

Functional and molecular features of the Id4⁺ germline stem cell population in mouse testes

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The maintenance of cycling cell lineages relies on undifferentiated subpopulations consisting of stem and progenitor pools. Features that delineate these cell types are undefined for many lineages, including spermatogenesis, which is supported by an undifferentiated spermatogonial population. Here, we generated a transgenic mouse line in which spermatogonial stem cells are marked by expression of an *inhibitor of differentiation 4 (Id4)-green fluorescent protein (Gfp)* transgene. We found that Id4-Gfp⁺ cells exist primarily as a subset of the type A_{single} pool, and their frequency is greatest in neonatal development and then decreases in proportion during establishment of the spermatogenic lineage, eventually comprising ~2% of the undifferentiated spermatogonial population in adulthood. RNA sequencing analysis revealed that expression of 11 and 25 genes is unique for the Id4-Gfp⁺/stem cell and Id4-Gfp⁻/progenitor fractions, respectively. Collectively, these findings provide the first definitive evidence that stem cells exist as a rare subset of the A_{single} pool and reveal transcriptome features distinguishing stem cell and progenitor states within the mammalian male germline.

[*Keywords:* germline stem cell; Id4; spermatogonia; spermatogonial stem cell; transcriptome]

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The functionality of tissues is provided by differentiated cells that are continually lost as a result of terminal differentiation or cytotoxic insult. Constant cycling of cell lineages to provide differentiated cells relies on the actions of undifferentiated subpopulations consisting of stem cells and progenitors. The tissue-specific stem cells self-renew to maintain a constant pool from which progenitors periodically arise and transiently amplify in number before committing to a pathway of terminal differentiation. Thus, mechanisms controlling these actions are critical for tissue homeostasis.

Identification of characteristics that distinguish stem cells from progenitors is of paramount importance for understanding the regulatory mechanisms governing their actions. For example, identification of Lgr5 expression in the intestinal epithelium has allowed for discovery of key mechanisms that sustain the crypt stem cell population (Barker et al. 2007). In addition, identification of Sox2

expression in a multitude of cell lineages has allowed for comparison of conserved stem cell and progenitor properties (Arnold et al. 2011). Despite these advancements, characteristics that distinguish stem cells and progenitors are undefined for many cell lineages.

For the mammalian spermatogenic lineage, the actions of spermatogonial stem cells (SSCs) provide the basis for continual generation of millions of genetically unique spermatozoa daily (de Rooij and Russell 2000; Oatley and Brinster 2008, 2012). In testes of rodents, germline regenerative activity is a property of the type A undifferentiated spermatogonial population that consists of A_{single} (individual cells), A_{paired} (cohorts of two cells), and A_{aligned} (cohorts of four, eight, and 16 cells) subtypes. Traditionally, A_{single} spermatogonia have been considered the SSC population that self-renews to maintain a foundational pool from which A_{paired} spermatogonia arise

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(Huckins 1971; de Rooij and Russell 2000; Oatley and Brinster 2012). Proliferation of A_{paired} generates A_{aligned} cohorts, and together these represent the progenitor spermatogonial population. During steady-state conditions, the majority of progenitor spermatogonia transition periodically into differentiating type A spermatogonia that are committed to terminal differentiation, eventually yielding spermatozoa. This transition occurs every 8.6 d in mice to sustain continual spermatogenesis (de Rooij 1998). Due to staggered timing in the initiation of terminal differentiation, spermatogenesis occurs in stages that make up a cycle of the seminiferous epithelium. In mice, 12 stages constitute a spermatogenic cycle, which takes 35 d to complete (Oakberg 1956). The actions of SSCs and progenitors during the cycle provide the foundation for continuity of spermatogenesis and are required for male fertility. However, despite their importance, little is known about the defining characteristics of SSC and progenitor spermatogonial subtypes in the mammalian male germline.

A widely accepted model is that, upon division, A_{single} spermatogonia have the capacity for two different fates. The cells can separate and form two A_{single} , thereby undergoing symmetric self-renewal to expand the SSC pool, or remain connected by an intercellular bridge due to incomplete cytokinesis, thereby forming A_{paired} spermatogonia (Oakberg 1971; de Rooij and Russell 2000; Oatley and Brinster 2012). In this model, formation of A_{paired} spermatogonia represents the initial step in commitment to terminal differentiation. Recent studies have begun to challenge this paradigm by suggesting that chained undifferentiated spermatogonia (A_{paired} and A_{aligned}) fragment to produce smaller chains and even single cells that contribute to the SSC pool (Nakagawa et al. 2010). While these findings are based solely on time-lapse imaging of select seminiferous tubules, they suggest that the stem cell population in mouse testes may be more flexible than the traditional A_{single} model implies. However, whether the newly formed putative A_{single} or smaller cohorts produced by fragmentation fulfill the definition of a stem cell remains to be clarified.

In the most basic sense, stem cells can be defined by a functional ability to regenerate and sustain a cycling cell lineage. Thus, the defining feature of SSCs is the capacity to regenerate and sustain spermatogenesis. In comparison, the progenitor population contributes to steady-state conditions but lacks regenerative capacity. Transplantation methodology is an unequivocal means to determine stem cell capacity of a cell population based on regenerative ability. Germ cell transplantation methodology is a well-established tool for mice to determine the SSC capacity of a cell population based on regenerated colonies of continual spermatogenesis (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). Previous studies suggest that testes of adult mice contain ~3000 cells with regenerative capacity (Nagano 2003), yet the number of A_{single} spermatogonia has been calculated to be ~35,000 (Tegelenbosch and de Rooij 1993); thus, SSCs likely represent a subset of A_{single} spermatogonia. Importantly, preparation of testis cell

suspensions for transplantation involves disassociation of spermatogonial chains to generate a single-cell suspension, but only a small portion (~3000) of the population has regenerative capacity. Thus, fragmentation in itself does not induce stem cell capacity for the mouse male germ cell lineage. At present, distinguishing morphological (chain identity) or molecular (expression of specific proteins) characteristics of the SSC and progenitor spermatogonial populations are undefined, and the only unequivocal measure is a functional ability for regeneration of spermatogenesis after transplantation.

