest age for which data were available in the cited studies); however, the data in the table is still indicative of the relative contribution of each cell type to the total transcriptome in this study, since the predominant change after day 31 is the appearance of vast numbers of transcriptionally inactive elongating spermatids and sperm.

Figure 1 shows the 24-cluster partitioning of the gene set. Individual clusters have been coloured according to supergroup, and sorted within each supergroup based upon the height above baseline at day 1. The starting height at day 1 is likely to indicate the degree of somatic expression of each of the genes, since the only germ cells present are comparatively low numbers of gonocytes. The supergroups identifiable within the data are as follows.

### 1: Cluster 16 (purple)
This cluster falls in expression over the first wave and is expected to contain genes expressed preferentially in the somatic component of the testis, which is diluted as germ cells proliferate.

### 2: Clusters 9, 5 and 20 (burgundy)
These remain approximately level throughout the first wave and are expected to contain housekeeping genes expressed in somatic and germ cells. There is a variable degree of ‘kink’ at day 15 (see below). The library clones in this supercluster represent only 157 of the 1905 different genes. This very low proportion of housekeeping genes demonstrates the success of the subtractive approach used to create the gene set.

### 3: Cluster 18 (green)
This cluster contains two profiles only, which are mostly flat except for a pronounced peak at day 35. This is unlikely to be germ cell expression, since the level at day 1 is relatively close to the final level, indicating significant somatic expression. This profile may reflect a transient response to hormonal changes during sexual maturation.

### 4: Cluster 6 (blue)
This cluster contains the very early up-regulating genes, showing marked change between days 1 and 5. These may thus be genes associated with the presence of early spermatogonial stages.

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Table II. Cellular proportions as a percentage of total cell number (uncorrected for nuclear size) in the seminiferous epithelium at various ages post partum (dpp = days post partum)

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Profile</th>
<th>1 dpp</th>
<th>5 dpp</th>
<th>10 dpp</th>
<th>15 dpp</th>
<th>23 dpp</th>
<th>31 dpp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sertoli</td>
<td>Somatic</td>
<td>86.9</td>
<td>88.5</td>
<td>75.8</td>
<td>40.9</td>
<td>21.9</td>
<td>12.0</td>
</tr>
<tr>
<td>A spermatogonia</td>
<td></td>
<td>8.0</td>
<td>8.0</td>
<td>19.0</td>
<td>12.7</td>
<td>5.7</td>
<td>2.2</td>
</tr>
<tr>
<td>IntB spermatogonia</td>
<td></td>
<td></td>
<td></td>
<td>3.8</td>
<td>11.6</td>
<td>7.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Early spermatocytes (preleptotene to zygotene)</td>
<td></td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Late spermatocytes (pachytene + secondary)</td>
<td></td>
<td>15.5</td>
<td>15.5</td>
<td>15.5</td>
<td>15.5</td>
<td>15.5</td>
<td>15.5</td>
</tr>
<tr>
<td>Haploid spermatids</td>
<td></td>
<td>14.1</td>
<td>14.1</td>
<td>14.1</td>
<td>14.1</td>
<td>14.1</td>
<td>14.1</td>
</tr>
</tbody>
</table>

Numbers in parentheses show the proportions relative to a level of 100% at day 31. Full details of the data used to generate this table are shown in Supplementary Table III.

Table III. Genes found via literature search to reproduce previously known expression profiles during the cluster validation process

<table>
<thead>
<tr>
<th>Cluster</th>
<th>Profile</th>
<th>Genes verified</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>Somatic</td>
<td>Anxa6, Itga5, Itga6, Itgb1, Maged1, Rbm3</td>
</tr>
<tr>
<td>9</td>
<td>Housekeeping</td>
<td>Atp5a1, Histone H3.3, Hurphil1, Mt-Co1, Mt-Nd4l, Mt-Nd5, Rpl44, Rp17, Rps7, Rps13, Rps25, Rps29</td>
</tr>
<tr>
<td>6</td>
<td>Very early up-regulating, putatively spermatogonia</td>
<td>Aard, DazP, Taf7P</td>
</tr>
<tr>
<td>2</td>
<td>Early up-regulating, putatively early spermatocyte genes</td>
<td>Sycp2, Hem1, Cct7</td>
</tr>
<tr>
<td>22</td>
<td>Steady up-regulation, putatively late spermatocyte genes</td>
<td>Adam2, Bak1, Clgn1, Hrp-111, Hsp90a2, Lyar1, Mns1, Nasp, Nphp16, Tep1, Ybx218</td>
</tr>
<tr>
<td>1</td>
<td>Up-regulating d15-23, putatively round spermatid</td>
<td>Acrvi1, Adams4, Akap3, Akap12, Bch2, Capp3, Cast, Can, Iba1, Ins162, Pkg-2, Pmnbp129, Ropn1, Shipp1, Sry2, Tep1, The5, Akap4, Gapd35, Hb116, Oaz3, Odj1, Odj2, Prm1, Prm2, Prm20, Top1, Top2</td>
</tr>
<tr>
<td>17</td>
<td>Late up-regulating, putatively late round spermatid</td>
<td>Ankap2, Gapd35, Hb116, Oaz3, Odj1, Odj2, Prm1, Prm2, Prm20, Top1, Top2</td>
</tr>
</tbody>
</table>

