Defining the spermatogonial stem cell

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Received for publication 22 September 2003, revised 21 January 2004, accepted 23 January 2004

Abstract

Through the use of donor cells from transgenic rats expressing GFP exclusively in the germline, we have defined culture conditions where male germ cells lose (on STO cells) or maintain (on MSC-1 cells) stem cell activity. A cadre of germ cell transcripts strikingly decrease in relative abundance as a function of testis age or culture time on STO cells, but only a subset of these transcripts (approximately 248) remain elevated when cultured on MSC-1 cells. If specific gene expression regulates stem cell activity, some or all of these transcripts are candidates as such regulators. We establish a spermatogonial stem cell index (SSCI) that reliably predicts relative stem cell activity in rat or mouse testis cell cultures, and through the use of an antibody to a robust signal (Egr3) within the index find intense signals in single or paired cells. As germ cells form longer interconnected chains (incomplete cytokinesis), the Egr3 signal disappears coincident with a loss of stem cell activity. Thus, molecular markers specific for spermatogonial stem cells establish a reliable and rapid means by which to define these cells in culture and alleviate the need for laborious testicular transfers in initial cell culture studies.

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Keywords: Stem cells; Male germ cells; Spermatogonia; Microarray; Testis colonization; Rat; Mouse; Transcript profile; Stem cell index; Spermatogonial stem cells

Introduction

An ability to culture and genetically manipulate spermatogonial stem cells in culture could lead to direct germ-line transmission of modified genomes, methods to correct male infertility and a means to rapidly screen for germ cell-directed contraceptives. The laboratory rat represents one of the most comprehensively studied mammals with more than a million publications in a wide range of medically relevant areas (Gill et al., 1989; Hedrich, 2000). Thus, expansion of technology to produce genetically modified rats through the use of the spermatogonial stem cell would represent a significant advance in biology and medicine (Hamra et al., 2002; Ogawa et al., 1999; Orwig et al., 2002a; Ryu et al., 2003; Zhang et al., 2003). In rats and mice, type A-single (A,) spermatogonia, formerly classified as “A-stem” spermatogonia (Oakberg, 1971) or “A-isolated” spermatogonia (Huckins, 1971c,d), are considered to contain the spermatogonial stem cell population, which functions to continually renew developing germ cells in the seminiferous epithelium (Brinster, 2002; Zhao and Garbers, 2002). The current means of evaluating spermatogonial stem cell character is the transfer of cells to recipient males and determination of testis colonization activity, a method that can take weeks or even months for evaluation (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Dobrinski et al., 1999; Nagano et al., 1999). Thus, the necessity of using colonization of a recipient testis as an assay method for spermatogonial stem cell activity has been a particularly slow process. The spermatogonial stem cell in mammals has not been unequivocally identified through the use of germ cell-specific molecular markers (de Rooij, 2001; Huckins,
Vasa and other molecular markers for germ cells also exist (e.g. other testicular cells (see Results and discussion). Various scatterlo, integrin-αv, integrin-β1, Thy-1, CD24, CD9, Kit, integrin-αv, MHC-Ia/β2M, Sca-1 and CD34 have been sorted, and are enriched in other stem cells, differentiated germ cells and other testicular cells (see Results and discussion). Various other molecular markers for germ cells also exist (e.g. Dazl, Vasa), but these genes are expressed at many stages of development (Noce et al., 2001; Reijo et al., 2000) and therefore are not unique signatures for the spermatogonial stem cell.

Whether a set of stem cell-specific genes exist or whether a general stem cell transcript profile, as has been suggested for stem cells as diverse as neural, hematopoietic and embryonic, is the case for the spermatogonial stem cell is not known (Evsikov and Solter, 2003; Fortunel et al., 2003; Ivanova et al., 2002, 2003; Ramalho-Santos et al., 2002). Here, we define conditions to maintain spermatogonial stem cells in vitro for long periods of time with no significant decrease in stem cell activity based on colonization of a recipient testis. We also define culture conditions where spermatogonial stem cell activity is lost in the face of retention of male germ cell character. This has allowed us to define gene transcripts that disappear before or coincident with a loss in stem cell activity, or to define transcripts that remain highly expressed when stem cell character is maintained. This approach, unlike steady-state profiling, identifies the kinetics of the change in gene transcript levels as stem cells move to a differentiated state. Thus, genes whose expression changes coincident with or before differentiation become candidate genes for maintenance of ‘stemness’. We develop a stem cell index for both rats and mice and use one of the robust index proteins to identify and characterize spermatogonial stem cells in culture.

Materials and methods

Materials and chemicals

Dispase, rat-tail collagen I-coated culture dishes and mouse laminin were from Fisher, Inc. Medium, trypsin solutions and antibiotic–antimylocotic solutions were from Gibco BRL, Inc. Fetal bovine serum (FBS) was from Atlanta Biologicals, Inc. Phosphate-buffered saline (PBS) was from JRH Biosciences, Inc. Protease inhibitors (EDTA-free tablets) were from Roche Applied Sciences, Inc. Mitomycin-C, 2-mercaptoethanol (ME) and the Cy3-conjugated, anti-vimentin IgG (clone V9) were from Sigma, Inc. Rabbit, non-immune IgG (SC-2027) and rabbit anti-Egr3 IgG (SC-191) were from Santa Cruz Biotechnology, Inc. AlexaFlour-594-conjugated, goat anti-rabbit IgG was from Molecular Probes, Inc.

Testicular cell cultures

To initiate testis cell cultures, seminiferous tubules were isolated from the testes of 22- to 23-day-old wild type (Harlan, Inc.) or homozygous SD-Tg(ROSA-EGFP)2-4Reh Sprague–Dawley rats, or from 19-day-old C57/BL6 mice (Harlan, Inc.). Testes from rats and mice of these ages are developmentally similar in that they contain late pachytenes 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 (λamnb), or laminin-non-binding (lamnb) germ cells and somatic cells by our 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). Rats in the SD-Tg(ROSA-EGFP)2-4Reh line were produced by pronuclear injection, and exhibit germ cell-specific expression of GFP (unpublished data, JTC). Therefore, we refer to the SD-Tg(ROSA-EGFP)2-4Reh line as GCS-GFP rats.

