

Endocrine Regulation of Spermatogonial Stem Cells in the Seminiferous Epithelium of Adult Mice

Kyle C. Cairnes,¹ Jeanene de Avila,² and Derek J. McLean²

Abstract

A balance between self-renewal and differentiation of spermatogonial stem cells (SSCs) is required to maintain sperm production throughout male life. The seminiferous epithelium is organized into stages of spermatogenesis based on the complement of germ cell types within a tubular section of the testis. The stages exist in close physical proximity and foster diverse phases of germ cell development despite exposure to a similar endocrine milieu that supports coordinated spermatogenesis. The objective of the current study was to identify the population dynamics of SSCs *in vivo*. We hypothesized that SSC populations and their niches are specifically distributed across the mature seminiferous epithelium in the mouse testis. To test this hypothesis, we conducted stem cell transplantation of germ cells obtained from stage-specific clusters of seminiferous tubules representing areas of high responsiveness to follicle-stimulating hormone (IX–I), androgen (II–IV), and retinoid (V–VIII) signaling. Similarly, we analyzed the expression of genes linked with SSC activity in these groups of stages. No stage-specific differences in the colonization efficiency or the colony number were detected after SSC transplantation, indicating that SSCs are equally distributed across all stages of the seminiferous tubule. In contrast, SSCs obtained from donor stages IX–IV established larger donor-derived colonies due to increased colony expansion. SSCs originating from different stages have varying degrees of stem cell activity *in vivo*, a notion consistent with *Gdnf*, *Ret*, and *Bcl6b* expression data. These results support the conclusion of a stage-specific, microenvironment-regulating SSC self-renewal and suggest the presence of a transit-amplifying population of undifferentiated spermatogonia *in vivo*.

Key words: gamete biology; spermatogenesis; spermatogonial stem cells; stem cells; testis

Introduction

ADULT STEM CELLS are organized in specialized microenvironments called niches that are composed of somatic cells and extracellular matrix components that provide intrinsic and extrinsic factors involved in regulating cell fate decisions. A coordinated balance between self-renewal and differentiation is required to meet the demands of tissue function and maintenance. Spermatogonial stem cells (SSCs) are responsible for continual sperm production in mammals, and are the only adult stem cell population capable of transmitting genes to offspring. SSCs exist in seminiferous tubules of the testes and are in close proximity to somatic Sertoli cells that are involved in supporting their function. In fact, Sertoli cells are equally capable of producing paracrine factors that either contribute to self-renewal of SSCs, in the case of glial cell line-derived neurotrophic factor (GDNF)^{1,2} and basic fibroblast growth factor (FGF2),³ or stimulate their differentiation via the actions of activin and bone morphogenetic

protein 4.⁴ This signaling coordinates a delicate balance between self-renewal and differentiation of SSCs that is required to maintain sperm production throughout life in males.

SSCs are a population of undifferentiated spermatogonia capable of self-renewal and producing donor-derived spermatogenesis in sterile recipients after transplantation.^{5,6} Although the activity of cells making up the undifferentiated spermatogonial population continues to be defined, single spermatogonia (A_s) are suggested to be SSCs that either self-renew or give rise to paired spermatogonia (A_{pr}) connected by cytoplasmic bridges. A_{pr} spermatogonia follow a clonal progression and differentiate into chains of 4, 8, and potentially 16 A-aligned (A_{al}) spermatogonia before becoming A1 spermatogonia. However, the identification of molecules and sources of intrinsic and extrinsic factors that regulate SSC cell fate remains incompletely explained due to the complex cell biology of the testis and the lack of *bona fide* SSC markers.⁷ Thus, germ cell transplantation is the

¹Department of Animal Sciences, Berry College, School of Math and Natural Sciences, Mount Berry, Georgia.

²Department of Animal Sciences, Center for Reproductive Biology, Washington State University, Pullman, Washington.

only reliable means to investigate factors that regulate *in vivo* SSC homeostasis.⁸

Morphological studies have suggested a nonrandom distribution of undifferentiated spermatogonia with the interstitial compartment.⁹ This area of testis contains blood vessels, lymphatics, and testosterone-producing Leydig cells. Similarly, researchers using a transgenic mouse expressing green fluorescent protein (GFP) under the control of a neurogenin 3 (NEUROG3) regulatory sequences suggest that SSCs exist within a vascular-associated niche.¹⁰ Interestingly, the preferential localization of undifferentiated spermatogonia in these studies has been linked with specific stages of the mature seminiferous epithelium. This leads to the question of whether a microenvironment enriched for SSCs and their respective niches are associated with particular stages or regions of the mature seminiferous epithelium in the testis.

The mature seminiferous epithelium is organized into stages of spermatogenesis based on the presence and precise complement of germ cell types within a tubular section of the testis, including (1) spermatogonial cell type present, (2) specific phase of meiosis observed, and (3) step of spermatid development.^{11,12} This unique organization allows for precise regulation of spermatogenesis, such that gene products can orchestrate specific events important for highly differentiated germ cells, without impairing less-mature germ cells during maturation or development. For instance, gradients of mitogenic and differentiation signals are found in a spatial and temporal manner that are directly correlated to cellular events such as mitosis, meiotic entry, and spermiation, so that efficient sperm production can occur. For example, regions of high responsiveness to follicle-stimulating hormone (FSH; stages IX–I),¹³ androgen (stages II–IV),¹⁴ and retinoic acid (stages V–VIII)¹⁵ have been described. Each portion of the seminiferous tubule will progress through all stages of spermatogenesis over time, and each stage of the cycle follows in an orderly sequence along the length of the tubule, a phenomenon called the spermatogenic wave, which is thought to initiate at postnatal day 10 in response to androgen and retinoid signaling in Sertoli cells.¹⁶ It is known that A_{al} spermatogonia differentiate into A1 spermatogonia during stages VII–VIII of the epithelial cycle¹⁷ concurrent with spermiation^{12,18,19} and high responsiveness to retinoid signaling.¹⁵

No functional studies have been used to identify the specific location and distribution of SSCs in the seminiferous tubule. Therefore, this study was designed to isolate and characterize specific regions of the mature seminiferous epithelium of the testes to determine if a microenvironment enriched for SSCs is associated with particular stages or regions of the seminiferous epithelium. To achieve this objective, we isolated clusters of seminiferous tubules using transillumination-assisted microdissection to obtain segments of tubules that represent areas of high responsiveness to FSH (IX–I), androgen (II–IV), and retinoid (V–VIII) signaling. We hypothesized that SSC populations are static and are randomly distributed across the mature seminiferous epithelium in the mouse testis. To test this hypothesis, we (1) evaluated gene expression of SSCs and SSC-niche-associated factors within specific stages using quantitative real-time polymerase chain reaction (PCR), and (2) assayed the SSC activity and SSC proliferation kinetics of SSCs obtained from these stage-specific clusters with the use of germ cell transplantation.