Bold type represents cases where multiple clones of the same gene fell in the same cluster, up to 67 clones in the case of Prm2. Superscript numbers refer to the Supplementary Bibliography.
5: Cluster 2 (dark blue)
This cluster contains genes that begin up-regulation before day 10 and reach their maximal expression by day 15. These may be genes associated with either late spermatogonial or early spermatocyte stages. Based on Table II, late spermatogonial genes are expected to show a higher day 15 peak than early spermatocyte genes.

6: Clusters 21, 3, 24 and 10 (black)
These contain genes that are up-regulated steadily throughout much of the first wave and also have significant day 1 expression levels (cluster 24 also shows the day 15 kink). These are expected to be genes associated with multiple somatic and germ cell types, though preferentially in germ cells.

7: Clusters 11, 22, 14 (olive)
These contain genes that are up-regulated between days 10 and 23, and have reached their final expression levels by day 23. The lack of any change in expression post day 23 shows that there is little or no haploid expression of these genes, thus these are likely to be genes expressed in association with spermatocyte stages.
8: Clusters 13, 7, 8 and 23 (dark green)
These contain genes that show little increase before day 15 (thus are not highly expressed prior to mid pachytene), which then increase markedly between days 15 and 23 but show little further change between days 23 and 35. These are likely to be genes expressed in association with late spermatocyte or early round spermatid stages.

9: Clusters 4, 1 and 12 (grey)
These contain genes that show little increase before day 15, a large increase to day 23, and which then continue to rise by a lesser amount through the subsequent ages. These genes are thus likely to be expressed in association with late spermatocyte through to middle round spermatid stages.

10: Clusters 19, 15 and 17 (red)
These show no increase before day 15, and a steady rise across the day 23 and day 35 time-points and even after day 35. These genes are expressed in association with later stage spermatids, especially cluster 17, which shows the largest continued change after day 35.

Examining the clusters, it can be seen that a few of the clusters follow very similar patterns, with profiles more or less identical except for a downward ‘kink’ at day 15, namely clusters 9/5/20 and 10/24. This day 15 kink may in part reflect the consequences of meiotic sex chromosome inactivation (MSCI) during the pachytene stage of germ cell development (McKee and Handel, 1993). As the cohort of pachytene spermatocytes expands between days 10 and 15, this will lead to a fall in the testis/adult expression ratio for germ cell expressed X and Y transcripts.

In support of this hypothesis, Supplementary Figure 5 shows example clones for the 27 genes in the gene set that are known to map to the X or Y chromosome. The great majority show evidence of a fall in measured expression at day 15, whereas the two that do not fall in expression correspond to HPRT and Rbm3. HPRT transcripts are known to be maintained at all germ cell stages (Shannon and Handel, 1993), while Rbm3 is a Sertoli cell gene (Danno et al., 2000) and as such should not be affected by the transcriptional inactivation in the germ line. Other clones exhibiting a day 15 kink may also correspond to X or Y genes on the array for which the mapping has not yet been confirmed, or to downstream targets of genes affected by MSCI. The kinking may also be in part artefactual, due to a chance reduction in background noise for the day 15 series of hybridizations. Clusters 5 and 20 in particular contain genes with near-zero expression in absolute terms. Further work using targets designed to differentiate between X or Y genes and autosomal retroposed homologues of these genes will be necessary in order to clarify this issue, while expansion of this study to include more time-points will allow smoothing of the derived expression profiles, reducing the impact of experimental error within any given time-point.