Feeder cell lines

MSC-1 Sertoli cells were a gift from Michael D. Griswold (McGuinness et al., 1994) and were maintained in Dulbecco’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% CO₂. SNL 76/7 STO fibroblasts (STO) were a gift from Allan Bradley (McMahon and Bradley, 1990) and were maintained in Dulbecco’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% CO₂. Before culture with testis cells, feeder cell lines were treated with growth medium containing 10 µg/ml of mitomycin C in 10 cm² dishes at 80–90% confluence for 3.75 h at 32.5°C/5.5% CO₂ (MSC-1) or for 3 h at 36.5°C/5.5% CO₂ (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 0.5 × 10⁴ cells/cm² for MSC-1 and 0.6 × 10⁴ cells/cm² 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% CO₂. Isolated testis cells from primary cultures were then plated at a density of 4 × 10⁵ cells/cm² (8 × 10⁴ cells/well) into cultures of mitomycin C-treated feeder cells in DHF12–10% FBS and then maintained at 32.5°C/5.5% CO₂ until the time of transplantation. Approximately every 2 days during maintenance, spent medium was removed from each culture and then replaced with fresh DHF12–10% FBS. Before 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.

**Microarray processing**

Total RNA was isolated from cultures of rat and mouse testis cells using RNAqueous (Ambion, Inc.). Total RNA (50 ng) was amplified and labeled in two rounds of amplification using the RiboAmp RNA Amplification Kit (Arcturus, Mountain View, CA) and the BioArray High-Yield RNA Transcript Labeling Kit (Enzo Life Sciences, Farmingdale, NY) for the final in vitro transcription and biotin labeling. The amplified RNA was purified using an RNeasy Mini column (Qiagen, Valencia, CA). Twenty micrograms of amplified RNA of each sample was 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 (Affymetrix, Santa Clara, CA). The arrays were hybridized and processed according to the manufacturer’s specifications.

**Data analysis and clustering**

Results were analyzed using Affymetrix Microarray Suite (MAS) 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. The complete data sets can be queried in the NCBI Gene Expression Omnibus (GEO) repository (www.ncbi.nlm.nih.gov/geo), GEO accession numbers GSE829 (mouse testis cell cultures), GSE830 (rat testis cell cultures), and GSE640 (developing mouse testis, Schultz et al., 2003). All sample comparisons were performed in GeneSpring 5.1 (Silicon Genetics, Redwood City, CA). Clustering was performed in ArrayMiner 4.0 (Optimal Design, Brussels, Belgium) using 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). Cluster C2 was derived by performing a second round of cluster analysis on nonclassified genes (Fig. 6B).

**Identification of mouse homologs to rat genes**

Transcribed mouse sequences homologous to transcripts exclusively expressed in cultures of rat testis cells (see legend to Fig. 3) and to rat genes listed in Table 1 were identified in the NCBI database using standard nucleotide blast searches. Apparent mouse homologs shared greater than 80% identity to queried rat sequences and had bit scores over 100. Supplemental Table 2 lists all 255 rat transcripts (248 genes) with their respective mouse homologs and Affymetrix mouse probe identifiers. Of the identified homologs for genes in Table 1, 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 (Schultz et al., 2003). Supplemental Table 3 lists the Affymetrix mouse probe identifiers with the detection calls in the postnatal developing mouse testis (A = not detected in the mouse testis time course, P = detected in at least one time point, I/NC = detected in at least one time point, but increased or no change in relative expression over time).

**Germ cell transplantation and progeny genotyping**

WT Sprague–Dawley rats at 12 days of age were injected intraperitoneally with 12.5 mg/kg busulphan (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 rat lamB cells (after culture for approximately 1 day on STO feeder cells) were paired at 12 weeks of age (approximately 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 by previous methods (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.

**Fluorometric analysis of spermatogonial 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 s 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 × g for 10 min at 4°C in a GPR tabletop centrifuge (Beckman, Inc.). The supernatant solutions were centrifuged at 15,800 × g for 10 min at 4°C in a microcentrifuge (Model# 5042, Eppendorf, Inc.), and the resultant supernatant fractions were then stored at −80°C. Frozen supernatant solutions were thawed on ice and then further clarified by centrifugation.
gation at 230,000 \( \times \) g, \( r_{av} \) (tl-100.3 rotor, TL1000 ultracentrifuge, Beckman, Inc.) for 30 min at 4°C. 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 fluorescent intensity using a FL600 fluorescence microtiter plate reader (BioTek, Inc.) 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, Roche, Inc.) into COS-7 cells. pcDNA6.0-EGFP-V5-His-B was produced by cloning the EGFP open reading frame (Fugene6 transfection reagent, Roche, Inc.) into pcDNA6.0-V5-His-B (Invitrogen, Inc.). 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 and GCS-GFP transgenic rats were processed similarly. Sections were counterstained for 25 min at 22–24°C in 1% Hoechst 33342. Following incubation in primary antibodies, the cells were washed three 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°C in conjugated, secondary antibody (0.4 ml/well) diluted to 1 µg/ml in PBS containing 5 µg/ml Hoechst 33342 dye (Molecular Probes, Inc.) and anti-vimentin IgG diluted 1:200 dilution. For direct visualization of EGFP expression in frozen cross sections and in seminiferous tubules, a Nikon SMZ1500 fluorescence stereomicroscope was used.

**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 per pooled sample per lane) was separated on SDS gels (10–20% acrylamide gradient, Invitrogen, Inc.) and transferred to nitrocellulose membranes. Nonspecific, protein-binding sites were blocked by incubating the cells in 1% w/v blocking reagent. 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 and GCS-GFP transgenic rats were processed similarly. Sections were counterstained for 25 min at 22–24°C in 1% Hoechst 33342 dye (Molecular Probes, Inc.) and anti-vimentin IgG diluted 1:200 dilution. For direct visualization of EGFP expression in frozen cross sections and in seminiferous tubules, a Nikon SMZ1500 fluorescence stereomicroscope was used.

**Immunocytochemistry**

Cultures of germ cells (2 cm²) were washed twice with serum-free DHF12 medium (0.6 ml/well) and then fixed for 7.5 min with 4% paraformaldehyde, 0.1 M sodium phosphate, pH 7.2 (0.4 ml/well). After fixation, the cells were washed three 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 three times in PBS (0.6 ml/well) and nonspecific, protein-binding sites were blocked by incubating the cells in 1% w/v blocking reagent (0.4 ml/well, Roche, Inc.) for 1.5 h at 22–24°C. The blocking reagent was then removed and the cells were incubated for 16 h at 22–24°C in primary antibodies (0.4 ml/well). The anti-Egr-3 IgG and the non-immune IgG fractions were each diluted to 167 ng/ml in blocking reagent. The anti-Dazl-3 IgG and the 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 three 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°C in conjugated, secondary antibody (0.4 ml/well) diluted to 1 µg/ml in PBS containing 5 µg/ml Hoechst 33342. Following incubation in secondary antibodies, the cells were washed three times for 5 min with TBST (0.6 ml/well) to remove unbound IgG and dye before viewing in fresh PBS (0.4 ml/well) using an inverted Olympus IX70 microscope (Olympus, Inc.).

