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Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal

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Self-renewal and differentiation of spermatogonial stem cells (SSCs) provide the foundation for testis homeostasis, yet mechanisms that control their functions in mammals are poorly defined. We used microarray transcript profiling to identify specific genes whose expressions are augmented in the SSC-enriched Thy1$^+$ germ cell fraction of mouse pup testes. Comparisons of gene expression in the Thy1$^+$ germ cell fraction with the Thy1-depleted testis cell population identified 202 genes that are expressed 10-fold or higher in Thy1$^+$ cells. This database provided a mining tool to investigate specific characteristics of SSCs and identify novel mechanisms that potentially influence their functions. These analyses revealed that colony stimulating factor 1 receptor (*Csf1r*) gene expression is enriched in Thy1$^+$ germ cells. Addition of recombinant colony stimulating factor 1 (Csf1), the specific ligand for Csf1r, to culture media significantly enhanced the self-renewal of SSCs in heterogeneous Thy1$^+$ spermatogonial cultures over a 63-day period without affecting total germ cell expansion. In vivo, expression of Csf1 in both pre-pubertal and adult testes was localized to clusters of Leydig cells and select peritubular myoid cells. Collectively, these results identify Csf1 as an extrinsic stimulator of SSC self-renewal and implicate Leydig and myoid cells as contributors of the testicular stem cell niche in mammals.

KEY WORDS: Csf1, Leydig cell, Myoid cell, Niche, Self-renewal, Spermatogonial stem cell

INTRODUCTION

The homeostasis of most, if not all, tissues is dependent on continual function of an undifferentiated cell population referred to as tissue-specific or adult stem cells. Spermatogonial stem cells (SSCs) are the tissue-specific stem cell population of mammalian testes and are tasked with establishing and maintaining male fertility. Similar to other adult stem cell populations, SSCs provide this function by undergoing both self-renewal and differentiation. Self-renewal continually provides a pool of SSCs (often referred to as A_{single} or A_s spermatogonia), whereas differentiation results in formation of $A_{paired(pr)}$ (A_{pr}) followed by $A_{aligned(al)}$ (A_{al}) spermatogonia, which continue to differentiate, eventually resulting in formation of spermatozoa (Oakberg, 1971; Huckins and Oakberg, 1978; Huckins, 1971). Collectively, A_s, A_{pr} and A_{al} spermatogonia are referred to as proliferating spermatogonia (Russell et al., 1990; de Rooij and Russell, 2000). Currently, the mechanisms controlling SSC functions are poorly understood.

Advancement of knowledge regarding specific characteristics of SSCs has been hampered by an inability to isolate these cells from the testis of any mammalian species, mostly due to lack of known specific phenotypic or molecular markers. However, isolation of SSC-enriched fractions from rodent testes has been achieved. Recent studies demonstrated that Thy1 (CD90) is expressed on the surface of mouse SSCs and nearly all SSCs are contained within the Thy1$^+$ cell fraction (Kubota et al., 2003; Kubota et al., 2004a). When isolated using magnetic activated cell sorting (MACS), the Thy1$^+$ cell fraction is enriched 5- to 30-fold for SSCs compared to the total cell population in pre-pubertal and adult mouse testes, respectively (Kubota et al., 2004a). Thus, isolation of the Thy1$^+$ testis cell fraction provides an experimental cell population that can be used to study specific characteristics of mammalian SSCs.

In general, adult stem cell functions are controlled extrinsically from influence of a niche microenvironment and intrinsically by expression of specific gene networks. Stem cell niches are composed of both architectural support and a milieu of growth factors (Spradling et al., 2001; Scadden, 2006). These specialized microenvironments are formed by contributions of surrounding support cells. In mammalian testes, Sertoli cells are thought to be the major contributor to the SSC niche (Tadokoro et al., 2002; Yomogida et al., 2003), but contributions by other testicular somatic cells including peritubular myoid and interstitial Leydig cells are also possible. Knowledge of niche growth factors produced by testicular somatic cells is limited. Previous studies have established that glial cell line derived neurotrophic factor (Gdnf) is a major regulator of mouse, rat and hamster SSC self-renewal in vitro (Kubota et al., 2004b; Ryu et al., 2005; Kanatsu-Shinohara et al., 2008) and essential for normal spermatogenesis in vivo (Meng et al., 2000; Naughton et al., 2006). Additionally, exposure to basic fibroblast growth factor (Fgf2) was shown to enhance the effects of Gdnf in vitro, but was unable to support SSC self-renewal alone (Kubota et al., 2004b). In vivo, both Gdnf and Fgf2 production in the testis has been localized to Sertoli cells (Tadokoro et al., 2002; Mullaney and Skinner, 1991). In long-term cultures of Thy1$^+$ testis cells supplemented with Gdnf and Fgf2, clumps of germ cells form which are composed of both SSCs and non-stem cells (Kubota et al., 2004b; Kanatsu-Shinohara et al., 2005). While SSC numbers expand for extended periods of time in these cultures, their proliferation rate is slow. Additionally, the percentage of SSCs within the germ cell clumps varies greatly throughout a given culture period and can be extremely small, estimated to be 0.02% in one instance (Kanatsu-Shinohara et al., 2005). These observations suggest that niche growth factors other than Gdnf and Fgf2 have important roles in supporting SSC self-renewal.

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The objective of the current study was to identify genes whose expressions are enriched in the Thy1$^+$ germ cell fraction of mouse testes to provide insights into potential extrinsic and intrinsic regulators of SSC self-renewal. We found that expression of colony stimulating factor 1 receptor (*Csf1r*) is highly enriched in this fraction and that exposure to the specific ligand for this receptor, the cytokine colony stimulating factor 1 (Csf1) enhances self-renewal of mouse SSCs in vitro. Additionally, we localized Csf1 expression to both interstitial Leydig and peritubular myoid cells in pre-pubertal and adult mouse testes, implicating these cell populations as contributors to the SSC niche.

MATERIALS AND METHODS

Isolation of Thy1$^+$ and Thy1-depleted testis cell fractions

All animal procedures were approved by the University of Pennsylvania or Pennsylvania State University Institutional Animal Care and Use Committees (IACUC). To collect testis cell fractions with different SSC contents from mouse testes we used a modified MACS isolation protocol of Thy1$^+$ cells (Fig. 1A) based on methodology previously described (Kubota et al., 2004b; Oatley and Brinster, 2006). Three replicate samples were collected for each fraction from independent donor preparations. Viability was >90% for all isolated cell populations based on assessment of Trypan Blue exclusion. The SSC contents of these cell fractions were determined using functional germ cell transplantation and gene expression profiles were evaluated using DNA microarray analysis.