In previous studies, we found that expression of the transcriptional repressor inhibitor of differentiation 4 (*Id4*) is selective for A_{single} cells within the spermatogonial population of mouse testes and plays an important role in the regulation of SSC self-renewal (Oatley et al. 2011a). Here, we succeeded in labeling the SSC population specifically by generating a novel transgenic mouse model in which *Id4*-expressing cells are marked by green fluorescent protein (Gfp). Using this model, we discovered that regenerative capacity is restricted to the *Id4*-expressing subpopulation of undifferentiated spermatogonia, supporting the traditional model of A_{single} representing the SSC pool. In addition, we describe for the first time other distinguishing features of this population, including (1) relative abundance during neonatal development and adulthood, (2) localization within regions of seminiferous tubules, and (3) unique aspects of the transcriptome.

Results

Generation of an Id4-Gfp reporter mouse line

In previous studies, we used immunostaining to localize *Id4* in testes of mice, which revealed selective expression by rare A_{single} spermatogonia (Oatley et al. 2011a). To label this population as live cells for in-depth analysis, we created a transgenic mouse line in which a Gfp reporter transgene is driven by *Id4* regulatory elements (Fig. 1A). A 17-kb fragment containing all exons and introns of the mouse *Id4* gene, including 5' (7512-base-pair [bp]) and 3' (9214-bp) flanking regions, was captured from a BAC clone. A yeast recombineering approach was then used to insert an enhanced Gfp (eGfp)-Ura3 cassette in-frame of exon 1, deleting base pairs 13–29. The transgene construct was then used with pronuclear injection to generate founder males with an FVB genetic background. A PCR strategy was devised to identify mice containing the *Id4-Gfp* transgene (Fig. 1B). Thus, expression of Gfp faithfully represents *Id4* protein expression. We identified five transgenic founder lines, and all possessed a similar pattern and level of Gfp expression in testes. One line was then chosen for expansion and backcrossed on the C57BL/6J genetic background to create a new line, referred to hereafter as LT-11B6 (Fig. 1A). Mice of this line containing the *Id4-Gfp* transgene are of normal viability and fertility, and transgene inheritance occurs at an expected Mendelian ratio. Expression of the transgene is detectable in numerous tissues, including the testes,

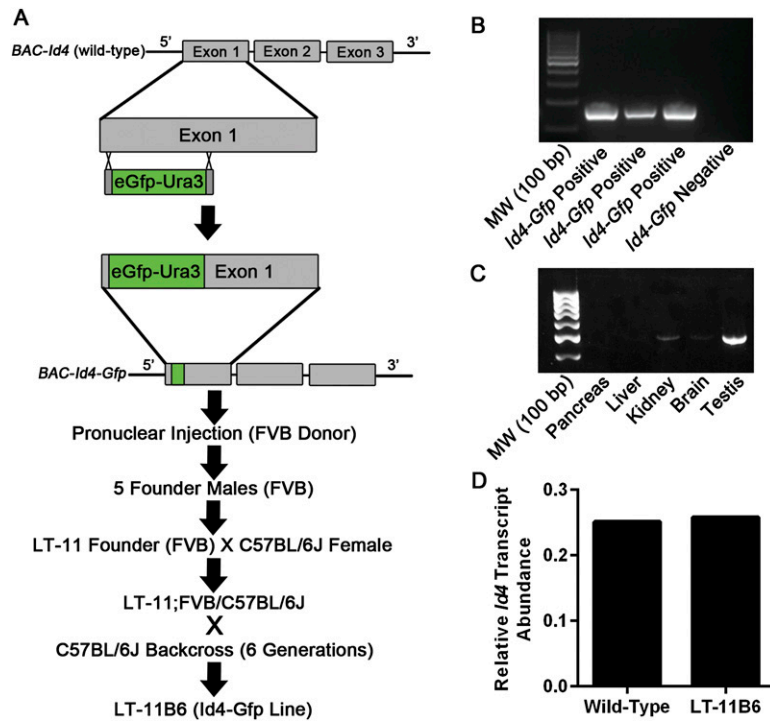


Figure 1. Generation of a transgenic *Id4-Gfp* reporter mouse line. (A) Schematic of the recombineering approach and breeding strategy used for generating the LT-11B6 mouse model that contains a *Gfp* reporter transgene representing expression of *Id4*. (B) Representative image of an agarose gel from genotyping analysis designed to identify LT-11B6 mice containing the *Id4-Gfp* transgene. MW is 100-bp ladder. The image displays products of PCR reactions with DNA samples from three transgenic littermates and one nontransgenic littermate. (C) Expression of the *Id4-Gfp* transgene in several different tissues of LT-11B6 mice as determined by RT-PCR analysis. (D) Quantitative comparison of *Id4* transcript abundance in testes of adult LT-11B6 mice and wild-type littermates using qRT-PCR analyses.

brain, and kidney (Fig. 1C). Also, presence of the transgene does not result in excessive *Id4* transcript abundance in testes of LT-11B6 mice (Fig. 1D).

Expression of Id4-Gfp selectively labels A_{single} cells of the spermatogonial population

In mammalian testes, the cycling spermatogenic lineage is established during a defined period of postnatal development, encompassing 0 and 35 d of age in mice (Drumond et al. 2011). At postnatal day (PD) 0, the germ cell population consists of quiescent gonocyte precursors that were formed during late embryonic development. The undifferentiated spermatogonial population materializes from these precursors during PD 3–8. In adulthood, at PD 35 and beyond, dynamics of the undifferentiated spermatogonial population are identical to that established during PD 3–8 (Drumond et al. 2011). To assess expression of *Id4-Gfp* in the germline during postnatal development, we used immunostaining of testis cross-sections with an antibody recognizing *Gfp* and whole-mount imaging for *Gfp* in live tissue. Immunostaining of cross-sections revealed expression in most gonocytes at PD 0 and then a subset of type A spermatogonia at PD 3, 6, 8, 10, and 12 (Fig. 2A). In addition, *Gfp* expression was detected in both a subset of type A spermatogonia and some pachytene spermatocytes at PD 20 and 35 but not in other spermatogonial subtypes, other spermatocytes, or spermatids. Next, we aimed to determine whether *Id4-Gfp* expression within the spermatogonial population is localized to *A_{single}* and/or chains of *A_{paired}*/*A_{aligned}*. To accomplish this, whole-mount imaging of dissected seminiferous tubules for live *Gfp*⁺ cells was performed at PD 6