In order to calibrate the assignment of genes to germ cell type, we selected an exemplary cluster from each supergroup that could be matched to a specific germ cell stage, using the cluster with best match to the germ cell proportions in Table II if more than one cluster was present in the supergroup. A literature search was then performed for each gene in each selected cluster. In every case where data were available for the testicular expression profile of a given gene, the array-generated expression profile matched prior knowledge, some 61
genes in all being verified (Table III and Supplementary Bibliography). The agreement with previous data for these previously characterized genes gives cause for high confidence in the profiles generated for the many hundreds of unknown genes contained in the clone set. Furthermore, the cluster validation bears out the success of the normalization strategy we used, since multiple known housekeeping genes were grouped into a cluster with a flat expression profile.

Validation of the array-derived profiles was also performed by using semi-quantitative real-time RT–PCR to analyse four genes (Aard, Tex101, Cklf1 and Ankrd5) never previously studied in mouse postnatal testis development. Aard has been reported to be up-regulated in embryonic XY indifferent gonads relative to XX indifferent gonads (Menke and Page, 2002), and is suggested to be of somatic origin in that context. Of the others, Tex101 is the transcript corresponding to a novel germ cell antigen TES101 (Kurita A et al., 2001), Cklf1 is the mouse homologue of a human cytokine related molecule (Han et al., 2001), and Ankrd5 is an unknown transcript containing putative ankyrin repeat domains. The elongation factor Eif3s7 was used as a control gene for the RT–PCR, thus array data was re-normalized based upon Eif3s7 rather than the control gene panel for the purposes of this comparison.

The results for the RT–PCR analysis are shown in Figure 2. Profile agreement is good, except that the dynamic range of array-based measurements is less than that of RT–PCR techniques, being 2–3 orders of magnitude rather than the 5–6 orders of magnitude possible with RT–PCR. Where the range of the expression profiles falls within the linear range of array data, agreement is very good, giving true quantitative data on fold change.

**Analysis of germ cell depleted model data**

Following the first wave cluster analysis, data from the four selected germ cell depleted models outlined in the Introduction were integrated into the data set and the clusters visualized as before. Figure 3 shows the clusters following the addition of the infertile model data (shown at the right hand side of each subgraph). Genes which were clustered together in the first wave analysis generally continue to group closely.

![Figure 3. Expression profiles for all genes across all first wave sample time-points and the four germ cell depleted models, broken down according to the 24-cluster $k$-means partitioning of the data set. Sets are coloured and ordered as in Figure 1. The first wave expression profile is plotted on the x-axis for each subgraph as before, with the depleted models appended to the right hand side of each subgraph in the order XXSxr, mshi, Bax $\text{-/-}$, bs. Expression relative to 8 week adult control is plotted on the y-axis. The y-axis range for each graph has been set to show the full range of the data in each set, thus the y-axis for the outlined sets runs from 0 to 10, while the y-axis for the other sets runs from 0 to 2. The dashed line in each subgraph represents the line of equal expression between the staged testes and the adult control.](image-url)
together in the four models, even though they are expressed at markedly altered levels in these models relative to the normal control.

It is noteworthy that the expression levels at day 1 very closely match the expression levels in the XXSxr\textsuperscript{b} model, both in terms of the average ratio for genes within a given cluster and in the breadth of variation of the ratio between genes in a cluster. These two samples, despite their extremely different aetiology, have a similar cellular make-up except for the presence of gonocytes in the day 1 testis (not detected by the arrays due to the lack of gonocyte genes in the gene set). Comparing the day 1 fluorescence ratio data to the XXSxr\textsuperscript{b} data, we observed an \( r^2 \) correlation of 0.7676, demonstrating the very high similarity between the two transcriptomes (Supplementary Figure 6). This close agreement strongly validates the hypothesis advanced above, that the expression ratio measured at day 1 is indicative of the degree of somatic expression of any given gene.

Figure 4 shows close-ups of individual clusters of interest. Here, a logarithmic scale is used on the y-axis to show more clearly the fine detail at the lower end of the expression ratio spectrum. Figure 4A shows cluster 14, a putative cluster of spermatocyte genes. This germ cell type is absent in the XXSxr\textsuperscript{b} model, very much reduced in the mshi and Bax \(-/\) models, and slightly reduced in the bs model, and this is reflected in the relative expression level of the clones within this cluster. Figure 4B shows the putative somatic cluster 16. Addition of the depleted model data clearly divides this cluster into two subsets, one of clones overexpressed in all four models, and one of clones showing normal expression in these models. The overexpressed clones correspond to the \( \text{Itga6, Itgb1, Maged, Rbm3 and Vim} \) genes, which the literature search reports are Sertoli cell specific. The remaining genes expressed at normal levels in this cluster (including alpha, beta and gamma actins), are likely to be expressed in multiple somatic cell types.