Germ cell transplantation and analysis

To determine the SSC content of Thy1$^+$ and Thy1-depleted cell fractions and cultured Thy1$^+$ germ cell clumps we conducted functional germ cell transplantation analyses using techniques previously described (Brinster and Avarbock, 1994; Oatley and Brinster, 2006). Briefly, for all transplantations, 8-10 μl of cultured or MACS isolated cell suspensions originally collected from Rosa (B6.129S7-Gtrosa26; The Jackson Laboratory) donor males, which express *lacZ* in germ cell types, were microinjected into testes of 129 × C57 recipient males that were treated with busulfan (60 mg/kg) at least 6 weeks earlier to deplete endogenous spermatogenesis. Two months after transplantation the number of donor-derived colonies of spermatogenesis in each recipient testis were counted using a dissecting microscope following staining with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Because each colony is clonally derived from a single SSC (Nagano et al., 1999; Kanatsu-Shinohara et al., 2006), counting donor-derived colonies of spermatogenesis provides a relative quantification of SSC number in an injected cell suspension. All donor cell suspensions were injected at a standard concentration of 1×10^6 cells/ml.

RNA isolation and DNA microarray processing/analysis

To conduct DNA microarray analyses of gene expression, RNA was isolated from MACS-isolated Thy1$^+$ and Thy1-depleted cell fractions collected from 6 days post-partum (dpp) C57BL/6 mouse pups. Highly pure samples of total RNA were isolated using a hybrid Trizol-DNeasy column protocol previously described (Oatley et al., 2006). Each sample was processed and used for hybridization onto Affymetrix whole genome 430 2.0 GeneChips (Affymetrix, Santa Clara, CA, USA). Three different replicate samples were collected for each Thy1$^+$ and Thy1-depleted cell fraction and each was hybridized onto a single array, totalling six GeneChips. All hybridizations and scanning of GeneChips were conducted by the University of Pennsylvania Microarray Core facility as previously described (Oatley et al., 2006). Briefly, 5 μg of total RNA was reverse transcribed using Superscript II reverse transcriptase and poly(T) oligomer priming that incorporated the T7 promoter. In vitro transcription was then conducted, followed by fragmentation of resultant cRNA and hybridization to Mouse Genome 430 2.0 arrays (Affymetrix). Chips were then washed and stained with streptavidin-phycoerythrin. A confocal scanner was then used to collect fluorescence signals, and the average signal from two sequential scans was calculated for each microarray. Affymetrix microarray suite 5.0 was used to quantify expression levels for target genes and default values provided by Affymetrix were applied to all analysis parameters. The number of probe pairs meeting the default discrimination threshold (t=0.015) was used to assign a call of absent, present or marginal for each assayed gene, and a P-value was calculated to reflect confidence in the detection call.

Microarray data analysis

Affymetrix CEL files for each genechip were imported into Stratagene Array Assist Lite software version 3.4 to calculate normalized GC Robust Microarray Analysis (GCRMA) expression levels for each probe set on each array. Data (CHP files from GCRMA calculations) were then imported into GeneSpring 7.2 software (Agilent Biotechnology; Santa Clara, CA, USA) to visualize expression patterns and data were normalized to the median. Filter flags were then applied including a Present call in three out of six samples and differential expression in Thy1$^+$ versus Thy1-depleted samples. These parameters resulted in the filtering out of 26,927 genes from the 45,101 genes present on the genechips. The GCRMA raw values for these filtered genes were then exported into Excel for statistical analysis. A two-class paired test using Statistical Analysis of Microarray version 2.23A tool, which included a 3% false discovery rate with P-value <0.05, was used to identify genes with differential expression at the 10-fold level between Thy1$^+$ and Thy1-depleted cell fractions. All microarray data are available from the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/), Accession No. GSE14222.

Quantitative RT-PCR analyses

For quantitative reverse transcriptase PCR (qRT-PCR) analyses 1 μg of total RNA for each sample was DNase-treated (DNA-free Kit; Invitrogen, Carlsbad, CA, USA) to remove possible contaminating genomic DNA, and reverse transcribed using oligo(d)T priming and Superscript II reverse transcriptase (Invitrogen). Expression levels of specific genes were measured using SYBR Green assays and an ABI 7300 sequence detection system (Applied Biosystems, Foster City, CA, USA). Specificity of amplicons was evaluated using melt curve analyses. Transcript levels for specific genes-of-interest were normalized to those of ribosomal protein S2 (Rps2) to make quantitative comparisons between different samples as previously described (Oatley et al., 2006; Oatley et al., 2007). All primer sequences were designed using Primer Express 3.0 (Applied Biosystems) and the nucleotide sequences were; 5′-TACTTCAAGGCTTCGCCTCTCT-3′, 5′-CTACGTGTTCCATCTGCAAATAGG-3′ for *Bcl6b*, 5′-AACTATGTTGTCAAGGGCAATGC-3′, 5′-GGACCACACATCACTCTGAACTG-3′ for *Csf1r*, 5′-CAACTTCATGCATATGGCTCTCA-3′, 5′-TCTGCTAAAGCACTGGGCTTCT-3′ for *Gfra1*, 5′-CCCAGCTTTCCCGAATCCT-3′, 5′-GCGGGACGTAAATAAATAAAATGG-3′ for *Lhx1*, and 5′-CCATGCCTCATCACTTACCCTAT-3′, 5′-GTCCGGAAGAGCTTGCAGAA-3′ for *Rps2*.

Thy1$^+$ germ cell cultures and assessment of Csf1r effects on SSC self-renewal in vitro

Germ cell cultures were established from MACS isolated Thy1$^+$ cells collected from 6-dpp Rosa mouse testes using methods previously described (Kubota et al., 2004b; Oatley and Brinster, 2006). Germ cells were seeded onto mitotically inactivated STO feeder monolayers and maintained in chemically defined mouse serum-free medium (mSFM) (Kubota et al., 2004b) supplemented with 20 ng/ml recombinant human Gdnf (R&D Systems, Minneapolis, MN, USA) and 1 ng/ml recombinant human Fgf2 (BD Biosciences; San Jose, CA, USA). To test its effects on SSC self-renewal, recombinant mouse Csf1 (R&D Systems) was added to the culture media at a concentration of 10 ng/ml. Control conditions for these Csf1 experiments consisted of supplementation with Gdnf and Fgf2 alone. To evaluate SSC self-renewing expansion in the different culture conditions, cells were collected from culture wells by trypsin-EDTA digestion of entire culture well contents (STO feeders + germ cell clumps) and an aliquot of the cell suspension was transplanted into recipient mouse testes to determine SSC content. The remaining cell suspensions were sub-cultured at a 1:2 ratio. Cells were transplanted on day 7 of culture and every 2 weeks thereafter throughout a 63-day culture period. Analysis of recipient testes was conducted 2 months after transplantation by X-Gal staining and using a dissecting microscope to count the number of blue-stained colonies. These colony numbers were used to determine SSC content in Csf1-treated and

control cultures at each time-point analysed throughout the 63-day culture period. SSC expansion in Csf1-treated and control cultures was evaluated by applying the following equation at each culture time-point analysed:

SSC number/10^5 Thy1$^+$ cells cultured=
[number of donor-derived colonies of spermatogenesis ×
(10^5 total cells harvested/10^5 cells transplanted)] ×
[1/10^5 Thy1$^+$ cells originally cultured]

For each culture, SSC numbers/10^5 Thy1$^+$ cells cultured were multiplied by sub-culture ratios in order to determine SSC expansion rates throughout the 63-day culture periods.