and 35. At both ages, individual *Gfp*⁺ cells were observed at the periphery of seminiferous tubules (Fig. 2B). Scanning along the length of a tubule revealed numerous *Id4-Gfp*⁺ cells at PD 6, but the occurrence declined upon aging, with only a few cells per seminiferous tubule fragment observed at PD 35. Also, in rare incidences (four observations out of >300 *Gfp*⁺ cells), a cohort of two *Id4-Gfp*⁺ cells was observed (Supplemental Fig. S1). Whether these were true *A_{paired}* or false pairs sometimes observed when *A_{single}* have not separated following division could not be determined. Taken together, these findings indicate that expression of *Id4* is restricted to some, if not all, *A_{single}* spermatogonia within the undifferentiated spermatogonial population of mouse testes.

Id4-expressing cells represent a subset of the A_{single} spermatogonial population

To explore further the expression of *Id4-Gfp* within the undifferentiated spermatogonial population specifically, we used coimmunofluorescent staining for the pan-undifferentiated marker *Plzf* and *Gfp* (Fig. 3A). While these analyses revealed overlap in expression of both markers, only a subset of the *Plzf*⁺ population was *Id4-Gfp*⁺ at all ages examined (Fig. 3B). At PD 3, when gonocyte precursors have transitioned to a postnatal spermatogonial state, the *Id4-Gfp*⁺ cells comprised 92.9% ± 0.5% of the *Plzf*⁺ undifferentiated spermatogonial population, decreasing to 46.1% ± 1.1% at PD 6, 19.6% ± 0.6% at PD 12, and 8.5% ± 0.4% at PD 20 (*n* = 3 different mice and 50–60 tubules for each age). In adulthood, the *Id4-Gfp*⁺ population comprised only 1.9% ± 0.3% of the *Plzf*⁺ undifferentiated spermatogonial population (*n* = 3

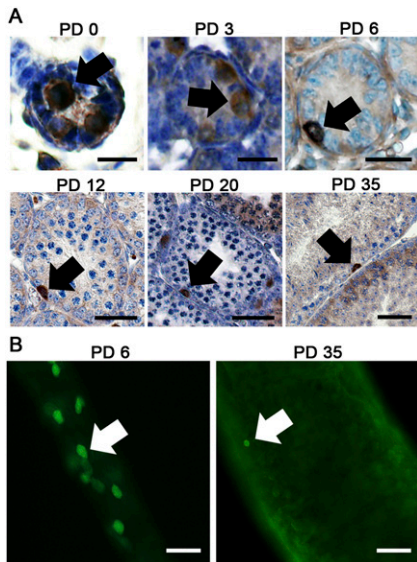


Figure 2. Identification of Id4-Gfp-expressing spermatogonia in testes of LT-11B6 mice at multiple stages of postnatal life. (A) Representative images of immunostaining for Id4-Gfp-expressing cells in cross-sections of seminiferous tubules from testes of mice at PD 0, 3, 6, 12, 20, and 35. Arrows indicate Id4-Gfp⁺ spermatogonia. Bars: PD 0, 3, and 6, 50 μ m; PD 12, 20, and 35, 100 μ m. (B) Representative pictures of live imaging for Id4-Gfp⁺ cells in whole-mount seminiferous tubules from LT11-B6 mice at PD 6 and 35. Arrows indicate single Id4-Gfp⁺ spermatogonia. Bars, 50 μ m.

different mice and 50 tubules). Overlap of staining for markers of differentiating spermatogonia, including Kit and Stra8, was not observed at any age examined (Supplemental Fig. S2), confirming that expression of Id4 is restricted to spermatogonia in the undifferentiated state of development. Next, we examined Id4-Gfp⁺ cells within the Gfra1-expressing undifferentiated spermatogonial population, which has been reported to be restricted to A_{single}, A_{paired}, and short chains (i.e., four cell cohorts) of A_{aligned} spermatogonial subsets (Suzuki et al. 2009; Nakagawa et al. 2010). Results of coimmunofluorescent staining revealed that, beginning at PD 6, the Id4-Gfp⁺ population represents a subset of individual cells within the more abundant Gfra1⁺ spermatogonial population (Fig. 3C). Taken together, these findings demonstrate that the A_{single} spermatogonial population is heterogeneous, supporting a notion that expression of Id4 selectively marks the SSC pool.

Relative abundance and distribution of Id4-Gfp⁺ spermatogonia in mouse testes during postnatal development and adulthood

Next, we determined changes in the relative abundance of Id4-Gfp⁺ spermatogonia in testes during postnatal development and their distribution within seminiferous tubules during steady-state spermatogenesis in adulthood. To achieve this, immunostaining for Gfp within cross-sections of testes was used, and the number of Id4-

Gfp⁺ cells was quantified (Fig. 4A). At PD 0 and 3, most seminiferous tubule cross-sections were found to contain Id4-Gfp⁺ spermatogonia at a rate of $98.9\% \pm 1.1\%$ and $88.1\% \pm 1.8\%$, respectively. During advancing postnatal development, the frequency decreased to $70.8\% \pm 4.8\%$, $61.6\% \pm 9.6\%$, $29.9\% \pm 7.8\%$, and $6.8\% \pm 4.4\%$ of seminiferous tubule cross-sections containing Id4-Gfp⁺ spermatogonia at PD 6, 12, 20, and 35, respectively. Interestingly, the number of Id4-Gfp⁺ spermatogonia within tubule cross-sections was relatively constant at all postnatal ages examined, with 2.5 ± 0.2 , 2.7 ± 0.3 , 2.0 ± 0.1 , 1.7 ± 0.2 , 1.3 ± 0.1 , and 1.1 ± 0.1 positive spermatogonia at PD 3, 6, 12, 20, and 35, respectively (Fig. 4B). These findings indicate that the Id4-Gfp⁺ spermatogonial population is of greatest abundance in neonatal life, decreases in both frequency and number as the spermatogenic lineage becomes established, and is then maintained in a cycling manner during adulthood.

