4 Methods

4.1 Mouse testis cDNA microarrays

4.1.1 Generation of testis-specific cDNA microarrays

For the development of a testis-specific microarray we obtained two different arrayed mouse testis cDNA libraries and five stage-specific germ cell libraries (McCarrey et al., 1999) from Incyte Genomics (Palo Alto, CA). The libraries are curated by the I.M.A.G.E. consortium (Lennon et al., 1996).

Full testis libraries:

- Barstead MPLRB11 testis (1920 clones)
- NCI_NCGAP_Te1 testis (1152 clones)

Stage-specific libraries:

- Barstead MPL-RB15 pachytene (2304 clones)
- McCarrey/Eddy type A spermatogonia (1536 clones)
- McCarrey/Eddy type B spermatogonia (2304 clones)
- McCarrey/Eddy round spermatids (2304 clones)
- McCarrey/Eddy spermatocytes (1920 clones).

At the time of acquisition of the libraries, sequence information and accession numbers were available for approximately one quarter of the clones. This sequence information indicated low redundancy within each library, and we observed a significant number of rare transcripts.

Clones from the libraries were amplified by polymerase chain reaction (PCR) in 96-well plates. Denatured bacteria were used as the template for the reaction (direct colony PCR). Purification of the PCR products was performed using 384-well Multiscreen filter plates. These plates were found to give excellent product recovery with no significant contamination at relatively low cost (Hegde et al., 2000). PCR results were analyzed by gel electrophoresis, and an aliquot of each PCR reaction was stored for DNA sequencing. The purified PCR products were dried down and redissolved in 3X SSC for spotting on poly-L-lysine-coated glass slides (1st print run) and Corning UltraGAPS slides (2nd print run). Preparation of clones was partly performed robotically using the Beckman Biomek 2000 pipetting robot.

The DNA was spotted at the UT Southwestern microarray facility using an arrayer constructed from a Toshiba high precision/reproducibility pick and place robot with a multichannel spotting head, operated with a pin-spotting tool. An ultra clean environment was maintained using a HEPA filter to pressurize robot operating volume and proper clean room practices. Plates were kept cool using a surface chiller to minimize evaporation loss.

The poly-L-lysine-coated arrays were post-processed to reduce background fluorescence. In this step the DNA was linked to the slide by UV crosslinking, and the slides were subsequently blocked using a succinic anhydride and 1-methylimidazole solution. UltraGAPS slides were not post-processed but incubated with 1% BSA prior to hybridization.

4.1.2 Use of testis-specific cDNA microarrays

Sample labeling

Ten micrograms of total RNA for each sample were primed using 2 µg of anchored oligo dT primers and labeled for 2 hours at 42 ºC in a 20 µl cDNA synthesis reaction using 20 U of Superscript II, 0.01 mM DTT, 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dTTP, 0.125 mM dCTP, and 2 nmol Cy-labeled dCTP (Cy3 or Cy5). RNA was subsequently degraded using 1.5 µl of 20 mM EDTA and 1 µl of 500 mM NaOH (incubated for 8 min at 68 ºC). Samples were neutralized with 1.5 µl of 500 mM HCl.

To remove the unincorporated nucleotides the samples were purified by using YM-30 micro-concentrators. The filters were washed with 500 µl of TE and then spun in a tabletop centrifuge at 11,000 g for 9 minutes. The samples were added to 450 µl of TE and 30 µg of mouse Cot-1 DNA, applied to the microcon and spun at 11,000 g for 8 minutes. The flow-through was discarded, and the filter was washed twice by adding an additional 500 µl of TE, spun at 11,000 g for 8 minutes. During the final wash step, the probe was concentrated to a target volume of 5 µl. The concentrator was then turned over, placed into a new collection tube, and spun at 800 g for 4 minutes to retrieve the purified sample.

Hybridization and washing

For hybridization, 40 µl of SlideHyb Glass Array Hybridization Buffer #3, and 20 µg of yeast tRNA were added to the purified sample. The hybridization mix was incubated at 100 ºC for 2 minutes and then at 42 ºC for 10 minutes.

Hybridization on the microarrays was performed under a lifted coverslip (Lifterslip). The hybridization mix was applied between microarray and coverslip by capillary force. The slides were placed in hybridization cassettes and submerged in a water bath at 61 ºC for 16-20 hours. The cassettes contained 100 µl of 3X SSC for humidification.