Testis cross-section immunofluorescence for Csf1 and Csf1r expression

To localize the expression of Csf1 and Csf1r in mouse testes immunofluorescent staining was used. Primary antibodies used were polyclonal sheep anti-mouse Csf1 (1:200; R&D Systems) or polyclonal rabbit anti-human Csf1r (1:200, Santa Cruz Biotechnology; Santa Cruz, CAUSA). Secondary antibodies were Alexa Fluor 488-conjugated donkey anti-sheep (1:1000; Invitrogen) or goat anti-rabbit (1:1000; Invitrogen). Negative controls were cross-sections incubated with normal sheep or rabbit IgG as primary antibody. All cross-sections were viewed using fluorescent microscopy and digital images were captured.

Flow cytometric analysis of Csf1r and F4/80 expression

To determine the percentage of cells expressing Csf1r and the macrophage-specific marker F4/80 within pup testes and cultured Thy1$^+$ germ cell clumps we used flow cytometric analysis. For mouse pup testes, single cell suspensions were generated by trypsin-EDTA digestion at 37°C for 10 minutes. Approximately 1×10^6 cells for each sample were then incubated for 20 minutes on ice in DPBS-S (DPBS with 0.1% FBS, 10 mM HEPES, 10 mM Na pyruvate, 1 mg/ml glucose, and penicillin/streptomycin) with specific antibodies. Primary antibodies used were mouse anti-human Thy1 IgG1 (Abcam, Cambridge, MA, USA), PE-conjugated rat anti-mouse Csf1r IgG1 (AbD Serotec, Raleigh, NC, USA), or APC-conjugated rat anti-mouse F4/80 IgG2a (Biolegend, San Diego, CA, USA). Detection of Thy1 involved secondary incubation with FITC-conjugated rat anti-mouse IgG1 (Biolegend). Isotype controls consisted of PE conjugated rat IgG1 (Biolegend), APC conjugated rat IgG2a (Biolegend), and FITC conjugated rat anti-mouse IgG1 (Biolegend). Cells were analysed with a Beckman-Coulter FC500 flow cytometer (Fullerton, CA USA). For Thy1$^+$ germ cell cultures, clumps were removed from STO feeders using gentle pipetting, collected into HBSS, and pelleted at 600 ***g*** for 7 minutes. Cell clumps were then digested with trypsin-EDTA at 37°C for 5 minutes to generate single cell suspensions. Approximately 1×10^5 cells for each sample were then incubated for 20 minutes on ice with rabbit anti-human Csf1r polyclonal antibody (Santa Cruz Biotechnology) or control immunoglobulin in DPBS-S. Secondary detection included incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG (Invitrogen) on ice for 20 minutes. Propidium iodide (1 µg/ml) was added to each sample just before analysis to distinguish live and dead cells. The percentage of Csf1r$^+$ cells in each sample was then evaluated using flow cytometric analysis with a Guava PCA 96 System (Guava Technologies; Hayward, CA, USA).

Statistical analyses

All statistical analyses were conducted using SPSS v.15 software (SPSS, Chicago, IL USA). Differences between means for functional germ cell transplantation analyses were examined using a general linear model (GLM) univariate ANOVA. Comparisons of total germ cell and SSC expansion rates between Csf1-treated and control Thy1$^+$ germ cell cultures were conducted using GLM regression analyses to test for homogeneity of slopes between growth lines.

RESULTS

Isolation of SSC-enriched and SSC-depleted cell populations from mouse pup testes

We reasoned that comparison of gene expression profiles between an SSC-enriched fraction and SSC-depleted population would provide insights into the extrinsic niche growth factors and intrinsic genes that control SSC self-renewal. MACS isolation of the Thy1$^+$ cell fraction and corresponding Thy1-depleted population was used to provide these different SSC-containing samples (Fig. 1A). Additionally, we used 6-dpp mice as donors because SSCs and A_{pr} and A_{al} spermatogonia are the only germ cell types in the testis at this age (Russell et al., 1990). Before assaying global gene-expression profiles we used functional germ cell transplantation with Rosa mice serving as donors to unequivocally examine relative difference of SSC contents in the cell fractions (Fig. 1B,C). The Thy1$^+$ cells produced 228.1±45.9 colonies of donor-derived spermatogenesis/10^5 cells injected (mean±s.e.m., n=3 individual replicate samples and 24 total testes), whereas Thy1-depleted cells produced 9.2±3.3 colonies of donor-derived spermatogenesis/10^5 cells injected (n=3 replications and 24 total testes). Each colony of donor-derived spermatogenesis is clonally derived from a single transplanted SSC (Nagano et al.,

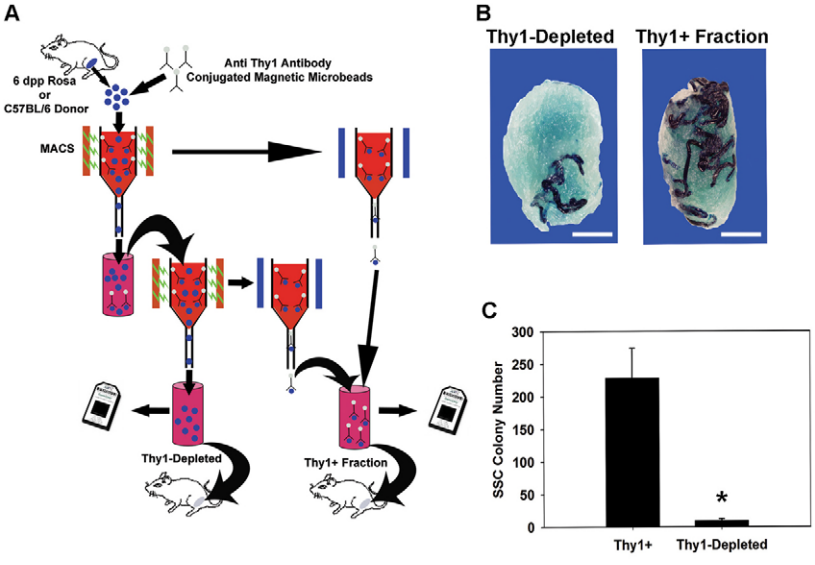

Fig. 1. Isolation of Thy1$^+$ and Thy1-depleted cell populations from 6-dpp mouse testes and quantification of SSC contents by functional germ cell transplantation. (**A**) Schematic of the experimental strategy using MACS to isolate Thy1$^+$ fractions. Following incubation with anti-Thy1 antibody conjugated to magnetic microbeads, testis cell suspensions were subjected to magnetic column selection. Rosa mice that express *lacZ* in all germ cells were used as donors to compare SSC content of the Thy1$^+$ and Thy1-depleted populations. Inbred C57BL/6 donors were used as a source of cell fractions for gene expression profiling, which was conducted using Affymetrix 430 2.0 GeneChips. (**B**) Representative recipient mouse testes transplanted with MACS-isolated Thy1$^+$ or Thy1-depleted cell fractions from 6-dpp Rosa donors. Scale bars: 2 mm. (**C**) Quantification of SSC content in Thy1$^+$ and Thy1-depleted cell fractions based on donor-derived colonies of spermatogenesis in recipient testes following functional germ cell transplantations. *P≤0.05.