To assess further the Id4-Gfp⁺ spermatogonial population during steady-state conditions in adulthood, we examined the relative abundance of these cells at different stages of the seminiferous epithelial cycle. In mammalian testes, spermatogenesis occurs in a staggered manner along the length of seminiferous tubules to provide constant generation of spermatozoa. This staggering is reflected by different stages of germ cell development, and all stages collectively constitute a cycle of the seminiferous epithelium. At present, the behavior of SSCs during these stages is undefined. While there are 12 defined stages in testes of adult mice, accurate identification of each relies on fixation and staining of nuclear material that is not compatible with immunostaining. Thus, to improve accuracy, we evaluated stages as early (II–VI), middle (VII–VIII), and late (IX–I) groupings (Fig. 4C). Results of these analyses revealed even distribution of the Id4-Gfp⁺ spermatogonial population across the groupings (Fig. 4D). These findings suggest that the SSC pool is distributed equally during steady-state conditions of a constant cycling spermatogenic lineage.

Last, we aimed to determine whether Id4-Gfp⁺ spermatogonia are localized preferentially within select regions of seminiferous tubules of adult testes during steady-state spermatogenesis. Results of previous studies suggest that undifferentiated spermatogonia and possibly SSCs are positioned preferentially within regions juxtaposed to interstitial tissue and the vasculature (Chiarini-Garcia et al. 2001; Yoshida et al. 2007). Quantification of Id4-Gfp⁺ spermatogonia in cross-sections of seminiferous tubules revealed that $49.6\% \pm 0.9\%$ and $46.9\% \pm 1.3\%$ ($n = 3$ different mice and 20 cross-sections) of the population was located in regions adjacent to other tubules or interstitial tissue not containing blood vessels, respectively (Fig. 4E; Supplemental Fig. S3). Importantly, only $3.6\% \pm 0.6\%$ of the Id4-Gfp⁺ spermatogonial population was observed in tubular regions juxtaposed to interstitial tissue containing blood vessels (Fig. 4E; Supplemental Fig. S3). These findings suggest that the SSC pool in testes of adult mice is distributed randomly in regions of seminiferous tubules that are avascular.

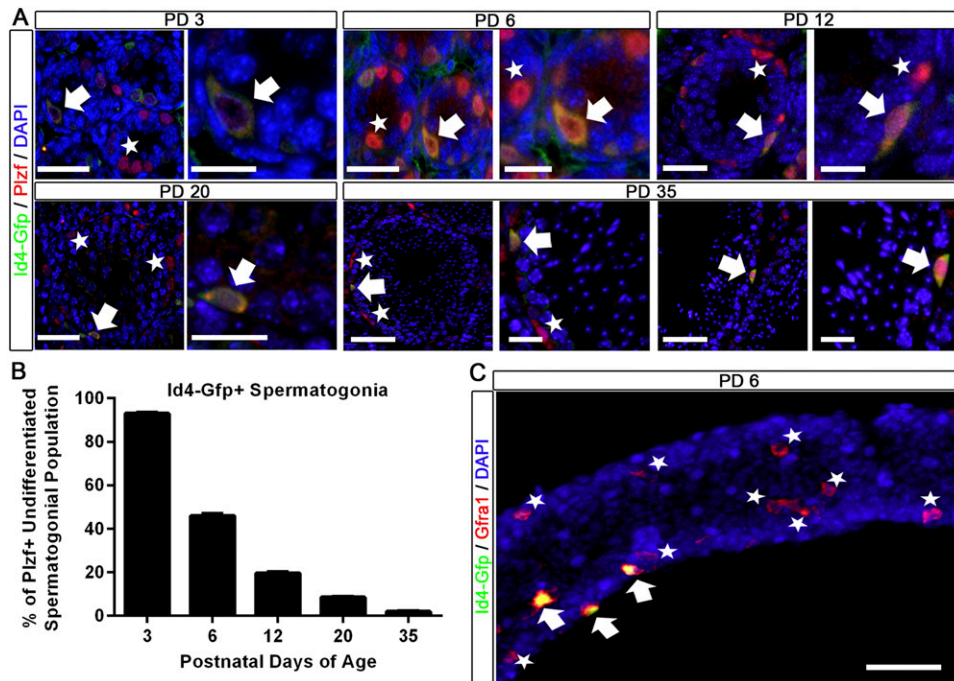


Figure 3. Proportionality of Id4-Gfp-expressing cells in the undifferentiated spermatogonial population of testes from LT-11B6 mice at multiple stages of postnatal life. (A) Representative images of coimmunofluorescent staining for Plzf (red) and Id4-Gfp-expressing cells in cross-sections of seminiferous tubules from testes of mice at PD 3, 6, 12, 20, and 35. DAPI (blue) was used to stain the nuclei of cells. At each age, the *right* image is a magnified view of the *left* image. Arrows indicate Plzf⁺/Id4-Gfp⁺ spermatogonia, and stars indicate Plzf⁺/Id4-Gfp⁻ spermatogonia. Note that four images are provided for PD 35 to demonstrate multiple different spermatogonia. Bars: *left* images, 100 μ m; *right* images, 50 μ m. (B) Quantification of the proportion of the undifferentiated spermatogonial population that is Id4-Gfp⁺ at multiple stages of postnatal development. Data are presented as mean \pm SEM percentage of the Plzf⁺ undifferentiated spermatogonial population and were generated from immunostained cross-sections of three different mice at each age point. (C) Representative image of coimmunofluorescent staining for spermatogonia expressing the markers Gfra1 (red) and Id4-Gfp (green) within whole-mount seminiferous tubules from mice at PD 6. DAPI (blue) was used to stain the nuclei of cells. Arrows indicate single spermatogonia that are Gfra1⁺/Id4-Gfp⁺, and stars indicate spermatogonia that are Gfra1⁺/Id4-Gfp⁻. Bar, 100 μ m.