**Electron microscopy**

Testis cells were fixed with 3% glutaraldehyde in phosphate buffer followed by postfixation with 1% osmium tetroxide, dehydrated with ethanol, embedded in Spurr resin in Beem capsules and polymerized overnight at 60°C. Semithin sections for light microscope were cut at 1 µm, placed on glass slides and stained with filtered 1% Toluidine Blue in 1% Sodium Borate. Ultra thin sections are cut at 80 nm, picked up on 200 mesh copper grids, stained with Uranyl Acetate and Lead Citrate and documented with a JEOL 1200EXII Transmission Electron Microscope. A blind 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 5000× from three separate cultures of the lamB and the lamNB cells. Then, the cells were categorized as type A spermatogonia, intermediate/type B spermatogonia, spermatocytes or somatic based on cellular morphologies previously established for different testis cell types (Chiarini-Garcia and Russell, 2001, 2002; Chiarini-Garcia et al., 2003; De Martino et al., 1979; Dettin et al., 2003; Huckins, 1971c; Russell et al., 1990).
Results and discussion

We have generated a line of transgenic rats that express GFP (EGFP variant) specifically in germ cells, termed GCS-GFP rats. The genomic locus of the Tg(GCS-EGFP) integration has not been defined. In cross sections (Fig. 1A), and in primary cultures (Fig. 1C) prepared from GCS-GFP rat testes (22 days old), only the germ cells (DAZL\(^+\), vimentin\(^-\) cells) expressed GFP when compared to cells from testes of wild-type rats (Figs. 1B, D). GFP was expressed in all germ cell types within the testis sections and in testis cell cultures prepared from the GCS-GFP rats (Figs. 1A, C). Somatic cells (DAZL\(^-\), vimentin\(^+\) cells) within the testis sections (Figs. 1A, B) or cultures (Figs. 1C, D) did not express detectable GFP. Therefore, germ cell-specific expression of GFP in the testes of the GCS-GFP animals provides a novel marker protein for the identification of spermatogenic cells within populations of somatic cells.

When germ cell-enriched suspensions from cultures of GCS-GFP rat testicular cells were added to collagen-coated culture dishes, cells that failed to bind to collagen (colNB cells) were highly enriched in GFP\(^+\) cells (Fig. 2A). When these freshly isolated germ cells were then transferred to laminin-coated culture dishes, two populations of GFP\(^+\) cells were obtained, those that bound to laminin (lamB) and those that did not bind (lamNB) (Fig. 2A). The cells at this initial time of selection on laminin are considered as day 0. Greater than 98% of the cells present in the lamB and lamNB fractions were GFP\(^+\), and about 95% of these distributed to the lamB group. In contrast, cells that initially bound to plastic and collagen (colB cells) were more than 98% GFP\(^-\) (Fig. 2A).

When the originally selected lamB and lamNB GFP\(^+\) germ cell populations were transferred to the testis of recipient males, ostensibly a measure of spermatogonial stem cell activity (Brinster and Zimmermann, 1994; Dobrinski et al., 1999; Nagano et al., 1999), only the lamB fraction substantially colonized (Fig. 2B). This finding verified previous results where the lamB fraction, which represented about 5% of the total germ cell population, was greatly enriched in spermatogonial stem cells (Hamra et al., 2002). This conclusion was also supported by three additional observations. First, the DNA content of the cells showed that a majority of cells in the initially selected lamNB fraction were 4C whereas most of the cells in the lamB fraction were 2C (Fig. 2C). The 2C cells are most likely germ cells at a stage of development before meiosis, whereas the 4C cells probably represent cells proceeding through meiosis (Malkov et al., 1998). Second, electron microscopy studies confirmed that the lamB population was enriched with cells displaying distinct patterns of heterochromatin staining and organelles ascribed to type A spermatogonia (91 \(\pm\) 3\%), with relatively few intermediate/ type B spermatogonia or spermatocytes (4 \(\pm\) 1\%), or somatic cells (5 \(\pm\) 1\%) (Fig. 2D). In contrast, the lamNB population was represented predominantly by spermatocytes plus intermediate/type B spermatogonia (89 \(\pm\) 6\%) and contained a much smaller percentage of type A-like spermatogonia (11 \(\pm\) 6\%) (Fig. 2D). Third, the profile of transcripts expressed in the initially selected lamB cells (Affymetrix microarrays) correlated strongly with transcripts expressed at early ages in the postnatal mouse testis (Fig. 3). As shown in a Venn diagram, about 7\% (906; 820) of the transcripts present in the originally selected lamB population are absent in either day 0 somatic or day 0 lamNB cultures of either rats or mice (Fig. 3A; see Materials and methods for calculation of present or absent scores for microarrays). About 10\% (rat) or 17\% (mouse) of transcripts present in day 0 lamNB cultures are absent in somatic or lamB populations, and about 12\% of transcripts present in cultures of mouse or rat testicular somatic cells are absent in either day 0 lamB or lamNB cultures (Fig. 3A). Thus, transcripts present exclusively within cultures of lamB, lamNB or somatic testis cells (Fig. 3A) provided molecular markers for type A spermatogonia, spermatocytes or testicular somatic cells, respectively (Fig. 2). When the profiles of these marker transcripts were evaluated in the developing mouse testis using available gene expression data (Schultz et al., 2003), transcripts marking the initially selected lamB cells (Affymetrix microarrays) dominate the testis at early ages (Fig. 3B) when gonocytes and type A spermatogonia are prevalent (Dettin et al., 2003; McLean et al., 2003). However, these same transcripts decrease in relative testicular abundance as the animal ages due to lamB-like cells in the testis becoming outnumbered by more-differentiated spermatogonia, spermatocytes and spermatids (Fig. 3B). In contrast to the situation with lamB cultures, transcripts that mark lamNB cells coincided with mRNA that substantially increased in relative abundance in the testis as a function of age (Fig. 3B), and thus, correlated strongly with the appearance of spermatocytes and spermatids. Transcripts exclusively present in testicular somatic cells decreased in relative abundance as a function of testis age (Fig. 3B). Thus, cultures of lamB germ cells represent a relatively pure population of type A spermatogonia that is highly enriched in spermatogonial stem cell activity.