Table 1. Functional classification of genes expressed 10-fold or greater in Thy1$^+$ cell fractions as compared with Thy1-depleted cell populations isolated from 6-dpp mouse testes

Function	Gene
Apoptosis	*Pmaip1*, *Aplp1*
Cell growth and proliferation	*Csf1r*, *Evia2*, *Gmfg*, *Ret*, *Fst*, *Il1b*
Chemotaxis and cell adhesion	*Cxcl4*, *C3ar1*, *Fermt3*, *Ccl2*, *Ccl3*, *Itgb2*, *Cxcl2*, *Xcl1*, *Itgal*, *Ccr2*, *Ccl21b*, *Rac2*, *Emilin2*, *Stab2*, *Selpl*, *Cdh1*, *Jam4*, *1 EST*
Cytoskeleton	*Ms4a11*, *Coro1a*, *Tubg2*, *Aif1*, *Nef3*, *Was*, *Crygs*, *Myo1f*, *Matn4*
Development	*Cd44*, *Ina*, *Fmn2*
Immune response	*Ly86*, *Lcp1*, *Clec4n*, *H2-Aa*, *Lst1*, *Tcrg*, *Cd14*, *H2-D1*, *C8b*, *H2-Eb1*, *Tcrb-V13*, *Il18*, *Clec4a2*, *Nalp4f*
Metabolism	*Lyzs*, *Lzp-s*, *Cbr2*, *Cybb*, *Alox5ap*, *Cad*, *Pld4*, *Ncf1*, *Ncf2*, *Sult4a1*, *Psmb9*, *Car6*, *Gldc*, *St8sia4*
Other	*Laptm5*, *Cd52*, *Cd68*, *Cd53*, *Arhgdib*, *Rgs5*, *Ii*, *Lcp1*, *Laptm5*, *Lgals3*, *ESTAW112010*, *Ctla2b*, *Prg1*, *Cd48*, *Tmem30b*, *Klrd1*, *ESTAI790276*, *Hemt1*, *Ifi205*, *Mageb1*, *Arhgap30*, *Cd84*, *Thy1*, *Ctla2a*, *Nckap1l*, *Sez6*, *1200013B08Rik*, *Wdfy4*, *AI467606*, *Nkg7*, *Loc545386*, *Gvin1*, *2610318N02Rik*, *AY078069*, *A630038E17Rik*, *ESTAW552008*, *AI662270*, *AI413582*, *Ccr1*, *Dock2*, *4933425L06Rik*, *Mgl2*, *Metrnl*, *1700011F14Rik*, *Cd83*, *Mpp4*, *Plvap*, *B230216G23Rik*
Proteolysis	*Ctss*, *Ctsc*, *Gzmc*, *Cpa3*, *Mcpt5*, *Mcpt4*, *Gzma*, *Ctsw*, *Mmp9*, *Prss19*, *Gzmb*, *Mcpt6*
Signal transduction	*Tyrobp*, *Fcer1g*, *Ptprc*, *Msr2*, *Ms4a6b*, *Rgs1*, *Gp49a*, *Fcgr3*, *Stab1*, *Mrc1*, *Gfra1*, *Cx3cr1*, *Ms4a6d*, *Emr1*, *Plek*, *Il1rl1*, *Cd36*, *Fcgr2b*, *Ms4a7*, *Cd3g*, *P2ry12*, *Inpp5d*, *Pik3cd*, *Xlkd1*, *Chn2*, *Gpr65*, *Clec4a3*, *Ptpns1*, *Gpr34*, *Prkch*, *Cd86*, *Tlr13*, *Mapk12*, *Rgs5*, *Lcp2*, *Rgs18*, *Cd300d*, *Gfra1*, *Tlr2*, *Kirc1*, *Mapk11*, *Ptger4*, *Sla*, *MGl*, *Rab32*, *Procr*
Transcription regulation	*Lyl1*, *T*, *Eomes*, *Tcl1*, *Lhx1*, *Pou5f1*, *Ovol2*, *Hoxb6*, *Id4*, *Cbx2*, *Sfmbt2*, *Glis3*, *1700020N01Rik*, *Ehox*, *Idb4*, *Ifi204*, *Zfpn1a1*, *Bcl6b*
Transport and ion binding	*F13a1*, *C1qa*, *C1qb*, *C1qg*, *S100a4*, *Apoe*, *Slc9a9*, *Slco2b1*, *Chrna4*, *Pfkp*, *Fxyd5*, *1700031F13Rik*, *Mgl1*, *Mbl2*, *Stx3*

1999; Kanatsu-Shinohara et al., 2006); thus, SSC content of the MACS isolated Thy1$^+$ cell fraction was approximately 25-fold higher than the Thy1-depleted cell population.

Identification of differential gene expression between Thy1$^+$ and Thy1-depleted testis cell populations

To identify genes whose expressions are enriched in SSCs we measured global gene expression profiles in MACS isolated Thy1$^+$ (n=3) and corresponding Thy1-depleted testis cell populations (n=3) from 6-dpp inbred C57BL/6 donor mice using DNA microarray analysis. Genes expressed 10-fold or higher in Thy1$^+$ compared with Thy1-depleted fractions were filtered, resulting in identification of 202 genes (see Table S1 in the supplementary material). To gain insight into possible biological functions of these genes they were sorted into 12 functional categories based on Gene Ontology (GO) Consortium biological process classifications provided by Affymetrix (Table 1). Genes without a clearly defined function were classified as other. We confirmed differential expression for three of these genes using qRT-PCR, including *Bcl6b*, *Lhx1* and *Gfra1* (see Fig. S1 in the supplementary material). Investigation of surface antigens implicated as SSC markers (Table 2) revealed that expression of *Thy1*, *c-Ret* (*Ret* – Mouse Genome Informatics), *Gfra1* and *Cdh1* were enriched 10-fold or greater in the Thy1$^+$ cell fraction. By contrast, expression of *Gpr125*, which has been suggested as an SSC marker in adult mouse testes (Seandel et al., 2007), was expressed 5.5-fold higher in the Thy1-depleted population than in the Thy1$^+$ cell fraction. Next, we examined the expression of transcription regulators implicated as having important roles in SSC functions (Table 3). Only *Bcl6b* and *Lhx1*, identified in our previous studies as important regulators of mouse SSC self-renewal (Oatley et al., 2006; Oatley et al., 2007), were expressed greater than 10-fold in the Thy1$^+$ cell fraction. Expression of *Plzf* (*Zbtb16*) (Bauus et al., 2004; Costoya et al., 2004) and *Taf4b* (Falender et al., 2005), both of which have been suggested as essential for SSC self-renewal, were marginally enriched (~2-3-fold) in Thy1$^+$ cells, similar to the expression levels of *Ngn3* (*Neurog3*) (Yoshida et al., 2004) and *Sohlh1* (Ballow et al., 2006), which have been implicated as regulators of spermatogonial differentiation. By contrast to the enriched genes, expression of *n-Myc* (*Mycn*) (Braydich-Stolle et al., 2007) and *c-Fos* (*Fos*) (He et al., 2008), which have been suggested as important for proliferation of testis cell populations with unproven SSC content, were more highly expressed in the Thy1-depleted testis cell population than in Thy1$^+$ cells. Overall, identification of differential gene expression between the Thy1$^+$

Table 2. Comparison of the expression of genes implicated as SSC surface markers in Thy1$^+$ and Thy1-depleted testis cell fractions isolated from 6-dpp mouse testes

Gene	Expression in Thy1$^+$	Expression in Thy1-depleted	Fold-difference
α6-integrin	1747.5±66.0	2792.4±119.4	0.6
Cd9	2997.1±145.7	1077.2±42.7	2.9
c-Ret	1194.6±122.9	95.8±9.1	12.5
E-cadherin (cadherin 1)	573.0±121.0	64.3±9.8	8.9
Epcam	1547.2±190.9	479.5±126.8	3.2
Gfra1	522.9±59.9	10.6±0.2	49.3
Gpr125	1302.7±144.8	7129.5±785.6	0.2
Thy1	73.7±6.9	4.6±0.4	15.9

Expression values (mean ± s.e.m.) were calculated from GC Robust Microarray Analysis (GCRMA) normalization of Affymetrix raw values from three different replicate samples.