Stem cell capacity of the undifferentiated spermatogonial population resides in the Id4-Gfp⁺ fraction

While expression of Id4-Gfp by a subset of A_{single} spermatogonia is indicative of stem cell specificity, regenerative capacity for derivation of the cycling spermatogenic lineage is the only unequivocal measure. Thus, we isolated Id4-Gfp⁺ cells from testes of adult mice (2 mo of age) using fluorescence-activated cell sorting (FACS) followed by transplantation into seminiferous tubules of sterile *W/W^v* recipient mice. Examination of cross-sections from recipient testes 3 mo after transplantation revealed seminiferous tubules containing normal spermatogenesis (Supplemental Fig. S4). Because *W/W^v* mice lack endogenous germ cells (Handel and Eppig 1979; Brinster and Zimmermann 1994), the spermatogenesis must have derived from transplanted donor cells. These findings demonstrated that at least some Id4-Gfp⁺ spermatogonia function as SSCs.

Next, we assayed whether regenerative capacity in the undifferentiated spermatogonial population resides with the Id4-Gfp⁺ fraction, the Id4-Gfp⁻ fraction, or both. Using the LT-11B6 line, this comparison could not be made by isolation of cells from testes directly because the

Id4-Gfp⁻ fraction was a mix of several germ cell and somatic cell types. Thus, to examine fractions in the context of the undifferentiated spermatogonial population specifically, primary cultures established from F1 double-transgenic *Id4-Gfp; RosaLacZ* hybrid mice were used (Fig. 5A). Primary spermatogonial cultures are comprised of SSCs with regenerative capacity (~20% of the population) and nonstem cell progenitors (Kubota et al. 2004; Oatley et al. 2006). Also, all cells of the hybrid donors possessed the *LacZ* transgene, including the various subtypes of germ cells, but the Gfp transgene was expressed selectively in Id4⁺ cells. Thus, colonies of donor-derived spermatogenesis could be detected in testes of recipient mice after transplantation by incubation with the substrate X-gal, which stained the donor cells blue. First, examination of the cultures using live imaging revealed that the Gfp⁺ cells comprised a subset of the population (Fig. 5A). Quantification using flow cytometric analysis revealed that 18.7% \pm 5.3% ($n = 3$ different cultures) of the population was Gfp⁺. Second, the Gfp⁺ and Gfp⁻ fractions were isolated using FACS and transplanted as separate cell populations into seminiferous tubules of 129XC57 recipient mice pretreated with busulfan to eliminate the endogenous germline. The FACS