Materials and Methods

Animal care and treatments

All animal experiments were approved by the Washington State University Animal Care and Use Committee and were conducted in accordance with the Guiding Principles for the Care and Use of Research Animals of the National Institutes of Health. Animals were housed in a standard animal facility with and provided *ad libitum* access to food and water.

Stage-specific tubule dissection of the adult seminiferous epithelium

Stage-specific seminiferous tubule clusters were collected from adult (postnatal 8–12 weeks) mice using the transillumination-assisted microdissection technique as described previously.¹² Briefly, donor testes were removed, placed in Hanks buffered salt solution, and then detunicated. Segments of tubule clusters that represent areas of high responsiveness to FSH (IX–I), androgen (II–IV), and retinoid (V–VIII) signaling were harvested and placed in an alpha-minimum essential medium (α -MEM). Tubule segments representing all stages (I–XII) in normal adult mice were used as a positive control. Randomly selected tubule segments from mice treated with busulfan (intraperitoneally; 40 mg/kg body weight) to eliminate endogenous germ cells were harvested as a negative control. Transgenic B6.129S7-GtRosa26 (Rosa26) mice from Jackson Laboratory (Bar Harbor, ME) that express β -galactosidase in all testicular germ cells were used as donors for all transplantation experiments. After dissection, tubule clusters from C57Bl/6 (C57) mice were left intact and processed for RNA isolation of cDNA production using TRIzol (Life Technologies, Grand Island, NY). At least three donors were used for each tubule cluster evaluated ($n > 3$), and multiple independent dissection replicates were performed for each donor.

Preparation of donor germ cells

Rosa26 seminiferous tubule clusters were enzymatically digested as described by McLean and coworkers,⁸ with minor modifications. Briefly, tubule clusters were transferred to a dish containing a digestion medium, which consisted of 0.18 mg/mL trypsin (GibcoBRL, Bethesda, MD), 0.16 mg/mL collagenase type IV (Sigma, St. Louis, MO), and 0.6 mg/mL DNase (Sigma) in an α -MEM, pH 7.4, and incubated for 10 min at 37°C. After incubation and addition of fetal bovine serum (10% v/v), tubule segments were dispersed by gentle pipetting, and the testis cell suspension was centrifuged at 600 g for 7 min at 4°C. After several washes, donor cells were resuspended in an MEM containing 0.03% trypan blue (GibcoBRL) to obtain germ cells for transplantation at a concentration of 10^7 cells/mL.

Germ cell transplantation and analysis of recipient testes

To determine stem cell activity, $\sim 7.8 \times 10^4$ donor cells were transplanted into seminiferous tubules of busulfan-treated immunologically compatible 129SvCP (129) \times C57BL/6 F1 hybrid recipient mice. Recipient mice were sacrificed 8 weeks after transplantation, and the colonization efficiency of Rosa26 SSCs was determined by counting the number

TABLE 1. TAQMAN POLYMERASE CHAIN REACTION PROBES USED FOR QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION ASSAYS

Assay ID	Gene symbol	Gene name
Mm00442688_m1	<i>Ar</i>	Androgen receptor
Mm00455914_m1	<i>Bcl6b</i>	B-cell CLL/lymphoma 6 member B protein
Mm01235198_m1	<i>Fshr</i>	Follicle-stimulating hormone receptor
Mm00599849_m1	<i>Gdnf</i>	Glial cell line-derived neurotrophic factor
Mm00833897_m1	<i>Gfra1</i>	Glial cell line-derived neurotrophic factor family receptor-alpha 1
Mm02525720_s1	<i>Nanos2</i>	Nanos homolog 2
Mm00437606_s1	<i>Neurog3</i>	Neurogenin 3
Mm00436304_m1	<i>Ret</i>	Ret proto-oncogene
Mm00475529_m1	<i>Rps2</i>	Mitochondrial ribosomal protein S2
Mm01165142_m1	<i>Stra8</i>	Stimulated by retinoic acid gene 8

and measuring the length of blue colonies after staining with 5-bromo-4-chloro-3-indoyl β -D-galactoside (Xgal).

Quantitative real-time reverse transcriptase-PCR analysis

Tubule clusters obtained from adult C57 mouse testis tissue were homogenized in TRIzol (Life Technologies) according to the manufacturer's protocol. RNA concentration and purity were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Schwerte, Germany), and only samples with a 260/280 ratio of ≥ 1.8 , but ≤ 2.1 , were selected for analysis of gene expression as described. cDNA was prepared from 1 μ g of total RNA, and real-time PCR was performed as previously described²¹ using an iCycler iQ (Bio-Rad, Hercules, CA) detection system. Samples were assayed in duplicate, and comparisons were made by normalizing the expression of the target gene to that of ribo-

somal protein S2 using the Q-Gene method.²² A list and description of TaqMan probes used in the current study are shown (Table 1). The PCR reaction mixture contained 100 ng cDNA, 12.5 μ L of 2 \times TaqMan Universal PCR Master Mix, 1.25 μ L 20 \times TaqMan gene-specific probes (Table 1), and 9.25 μ L of nuclease-free water for a total volume of 25 μ L. Data are presented as fold change (mean expression levels \pm standard error of the mean [SEM]) relative to the expression levels of seminiferous tubules with all stages of the mature seminiferous epithelium present.

Immunohistochemistry methods

Immunohistochemistry was performed on paraffin-embedded testis tissue sections (5- μ m thickness) obtained from normal and busulfan-treated (intraperitoneally; 40 mg/kg body weight) intact mice using the avidin-biotin complex method and sodium citrate antigen retrieval (pH=6.0), as described.²³ After quenching endogenous peroxidase activity and washing slides in phosphate-buffered saline (PBS), slides were incubated for 30 min in a blocking solution (10% normal serum v/v in PBS). After blocking, tissue sections were incubated Armenian hamster anti-promyelocytic leukemia zinc finger (PLZF) monoclonal antibody (1:600; a gift from the Hobbs Lab) and rabbit anti-RET-phospho^{Y1062} antibody (1:200) for 12 h at 4°C. After washing, sections were incubated with biotinylated secondary antibodies for 60 min at 25°C, then washed, and incubated with a streptavidin-horseradish peroxidase substrate. Immunoreactivity was detected after a 5-min incubation in 3,3'-diaminobenzidine, and sections were counterstained with hematoxylin. As a negative control, serial sections were processed without primary antibody.