Table 3. Comparison of the expression of genes implicated as important regulators of mouse SSC self-renewal in Thy1⁺ and Thy1-depleted testis cell fractions isolated from 6-dpp mouse testes

Gene	Expression in Thy1⁺	Expression in Thy1-depleted	Fold-difference
Atm	1555.6±211.7	951.9±255.8	1.6
Bcl6b	778.4±15.9	74.4±3.7	10.5
c-Fos	13999.7±1954.6	24130.4±2736.3	0.6
Etv5	499.3±17.6	87.1±15.8	5.7
Lhx1	106.9±27.8	7.1±1.8	13.9
Ngn3 (*Neurog3*)	155.9±55.6	50.1±30.1	3.1
n-Myc (*Mycn*)	1681.9±50.5	2192.5±251.5	0.8
Plzf (*Zbtb16*)	104.9±6.7	53.4±12.4	1.9
Sohlh1	1249.4±147.6	347.2±132.2	3.6
Taf4b	433.9±25.7	118.1±38.5	3.7

Expression values (mean±s.e.m.) were calculated from GC Robust Microarray Analysis (GCRMA) normalization of Affymetrix raw values from three different replicate samples.

and Thy1-depleted cell fractions provides a database to better understand characteristics of mouse SSCs and mechanisms regulating their functions.

Expression of *Csf1r* is highly enriched in Thy1⁺ germ cell fractions

Currently, understanding of extrinsic niche factors that stimulate SSC functions is limited. We used the Thy1$^+$ germ cell microarray database to mine for enriched expression of growth factor receptors whose corresponding ligand could potentially influence SSC functions. Coincidentally, the gene detected to be most enriched in the Thy1$^+$ testis cell fraction was colony stimulating factor 1 receptor (*Csf1r*). Transcript levels were determined to be 478.5±3.9-fold higher in the Thy1$^+$ cell fraction compared with the Thy1-depleted testis cell population using microarray analysis (Fig. 2A). Validation of this differential expression level using qRT-PCR revealed a 953.8±104.9-fold higher expression of *Csf1r* in Thy1$^+$ germ cells (Fig. 2B). Next, we examined whether this characteristic was maintained in adult testis cell populations. Using qRT-PCR a 389.4±58.1-fold enriched expression of *Csf1r* in the Thy1$^+$ germ cell fraction compared to the Thy1-depleted cell population from 2-month-old mouse testes was measured (Fig. 2C). This observation suggests a conserved characteristic of enriched *Csf1r* expression by SSCs during both pre- and post-pubertal spermatogenesis. Expression of Csf1r by macrophages has been well established and previous studies have indicated that Csf1r expression in the adult mouse testis is localized to macrophages (Cohen et al., 1996). Thus, we used flow cytometric analysis (FCA) to examine whether enriched *Csf1r* expression by the isolated Thy1$^+$ cell fraction from mouse testes could be due to co-enrichment of macrophages. Examination of the F4/80$^+$ cell population, a macrophage marker, in 8-dpp mouse pup testes revealed complete absence of Thy1 expression, indicating that testicular macrophages are not Thy1$^+$ (see Fig. S2 in the supplementary material). Thus, we were confident that enriched *Csf1r* expression was restricted to the Thy1$^+$ germ cell fraction.

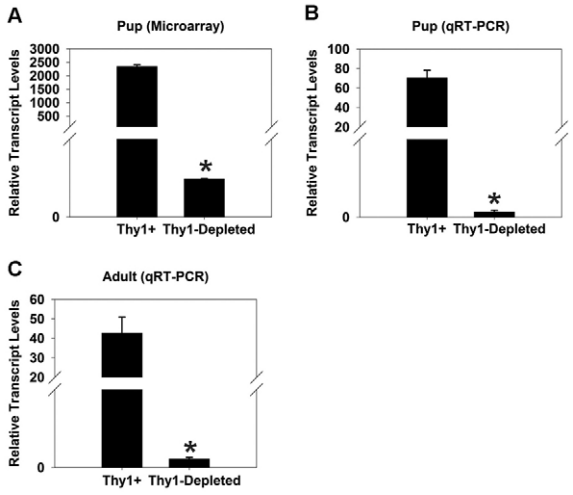

Fig. 2. Identification of differential *Csf1r* gene expression in Thy1⁺ versus Thy1-depleted testis cell fractions. (**A**,**B**) Relative *Csf1r* transcript expression in cell fractions isolated from 6-dpp mouse pup testes measured by microarray analysis (A) and qRT-PCR (B). (**C**) Relative *Csf1r* transcript expression in cell fractions isolated from adult mouse testes measured by qRT-PCR. All data are mean±s.e.m. for three different replicates. *$P \leqslant 0.05$.

Csf1r is expressed by a sub-population of spermatogonia in mouse pup testes and cultured Thy1⁺ germ cells

Previous studies indicate that Csf1r is not expressed by any cells within seminiferous tubules of mouse testes and localized specifically to macrophages present in the interstitial space and occasionally intermingled with peritubular myoid cells (Cohen et al., 1996; Pollard et al., 1997). Lack of observed Csf1r expression by germ cells in those studies is not surprising if expression is restricted to SSCs in the seminiferous epithelium, because their rarity could prove very difficult to detect. However, studies by Johnson et al. (Johnson et al., 2007) detected enriched *Csf1r* transcript expression by spermatogonia within the rat testis. Using immunofluorescence, we observed Csf1r expression by individual spermatogonia located on the basement membrane within some seminiferous tubules of 10-dpp mouse testes that contain the full complement of spermatogonia (Fig. 3A). These observations were rare, as only two to three tubules containing Csf1r$^+$ germ cells were observed per cross-section (n=6 testes examined). Negative controls with normal IgG as the primary antibody showed minimal background staining (see Fig. S3 in the supplementary material). Further examination of Csf1r expression using FCA revealed the presence of an F4/80$^-$/Csf1r$^+$ cell population in testes of mouse pups (Fig. 3B), and this population was weakly positive for Thy1 (Fig. 3C), similar to previous reports of Thy1 expression by the SSC population in mouse pup testes (Kubota et al., 2004a). Collectively, these results identify a Csf1r$^+$ spermatogonia population within mouse testes.