To evaluate the effectiveness of various culture conditions to maintain spermatogonial stem cells within the lamB population, we developed a quantitative assay for assessment of colonization of rat testes by GFP\(^+\) cells from the GCS-GFP rats. After transfer of GFP\(^+\) lamB cells (maintained for 1 day on STO feeder layers) to recipient testes, followed by solubilization of testes 32 days later, a nearly linear relationship was evident between numbers of cells transplanted and subsequent GFP fluorescence (Fig. 4). On average, extracts prepared from rats transplanted with 1.5 \(\times\) 10\(^5\) GFP\(^+\) cells/testis contained 1.8 \(\pm\) 0.3 ng rEGFP/testis \((n = 3)\). Based on this assay, we could quantify spermatogonial stem cell activity, which was at least 60-fold higher in day 1 lamB (221 \(\pm\) 59 ng rEGFP/testis/3 \(\times\) 10\(^4\) donor cells, \(n = 5\), SEM), than in day 1 lamNB cultures (3.5 \(\pm\) 4.9 ng rEGFP/testis/3 \(\times\) 10\(^3\) donor cells, \(n = 3\), SEM). The stem cell activity measured in
day 1 lamNB cultures was not significantly above background levels for the assay as measured in non-transplanted, contralateral testes (1.7 ± 3.1 ng rEGFP/testis/donor cells, n = 8, ±SEM). Cultures of donor lamB cells (day 1 on STO) that colonized the seminiferous tubules of recipients in these studies also maintained the ability to differentiate into GFP+ spermatocytes and spermatids (Fig. 4D). The recipient males transplanted with 5 × 10^4 and 1.5 × 10^5 GFP+, day 1 lamB cells/testis transmitted the donor Tg(GCS-EGFP) transgene to 74 ± 5% (20 of 26 total pups, n = 3 litters, ±SEM) and 86 ± 8% (28 of 33 total pups, n = 3 litters, ±SEM) of pups born/litter from wild-type females, respectively (both testes transplanted/recipient). Therefore, similar to lamB germ cells isolated from MT-lacZ transgenic rats (Hamra et al., 2002), cultures of lamB germ cells from GCS-GFP transgenic rats can develop into functional spermatozoa within recipient rat testes. Thus, the cultures of rat lamB cells used to establish the colonization assay are highly enriched in functional spermatogonial stem cells.
We then investigated various cell feeder layers for ability to maintain spermatogonial stem cell activity. When the initially selected rat lamB cells were incubated on STO fibroblasts, they remained GFP+ (Fig. 5A), but lost ability to colonize a testis by day 10 in culture (Figs. 5B, C). In contrast, when lamB GFP+ cells were cultured on MSC-1 cells, colonization activity was maintained for at least 20 days (Figs. 5B, C). A potentially important characteristic of male germ cells cultured on STO feeder layers was the common appearance of long chains of 8–64 interconnected cells (Fig. 5A), this is similar to germ cell development characteristics in vivo (Huckins, 1990).

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In contrast, GFP+ cells grown on MSC-1 cells continued to grow predominantly as either single or paired cells (Fig. 5A); this characteristic is consistent with various stem cell models (Huckins, 1971c,d; Lok and de Rooij, 1983b; Lok et al., 1983; Oakberg, 1971). We then determined the transcript profile of rat lamB GFP+ cells as a function of culture time on STO feeder layers. Initially, isolated (day 0) GCS-GFP rat lamB cells were plated onto monolayers of STO fibroblasts and then maintained in culture for 1, 5, 10 or 20 days. In all cases, after each time point, GFP+ cells were sorted (FACS) and their transcript profile was determined as a function of culture time on STO feeder layers.
from the GFP− feeder cells before isolation of RNA. One-half of the GFP+ cells harvested from each culture were also transplanted to recipient rat testes to measure stem cell activity (see legend to Fig. 6B). In terms of exclusively expressed marker transcripts for day 0 germ cell cultures, the relative abundance of 381 transcripts initially expressed in the lamB cells (see legend to Fig. 3B) markedly decreased whereas the relative abundance of 462 transcripts initially expressed in the lam NB cells (see legend to Fig. 3B) markedly increased as a function of culture time on STO cells (Fig. 6A). Thus, the GFP+ cells on STO feeders appeared to be moving towards the day 0 lamNB transcript profile. This is supported by the time-dependent loss of stem cell activity within highly enriched cultures of type A spermatogonia (Fig. 5, legend to Fig. 6B), and as reported using suspensions of wild-type or cryptorchid mouse testis cells (Nagano et al., 1998, 2000, 2001, 2003), or rat pup testis cells (Orwig et al., 2002a).

Our next goal was to determine whether we could identify genes whose expression would decrease before or coincident with a loss of spermatogonial stem cell activity. These gene products could be critical for the maintenance of stem cell activity and could also then serve as reliable molecular markers for the spermatogonial stem cell. Transcripts present in GFP+ cells that yielded a time-dependent change in relative abundance by at least 2-fold (6639 of 12833) were grouped into six gene clusters (C1–C6; Fig. 6B). Clusters C1–C4 define 2167 transcripts that are detected at higher signal intensities in lamB germ cells at

Fig. 4. Fluorometric assay for GCS-GFP rat spermatogonial stem cell activity. (A) Images of WT testes transplanted with increasing numbers (0, 1.5 × 10⁴, 5 × 10⁴, 1.5 × 10⁵ GFP+ cells/testis) of GCS-GFP rat lamB cells cultured for approximately 1 day on STO cells. (Top) Green fluorescence observed in the testis correlates with the number of donor cells transplanted. (Bottom) Bright field microscopy images of testes in top. (B) Fluorescence units measured in extracts made from testes transplanted with increasing numbers of GFP+, lamB cells after approximately 1 day in culture on STO cells (closed circles). Values represent the mean number of fluorescence units/10 μl from 1.5-ml testicular extracts (± SEM, n = 3 rats/cell concentration transplanted). Also shown are fluorescence units plotted from increasing amounts of rEGFP-His (open circles). (C) Immunoblot detection of GFP in extracts from a 23-day-old GCS-GFP rat (GCSR). WT recipient testes transplanted with increasing numbers of GCS-GFP lamB cells after 1 day of culture on STO cells (0, 1.5 × 10⁴, 5 × 10⁴, 1.5 × 10⁵ GFP+ donor cells/testis) and of increasing amounts of rEGFP-His. Equal volumes from triplicate testis extracts were loaded per lane, which represent the same extracts used to generate the fluorometric data in panel B. (D) Germline-specific expression of the GCS-GFP rat transgene in a recipient rat testis. Shown is a frozen cross section from the testis of a wild-type recipient rat that was transplanted with GCS-GFP rat lamB germ cells (5 × 10⁴ GFP+ cells/testis, day 1 culture on STO) approximately 65 days earlier. (Left) Nuclei of all cells in the cross section are labeled with Hoechst 33342 (blue fluorescence). (Center) Expression of the GCS-GFP transgene in the same cross section shown in the left panel identifies the donor cell population and shows GFP+ germ cells (green fluorescence) that have developed up to the spermatid stage. (Right) Overlay of the same images shown in the left and center panels.
day 1 relative to day 20 (STO feeder cells; Fig. 6B). Clusters C5 and C6 contain a total of 1145 transcripts that are detected at lower signal intensities in lamB germ cells at day 1 relative to day 20 (STO feeder cells; Fig. 6B). Many transcripts (3327) did not fit into clusters C1–C6 and remain unclassified. The clusters containing genes of particular interest were those in C1 and C2, in that the relative abundance of these transcripts (C1, n = 954; C2, n = 231) decreased very rapidly and correlated closely with the time-dependent decrease in lamB stem cell activity (see legend to Fig. 6B).