Statistical analysis

All datasets are presented as the mean \pm SEM, and differences between tubule clusters were considered significant at $p < 0.05$. The effect of seminiferous tubule microenvironment on stem cell activity and expression of SSC-niche-associated factors were analyzed using a one-way analysis of variance, and pairwise comparisons were evaluated with a Dunn's

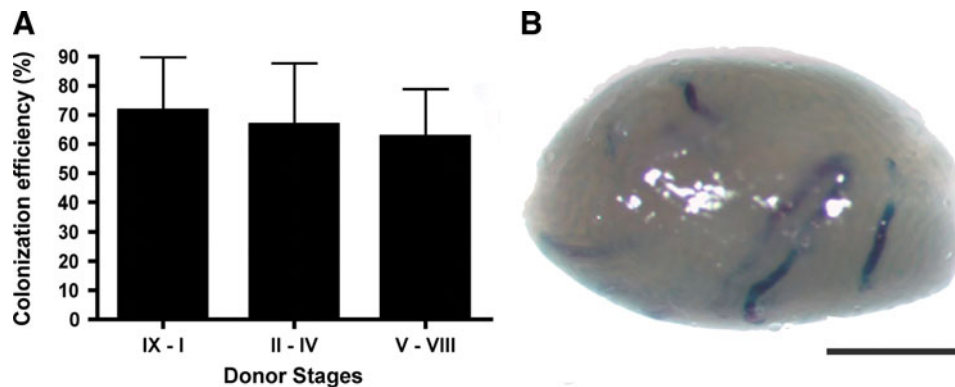


FIG. 1. Germ cell transplantation analysis of stage-specific SSCs obtained from adult seminiferous tubules of mice. (A) The colonization efficiency of SSCs originating from stages IX-I, II-IV, and V-VIII was similar after transplantation. (B) Light micrograph representative of SSC colony formation in recipient testis 8 weeks after transplantation. Data are representative of at least three independent experiments. Scale bar = 0.5 mm. SSCs, spermatogonial stem cells.

multiple-range test. Nonparametric data were analyzed using a Kruskal–Wallis test.

Results

Isolation and germ cell transplantation of stage-specific segments of the seminiferous epithelium obtained by transillumination-assisted microdissection

Stage-specific clusters of the adult seminiferous tubules were isolated based on the transillumination pattern described by Kotaja et al.²⁰ These groups of tubules were (1) stages IX–I, (2) stages II–IV, and (3) stages V–VIII. Transillumination can be employed to dissect seminiferous tubules based on stages due to the different germ cells present at each stage. The different morphology of differentiating germ cells, primarily spermatids, alters the amount of light passing through the tissue such that consistent sections can be obtained (Supplementary Fig. S1). Seminiferous tubules with branches were not used to avoid transitional regions without segmental stages (Supplementary Fig. S1). After dissection, stage-specific tubule segments from adult Rosa26⁺ mice were digested to obtain a single-cell suspension of germ cells for transplantation into the testes of infertile, busulfan-treated recipient mice. The cell suspensions obtained from each group of seminiferous stages contained cells of consistent size and morphology (Supplementary Fig. S1). Germ cell transplantation provides the ability to directly determine the SSCs in a cell suspension that are capable of generating donor-derived spermatogenesis. Eight weeks after transplantation, recipient mice were sacrificed, and testes were stained with Xgal, and the number of donor-derived stem cell colonies was determined. Germ cells obtained from stage-specific clusters (IX–I, II–IV, and V–VIII) colonize recipient testis and generate donor-derived spermatogenesis at similar rates (Fig. 1). These data indicate that adult SSC populations and their niches are equally distributed throughout the seminiferous epithelium.

The colonization efficiency of testicular cells isolated from randomly selected seminiferous tubules was not different from the germ cells isolated from the selected stage groups. We also transplanted testicular cells isolated from busulfan-treated mice as a second control. Busulfan treatment eliminates differentiating germ cells from the testis, resulting in seminiferous tubules devoid of germ cells, and donor cells for transplantation were obtained 4 weeks after busulfan treatment (Supplementary Fig. S1). Not surprisingly, the colonization efficiency of cells obtained from busulfan-treated donors was significantly impaired compared to cells from randomly selected seminiferous tubules and groups of stages, respectively (Fig. 2). Although busulfan treatment eliminates many SSCs, some do survive the treatment and can initiate donor-derived spermatogenesis after transplantation. However, the SSC recovery we observed was lower than reported results.²⁴

The extent of colony expansion was also evaluated to ascertain the proliferation kinetics and regeneration potential of SSCs obtained from specific groups of stages within the mature seminiferous tubule. Colony expansion of donor-derived spermatogenesis was significantly greater for donor cells obtained from stages IX–I and II–IV when compared to stages V–VIII (Fig. 3). Therefore, specific stages within seminiferous tubules support SSCs or undifferentiated spermatogonia, such that the colonization potential of these cells varies between stages. These data suggest that a dynamic SSC niche