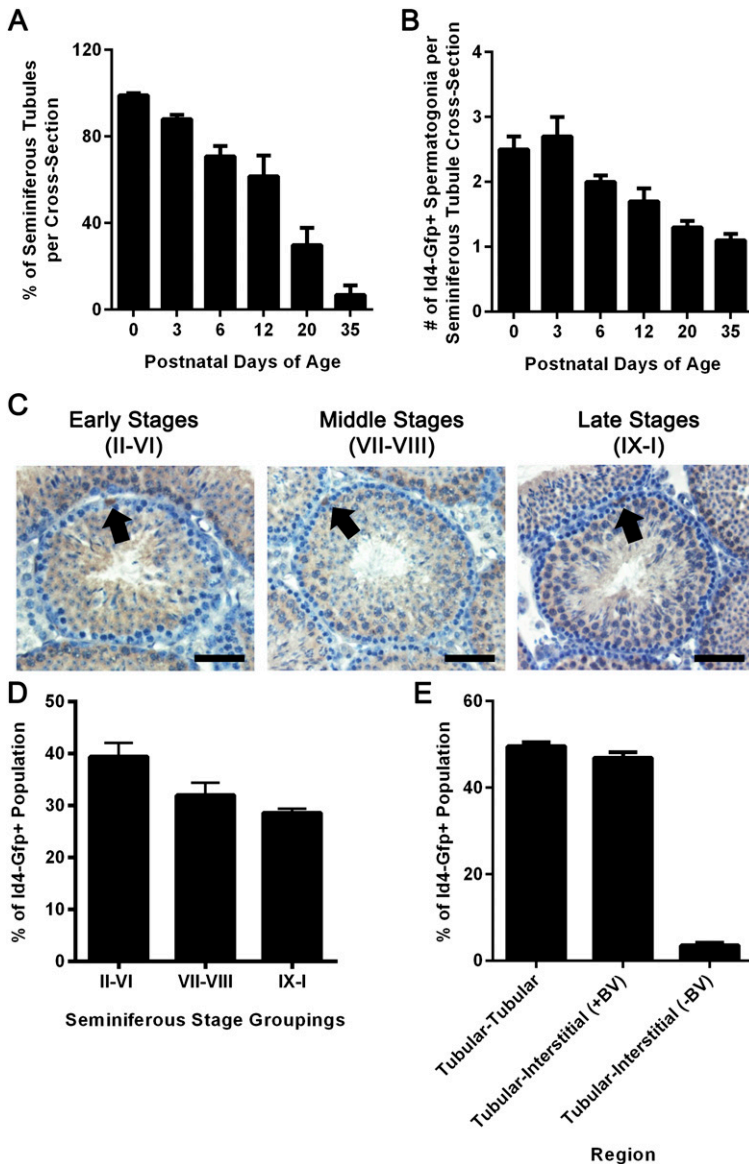


Figure 4. Frequency and distribution of Id4-Gfp-expressing spermatogonia in testes of LT-11B6 mice during postnatal development and adulthood. (A) Quantification of seminiferous tubule cross-sections from testes of mice at PD 0, 3, 6, 12, 20, and 35 containing at least one Id4-Gfp⁺ spermatogonia. Data are presented as the mean \pm SEM percentage of total seminiferous tubule cross-sections. (B) Quantification of Id4-Gfp⁺ spermatogonial numbers in cross-sections of seminiferous tubules from testes of mice at PD 0, 3, 6, 12, 20, and 35. Data are presented as the mean \pm SEM number of positive cells per seminiferous tubule cross-section. (C) Representative images of immunostaining for Id4-Gfp-expressing cells in cross-sections of seminiferous tubules from testes of adult mice grouped as early (II–VI), middle (VII–VIII), or late (IX–I) stages of the seminiferous epithelial cycle. Arrows indicate Id4-Gfp⁺ spermatogonia. Bars, 50 μ m. (D) Quantification of seminiferous tubule cross-sections at early, middle, and late stages of the seminiferous epithelial cycle containing at least one Id4-Gfp⁺ spermatogonia. Data are presented as mean \pm SEM percentage of the total Id4-Gfp⁺ spermatogonial population. (E) Quantification of regions within seminiferous tubule cross-sections where Id4-Gfp⁺ spermatogonia reside. Regions were defined as being juxtaposed to other tubules, interstitial tissue not containing blood vessels (–BV), and interstitial tissue containing blood vessels (+BV). Data are presented as mean \pm SEM percentage of the total Id4-Gfp⁺ spermatogonial population.

approach allowed for isolating fractions that were highly enriched for the cell type of interest (Fig. 5B); however, some Id4-Gfp⁺ cells were still present in the Id4-Gfp[–] fraction and vice versa. Examination of the recipient testes 2 mo after transplantation for colonies of donor-derived spermatogenesis by X-gal staining revealed extensive colonization by the Id4-Gfp⁺ cell population in all testes (Fig. 5C). In contrast, the Id4-Gfp[–] fraction generated only a small number of colonies in some recipient testes (Fig. 5C). In total, the Id4-Gfp⁺ population generated 516.6 \pm 98.1 colonies per 10⁵ cells transplanted, whereas, the Id4-Gfp[–] population produced 35.6 \pm 7.9 colonies per 10⁵ cells transplanted (Fig. 5D). Thus, \sim 95% of the SSC pool was captured in the FACS-isolated Id4-Gfp⁺ fraction, and \sim 5% of the total SSC population was present in the Id4-Gfp[–] fraction. The possibility that colonies from the Id4-Gfp[–] fraction were produced from the contaminating Id4-Gfp⁺ cells could not be ruled out.

However, the extent of colonization by Id4-Gfp⁺ cells suggests potent SSC capacity. Collectively, these findings indicate that most, if not all, SSCs of the undifferentiated spermatogonial population are present in the Id4-Gfp⁺ fraction, and the Id4-Gfp[–] fraction represents progenitors that lack stem cell capacity.

Unique transcriptome features of the Id4-Gfp⁺ spermatogonial population

A major bottleneck in defining stem cell and progenitor states in the mammalian spermatogonial lineage has been an inability to examine the cell populations separately. Based on results of our transplantation analyses, we were confident that a means had been devised to overcome this limitation using FACS separation of Id4-Gfp⁺ and Id4-Gfp[–] populations from primary cultures of undifferentiated spermatogonia. Using this approach, we

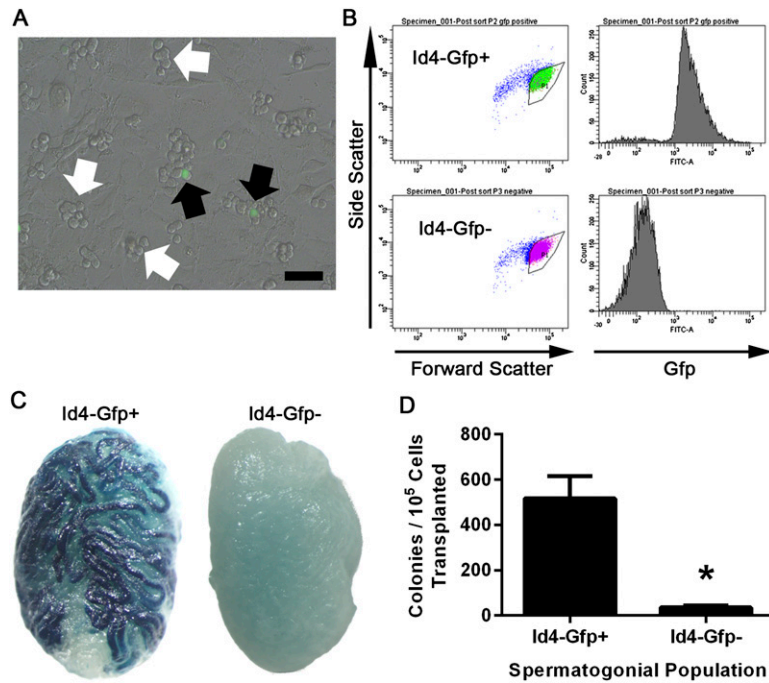


Figure 5. Regenerative capacity of Id4-Gfp⁺ and Id4-Gfp⁻ spermatogonial populations from testes of hybrid LT-11B6/RosaLacZ mice. (A) Representative image of overlaid fluorescent and phase-contrast views of primary cultures of undifferentiated spermatogonia established from LT-11B6/RosaLacZ mice. Black arrows indicate clumps of spermatogonia containing Id4-Gfp⁺ cells, and white arrows indicate clumps not containing Id4-Gfp⁺ cells. Note that all spermatogonia (i.e., Gfp⁺ and Gfp⁻) express LacZ. (B) Representative post-sort scatter plots of Id4-Gfp⁺ and Id4-Gfp⁻ populations isolated from primary cultures of undifferentiated spermatogonia using fluorescence-activated cell sorting (FACS). (C) Representative images of testes from recipient mice 2 mo after transplantation with FACS-isolated Id4-Gfp⁺ and Id4-Gfp⁻ subpopulations that have been stained for LacZ-expressing colonies of donor-derived spermatogenesis. Note that each blue segment is clonally derived from a single SSC. (D) Quantitative comparison of donor-derived colonies of spermatogenesis in testes of recipient mice that were generated from 10⁵ FACS-isolated Id4-Gfp⁺ or Id4-Gfp⁻ cells 2 mo after transplantation. Data are mean ± SEM for three different cultures. (*)Significantly different at $P < 0.05$.