If clusters C1 and C2 represent genes critical for maintenance of stem cell activity, then these gene transcripts should be maintained in lamB germ cells cultured on MSC-1 cells. Two hundred forty-eight of the putative genes (255 transcripts) in clusters C1 and C2 remained enriched (2-fold or greater relative to day 0 lamB germ cells) in GFP+ cells after maintenance for 20 days in culture on MSC-1 cells (Supplemental Table 2). Apparent mouse homologs or orthologs for 223 of the 248 rat genes were identified in the NCBI database (Supplemental Table 2). Based on mouse gene ontology, these candidate stemness genes were divided into 10 functional categories, and a group of transcripts with unknown function or expressed sequence tags (ESTs) (Table 1). The ESTs with unknown function represent the largest, single category of genes in Table 1 (48%).

Although previous work on other stem cells has not analyzed the kinetics of gene transcript changes relative to
variations in stem cell activity, the gene products listed in Table 1 can be compared to gene transcripts that are enriched in cells such as neural, hematopoietic and embryonic stem cells, relative to a select number of enriched tissue fractions (Ramalho-Santos et al., 2002). Transcriptional regulators and signal transduction modulators seem more highly expressed in all of these stem cells, as well as in spermatogonial stem cells; however, only a few of the molecules within these functional categories are represented by the same gene transcripts. In fact, only five genes among the signal transduction and transcriptional regulation functional groups listed in Table 1 (Elovl6, F2r, Rpl22, Sh3d19, 5730599O09Rik) are also enriched in neuronal, embryonic and hematopoietic stem cell populations (Ramalho-Santos et al., 2002). F2r encodes a proteinase-activated receptor (PAR1; thrombin receptor) which has established roles in platelet aggregation, chemotaxis, proliferation and differentiation (Macfarlane et al., 2001). Elovl6 encodes a recently discovered long-chain fatty acid elongase postulated to convert C16:0 palmitic acids to C18:0 steric acids (Moon et al., 2001). The nucleolar, ribosomal protein encoded by Rpl22 is postulated to modulate viral gene expression in eukaryotic hosts (Toczyński et al., 1994). The human homolog for the protein encoded by 5730599O09Rik, TGIF2, interacts with histone deacetylase 1 and functions as a transcriptional silencer (Melhuish et al., 2001), whereas Sh3d19, based on conserved domains, is predicted to encode a protein that signals organization of the cytoskeleton (Shimomura et al., 2003). When all spermatogonial stem cell genes in Table 1 are compared to genes enriched in individual populations of these other stem cells, only 25, 22 and 26 genes are enriched in neuronal, hematopoietic and embryonic stem cells, respectively (Ramalho-Santos et al., 2002). These results imply that most genes functioning in spermatogonial stem cells that maintain or correlate with stem cell activity are different across the various types of stem cells. Such a hypothesis is supported by recent studies that identified genes commonly expressed by diverse stem cell populations of embryonic and somatic origin; these stemness transcripts displayed little overlap when data were analyzed.}

Fig. 6. Transcriptional profile of lamB germ cells in culture on STO fibroblasts. (A) Expression of germline marker transcripts within rat lamB germ cells as a function of time in culture on STO fibroblasts. Freshly isolated (day 0) GCS-GFP rat lamB cells were plated onto monolayers of STO fibroblasts, and then maintained for 1, 5, 10 or 20 days. After each culture period, the GFP+ germ cells were sorted from the GFP- fibroblasts. Then, each purified germ cell fraction was used to isolate RNA. Microarray analysis was performed using the RNA samples to identify transcripts expressed by each germ cell fraction. The relative expression level for two sets of germline markers was then determined in each of the germ cell fractions. One set of markers contained 381 transcripts (375 genes) identified as being exclusively expressed in day 0 cultures of lamB germ cells (green bars, lamB germ cell markers) and the second set contained 462 transcripts (453 genes) identified as being exclusively expressed in day 0 cultures of lamNB germ cells (red bars, lamNB germ cell markers). Each set of germline markers is fully described in the legend to Fig. 3. Plotted are average signal intensities for all transcripts/marker set normalized to a ratio of 1 at the day 1 time point, and which are detected in at least one time point. (B) Transcriptional profile of rat lamB germ cells after their maintenance in culture on STO feeder cells. As described above in panel A, RNA was isolated from FACS-purified GFP+ germ cell fractions for microarray assays after maintenance of initially isolated GCS-GFP rat lamB germ cells for 1, 5, 10 and 20 days in culture on STO fibroblasts. A total of 12,833 transcripts were detected on the microarrays as present from at least one of the germ cell fractions; 6639 of these transcripts exhibited a time-dependent change in signal intensity by at least 2-fold, and were therefore clustered using the program ArrayMiner 4.0. Heat maps illustrate that 3312 of the 6639 transcripts fit into one of six clusters (C1, C2, C3, C4, C5, C6) representing distinct patterns of gene expression. Green and red symbols indicate time points in which expression levels of transcripts are decreased and increased, respectively, relative to the day 1 time point on STO cells. Intensities of the green and red symbols are proportional to changes in transcript expression (scale bar). Also, GFP+ germ cells were harvested from parallel cultures after each time point and then transplanted into rat testes to determine the relative levels of stem cell activity in each of the cultures analyzed by microarray. The equivalent rEGFP in testis extracts prepared from rats transplanted with approximately 5 × 10^6 GFP+ cells/testis/time point dropped over time in culture on STO cells (day 1, 381 ± 110; day 5, 88 ± 75; day 10, 2.9 ± 4, ng rEGFP-His/testis, n = 3 testes/time point, ±SEM).
between different groups were compared (Evsikov and Solter, 2003; Fortunel et al., 2003; Ivanova et al., 2003; Vogel, 2003). Thus, steady-state transcript abundance, although a measure of the character of stem cells, fails to specifically define those genes primarily critical to stem cell maintenance. However, these studies also indicated that differences in experimental design and data analysis complicate comparisons between groups (Fortunel et al., 2003; Ivanova et al., 2003). Here, we set forth a hypothesis that if specific genes are required for maintenance of stem cell activity, then in the process of moving from a stem cell to a differentiated cell, candidate genes directly linked to stemness should be represented by transcripts that change in abundance before or coincident with a loss of stemness. For example, based on these criteria, in clusters C3 and C4 of Fig. 6B are eliminated as stem cell maintenance candidates. In these groups, most transcripts decrease in lamB germ cells well after a loss of stem cell activity on STO feeder layers.