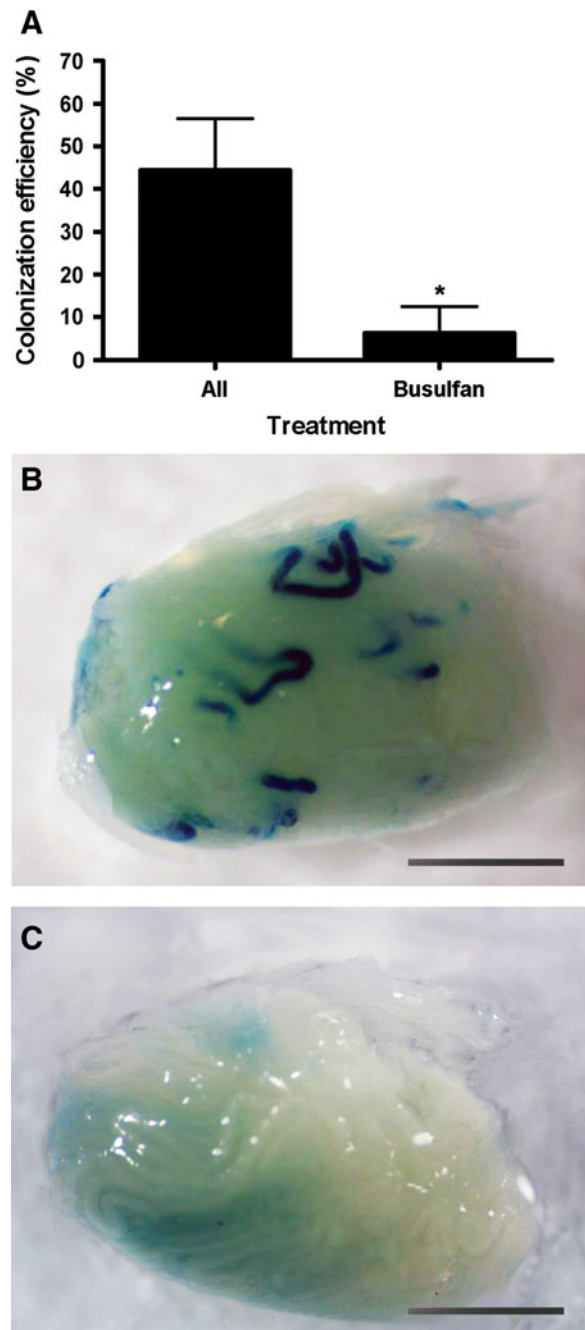


FIG. 2. Germ cell transplantation analysis of stage-specific SSCs obtained from randomly selected seminiferous tubules of normal and busulfan-treated donor mice. **(A)** The colonization efficiency of SSCs from randomly selected seminiferous tubules and busulfan-treated donors after transplantation. **(B)** Light micrograph representative of SSC colony formation in recipient testis post-transplantation of cells from randomly selected, nontreated donor seminiferous tubules. **(C)** Light micrograph representing SSC colony formation in recipient testis post-transplantation of cells from seminiferous tubules in busulfan-treated mice. Data are representative of at least three independent experiments. *Significant differences between means ($p < 0.05$). Scale bar = 0.5 mm **(B, C)**.

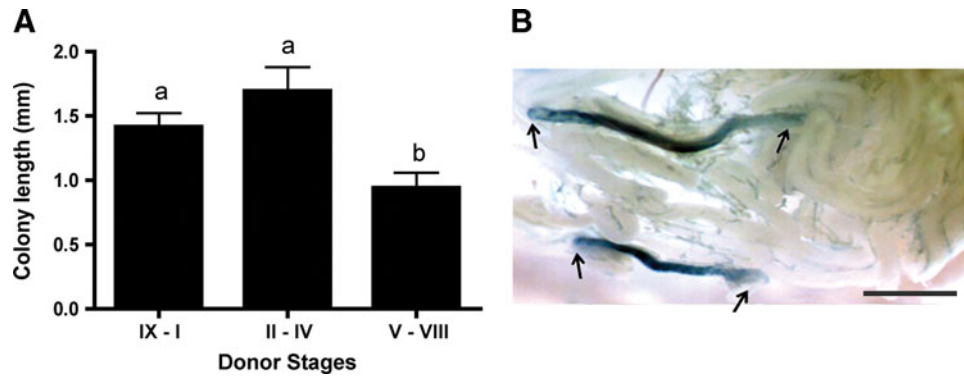


FIG. 3. (A) Colony growth and expansion were significantly greater in SSCs originating from stages IX–I and II–IV, when compared to stages V–VIII. (B) A light micrograph representative of differences in SSC colony expansion after transplantation due to the donor stage group. Arrows are used to show the length of donor-derived spermatogenesis and colonization in recipient testes. Data are representative of at least three independent experiments. ^{ab}Different letters indicate significant differences between means ($p < 0.05$). Scale bar = 0.5 mm.

may be present in the testis that is important for regulating the biological activity of SSCs and their ability to proliferate and self-renew. Busulfan treatment had no effect on the expansion rate of SSCs after transplantation, despite significantly reducing their numbers (data not shown).

Analysis of SSC-niche-associated factors in stage-specific segments of the seminiferous epithelium

The differential germ cell-derived colony expansion observed after transplantation of SSCs from different stages of the seminiferous epithelium suggests that the SSC niche or regulation may vary in different stages. To test this hypothesis, we investigated the expression of SSC-niche-associated factors and potential regulation of these factors. Germ cell differentiation and thus the stages of the seminiferous epithelium are primarily regulated by FSH, testosterone, and retinoic acid. Our first step was to determine the expression of *Fshr* and *Ar* mRNA transcripts using quantitative real-time reverse transcriptase-PCR in the groups of stages we dissected for germ cell transplantation to determine the prevailing regulatory factor for each group of stages. Although not statistically significant, *Fshr* expression was biologically highest in stages IX–I, whereas AR expression was biologically highest in stages II–IV (Fig. 4A). *Stra8* is induced by retinoic acid, so the expression of this gene was used to evaluate retinoic acid signaling in the groups of stage. The expression of *Stra8* was biologically higher in stages V–VIII when compared to the other groups of stages (Fig. 4). High cellular responsiveness to retinoic acid is associated with stages in which SSCs have lower colony expansion potential (Fig. 3).

A link between SSC homeostasis associated with SSC colony expansion and endocrine regulation of the seminiferous epithelium is lacking. Thus, we investigated the expression of SSC-niche-associated factors produced by germ and Sertoli cells in stage-specific clusters of the adult seminiferous epithelium. The GDNF signaling pathway, including its receptors RET and GFRA1, supports the maintenance and self-renewal of SSCs both *in vitro* and *in vivo*.^{1,25} The mRNA expression of *Gdnf* was significantly higher in stages IX–I and II–IV of the seminiferous epithelium compared to stages V–VIII (Fig. 4). *Ret* expression was significantly lower in stages V–VIII of the seminiferous epithelium compared to

all stages (not shown), but not when compared to stages IX–I and II–IV (Fig. 4). No stage-specific expression pattern was observed for *Gfra1* (Fig. 4).

To determine specific locations of the seminiferous tubule that may be associated with cell fate decisions and mechanisms directing SSC homeostasis *in vivo*, we assayed the mRNA expression of intrinsic regulators of SSC self-renewal, including B-cell CLL/lymphoma 6 member B protein (BCL6B) and nanos homolog 2 (NANOS2). The expression of *Bcl6b* was significantly higher ($p < 0.01$) in stages IX–I when compared to other tubule clusters (Fig. 4). Specifically, a stage-specific decline in *Bcl6b* expression was detected between tubule segments obtained from stages II–IV and V–VIII. No stage-specific mRNA expression patterns were observed for *Nanos2* (Fig. 4).