aimed to define the mRNA transcriptome of SSC and progenitor spermatogonial populations with RNA sequencing (RNA-seq) technology. Matched samples of RNA were collected from FACS-isolated Id4-Gfp⁺ and Id4-Gfp⁻ populations ($n = 3$ different cultures) and cDNA libraries generated from polyA mRNA. The Illumina HiSeq system was used to generate 24 million to 30 million paired-end reads of 100 bp in length followed by mapping to the mouse genome (mm9 build) with the TopHat alignment tool (Trapnell et al. 2012). For each sample, transcript assembly of confidently mapped reads was performed with the Cufflinks bioinformatics program, and fragments per kilobase of transcript per million mapped reads (FPKM) values were calculated to provide a quantitative value of abundance using the Cuffdiff program (Trapnell et al. 2012). Differential abundance of transcripts between Id4-Gfp⁺ and Id4-Gfp⁻ populations was then determined statistically with the Cuffdiff program. The outcome of these analyses revealed that most genes are not differentially expressed between the two cell populations (Supplemental Data Set S1). Examination of genes known to be essential for primordial germ cell specification and germ cell licensing (Lesch and Page 2012) revealed no difference in expression between the two cell populations (Fig. 6A). Moreover, examination of a specific subset of genes previously reported to be expressed by the undifferentiated spermatogonial population revealed that transcript abundance for only *Id4* and *Utf1* was significantly different between the two populations (Fig. 6A). In total, 11 transcripts were determined to be of significantly (Q -value < 0.05) greater abundance in the Id4-Gfp⁺ population compared with the Id4-Gfp⁻ population (Fig. 6B). Also, the abundance of 25 transcripts was significantly greater in the Id4-Gfp⁻ population

compared with the Id4-Gfp⁺ population (Fig. 6B). Last, we used quantitative RT-PCR (qRT-PCR) analysis to validate differential abundance of the 11 transcripts in the Id4-Gfp⁺ cell population (Supplemental Fig. S5). In all cases, outcomes of both experimental approaches were in agreement. Collectively, these findings reveal attributes of the mRNA transcriptome that distinguish the stem cell and progenitor states in the mammalian spermatogonial lineage.

Discussion

The undifferentiated spermatogonial population in mammalian testes is heterogeneous, containing both stem cells and transiently amplifying progenitors (de Rooij and Russell 2000; Oatley and Brinster 2012; Griswold and Oatley 2013). For decades, the only means to unequivocally identify the SSC component has been retrospective determination of regenerative capacity following transplantation (Brinster and Zimmermann 1994; Brinster and Nagano 1998; Brinster 2002, 2007; Oatley and Brinster 2008, 2012). Until now, molecular markers that distinguish SSC and progenitor spermatogonial subsets were undefined. Thus, studying the SSC population specifically to define molecular mechanisms that influence fate decisions and characteristics of these cells in vivo or during in vitro maintenance has been challenging. For the first time, the SSC population can be examined specifically in a range of in vivo conditions, including postnatal development of the spermatogenic lineage, steady-state conditions in adulthood, and regeneration of spermatogenesis following cytotoxic damage or transplantation, by using the LT-11B6/Id4-Gfp mouse model. Furthermore, the relationship between SSCs and somatic support cells