Because expression is exclusively in the testis or because expression is correlated with differentiation in various other cellular systems, other genes of interest include Ret and Gfra1, which encode membrane-bound receptors previously reported as present in undifferentiated spermatogonia (Meng et al., 2000; Tadokoro et al., 2002). These receptors appear to signal spermatogonial stem cell fate in response to GDNF (Meng et al., 2000; Tadokoro et al., 2002). Several genes encode transcription factors, such as Hey-1, which regulates maintenance of neuronal precursors (Sakamoto et al., 2003); Egr3 and Egr4, which are members of an early growth response family (O’Donovan et al., 1999; Tourtellotte et al., 1999); Aebp1, a transcriptional repressor with carboxypeptidase activity that blocks differentiation of cultured preadipocytes (Kim et al., 2001); the forkhead transcription factor (FoxO1) that stimulates skeletal muscle satellite cell proliferation and blocks differentiation of cultured preadipocytes (Michida et al., 2003; Nakae et al., 2003); Foxa2, a second forkhead family member expressed coincident with endodermal differentiation of embryonic stem cells (pathway is repressed by Oct4 and FoxD3) (Guo et al., 2002); and Pou3f1 (Oct6/Ts1/SCIP), an octamer-binding protein expressed by regenerating neuronal precursor cells (Kawasaki et al., 2003). Oct6 is expressed at high levels in cultures of undifferentiated embryonic stem cells or oligodendrocytes and is rapidly down-regulated upon differentiation (Collarini et al., 1992; Meijer et al., 1990). In the mouse testis, Oct4 is a transcription factor known to be expressed by gonocytes and type A spermatogonia, but which is then down-regulated in more differentiated male germ cells entering meiosis (Pesce et al., 1998). Oct4 was not detected as present in the germ cell cultures or in the testis using the rat and mouse arrays. In contrast, the Oct4 transcript is detected as present at substantial levels in mouse embryonic stem cells using mouse arrays (data not shown), suggesting that the Oct4 transcript is more abundant in

Table 1
Genes closely associated with spermatogonial stem cell activity

<table>
<thead>
<tr>
<th>Category</th>
<th>Genes</th>
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<tbody>
<tr>
<td>Apoptosis (4)</td>
<td>Casp7, Casp10a, Pmaip1, Sgk3</td>
</tr>
<tr>
<td>Cell adhesion and cytoskeleton</td>
<td>EFemp2, Unc1, Myp, Nid2, Punc, Reln, Sdc2, Sdc4, Thbs4, Tubb5, Univ,</td>
</tr>
<tr>
<td></td>
<td>Wasip, Cldn4, Cryga, Emb, Ephb4.13a, Ephb4.144a, Lamb2a, Myoif1b,</td>
</tr>
<tr>
<td></td>
<td>Myod1, Plec1, Scarb1a, Snap91a, Spock1a, Tpp2a, and one EST cluster</td>
</tr>
<tr>
<td>Cell cycle (1)</td>
<td>Cables1b</td>
</tr>
<tr>
<td>DNA modification and repair (3)</td>
<td>Dmnt3a, Hist4, Polg2b</td>
</tr>
<tr>
<td>Metabolism and biosynthesis (16)</td>
<td>Abcd4, Cacna1g, Slc25a13, Ephb4.144b, Nsft1, Rbip1a, Slc22a3a,</td>
</tr>
<tr>
<td></td>
<td>Slc6a4a, and one EST cluster</td>
</tr>
<tr>
<td>RNA binding and modification (7)</td>
<td>Rbmns, Rbmnsf, Smnfa, and four EST clusters</td>
</tr>
<tr>
<td>Signal transduction (36)</td>
<td>Adey5, Dasp6, F2r (thrombin receptor), Gfra1 (GDNF receptor alpha 1),</td>
</tr>
<tr>
<td></td>
<td>H2-M3, Igbp1, Ldb1, mSSH-1L, Pdk1, Pprg, Pipk, Rab12, Sema4d,</td>
</tr>
<tr>
<td></td>
<td>Skad19, Chnva4c, Cnp1b, Dast, Ephpb3a, Fgfr3, H2-Q10b,</td>
</tr>
<tr>
<td></td>
<td>Hak-pending, Ick, Peli2, Pp33ca, Pigfna, Ret, Rims4a, Spry4a,</td>
</tr>
<tr>
<td></td>
<td>Stard13a, statute, Tbc1d8, and five EST clusters</td>
</tr>
<tr>
<td>Transcriptional regulation (37)</td>
<td>Aebp1, Bcl6b, Bhlbd3, Egr3, Foxo1, Irf1, Lmo4, Mndn, Nr1d1, Pbx2,</td>
</tr>
<tr>
<td></td>
<td>Satb1, Ski, Sox13, Zif278, Ank2a, Ankds6a, Bhlhb3a, D3Jfr1a, Drl2b,</td>
</tr>
<tr>
<td></td>
<td>Egr4b, Foxa2b, Hdac5a, Hey1a, Kif3b, Pou3f1a, Sall4a, Tcf23b, and 10 EST</td>
</tr>
<tr>
<td></td>
<td>clusters</td>
</tr>
<tr>
<td>Translation and posttranslational modification (3)</td>
<td>Galnt2 and two EST clusters</td>
</tr>
<tr>
<td>Other (107)</td>
<td>Ash13b, Bhlbd1, Gig1-pending, H19, Lphn1, Tafa5-pending, Tera-pending,</td>
</tr>
<tr>
<td></td>
<td>Tpgb, Rtn1a, Upk1b, and 98 unknown EST clusters</td>
</tr>
</tbody>
</table>