Busulfan treatment significantly reduces the number of differentiating germ cells and SSCs in the testis (Fig. 2). In addition, Sertoli and germ cell interactions are disrupted after busulfan treatment, causing the loss of seminiferous epithelial stage organization. We investigated how the loss of germ cells and stage organization affected the expression of the SSC niche factors to evaluate the stage-specific SSC colony expansion. Interestingly, *Ret* expression was not different in the testes of busulfan-treated mice compared to stages I–XII from untreated controls (Fig. 5). In contrast, expression of *Bcl6b* and *Gfra1* was significantly lower in busulfan-treated samples compared to controls (Fig. 5). *Gdnf* expression was significantly higher in the seminiferous tubules of busulfan-treated animals compared to controls (Fig. 5). The similarity of the *Ret* expression in the seminiferous tubules of busulfan-treated and control samples suggests that somatic cells express RET. Immunohistochemical analysis confirmed the Sertoli cell expression of RET (not shown) and activated p-RET^{Y1062} (Ret phosphorylated on Y-1062) in testis tissue of normal (Fig. 5) and busulfan-treated (Fig. 5) mice. These data suggest that GDNF signaling is active in regulating the SSC niche through both paracrine and autocrine mechanisms.

Discussion

The *in vivo* mechanisms regulating the biological activity and cell fate decisions of SSCs remain poorly understood.