After hybridization, slides were washed under light agitation for 10 minutes each in 2X SSC, 0.1% SDS (at 62 ºC), in 0.4X SSC (room temperature) and in 0.2X SSC (room temperature). After the final wash, slides were quickly transferred into dry 50 ml conical tubes and spun at 500 rpm for 5 minutes to remove all liquid.

Microarray scanning

After drying, slides were scanned using an Axon GenePix 4000B scanner and the GenePix Pro 4.0 software package. Slides were scanned at a resolution of 10 micron using 532 nm (17 mW) and 635 nm (10 mW) lasers. For each experiment, the photomultiplier tube (PMT) settings were adjusted to levels that limit signal saturation and produce similar average signal intensities for both dyes.

The raw data was normalized and analyzed using the Microarray Calculation and Visualization (Marc-V) software tool (Schageman et al., 2002).

4.2 Gene expression analysis of mouse spermatogenesis

4.2.1 Biological material and RNA isolation

GCS-GFP rat

The Garbers Lab has generated a line of transgenic Sprague Dawley rats that express GFP (EGFP variant) specifically in germ cells, termed *SD-Tg*(*ROSA-EGFP*)*2-4Reh* rats (J. Cronkhite, unpublished). The rats will be referred to as GCS-GFP in this manuscript. The genomic locus of the *Tg*(*GCS-GFP*) integration has not yet been defined. That only germ cells express GFP within our cultures is confirmed by the previous observation that DAZL-positive and vimentin-negative cells exclusively express GFP (Hamra et al., 2002). Furthermore, in sections of the rat testis, GFP expression is detectable only within the testicular germ cell population (data not shown).

Testis samples

Testes were individually collected from C57BL/6 mice at days one, four, eight, 11, 14, 18, 21, 26, 29, 60 (adult) *post partum* and immediately frozen in liquid nitrogen. Total RNA was isolated from individual testis using RNA Stat-60 according to the manufacturer's protocol.

Testicular cell cultures

To initiate testis cell cultures, seminiferous tubules were isolated from the testes of 22-23 day old wild-type or homozygous *SD-Tg*(*ROSA-EGFP*)*2-4Reh* Sprague Dawley rats or from 19 day old C57/BL6 mice. Testes from rats and mice of these ages are developmentally similar in that they contain late pachytene spermatocytes, which predominate as the most advanced germ cell type (Malkov et al., 1998). The tubules were enzymatically and mechanically dissociated into a cellular suspension to generate cultures of testis cells (Hamra et al., 2002). The testis cell cultures were then used as a source for isolating enriched populations of laminin-binding (lam_B), or laminin-non-binding (lam_{NB}) germ cells and somatic cells by previously established procedures (Hamra et al., 2002). Cultures of interstitial cells were prepared from 23 day old rats and 19 day old mice (Mather et al., 1981). Total RNA from all cultures of rat and mouse testis cells was isolated by using RNAqueous.

4.2.2 Microarray processing

Five micrograms of total RNA from the full testis samples were reverse transcribed into double-stranded cDNA containing a T7 promoter using the RiboAmp RNA Amplification Kit and *in vitro* transcribed and biotin-labeled using the BioArray HighYield RNA Transcript Labeling Kit. RNA from the mouse and rat somatic cell samples was amplified and labeled from 50 ng starting material using two rounds of amplification. The target aRNA was purified using an RNeasy Mini column. Twenty micrograms of aRNA of each sample were fragmented for hybridization to each microarray. The Affymetrix Rat Expression Set 230 (A and B chips) was used for all rat samples, and Murine Genome U74v2 A, B, and C arrays were used for the mouse samples. The arrays were hybridized and processed according to the manufacturer's specifications.

4.2.3 Data analysis and clustering

Results were analyzed by using the Affymetrix Microarray Suite (MAS), Ver. 5.0. Signals on each chip were scaled to a mean intensity of 250. Present, marginal and absent calls were calculated by MAS using a tau value of 0.015. All sample comparisons were performed in GeneSpring 5.1. ArrayMiner 4.0 was used for clustering, applying the Gaussian clustering model (Distance measure: Pearson coefficient), an algorithm that takes cluster variance into account and has the ability to recognize outliers (www.optimaldesign.com/Download/ArrayMiner/AM2whitepaper.pdf).