Genes listed represent transcripts with the following properties: present in lamB germ cells, both before (day 0) and after maintenance for 20 days in culture on MSC-1 cells, enriched at least 2-fold in lamB cultures relative to cultures of lamB germ cells and present in cluster 1 or 2 (transcripts that decrease before or coincident with loss of stem cell activity). Transcripts present in cultures of testis somatic cells were not excluded. Mouse gene symbols are presented where mouse homologs or orthologs to rat transcripts could be identified (see Supplemental Tables 2 and 3).

a No change of expression or up-regulated during postnatal mouse testis development.

b Not detected on the mouse arrays.

c No oligonucleotide probe set for transcript on mouse arrays.

cultures of embryonic stem cells than in cultures of type A spermatogonia. Another transcription factor, Stra8, is known to be expressed by mouse spermatogonial (Oulad-Abdelghani et al., 1996) and oogonial (Menke et al., 2003) cells before their entering meiosis, and was present at
highest levels in cultures of mouse lamB cells (signal intensities = lamB cell, 1457; lamNB cells 260, somatic cells, 44). An apparent rat homologue for Stra8 was not found in the NCBI database. Thus, it is not known if transcripts such as Stra8, which are more abundant in mouse lamB cells than in lamNB cells, and which are not identified on the rat microarrays, would meet the criteria established for Table 1. In the mouse testis, the Stra8 transcript peaks in relative abundance at day 11 and then dramatically drops, consistent with a peak expression of Stra8 protein in differentiated spermatogonia (Oulad-Abdelgani et al., 1996).

In contrast to other stem cells, spermatogonial stem cells in culture appear to express relatively low levels of cell cycle progression transcripts, suggesting that these cells are relatively quiescent. In vivo, the duration of the cell cycle (approximately 56 h) for rat type A spermatogonia is longer than for type A spermatogonia committed to differentiation (approximately 42 h; types A2 to A4) (Huckins, 1971a,d). This is also the case in hamsters, where the cell cycle duration is approximately 90 h for type A spermatogonia, but is shortened to approximately 60 h in differentiating type A spermatogonia (types A2 to A4) (Lok and de Rooij, 1983a; Lok et al., 1983). More recent colonization assays have also demonstrated a relatively long doubling time of approximately 67 h for donor populations of rat spermatogonial stem cells (Ryu et al., 2003). However, due to the lack of molecular markers specific for authentic spermatogonial stem cells across different species, there is still debate about which populations of spermatogonia have the potential to function as a stem cell. The relative low abundance of transcripts involved in cell cycle kinetics listed in Table 1 further suggests similarities between type A spermatogonia within cultures of lamB germ cells (before differentiation on STO cells) and the slow-cycling stem cell populations previously studied in the testis (Clermont and Bustos-Obregon, 1968; Huckins, 1971b; Lok et al., 1984).

Spermatogonial stem cells also express numerous genes involved in extracellular matrix production and attachment. Genes in this category may function to help spermatogonia colonize their niche on the basement membrane of the seminiferous tubules (Chiarini-Garcia et al., 2003; Ryu et al., 2003). Here (Fig. 2), and in earlier studies (Hamra et al., 2002; Orwig et al., 2002b; Shinohara et al., 1999, 2000a), the attachment of spermatogonial stem cells to the extracellular matrix protein, laminin, has been used for enrichment during isolation. In fact, the first defined surface antigens on functional spermatogonial stem cells were laminin receptor subunits encoding the integrins, Itga6 and Itgb1 (Shinohara et al., 1999). Similar to selection on laminin matrix, these and other cell surface antigens have been successfully used in cell sorting and subsequent transplantation assays to enrich spermatogonial stem cell activity. The cells isolated were characterized as side-scatter\(^{lo}\), integrin-\(\alpha 6\), integrin-\(\beta 1\), Thy-1\(^+\), CD24\(^+\), integrin-\(\alpha v\), MHC-Ia/\(\beta 2 M\), Sca-1\(^+\), CD34\(^-\) and Kit\(^-\) (Kanatsu-Shinohara et al., 2003; Kubota et al., 2003; Shinohara et al., 1999, 2000b). Transcripts encoding such prominent marker proteins, including, Itga6 (integrin-\(\alpha 6\)), Itgb1 (integrin-\(\beta 1\)) (Shinohara et al., 1999, 2000b) and Thy1 (thymus cell surface antigen, Thy-1) (Kubota et al., 2003) were also detected as present in rat lamB cells before (day 0) and after 20 days in culture on MSC-1 cells. However, we find the mRNA for these genes to be relatively abundant in cultures of rat lamNB germ cells (Fig. 7), or to persist in lamB cells after 20 days of culture on STO feeders (legend to Fig. 7). Since stem cell activity is almost undetectable by this time in culture, these transcripts do not fit our criteria for inclusion in Table 1. Furthermore, transcripts for these molecules are detected at similar or in even higher relative abundance in cultures of testis somatic cells (Fig. 7). It should also be noted that enrichment of stem cell activity through cell sorting using the above markers has in part relied on the generation of cryptorchid animals as a source of testicular cells. With this treatment plus subsequent FACS to remove testicular somatic cells and differentiated germ cells, a preparation of mouse spermatogonial stem cells can be enriched 166-fold (Shinohara et al., 2000b) and potentially up to 600-fold (Kubota et al., 2003) relative to adult non-cryptorchid testis. However,
with respect to cell surface antigens specifically expressed on spermatogonial stem cells in the testis, Sema4D, Cacna1g, Ret and Gfra1 were the only such transcripts in Table 1 that appeared exclusively in mouse and rat lamB cells; these transcripts were absent in cultures of mouse or rat somatic (interstitial and tubular) or differentiated germ cells. These gene products, therefore, could also be important environmental detectors within the spermatogonial stem cell niche.

Mouse spermatogonial stem cells also have been enriched by cell sorting, based on germline-specific expression of transgenes. A population of GFP⁺, Kit⁺, integrin-α6⁺ and EE-2 antigen⁺ spermatogonial stem cells have been enriched by about 50-fold from testicular cords of 7-day-old transgenic mice. In testes of these animals, GFP is expressed specifically in the germline under control of a 18-kb region of the Oct4 promoter (Ohbo et al., 2003). In comparison to adult animals, a 700-fold enrichment in spermatogonial stem cell activity was achieved through the use of transgenic mice that ectopically express a nonfunctional CD4 antigen on premeiotic and meiotic germ cells under control of a 400-bp region of the Stra8 promoter (Giulii et al., 2002). Thus, germline-specific transgene expression in the mouse is a means by which to dramatically simplify the isolation of large numbers of highly purified spermatogonial stem cells.