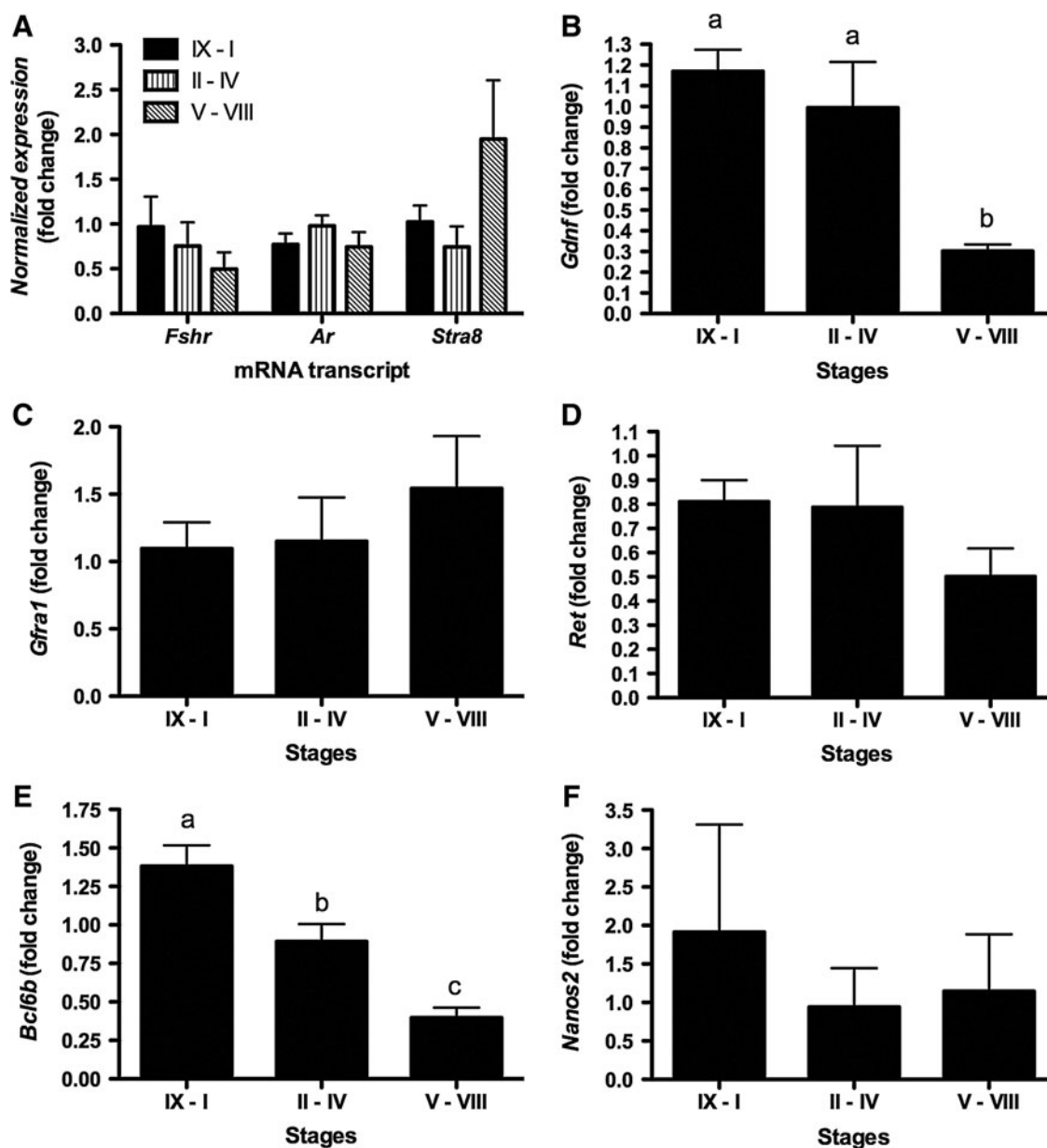


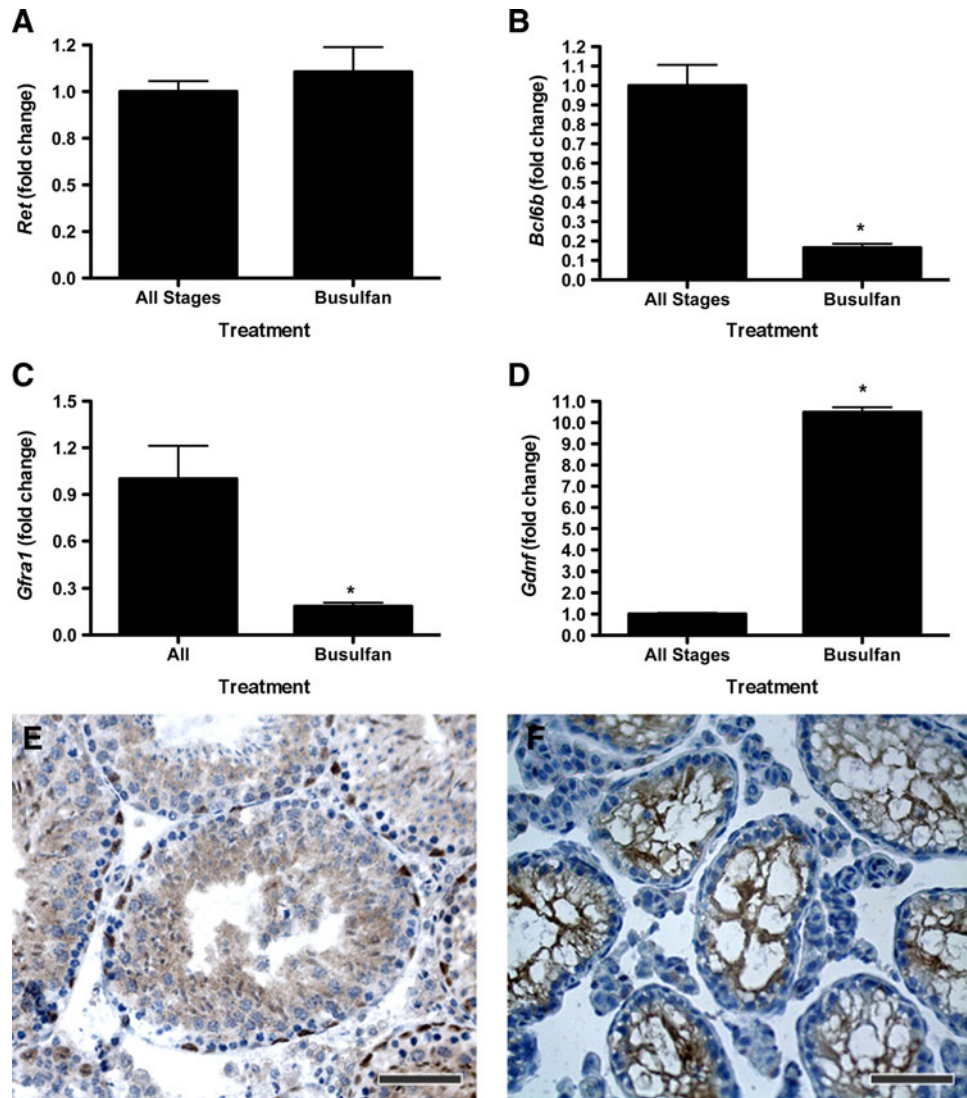
FIG. 4. Gene expression analysis of endocrine and SSC-niche-associated factors in stage-specific segments of the seminiferous epithelium. The mean expression values of (A) expression of *Fshr*, *Ar* and *Stra8*, (B) *Gdnf*, (C) *Gfra1*, (D) *Ret*, (E) *Bcl6b*, and (F) *Nanos2*, normalized to the levels of all stages (I–XII) in the normal adult testis. Data are representative of at least three independent experiments. ^{abc}Different letters indicate significant differences between means ($p < 0.05$).

SSCs, like other adult stem cells, are thought to reside in niches, yet the structural and functional components of the niche microenvironment are undefined in the testis. The adult seminiferous epithelium is organized into stages of spermatogenesis classified based on the precise complement of differentiating germ cells within a specific segment of a tubule, and occurs in a spatial and temporal pattern of development. Further, the seminiferous epithelium cycle is associated with areas of high responsiveness to FSH, androgen, and retinoid signaling, despite exposure to a similar endocrine milieu. Several reports suggest that undifferentiated spermatogonia are preferentially located near the interstitial compartment⁹ and vasculature¹⁰ within the testis in a stage-specific (IV–VI) manner. This raises the question as to whether SSCs and their respective niches are

randomly distributed or preferentially located within areas of the seminiferous tubule.

No functional studies have been used to (1) identify the specific location and distribution of SSCs in the seminiferous tubule, or (2) investigate the specific *in vivo* effects of local microenvironment on the biological activity of SSCs to self-renew or differentiate. In this study, we demonstrate that SSCs are randomly distributed across stages of the mature seminiferous epithelium in mice. We also observed stage-specific differences in the rate of colony growth and expansion after transplantation. Donor cells obtained from stages IX–IV produced larger colonies as characterized by a greater rate of postcolonization expansion when compared to SSCs from stages V–VIII. This suggests that the adult SSC

FIG. 5. Busulfan treatment eliminates stages of the seminiferous epithelium and germ cell–Sertoli cell interactions altering expression of SSC niche factors. Real-time reverse transcriptase–polymerase chain reaction analysis was performed with RNA from busulfan-treated testes and randomly selected seminiferous tubules of untreated control mice. Expression analysis of (A) *Ret*, (B) *Bcl6b*, (C) *Gfra1*, and (D) *Gdnf* transcripts. Immunohistochemistry to localize phospho-RET^{Y1062} demonstrates that endogenous GDNF signaling and RET activation are present in both germ and Sertoli cells in (E) untreated adult control testes and (F) the testes of busulfan-treated mice. Data are representative of at least three independent experiments. *Significant differences between means ($p < 0.05$). Scale bar = 50 μm (E, F). GDNF, glial cell line-derived neurotrophic factor.



population is characterized by varying degrees, or subsets, of stem cell activity, and indicates that a specific tubule microenvironment is associated with SSC activity *in vivo*.

Factors and protein markers associated with SSCs have been used to investigate the transition of a SSC into differentiating spermatogonia. For example, Sertoli cells produce GDNF in response to FSH,^{26,27} whereas testosterone stimulates the expression of GDNF by peritubular myoid cells.^{28,29} Responsiveness to FSH, androgen, and retinoic acid is highest between stages IX–I,^{13,30} II–IV,¹⁴ and V–VIII¹⁵ respectively, and this pattern correlated with the stage-specific expression of GDNF. A strict, stage-specific expression pattern was also observed for *Bcl6b*, a GDNF-inducible regulator of SSC maintenance and self-renewal *in vitro*. *Bcl6b* expression was observed with high, intermediate, and low expression in stages IX–I, II–IV, and V–VIII, respectively. Thus, the stage-specific responsiveness to FSH, androgen, and retinoids may provide local gradients of signal transduction necessary to precisely direct the cell fate decisions of SSCs, either directly or through the somatic microenvironment (see model; Fig. 6).

The differential SSC expansion means that undifferentiated spermatogonia (actual SSCs) may signal and stimulate Sertoli

cells to produce factors important for establishing a niche microenvironment for SSC expansion and self-renewal. Another population of undifferentiated spermatogonia (transit-amplifying SSCs) may have a reduced ability to exert feedback on Sertoli cells, thus negatively impacting or delaying their expansion after colonization. In support, donor cells obtained from stages IX–I and II–IV demonstrated larger post-colonization expansion than cells originating from V–VIII. This indicates the population of actual SSCs from stages IX–IV colonizes and initiates expansion before differentiation, accounting for the larger colonies observed in recipients. In contrast, the population of transit-amplifying spermatogonia from stages V–VIII may rapidly initiate differentiation after colonization and contribute toward a new population of A1 spermatogonia.