Figure 4C shows the early up-regulating cluster 6. The depleted model data split this cluster into two subsets also, one underexpressed in XXSxr\textsuperscript{b} and overexpressed in Bax \(-/\), the other vice versa. The former contains those genes (\( \text{Taf7l, Dazl} \) known to be spermatogonial genes, while the latter contains genes known or hypothesized to be of somatic origin such as clusterin and Aard, together with the previously uncharacterized \( \text{Akr1b3 and Defb119} \). These putative somatic genes show marked change across the first few days of spermatogenesis and thus mimic the profile of spermatogonial genes in this study.

Finally, Figure 4D shows the putative late spermatogonia/early spermatocyte cluster 2, where again many genes are underexpressed in XXSxr\textsuperscript{b} and overexpressed in Bax \(-/\). Note also that in Figure 4C and D the expression levels are near normal in mshi and bs, reflecting the near normal proportion of early germ cell stages in these two models. Other features visible in Figure 3 include four clones highly overexpressed in all germ cell depleted models, visible in clusters 5, 9 and 19. These correspond to the known Leydig cell genes beta-
hydroxysteroid dehydrogenase delta (two clones) and relaxin-like factor (RLF), and the uncharacterized aldo-keto reductase NM_027582. RLF (in cluster 19) mimics the expression pattern of a round spermatid gene in the first wave experiment due to its marked up-regulation at the time of sexual maturity. This overexpression reflects a proportional regression of the seminiferous epithelium in these models, and does not necessarily indicate up-regulation within any given cell type.

Two further models were also examined in this study; the azh teratozoospermia and subfertility model (Hagenholtz, 1984) and the Bcl-w ±/- targeted knockout model (Print et al., 1998). The latter is especially interesting, as the anti-apoptotic action of Bcl-w may promote survival of germ cells at the end of the first spermatogenic wave and in adulthood by opposing pro-apoptotic partners such as Bax (Meehan et al., 2001). However, both these models failed to show any significant expression differences from control animals at the age analysed, this being 8 weeks for azh and 35 days for Bcl-w ±/- (data not shown, accession number E-MEXP-35 in the ArrayExpress database).

Discussion

This paper details the construction and validation of two subtracted cDNA libraries, and demonstrates their usefulness in analysing a number of different models of murine spermatogenesis using cDNA microarray technology. Between them the two libraries contain ~2000 different genes, thus representing a significant proportion of the testis transcriptome, larger than any other testis gene set described to date, which include a Drosophila gene set (Andrews et al., 2000) and a 950-clone cherry-picked set (Rockett et al., 2001). This allows for large-scale screening studies for genes of interest in a wide variety of phenotypes. Tantalizingly, the gene set includes a large proportion of as yet uncharacterized genes, >50% being known only by RIKEN identifiers or as ESTs.

Both cDNA-based and oligonucleotide-based microarray approaches have been used to study aspects of spermatogenesis in a limited number of other studies, predominantly cell culture based (Sha et al., 2002; Cheng et al., 2002; McLean et al., 2002; Ohta et al., 2002; Wu et al., 2003), and more rarely in whole testis tissue (Maratou et al., 2004). A recent study (Yu et al., 2003) used separated cell types to search for genes differentially regulated during spermatogenesis; however, only 260 genes were shown to be expressed in the separated cells, and fewer still shown to be regulated during spermatogenesis. This study analyses almost an order of magnitude more genes, with 1748 different genes shown to vary markedly across the course of the first wave. This underscores the need to use a testis-focused gene set when analysing spermatogenesis, due to the very high number of genes specific to this process that are unlikely to be represented in other gene sets.

The number of genes in this gene set also compares favourably with the 1652 transcripts on the Affymetrix U74v2 array recently shown to be involved in meiotic and post-meiotic germ cell development (Schultz et al., 2003).

k-Means cluster analysis of the first wave data was used to generate groups of co-expressed genes that correlate well with the proportions of different germ cell types present during the course of the first wave of spermatogenesis. Genes known to be expressed specifically in a given cell type were partitioned into appropriate clusters, greatly strengthening confidence in the assignment of uncharacterized genes to cell types. The supergroups of clusters with similar profiles, taken as a whole, represent the genetic contribution of each cell type in the adult testis.