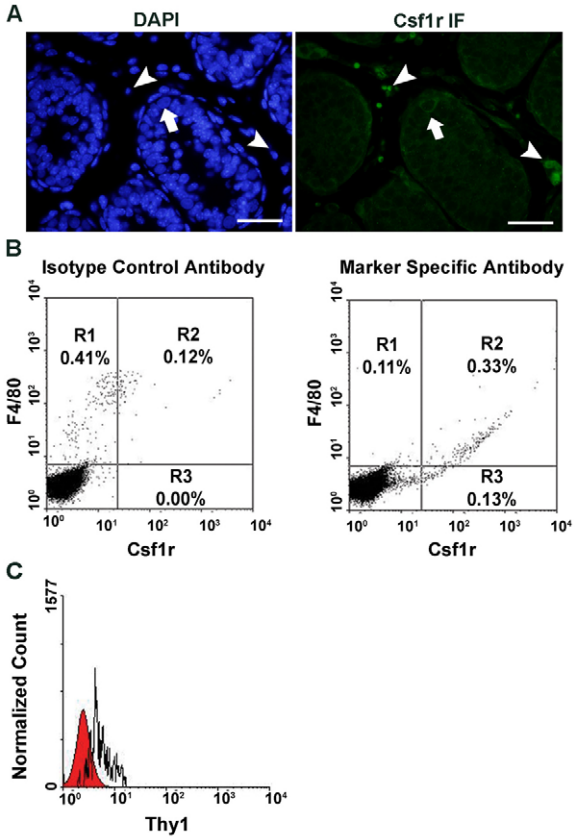

Fig. 3. Expression of Csf1r protein in mouse pup testes. (**A**) Immunofluorescence localization of Csf1r expression in testes of 10-dpp mouse pups. Expression was observed in individual spermatogonia within a few seminiferous tubules (arrow), and in macrophages and other blood cells in the interstitial space (arrowheads). (**B**) FCA of the Csf1r$^+$ cell populations in testes of 8-dpp mouse pups. F4/80 was used as a macrophage-specific marker to distinguish Csf1r$^+$ macrophages (region R2) from other Csf1r$^+$ populations. A population of cells that is F4/80$^-$/Csf1r$^+$ was identified (region R3). (**C**) Examination of Thy1 expression in pup R3 cell populations using FCA. Red fill, isotype control immunoglobulin; black line (open fill), marker-specific antibody. Data are mean±s.e.m. for three separate testis cell preparations.

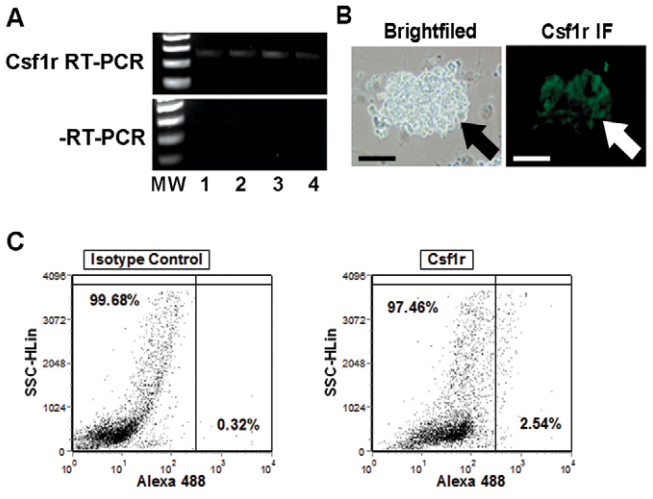

Fig. 4. Expression of Csf1r in cultured Thy1$^+$ germ cell clumps. Serum-free media with Gdnf and Fgf2 as the only growth factor supplements supports formation of germ cell clumps consisting of both SSCs and non-stem cell spermatogonia. (**A**) RT-PCR analysis of *Csf1r* transcript expression. (Top) Csf1r expression analysis of four different Thy1$^+$ germ cell cultures (lanes 1-4). (Bottom) Control expression analysis of identical RNA samples subjected to mock reverse transcription. MW, 100 bp DNA ladder. (**B**) Immunofluorescence evaluation of Csf1r protein expression in cultured Thy1$^+$ germ cell clumps (arrows). Scale bars: 100 μm. (**C**) Representative scatter plot for FCA of the percentage of cultured Thy1$^+$ germ cell clumps expressing Csf1r protein. The left-hand plot is cells incubated with isotype control immunoglobulin and the right-hand plot is cells incubated with anti Csf1r primary antibody.

Next, we examined if Csf1r is expressed in cultures of Thy1$^+$ germ cell clumps maintained in serum-free conditions with Gdnf and Fgf2 as the only growth factor supplements. Self-renewing expansion of SSCs is supported in these conditions and germ cell clumps are composed of both stem cells and, to a greater extent, non-stem germ cells. Thus, we reasoned that if Csf1r expression is restricted to SSCs only a portion of the Thy1$^+$ germ cells clumps would be Csf1r$^+$. Expression of Csf1r transcript could be detected using standard RT-PCR analysis (Fig. 4A) and expression of Csf1r protein was detected using immunofluorescence (Fig. 4B). However, staining was not homogenous throughout germ cell clumps, suggesting that only a portion of the cells express Csf1r. To further explore this possibility we evaluated the expression of Csf1r by germ cell clumps using FCA (Fig. 4C). These analyses revealed that ~2.2% of the cultured Thy1$^+$ germ cells (n=2 different primary cultures) expressed Csf1r, suggesting that this characteristic may be specific to SSCs in the heterogeneous cultured Thy1$^+$ spermatogonial cell population.

Addition of recombinant soluble Csf1 in serum-free chemically defined culture media enhances SSC self-renewal in vitro

Collectively, identification of enriched *Csf1r* gene expression by Thy1$^+$ germ cells, and expression of Csf1r protein by a sub-population of spermatogonia in mouse testes and cultured Thy1$^+$ germ cell clumps, suggested that exposure to the pleiotropic cytokine colony stimulating factor 1 (Csf1), the ligand for Csf1r, may influence SSC fate decisions. To investigate this possibility, recombinant soluble Csf1 was added to primary cultures of Rosa Thy1$^+$ germ cells maintained in serum-free chemically defined media also supplemented with Gdnf and Fgf2. These cultures were compared to controls receiving only Gdnf and Fgf2. To explore general effects of Csf1 on germ cell proliferation we examined the overall expansion rate of clump-forming germ cells (SSCs and non-stem cells) in Csf1-treated and control cultures. Additionally, specific examination of SSC proliferation in both Csf1-treated and control cultures was conducted using functional germ cell transplantation as an assay. The appearance of germ cell clumps with embryonic stem (ES)-like morphology was not observed in any control or Csf1-treated culture, and no teratomas were found in recipient testes following transplantation, indicating that pluripotent cells were not present in any cultured cell population examined. The morphology of germ cell clumps was unaffected by exposure to Csf1 (Fig. 5A), and total germ cell expansion was not altered in Csf1-treated cultures compared to controls over a 63-day culture period (Fig. 5B). Regression analysis for homogeneity of slopes between the growth lines revealed no significant (P=0.80) effect of Csf1 treatment. By