Additional alternative explanations for the increase in size of colonies from transplanted SSCs obtained from stages IX–IV include that these SSCs have enhanced proliferation capacity or accelerated migration along the length of the seminiferous tubule after transplantation. These differential SSC colonization kinetics could be due to the combination of the atypical seminiferous epithelial environment in busulfan-treated recipients and unique characteristics of SSCs from

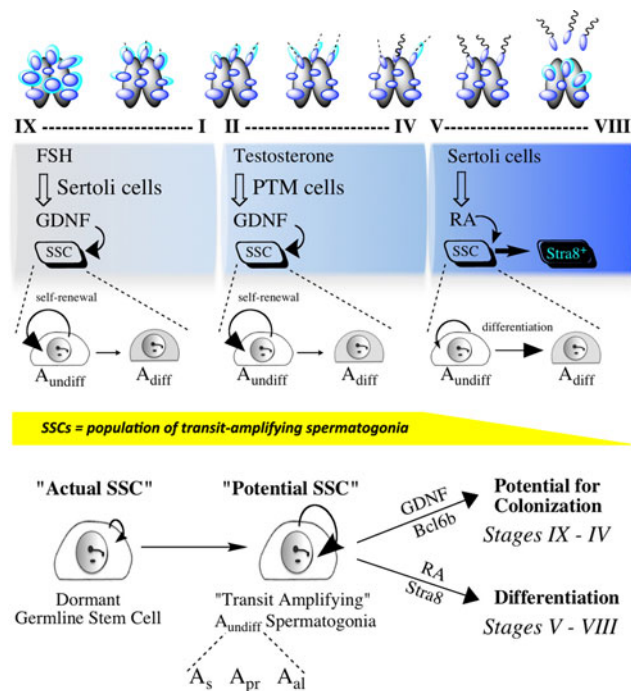


FIG. 6. Model of SSC cell fate decisions within the seminiferous tubule of adult mice developed with the use of the data presented in this article. The stage-specific microenvironment regulates SSC self-renewal and differentiation, but regulates production and potentially bioavailability of SSC niche factors such as GDNF. Factors such as retinoic acid stimulate spermatogonial differentiation and alter the stem cell potential of the undifferentiated pool of spermatogonia, such that spermatogonia may have actual and potential stem cell activity resulting in differential colonization and expansion capability after transplantation. Large gray ovals represent Sertoli cells, and blue circles represent differentiating germ cells (large round: spermatocytes; small round: round spermatids; slender elliptical: elongating spermatids) and spermatozoa.

stages IX–IV. For example, *Gdnf* expression is significantly higher in the testis of busulfan-treated mice than untreated controls, whereas *Ret* expression is not different in these groups. The observed increase in GDNF expression is likely due to the loss of differentiating germ cell RNA resulting in a proportional increase in Sertoli cell-expressed messages, but this does provide a potentially highly receptive environment for SSC colonization. Similar expression of *Ret* in testes from busulfan-treated and control mice explains why we did not observe stage-specific expression of *Ret*. Sertoli cell expression of *Ret* suggests that GDNF acts through paracrine and autocrine mechanisms in the seminiferous tubule. The combination of high expression of GDNF and RET in the seminiferous tubules of busulfan-treated mice may provide an environment where SSCs from stages IX–IV have enhanced proliferation or self-renewal, while SSCs from stages V–VIII have enhanced migration or differentiation.

The development of protocols to enrich for SSCs demonstrates that heterogeneity exists in the pool of undifferentiated spermatogonia. For example, GFRA1 is expressed on the surface of undifferentiated spermatogonia⁸ and is necessary to mediate the effects of GDNF-RET signal transduction.³¹ However, Thy1⁺, (selected) GFRA1-negative spermatogonia dem-

onstrate enriched stem cell activity when compared to GFRA1⁺ population after transplantation, and ~50% of the GFRA1⁺ population are also positive for KIT,³² a marker of spermatogonial differentiation associated with decreased stem cell potential.³³ Further, the expression of PLZF (ZBTB16), a transcription repressor whose expression is restricted to undifferentiated spermatogonia,³⁴ was similar across stages (data not shown). Quantitative real-time reverse transcriptase-PCR indicated no stage-specific effect on the expression *Gfra1* and *Nanos2*, two genes associated with SSCs. The significant stage-specific expression difference for *Gdnf* and *Bcl6b* supports the conclusion that the regulation of the SSC activity is at the level of GDNF production or bioavailability.

Germ cell transplantation analysis signifies a strong, functional relationship between SSC microenvironment and stem cell activity *in vivo*. The equal distribution of SSCs (and their niches) across the mature seminiferous epithelium was confirmed by transplantation, as no stage-specific effect on colonization efficiency was observed, and is supported by morphometric analysis.⁸ Together, these findings indicate that stem cell activity is independent of a static niche, as SSCs and their niches are equally distributed across all portions of the seminiferous tubule in the adult testis. The findings of Yoshida et al.³⁵ suggest that a population (subset) of A_{undiff} spermatogonia retains stem cell activity as a transit-amplifying cell. These, taken together with our data here, indicate that the pool of transit-amplifying A_{undiff} spermatogonia is largest in stages IX–I and II–IV (where GDNF expression is highest), whereas extrinsic signals in stages V–VIII stimulate the pool of transit-amplifying A_{undiff} spermatogonia toward differentiation. Therefore, the cycle of the seminiferous epithelium may regulate the dynamics of transit-amplification and stem cell activity in populations of A_{undiff} spermatogonia. Based on these data, we present a revised model summarizing the cellular kinetics of SSCs and their microenvironment within the mature seminiferous epithelium in the mouse (Fig. 6).

To our knowledge, this is the first functional study regarding the population dynamics and stem cell activity of adult SSCs *in vivo*. Moreover, our findings provide evidence that SSC self-renewal is regulated by a distinct microenvironment independent of a resident stem cell niche in the seminiferous tubule of sexually mature mice. The complex cell biology of the testis necessitates a better understanding of the regulatory networks supporting and maintaining stage-specific microenvironments for SSCs. Elucidation of these mechanisms may offer new insights into the regulation of other tissue-specific, adult stem cell populations. Identifying the balance of extrinsic and intrinsic cues that directly regulate the biological activity of SSCs and functional and structural components of the tubule microenvironment will be the focus of future *in vivo* experimentation and may provide novel approaches for treating male-factor infertility for agricultural and human biomedical purposes.

Acknowledgment

We extend our appreciation to Dr. Robin Hobbs for the PLZF antibody.

Author Disclosure Statement

The authors have nothing to disclose.