The majority of clones showed their largest changes in expression between days 10 and 23, suggesting that most of the clones on the array are associated with the presence of primary spermatocytes or round spermatids. No genes show the profile expected of gonocyte genes (high at day 1, dropping sharply by day 5, zero thereafter), due to the absence of this cell type in the library source tissue. The abundance of meiotic and post-meiotic transcripts may be due simply to the relative proportions of these germ cell stages in the total adult RNA used to make the subtracted libraries; however, a recent study using Affymetrix arrays (Schultz et al., 2003) suggests that the majority of testis specific genes are involved in meiotic and post-meiotic stages of germ cell development.

In keeping with this, the spermatid-associated clusters (supergroups 9 and 10) contain the largest number of different genes, and also show the highest proportion of uncharacterized genes, amply demonstrating the current poor state of understanding of the incredible complexity involved in the formation of a mature spermatozoon. This is in agreement with another recent cDNA array study (Maratou et al., 2004), which also showed the postmeiotic transcriptome to be the most distinct, with both the greatest diversity and also the least overlap with other cell types.

Within the framework established by analysis of the first wave data, library clone expression levels were analysed in genetic models of infertility. While the adult transcriptomes of the models will reflect secondary changes within the testes as well as the initiating pathological events, these models are nevertheless highly useful since they contain combinations of cell types not found in non-pathological states and hence allow a more precise assignment of transcripts to defined cell types. Four different models are addressed in detail in this study: the XXXSxr, mshi, Bax ±/- and bs models, which have been selected to represent a spectrum of pathogenic phenotypes affecting different stages of spermatogenesis.

Integrating data from these models shows that genes that cluster together across the first wave continue to exhibit similar expression patterns across all four depleted models. Such changes in abundance of whole groups of transcripts reflect the underlying gross changes in testicular histology and cell numbers. Where genes with similar first wave expression patterns no longer cluster together after integration of the mutant model data, this allows refinement of both the cluster analysis and the assignment of transcripts to cell type.

In particular, data from the completely germ cell deficient XXXSxr model not only highlight those genes that are preferentially expressed and/or induced in the somatic component of the testis, but also confirms that there is detectable low level somatic expression of many genes primarily expressed in specific or multiple germ cell types. Such somatic expression may well have been missed by other studies taking a less global approach, or which focus solely on genes specific to germ cells (Schultz et al., 2003).

Integration of genetic model data has allowed discrimination among the earliest-onset cluster, dividing it into somatic cell genes and spermatogonial/early spermatocyte genes. The very early up-regulation of Aard seen on the arrays and confirmed by RT–PCR is in agreement with the observed up-regulation of the rat homologue (Blomberg et al., 2002), and the depleted model data is in accordance with the suggested somatic expression of this gene during fate specification of the bipotential gonad (Menke and Page, 2002). Similarly, the clustering of integrins alpha 6 and beta 1 into a putative Sertoli cell group indicates that these may play a role in Sertoli cell junctional complexes, as suggested by other studies in rat and marmoset (Palombi et al., 1992; Salanova et al., 1998; Husen et al., 1999).

Comparison of models (mshi, Bax) with a complete or partial block in germ cell development demonstrates presence of near normal levels
of the early onset transcripts (and thus early germ cell stages) but absence of later onset genes. The *Bax* knockout model is of special interest, since the mature phenotype involves not only a reduction in the number of certain germ cell stages but also a massive amplification of arrested abnormal cells. Both these aspects of the phenotype are clearly detected by the array, with a marked reduction in transcripts associated with late spermatocytes and spermatid stages, an over-expression of several spermatogonial genes, including *Dazl*, *Taf7l* and *Sydc3*, and approximately normal levels of early spermatocyte genes such as *Hemt*. This combination of both premeiotic and early meiotic genes reflects the ambiguous phenotype of the arrested cells.

Interestingly, several of the transcripts in the late spermatogonial/early spermatocyte cluster 2 are not overexpressed in the *Bax* −/− model. Close analysis of the *Bax* −/− phenotype, along with analysis of other mutants triggering apoptosis at the early leptotene stage such as the *Spo11* knockout model (Baudat et al., 2000), may reveal details of quality control procedures during the initiating events of meiosis, perhaps uncovering novel checkpoints at this stage.