Analysis of testis samples

Triplicates and duplicates were averaged, and all data were normalized to day one. For subsequent data analysis all genes were filtered as follows: the transcripts had to have a present call and a minimum signal intensity of 75 in at least one time point. Transcripts also had to be up- or down-regulated more than 3-fold in at least one time point compared to the day 1 samples. In order to enrich for germ cell genes, transcripts also had to be absent in a testicular Sertoli cell sample (Hamra et al., 2002) and in an interstitial cell sample (Mather et al., 1981). Genes fulfilling these requirements were clustered by using the Gaussian clustering model. Annotations of all the filtered transcripts were updated by using Affymetrix Netaffx (www.netaffx.com), based on the March 2003 annotation update (Liu et al., 2003). All corresponding Unigene clusters were then screened for reported tissue expression using the NCBI Unigene database (Build no. 122 *Mus musculus*, May 1, 2003; www.ncbi.nlm.nih.gov/UniGene).

Analysis of cultured rat germ cells

Genes expressed in rat germ cells cultured on SNL 76/7 STO fibroblasts were filtered as follows: the transcripts had to have a present call in at least one of the germ cell fractions. Transcripts also had to be up- or down-regulated more than 2-fold in at least one time point compared to the day 1 samples. Genes fulfilling these requirements were clustered by using the Gaussian clustering model.

Annotations of all filtered transcripts, including gene ontology assignments for the categories in Table 6, were updated by using Netaffx, based on the October 2003 annotation update (Liu et al., 2003).

Identification of mouse homologues to rat genes

Transcribed mouse sequences homologous to transcripts exclusively expressed in cultures of rat testis cells (see legend to Figs. 17 and 18) and to rat genes listed in Table 6 were identified in the NCBI database using standard nucleotide blast searches. Apparent mouse homologues shared greater than 80% identity to queried rat sequences and had bit scores over 100. Of the identified homologues for genes in Table 6, 223 had matching oligonucleotide probe sets on the mouse arrays, and of these, 202 transcripts were detected as present in at least one time point in the developing mouse testis.

4.2.4 Quantitative real-time PCR

Real-time PCR analysis was performed by using the ABI PRISM 7900HT Sequence Detection System. All primers were designed in Primer Express 2.0 by using the MGB primer design method, and runs were analyzed by using the Sequence Detection System software (Ver. 2.1).

One microgram of total RNA of each sample was reverse transcribed in a 20-µl reaction by using 150 ng of random primers and the Superscript II reagents. Samples were diluted 1:10, and 0.5 µl was used for the PCR reaction. PCR was performed by using the SYBR Green PCR Master Mix reagents. For expression ratio verifications, all samples were normalized to the 18S signal. Ranges of fold changes were calculated according to the Applied Biosystems Comparative C_T method by using standard deviations (n=2).

4.2.5 In situ *hybridization*

Gene-specific PCR products of 200-350 bp were generated and linked to a T7-promoter-containing fragment by using the Lig'nScribe No-Cloning Promoter Addition Kit. Sense and antisense constructs were then generated by PCR by using a primer complementary to the T7 promoter fragment and the 3' or 5' gene primer, respectively. PCR products were gel purified and *in vitro* transcribed by using MAXIscript T7 Kit incorporating ^{35}S-UTP. Probes were subsequently DNAse treated and purified by using NucAway Spin Columns. To assess incorporation efficiency and transcription product quality, aliquots for scintillation counting and polyacrylamide gel electrophoresis analysis (PAGE; 5%) were collected prior to and after column purification. *In situ* hybridizations were performed by the Molecular Pathology Core Facility at the University of Texas Southwestern Medical Center, as described (Shelton et al., 2000).