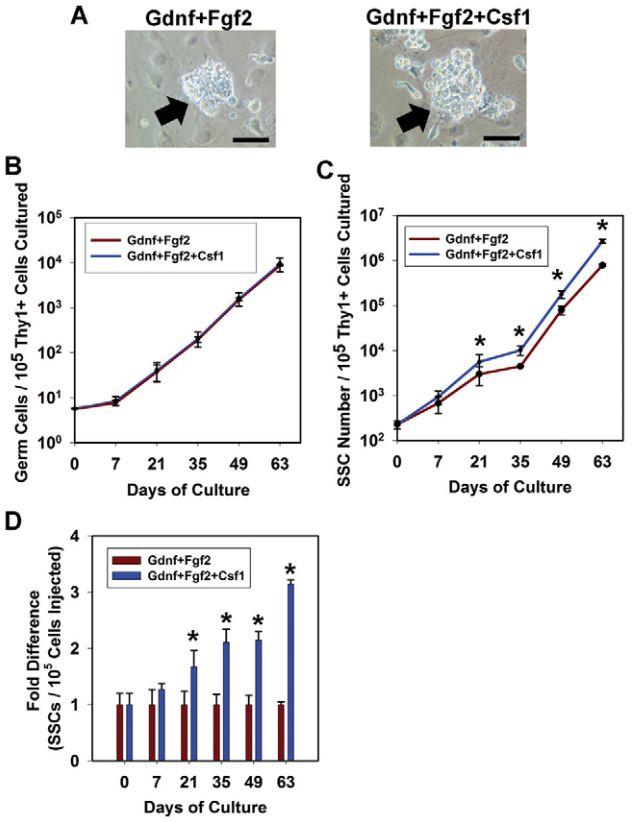

Fig. 5. Effects of Csf1 exposure on germ cell proliferation and SSC self-renewal in vitro. (**A**) Morphology of Thy1$^+$ germ cell clumps (arrows) in control (Gdnf + Fgf2) and Csf1-treated (Gdnf + Fgf2 + Csf1) cultures. Scale bars: 50 μm. (**B**) Total germ cell expansion in control and Csf1-treated cultures during a 63-day culture period. Graph lines for both conditions are nearly identical and overlap. Regression analysis for homogeneity of slopes revealed no difference (P=0.800) between control (y=112.1 × –1519.7) and Csf1-treated (y=120.8 × –1642.1) cultures. (**C**) SSC expansion in control and Csf1-treated Thy1$^+$ germ cell cultures as determined by functional germ cell transplantations. Regression analysis for homogeneity of slopes revealed a significant difference (P=0.011) between control (y=9568 × –135,198) and Csf1-treated (y=32,056 × –461,164) cultures. (**D**) Difference in SSC content within cultured Thy1$^+$ germ cell clumps at specific time-points of analysis throughout a 63-day period in control and Csf1-treated cultures. The concentration of SSCs in the cultured germ cells was significantly higher in cultures treated with Csf1 beginning at day 21 and progressively increased with time in culture. SSC numbers were determined using functional germ transplantation and data are presented as fold difference in SSCs/10^5 cells injected between Csf1-treated and control cultures. In all graphs, data are mean±s.e.m. for three different independent cultures. Red lines are control cultures (Gdnf + Fgf2) and blue lines are Csf1-treated cultures (Gdnf + Fgf2 + Csf1). *P≤0.05 between control and Csf1-treated cultures.

contrast, SSC expansion in Csf1-treated cultures was enhanced compared with control cultures (Fig. 5C). Regression analysis of growth lines for homogeneity of slopes revealed that exposure to Csf1 significantly (P=0.011) enhanced the expansion rate of SSCs. The doubling rate of SSCs in Csf1-treated cultures was calculated to be approximately 4.7 days {63 days of culture/[Log_2 of 11,749.4 (fold increase of SSCs from day 0 to day 63 of culture)]}, which was nearly 1 day less than the 5.4-day doubling rate calculated for control cultures {63 days of culture/[Log_2 of 3441.9 (fold increase of SSCs from day 0 to day 63 of culture)]}. The relative number of SSCs in Csf1-treated cultures was significantly (P≤0.05) greater compared with controls beginning at day 21 of culture and continuing throughout the 63-day culture period (Fig. 5D). At day 35 of culture the number of SSCs was determined to be 2.1-fold higher in Csf1-treated cultures compared with controls, but the total number of germ cells only differed by a factor of 1.1-fold. This trend continued throughout the remainder of the culture period, with the number of SSCs being 3.2-fold higher in Csf1-treated cultures at day 63 when the total number of germ cells differed by only 1.1-fold. Collectively, these results indicate that exposure to Csf1 alters the fate decision of self-renewal versus differentiation in vitro, resulting in greater concentration of SSCs being maintained in the heterogeneous mix of Thy1$^+$ germ cell clumps, and indicate that Csf1 is an extrinsic stimulator of mouse SSC self-renewal.

Interstitial Leydig cells and select peritubular myoid cells express Csf1 within mouse testes

To gain a better understanding of the potential contribution that Csf1 has on SSC niche function, its expression in vivo was further studied. First, we examined expression of Csf1 transcript in MACS-isolated Thy1$^+$ and Thy1-depleted fractions from mouse pup testes. Using RT-PCR, Csf1 transcript was detected only in the Thy1-depleted fraction (see Fig. S4 in the supplementary material), suggesting that Csf1 influence on SSC self-renewal does not occur via an autocrine manner. Subsequent examination revealed Csf1 expression by Leydig cells in cross-sections of testes from both 10-dpp pups and 2-month-old adults (Fig. 6A). Interestingly, Csf1 expression was also localized to select peritubular myoid cells (Fig. 6B), although this staining was much less prevalent than that of Leydig cells. Negative controls with normal IgG as the primary antibody showed minimal background staining (see Fig. S3 in the supplementary material). Previous studies suggest that Sertoli cells are the major contributor of the testicular stem cell niche; however, Csf1 staining was not observed in Sertoli cells within either pup or adult testes. These observations indicate that both Leydig and myoid cells are contributors to the SSC niche in mammalian testes.

DISCUSSION

In this study we were interested in identifying novel growth factors that influence SSC self-renewal. We reasoned that identification of growth factor receptors with highly enriched expression in the Thy1$^+$ testis cell fraction would reveal potential growth factors that influence SSC fate decisions. Identification of enriched *Csf1r* expression by Thy1$^+$ cells was the catalyst for identifying Csf1 as a novel factor that influences SSC self-renewal. The addition of recombinant Csf1 in culture media also supplemented with Gdnf and Fgf2 enhanced maintenance of SSCs in heterogeneous Thy1$^+$ germ cell clumps compared with Gdnf and Fgf2 exposure alone. At each time-point analysed over the 63-day culture period, the number of SSCs in Csf1-treated cultures was greater than in controls and the difference was significant beginning on day 21. Importantly, total germ cell expansion in these cultures was not affected by Csf1 treatment. These observations indicate that exposure to Csf1 alters the ratio of SSC self-renewal versus differentiation occurring within Thy1$^+$ spermatogonial cultures, resulting in a greater percentage of the germ cell clumps being composed of SSCs compared with cultures exposed to only Gdnf and Fgf2. In pilot studies, Csf1 addition without Gdnf did not support germ cell clump formation