Comparison across all of the germ cell depleted models also allows identification of further patterns among genes expressed in the somatic components of the testis, which in germ cell depleted mice constitute a relatively larger proportion of the testis. Notably, there is marked overexpression of a number of known Leydig cell genes and a novel aldo-keto reductase. This is suggestive of Leydig cell expression for the latter, which may thus be part of a novel steroid synthetic pathway. While these changes in somatic gene expression level may simply reflect changes of cell number due to regression of the seminiferous epithelium, this may have functional consequences in terms of ratio of hormone signal to target receptor molecules. It is clear that any study seeking to take an overview of spermatogenesis cannot limit itself to considering only the events within the germ cells and Sertoli cells.

One important drawback in examining the later stages of spermatogenesis using mRNA expression analysis is that owing to the transcriptional shutdown as chromatin is repackaged, with transition proteins and then protamines replacing histones, mRNA transcripts used in the later stages of spermiogenesis are believed to be pre-expressed and stored. Thus, the mRNA transcript abundance reported by microarray expression profiling or other forms of mRNA expression analysis is unlikely to correlate with protein expression in germ cells beyond the round spermatid stage of spermatogenesis. In line with this, no expression differences between mutant and control were found in the *ach* model (Hugenholz, 1984). This phenotype has since been shown to result from a microdeletion within the *Hook1* gene with downstream effects on protein complex assembly during spermiogenesis (Mendoza-Lujambio et al., 2002), thus neither the initiating event nor the downstream events are observable at the level of mRNA expression.

Further analysis of the mRNA expression data generated in this study, particularly those cases where the expression in the germ cell depleted models is at odds with the cell type assignments derived from the first wave expression analysis, may uncover genetic effects important to the pathogenesis of the various mutant phenotypes. In view of the close similarity between human and murine spermatogenesis (Russell et al., 1990), patterns of gene expression associated with defined defective spermatogenic phenotypes in the mouse may then serve to identify genes important in certain categories of infertility found in men. It should be borne in mind, however, that patients generally present with infertility at a relatively late stage of pathological progression, when the initial testicular phenotype has been considerably altered by secondary degenerative changes. It may therefore be prudent to examine both the initial and final stages of the pathological progression in the model system in order to draw clinically relevant parallels.

This is clearly demonstrated in the case of the *Bcl-w* knockout mouse, where the first wave of spermiogenesis is largely unaffected, yet males are infertile (Print et al., 1998). In the adult animal there is massive germ cell loss, the severity of the loss increasing with later germ cell stage. For this model, there are no detectable significant changes in expression of the genes in this gene set at the age of 35 days. It may be that expanding the gene set to include more apoptosis related and spermatogonial genes will show changes at this age, or it may be that later time-points are required to elucidate the mechanisms of this particular pathology.

By contrast, in the *Bax* knockout mouse, apoptotic responses are disordered throughout the first wave, resulting in reduced apoptosis as early as 7 dpp, and to massive abnormalities in cellular proportions by 25 dpp (Russell et al., 2002). The difference in age of onset of the phenotype between the *Bcl-w* and *Bax* knockouts is striking, given the potential opposing involvement of both proteins in the same apoptotic pathways.

This study details the production and comprehensive characterization of a testis-focused gene set for the analysis of spermatogenesis. It contains more genes differentially expressed during meiotic and postmeiotic differentiation than any other gene set described to date, including the ~20 000 gene Affymetrix U74av2 set. The subtractive process used to generate the libraries enriches for low expressed genes that may be excluded from other libraries. In this context it is significant that >1000 of the genes in the gene set are known only as EST or RIKEN full length sequences, showing that this gene set contains a very large proportion of novel genes. As such, it constitutes a key resource for future studies in male reproductive biology.

Analysis of the first wave expression data, together with the use of four different germ cell depleted models of radically different cellular composition and aetiology, demonstrates the ability of the cluster analysis to group together co-expressed genes. This suggests the likely cellular expression patterns of genes in the library, which includes >1000 previously uncharacterized in the testis. This in turn gives clues to their roles during the spermatogenic process, laying down a vital framework within which to interpret the results of future studies. Future work will include expansion of the gene set to include more genes expressed in gonocytes and in the various supporting cell lineages, followed by a finer grained study of the first wave with higher temporal resolution. Such a study will assist in deciphering the detail of the genetic mechanisms involved in the cellular processes during each germ cell stage. In concert with this, similar first wave studies on these and other mutant models may reveal when and how the program of gene expression becomes derailed in each model, casting light on the pathogenesis and progression of these phenotypes, and potentially on clinically related infertility phenotypes in humans.

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Array analysis of juvenile and mutant mouse testes

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