4.2.6 *Feeder cell lines*

MSC-1 Sertoli cells (McGuinness et al., 1994) were maintained in Dulbbeco's modified Eagle's medium / Ham's F12 medium 1:1 supplemented with 100 units/ml penicillin G sodium, 100 µg/ml streptomycin sulfate, 250 ng/ml amphotericin B (DHF12) and 8.5% FBS at 32.5 °C/5.5% CO_2. SNL 76/7 STO fibroblasts (STO) (McMahon and Bradley, 1990) were maintained in Dulbbeco's modified Eagle's medium supplemented with 10% FBS, 100 units/ml penicillin G sodium and 100 µg/ml streptomycin sulfate (DMEM) at 36.5 °C/5.5% CO_2. Prior to culture with testis cells, feeder cell lines were treated with growth medium containing 10 µg/ml of mitomycin C in 10 cm^2 dishes at 80-90% confluence for 3.75 h at 32.5 °C/5.5% CO_2 (MSC-1) or for 3 h at 36.5 °C/5.5% CO_2 (STO). Following treatment with mitomycin C, the feeder cells were passaged (0.05% trypsin-0.53 mM EDTA) into 24-well, collagen-I-coated dishes at 0.5x10^4 cells/cm^2 for MSC-1 and 0.6x10^4 cells/cm^2 for STO in DHF12 supplemented with 10% FBS and 30 µM ME (DHF12-10% FBS) and maintained for 6 h at 32.5 °C/5.5% CO_2. Isolated testis cells from primary cultures were then plated at a density of 4x10^4 cells/cm^2 (8x10^4 cells/well) into cultures of mitomycin C-treated feeder cells in DHF12-10% FBS and then maintained at 32.5 °C/5.5% CO_2 until the time of transplantation. Prior to transplantation, the testis cell and feeder cell co-cultures were harvested by trypsinization (0.25% trypsin-1-mM EDTA) and then suspended to the desired numbers of GFP$^+$ cells using ice-cold DHF12-10% FBS. The cell suspension was adjusted to contain 0.05% trypan blue and maintained on ice until transplanted or separated by FACS.

4.2.7 Germ cell transplantation and progeny genotyping

WT Sprague Dawley rats at 12 days of age were injected (i.p.) with 12.5 mg/kg busulfan (4 mg/ml in 50% DMSO) and then used as recipient males at 24 days of age. Donor cells were loaded into injection needles fashioned from 100 µl glass capillary tubes and then transplanted into the seminiferous tubules of anesthetized rats by retrograde injection through the rete testes (Ogawa et al., 1999). Recipient males transplanted with GCS-GFP lam_B cells (after culture for ~1 day on STO feeder cells) were paired at 12 weeks of age (~60 days post transplantation) with wild-type female Sprague Dawley rats of similar age. Genotypes of all pups were determined by dot blot hybridization and PCR analysis using rat tail genomic DNA to determine the presence of the GCS-GFP transgene (Hamra et al., 2002). Genotyping results were also verified in male progeny by direct visualization of transgene expression in testes using a Nikon SMZ1500 fluorescence stereomicroscope.

4.2.8 Fluorometric analysis of germ stem cell activity

At 32 days post-transplantation the seminiferous tubules of recipient animals were dissected from the testes and then homogenized in 1.5 ml of ice-cold lysis buffer (50 mM HEPES pH 8.0, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 1 protease inhibitor tablet/12.5 ml) for 30 seconds using a PTA-7 probe on setting 5 of a PT10-35 Polytron (Kinematica). The homogenates were incubated on ice for 15-20 min and then centrifuged at 3000 x g for 10 min at 4 ºC in a GPR table-top centrifuge (Beckman). The supernatant solutions were centrifuged at 15,800 x *g* for 10 min at 4 ºC in a micro-centrifuge (Model 5042, Eppendorf), and the resultant supernatant fractions were then stored at -80 ºC. Frozen supernatant solutions were thawed on ice and then further clarified by centrifugation at 230,000 x *g*, r_{av} (TLA-100.3 rotor, TL1000 ultracentrifuge, Beckman) for 30 min at 4 ºC. Standards and supernatant solutions from the final centrifugation step were diluted into assay buffer (100 mM sodium bicarbonate, pH 9.6) and then analyzed for fluorescence intensity using a FL600 fluorescence micro-titer plate reader (BioTek) equipped with filter wheel sets for maximal excitation at 485 nm and maximal emission at 516 nm. Affinity purified recombinant EGFP with a carboxyl-terminal histidine tag (rEGFP-His) was used as a standard for determining equivalents of EGFP in lysates prepared from testes of recipient animals. The rEGFP-His was produced by transient expression from the vector pcDNA6.0-EGFP-V5-His-B following transfection (Fugene6 transfection reagent) into COS-7 cells. The pcDNA6.0-EGFP-V5-His-B was produced by cloning the EGFP open reading frame (NheI, BamHI fragment from pEGFP-1) into pcDNA6.0-V5-His-B. Recipient rats in select

studies were also analyzed for donor cell colonization by monitoring the presence of green fluorescent germ cells in cross-sections of frozen testes prepared as described (Kisseberth et al., 1999), except that the testes were fixed in 4% paraformaldehyde at 4 ºC for 4 h and then sequentially equilibrated overnight in 10% and 20% sucrose solutions prior to sectioning. For direct visualization of EGFP expression in frozen cross-sections and in seminiferous tubules a Nikon SMZ1500 fluorescence stereomicroscope was used.