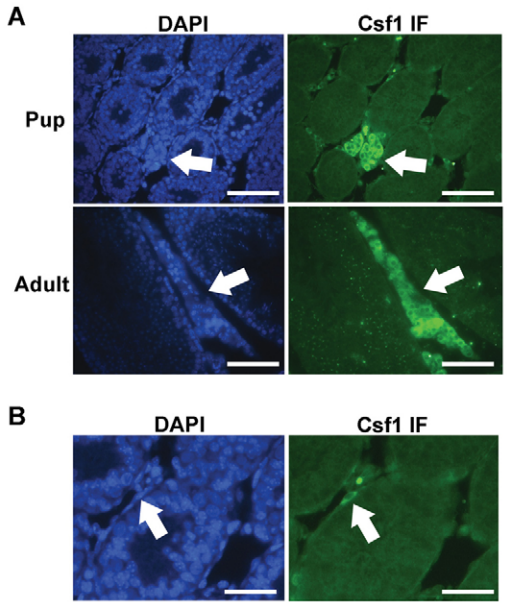

Fig. 6. Expression of Csf1 protein in mouse testes.
(**A**) Immunofluorescent localization of Csf1 expression in 10-dpp pup (top row) and adult (bottom row) mouse testes. Intense expression was observed in clusters of Leydig cells (arrows) located in the interstitial space at both ages. (**B**) Localization of Csf1 expression in myoid cells (arrow) lining the basement membrane of some seminiferous tubules in pup testes. Scale bars: 50 μm in A; 25 μm in B.

(data not shown), thus Csf1 is likely to act in synergy with Gdnf to promote SSC self-renewal. There are probably many other growth factors that also cooperate with Gdnf, Fgf2 and Csf1 to form the entire milieu of niche factors that regulate SSC self-renewal. Identifying these factors may result in derivation of a culture system that promotes only SSC self-renewal, allowing for rapid expansion of SSCs and in depth studies of intrinsic pathways influencing their fate decisions.

Self-renewal can be defined as the ability of a cell to undergo mitosis resulting in two new daughter cells, at least one of which does not enter a differentiation pathway, is identical to the parent cell and is capable of undergoing further self-renewing divisions. One major regulator of this process is response to growth factors such as Gdnf and Csf1, which occurs upon binding of these molecules to specific receptor complexes. Although Gdnf has been shown to be essential for SSC self-renewal, its receptor complex consisting of Gfra1 and c-Ret is expressed by several sub-populations of spermatogonia, including SSCs and A_{pr} and A_{al} spermatogonia (Dettin et al., 2003; Naughton et al., 2006). SSC content of the Gfra1$^+$ cell fraction is only marginally enriched (<2-fold) compared to the Gfra1-depleted cell population in mouse pup testes (Baugeaw et al., 2005), and depleted of SSCs in the adult testis (Ebata et al., 2005). Additionally, the c-Ret$^+$ cell fraction contains fewer SSCs than the total cell population in both pup and adult testes (Ebata et al., 2005). Also, expression of Gfra1 and c-Ret is homogenous in Thy1$^+$ germ cell clumps in vitro, which are composed of both SSCs and non-stem germ cells (Kubota et al., 2004b). These observations suggest that Gdnf action is not restricted to SSCs and may stimulate proliferation of multiple spermatogonia populations. Whereas proliferation is required for self-renewal, proliferation itself does not dictate a self-renewal event, because differentiation whether from symmetric or asymmetric stem cell division also involves mitosis. Thus, it is possible that Gdnf stimulates general spermatogonial proliferation and cues from other factors are needed to direct a self-renewal rather than differentiation pathway. Results of the present study demonstrate that exposure to Csf1 influences SSC self-renewal in vitro without affecting proliferation of non-SSCs. We observed expression of Csf1r by individual spermatogonia in only a few seminiferous tubules and could detect its expression on the cell surface by sub-populations of cells in mouse pup testes and Thy1$^+$ germ cell clumps, suggesting that Csf1r expression may be restricted to SSCs within mouse testes. These observations, in conjunction with in vitro analysis of SSC self-renewal, strongly suggest that at least for mice Csf1 action is restricted to SSCs in the male germline and does not affect other spermatogonial populations. Thus, Csf1 may be a specific regulator that maintains a stem cell phenotype while acting in concert with Gdnf, which promotes proliferation, and these two influences ultimately stimulate self-renewing division. Because of the heterogeneous nature of cultured Thy1$^+$ spermatogonia, consisting of SSCs and non-stem cell spermatogonia, it is possible that exposure to Csf1 also indirectly effects SSC self-renewal by acting on non-stem cell spermatogonia. Collectively, our results suggest that Csf1 is an integral component of the growth factor milieu that regulates self-renewal of mouse SSCs.

In vivo, interaction of stem cells with their cognate niche microenvironment is crucial for continual self-renewal and differentiation. In mammalian testes, the SSC niche is believed to be formed primarily by contributions of Sertoli cells. To date, production of Gdnf by these cells has been the only suggested mechanism of the SSC niche (Tadokoro et al., 2002; Yomogida et al., 2003). However, contributions by Leydig and myoid cells are also possible. Importantly, recent studies by Yoshida et al. (Yoshida et al., 2007) have revealed a biased localization of the proliferating spermatogonial population in regions of seminiferous tubules adjacent to the vascular network and clusters of Leydig cells. Because SSCs are present in this germ cell population, these observations suggest that Leydig cells may influence SSC function. We observed expression of Csf1 by Leydig cells and select myoid cells in both pre-pubertal and adult mouse testes. In agreement, studies by Shima et al. (Shima et al., 2004) identified elevated *Csf1* transcript expression by myoid cells and Ryan et al. (Ryan et al., 2001) localized Csf1 expression to Leydig cells in adult mouse testes. While our studies demonstrate that Csf1 influences SSC self-renewal in vitro, the importance of this factor on SSC functions in vivo remains undetermined. Studies by Cohen et al. (Cohen et al., 1996) revealed an impairment of spermatogenesis in *op/op* mutant mice that are deficient for Csf1 expression. In these animals, the number of sperm produced is approximately 60% fewer than in wild-type males, a phenotype that has been attributed to the dramatic (~86%) reduction of serum testosterone concentration in *op/op* mice. Spermatogenesis is sensitive to intratesticular testosterone concentrations, which is only modestly (~28%) diminished in *op/op* males. Additionally, in other models of testosterone deficiency, such as androgen receptor null mice, spermatogenesis is impaired due to a block in germ cell development during meiosis (Yeh et al., 2002; De Gendt et al., 2004), and this phenotype is not observed in *op/op* mice. Thus, decreased testosterone production does not completely explain the dramatic reduction in sperm production that occurs due to Csf1 deficiency in male *op/op* mice, and could be due at least in part to impaired SSC functions. Results of in vitro experiments in the current study suggest that Csf1 acts in synergy with Gdnf to

regulate SSC self-renewal in vitro. A similar mechanism may also function in vivo; thus Csf1 deficiency in the testis may result in sub-fertility rather than dramatic infertility, and it is a sub-fertility phenotype that may be of greatest importance when relating findings in mice to men.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/136/7/1191/DC1

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