References

1. Meng X, Lindahl M, Hyvonen ME, et al. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*. 2000;287:1489–1493.
2. Oatley JM, Avarbock MR, Telaranta AI, et al. Identifying genes important for spermatogonial stem cell self-renewal and survival. *Proc Natl Acad Sci USA*. 2006;103:9524–9529.
3. Goriely A, McVean GAT, van Pelt AMM, et al. Gain-of-function amino acid substitutions drive positive selection of FGFR2 mutations in human spermatogonia. *Proc Natl Acad Sci USA*. 2005;102:6051–6056.
4. Nagano M, Ryu BY, Brinster CJ, et al. Maintenance of mouse male germ line stem cells *in vitro*. *Biol Reprod*. 2003;68:2207–2214.
5. Brinster RL, Zimmermann JW. Spermatogenesis following male germ-cell transplantation. *Proc Natl Acad Sci USA*. 1994;91:11298–11302.
6. McLean DJ. Spermatogonial stem cell transplantation and testicular function. *Cell Tissue Res*. 2005;322:21–31.
7. McLean DJ. Spermatogonial stem cell transplantation, testicular function, and restoration of male fertility in mice. *Methods Mol Biol*. 2008;450:149–162.
8. Caires K, Broady J, McLean D. Maintaining the male germline: regulation of spermatogonial stem cells. *J Endocrinol*. 2010;205:133–145.
9. Chiarini-Garcia H, Hornick JR, Griswold MD, et al. Distribution of type A spermatogonia in the mouse is not random. *Biol Reprod*. 2001;65:1179–1185.
10. Yoshida S, Sukeno M, Nabeshima Y. A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. *Science*. 2007;317:1722–1726.
11. Leblond CP, Clermont Y. Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the periodic acid-fuchsin sulphurous acid technique. *Am J Anat*. 1952;90:167–216.
12. Leblond CP, Clermont Y. Definition of the stages of the cycle of the seminiferous epithelium in the rat. *Ann NY Acad Sci*. 1952;55:548–573.
13. Heckert LL, Griswold MD. Expression of follicle-stimulating hormone receptor mRNA in rat testes and Sertoli cells. *Mol Endocrinol*. 1991;5:670–677.
14. Bremner WJ, Millar MR, Sharpe RM, et al. Immunohistochemical localization of androgen receptors in the rat testis: evidence for stage-dependent expression and regulation by androgens. *Endocrinology*. 1994;135:1227–1234.
15. Kim KH, Griswold MD. The regulation of retinoic acid receptor mRNA levels during spermatogenesis. *Mol Endocrinol*. 1991;4:1679–1688.
16. Kluin PM, Kramer MF, de Rooij DG. Spermatogenesis in the immature mouse proceeds faster than in the adult. *Int J Androl*. 1982;5:282–294.
17. Russell LD, Ethlin RA, Sinha Hikim AP, et al. *Histological and Histopathological Evaluation of the Testis*. Cache River Press: Clearwater, FL, 1990.
18. Oakberg EF. A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am J Anat*. 1956;99:391–414.
19. Oakberg EF. Duration of spermiogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *Am J Anat*. 1956;99:507–516.
20. Kotaja N, Kimmins S, Brancorsini S, et al. Preparation, isolation and characterization of stage-specific spermatogenic cells for cellular and molecular analysis. *Nat Methods*. 2004;1:249–254.
21. Caires KC, de Avila J, McLean DJ. Vascular endothelial growth factor regulates germ cell survival during establishment of spermatogenesis in the bovine testis. *Reproduction*. 2009;138:667–677.
22. Muller PY, Janovjak H, Miserez AR, et al. Processing of gene expression data generated by quantitative realtime RT-PCR. *Biotechniques*. 2002;32:1372–1379.
23. Caires KC, Schmidt JA, Oliver AP, et al. Endocrine regulation of the establishment of spermatogenesis in pigs. *Reprod Domest Anim*. 2008;43 Suppl 2:280–287.
24. Zohni K, Zhang X, Tan SL, et al. The efficiency of male fertility restoration is dependent on the recovery kinetics of spermatogonial stem cells after cytotoxic treatment with busulfan in mice. *Hum Reprod*. 2012;27:44–53.
25. Jijiwa M, Kawai K, Fukihara J, et al. GDNF-mediated signaling via RET tyrosine 1062 is essential for maintenance of spermatogonial stem cells. *Genes Cells* 2008;13:365–374.
26. Tadokoro Y, Yomogida K, Ohta H, et al. Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. *Mech Dev*. 2002;113:29–39.
27. Simon L, Ekman GC, Tyagi G, et al. Common and distinct factors regulate expression of mRNA for ETV5 and GDNF, Sertoli cell proteins essential for spermatogonial stem cell maintenance. *Exp Cell Res*. 2007;313:3090–3099.
28. Welsh M, Saunders PT, Atanassova N, et al. Androgen action via testicular peritubular myoid cells is essential for male fertility. *FASEB J*. 2009;23:4218–4230.
29. Spinnler K, Köhn FM, Schwarzer U, et al. Glial cell line-derived neurotrophic factor is constitutively produced by human testicular peritubular cells and may contribute to the spermatogonial stem cell niche in man. *Hum Reprod*. 2010;25:2181–2187.
30. Kliesch S, Penttilä TL, Gromoll J, et al. FSH receptor mRNA is expressed stage-dependently during rat spermatogenesis. *Mol Cell Endocrinol*. 1992;84:R45–R49.
31. He Z, Jiang J, Hofmann MC, et al. Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase. *Biol Reprod*. 2007;77:723–733.
32. Hofmann MC, Braydich-Stolle L, Dym M. Isolation of male germ-line stem cells; influence of GDNF. *Dev Biol*. 2005;279:114–124.
33. Ohbo K, Yoshida S, Ohmura M, et al. Identification and characterization of stem cells in prepubertal spermatogenesis in mice. *Dev Biol*. 2003;258:209–225.
34. Costoya JA, Hobbs RM, Barna M, et al. Essential role of Plzf in maintenance of spermatogonial stem cells. *Nat Genet*. 2004;36:653–659.
35. Yoshida S, Nabeshima Y, Nakagawa T. Stem cell heterogeneity: actual and potential stem cell compartments in mouse spermatogenesis. *Ann NY Acad Sci*. 2007;1120:47–58.

Address correspondence to:
 Derek J. McLean, PhD
 Department of Animal Sciences
 Center for Reproductive Biology
 Washington State University
 P.O. Box 646353
 Pullman 99164-6353, WA
 E-mail: dmclean@wsu.edu