4.2.9 Western blot analysis

Proteins were extracted from testes as described above for fluorometric analysis of spermatogonial stem cell activity. Equal volumes of testis extracts from triplicate recipient rats/experimental condition were pooled to provide samples used for Western blotting. Protein (100 µg/pooled sample per lane) was separated on SDS gels (10-20% acrylamide gradient) and transferred to nitrocellulose membranes. Nonspecific, protein-binding sites were blocked by incubating membranes overnight at 4 ºC in blocking buffer: TBST (Tris-buffered saline with Tween 20: 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% non-fat dry milk. Membranes were washed 3 times in TBST and incubated 1 h, 22-24 ºC with a 1:1000 dilution of primary antibody (mouse monoclonal anti-GFP IgG cocktail) in blocking buffer. Membranes were washed 3 times in TBST (0.3% Tween 20) and incubated for 45 min at 22-24 ºC with peroxidase-conjugated, goat anti-mouse IgG diluted 1:50,000 in blocking buffer. Membranes were washed 3 times in TBST and protein bands detected using the enhanced chemiluminescence detection method (ECL, Amersham).

4.2.10 Immunocytochemistry

Cultures of germ cells (2 cm^2) were washed twice with serum-free DHF12 medium (0.6 ml/well) and then fixed for 7.5 min with 4% paraformaldehyde, 0.1M sodium phosphate, pH 7.2 (0.4 ml/well). After fixation the cells were washed 3 times with PBS (0.6 ml/well) and then incubated for 15 min in PBS containing 0.1% (v/v) Triton X-100 (0.4 ml/well). The cells were then washed 3 times in PBS (0.6 ml/well), and nonspecific, protein-binding sites were blocked by incubating the cells in 0.1% w/v blocking reagent (0.4 ml/well) for 1.5 h at 22-24 ºC. The blocking reagent was then removed, and the cells were incubated for 16 h at 22-24 ºC in primary antibodies (0.4 ml/well). The anti-Egr3 IgG and the non-immune IgG fractions were each diluted to 167 ng/ml in blocking reagent. The rabbit anti-Dazl-3 IgG and the rabbit preimmune-3 IgG fractions (Hamra et al., 2002) were diluted to 250 ng/ml in blocking reagent. Following incubation in primary antibodies, the

cells were washed 3 times for 5 min with TBST (0.6 ml/well) to remove unbound IgG. The cells were then incubated for 40 min at 22-24 ºC in conjugated, secondary antibody (0.4 ml/well) diluted to 1 µg/ml in PBS containing 5 µg/ml Hoechst 33342 dye. Following incubation in secondary antibodies, the cells were washed 3 times for 5 min with TBST (0.6 ml/well) to remove unbound IgG and dye prior to viewing in fresh PBS (0.4 ml/well) using an inverted Olympus IX70 microscope.

4.2.11 Electron microscopy

Testis cells were fixed with 3% glutaraldehyde in phosphate buffer followed by post fixation with 1% osmium tetroxide, dehydrated with ethanol, embedded in Spurr resin in Beem capsules and polymerized overnight at 60 ºC. Semi-thin sections for light microscopy were cut at 1 micron, placed on glass slides and stained with filtered 1% Toluidine Blue in 1% sodium borate. Ultra-thin sections were cut at 80 nanometers, picked up on 200 mesh copper grids, stained with uranyl acetate and lead citrate, and documented with a JEOL 1200EXII transmission electron microscope. A blinded operator, with respect to the cell types being studied, gathered images randomly from cultures of each testis cell population. Thirty to forty cells per culture were scored at a magnification of 5000x from three separate cultures of the lam_B and the lam_{NB} cells. Then, the cells were categorized as either type A spermatogonia, intermediate/type B spermatogonia, spermatocytes or somatic based on cellular morphologies previously established for different testis cell types (Huckins, 1971b; De Martino et al., 1979; Russell et al., 1990; Chiarini-Garcia and Russell, 2001; Chiarini-Garcia and Russell, 2002; Chiarini-Garcia et al., 2003; Dettin et al., 2003).