We developed a unit of measure for spermatogonial stem cell activity, which we named spermatogonial stem cell index (SSCI). SSCI values for cultures of rat germ cells (rSSCI) were determined using the mean signal intensity for the 248 rat genes listed in Supplemental Table 2. The rSSCI for day 0 lamB GFP⁺ cells dropped dramatically with time in culture on STO cells (810 at day 1 to 187 at day 20; Fig. 8A). The rSSCI at day 20 is similar to that seen with day 0 lamNB germ cells (rSSCI = 144; Fig. 8A). In contrast, a rSSCI of 650 for cultures of day 0 lamB GFP⁺ cells was maintained at 786 after 20 days of culture on MSC-1 feeder cells (Fig. 8A).

To establish an SSCI in the mouse (mSSCI), we further analyzed the mouse homologs identified to the rat gene transcripts in Table 1. Homologs or orthologs for 202 of the genes in Table 1 (81% of genes) were identified on the Affymetrix Murine Genome U74v2 microarray set, of which 175 were detected as present in the mouse testis. Cluster analysis demonstrated that transcripts for 115 of these 175 genes (68%) showed a time-dependent decrease in relative abundance after postnatal days 1–8 in the mouse testis. The remaining 56 homologs showed a time-dependent increase or did not change in relative abundance in the mouse testis. This could be potentially explained by species differences.
differences in gene expression or by incorrect assignments of the authentic rat and mouse homologs, and therefore such transcripts were not used in mSSCI calculations. Based on mSSCI values, these 115 genes (145 transcripts) were most abundantly expressed in the testis at early ages, with a peak at day 4 (mSSCI = 701); this was followed by a rapid decline as a function of testis age (mSSCI = 122 in the adult testis; Fig. 8B). Thus, the mSSCI is high in the early testis at times when spermatogonial stem cells are prevalent in the mouse or rat. As the testis develops, differentiated germ cell populations dominate and mSSCI values markedly decrease as expected (Fig. 8B). In cultures of mouse testis cells, the mSSCI was 508 for lamB cells and 120 for lamNB cells (Fig. 7B), which is in agreement with functional data reporting that mouse testis cells selected for on laminin are enriched in spermatogonial stem cell activity (Shinohara et al., 1999, 2000a).

If the mSSCI is an excellent predictor of spermatogonial stem cell activity, then it should also define spermatogonial stem cell activity in another species. When the 115 genes of the mSSCI were now used for the rat germ cell cultures, a SSCI value of 709 for the GFP+ day 0 lamB cells was maintained at 898 after 20 days on MSC-1 feeder cells. Furthermore, SSCI values for the GFP+ day 0 lamB cells decreased dramatically as a function of time in culture on STO cells (day 1 = 850, day 5 = 668, day 10 = 415, day 20 = 196). The mSSCI, therefore, appears as an excellent predictor of spermatogonial stem cell activity across both rat and mouse.

We then selected a protein encoded by one of the robust signals in the SSCI to ask whether we could identify the spermatogonial stem cell in culture. Egr3 represents a gene transcript that dramatically decreases in a manner similar to that for the average transcript expression pattern observed for all genes in Table 1 (Figs. 8A, 9A). The Egr proteins, Egr1, Egr2, Egr3 and Egr4, are closely related members of a subclass of immediate early gene-encoded, inducible transcription factors that share a similar DNA-binding domain (Beckmann and Wilce, 1997). LamB cells grown on STO cells retain GFP fluorescence and Dazl immunoreactivity after 10 days in culture, but lose detectable Egr3 immunoreactivity (Fig. 9B). In contrast, when grown on MSC-1

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Fig. 9. Egr3 expression in lamB germ cells after culture on STO or MSC-1 feeder cells. In all experiments, day 0, GCS-GFP rat lamB germ cells were used to initiate the cultures. (A) Time-dependent decreases in the relative abundance of the germ cell Egr3 transcript (reference i.d. AA964492) after culture on STO cells. RNA was isolated from GFP+ germ cells (FACS-purified) after culture for 1, 5, 10 or 20 days on STO fibroblasts. Plotted are signal intensities for Egr3 on Affymetrix Rat Expression Set 230. (B) (top left and right) Expression of GFP (green) in germ cells derived form lamB cells after culture for 10 days on MSC-1 feeder cells. (middle left) Anti-Dazl immunolabeling (red) of germ cells shown at top left. (middle right) Anti-Egr3 immunolabeling (red) of germ cells shown at top right. (Lower left and lower right) Bright field microscopy image overlay of Hoechst 33342-labeled nuclei (blue fluorescence) in cells shown at top left and right. (C) (top left and right) Expression of GFP (green) in germ cells after 10 days in culture on MSC-1 Sertoli cells. (middle left) Anti-Dazl immunolabeling (red) of germ cells shown at top left. (middle right) Anti-Egr3 immunolabeling (red) of germ cells shown at top right. (lower left and lower right) Bright field microscopy image overlay of Hoechst 33342-labeled nuclei (blue fluorescence) in cells shown at top left and right.
cells for 10 days, the germ cells retain GFP fluorescence and Dazl and Egr3 immunoreactivity (Fig. 9C). Strongly positive Egr3 immunoreactive signals are restricted in most cases to either single or paired germ cells. Although it might be expected that one or both of the paired cells would normally commit to differentiation (Huckins, 1971c,d; Lok and de Rooij, 1983b; Lok et al., 1983; Oakberg, 1971), that the MSC-1 culture appears to lack differentiation-inducing factors may explain the high Egr3 signals in both cells of a pair. On STO cells, in contrast, differentiation factors seem to exist, driving germ cells to form long chains, upon which Egr3 immunoreactivity is lost (Fig. 9B).

Thus, these studies further define the spermatogonial stem cell in both mice and rats, and provide a list of transcripts that not only correlate strongly with spermatogonial stem cell activity at steady state, but also identify gene transcripts that change coincident with or before a loss of stem cell activity. These results suggest that it is now possible to study spermatogonial stem cells in culture without the primary need to initially perform testicular transfers. Such transfers could now become a secondary means by which to define stem cell activity.

Acknowledgments

We thank Sasha C. Tindle and Guang-Quan Zhao for assistance with cell culture experiments, Dennis J. Bellotto for assistance with electron microscopy, and Daniel J. Garry and Timothy L. Macatee for performing FACS, and we acknowledge the contributions of the University of Texas Southwestern Medical Center Microarray Facility. This work was supported by the Howard Hughes Medical Institute, and the Cecil H. and Ida Green Center for Reproductive Biology Sciences.

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