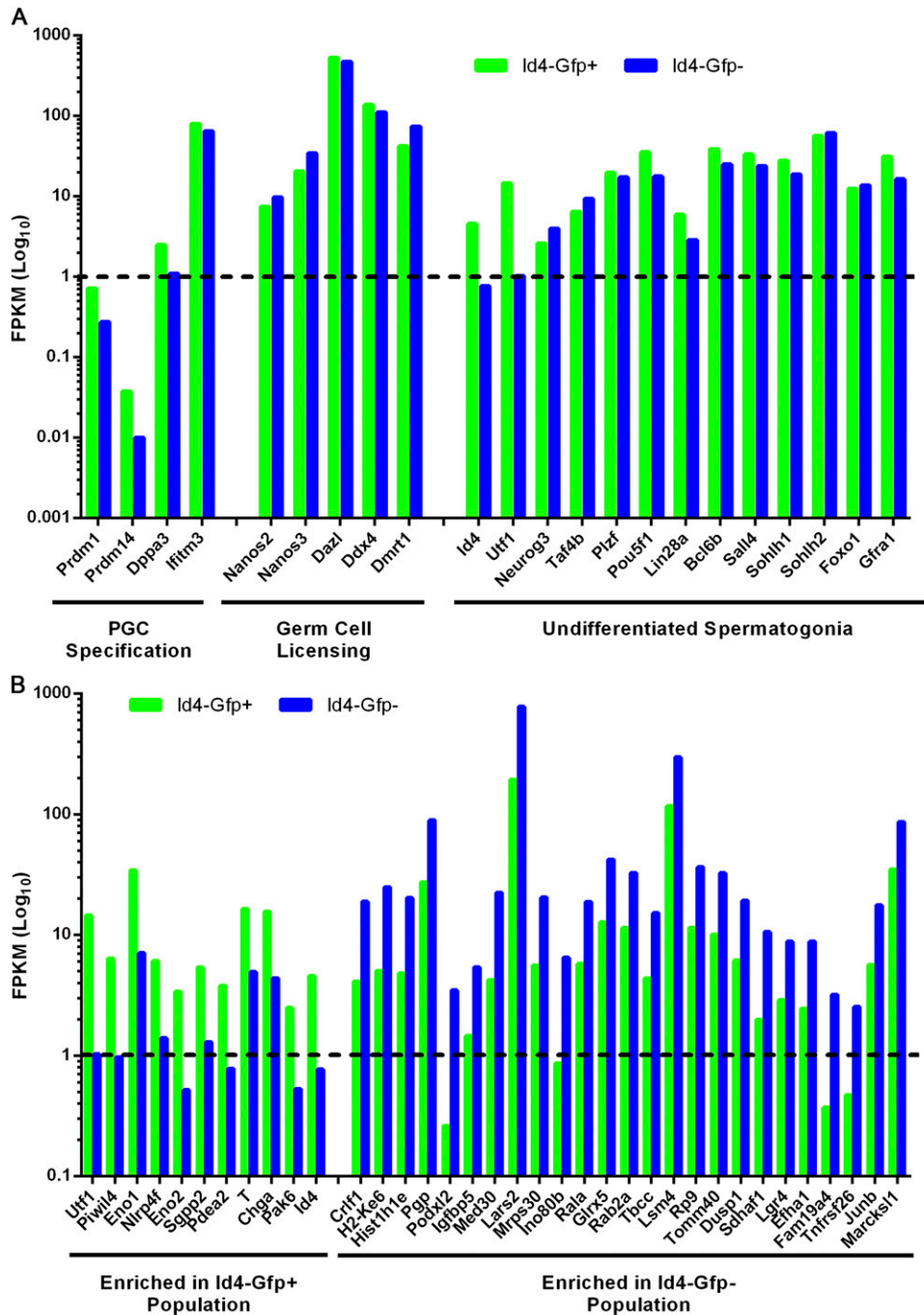


Figure 6. Transcriptome analysis of Id4-Gfp⁺ and Id4-Gfp⁻ spermatogonial populations from testes of LT-11B6 mice. (A) Quantitative comparison of transcript abundance for genes involved in specification of primordial germ cells (PGCs) and germ cell licensing and several genes reported previously to be expressed by undifferentiated spermatogonia. (B) Quantitative comparison of transcripts with significantly ($Q < 0.05$) different abundance. For both data sets, Id4-Gfp⁺ and Id4-Gfp⁻ fractions were isolated from primary cultures of undifferentiated spermatogonia, and transcript abundances were determined using RNA-seq analysis. Data are presented as log₁₀ of the mean FPKM generated from three different cultures. Differential abundance in B was determined statistically using Cuffdiff analysis. Note that dashed lines in A and B are set at 1, which was considered the cutoff of a transcript being considered present or absent.

can be investigated specifically rather than relying on studying the heterogeneous undifferentiated spermatogonial population as a whole. Moreover, the *in vitro* behavior of SSC and progenitor populations can be compared with

defined proliferation kinetics and responses to a variety of environmental conditions.

A limitation of the LT-11B6/Id4-Gfp mouse model is apparent expression of the transgene in pachytene sper-

matocytes beginning at PD 20 and persisting into adulthood. Expression in other germ cell types, including differentiating spermatogonia, other spermatocytes, and round/elongate spermatids, was not observed. The expression in pachytene spermatocytes may be nonspecific. Indeed, expression of reporter transgenes consisting of regulatory elements from genes normally not expressed in spermatocytes or even the male germ cell lineage in postnatal life, including *Nanog*, *Oct4*, and *Blimp-1*, is observed at the pachytene stage of spermatocyte development (Wang et al. 2008; Kuijk et al. 2010). Furthermore, immunostaining analysis for expression of endogenous *Id4* in previous studies did not reveal the presence of the protein in pachytene spermatocytes (Oatley et al. 2011a). Regardless, findings of the current study demonstrate that expression of the *Id4-Gfp* transgene in the undifferentiated spermatogonial population specifically is restricted to a rare subset of cells. Importantly, the *Id4-Gfp*⁺ spermatogonia possess regenerative capacity, thereby classifying the population as bona fide male germline stem cells.

Results of quantitative analyses using cross-sections of seminiferous tubules and imaging of whole-mount preparations indicate that *Id4-Gfp*⁺ spermatogonia represent a subset of the A_{single} spermatogonial population. During postnatal development, the proportion of the undifferentiated spermatogonial population characterized as *Id4-Gfp*⁺ was found to decline to the point of being a rare subset of cells in adulthood. Also, *Id4-Gfp*⁺ spermatogonia were found to represent a subset of the *Gfra1*⁺ pool from PD 6 to adulthood. These findings confirm those of previous studies indicating heterogeneity of the A_{single} spermatogonial population in testes of mice (Suzuki et al. 2009; Zheng et al. 2009; Gassei and Orwig 2013). Taken together, these observations suggest that the bona fide stem cell population of the mammalian male spermatogenic lineage is a subset of the A_{single} spermatogonial population. Moreover, these findings indicate that a hierarchy of A_{single} spermatogonia exists (Fig. 7). We propose

a model in which a subset of the A_{single} pool is self-renewing SSCs, and a second subset are progenitors poised to become A_{paired} spermatogonia upon the next cell division. Importantly, these subsets can be distinguished based on expression of *Id4* and possibly other unique markers that await validation.

During steady-state conditions in adulthood, the entire A_{single} population must be replenished throughout a cycle of the seminiferous epithelium. Considering that there are ~35,000 A_{single} in testes of adult mice (Tegelenbosch and de Rooij 1993), but the regenerative SSC population has been estimated to be only ~3000 in number (Nagano 2003), homeostasis of the pool requires vigorous regulation of division kinetics. In general, the undifferentiated spermatogonial population is estimated to undergo two to three doublings during a cycle of the seminiferous epithelium (Lok et al. 1983). Obviously, this level of division by a small number of SSCs would not be sufficient to replenish the entire A_{single} pool, and there are likely several possible mechanisms that could provide the infrastructure for replenishment. One possibility is that some A_{single} progenitors (i.e., *Id4*⁻ spermatogonia) retain limited self-renewal potential similar to short-term repopulating cells of the hematopoietic lineage but lack regenerative capacity, whereas most of the population forms A_{paired} spermatogonia upon the next division (Fig. 7). Another potential mechanism is that the regenerative SSC pool (i.e., *Id4*⁺ spermatogonia) is larger than has been estimated in previous studies, thus comprising >10% of the A_{single} population. For both of these scenarios, two to three divisions during a cycle of the seminiferous epithelium would be sufficient to sustain robustness. Clearly, the cell division kinetics and heterogeneous nature of the A_{single} population is quite dynamic and warrants further investigation.

The molecular phenotypes that distinguish SSC and progenitor spermatogonial subtypes have been difficult to define due to the lack of conclusive markers and the apparent heterogeneity in A_{single} , A_{paired} , and A_{aligned}

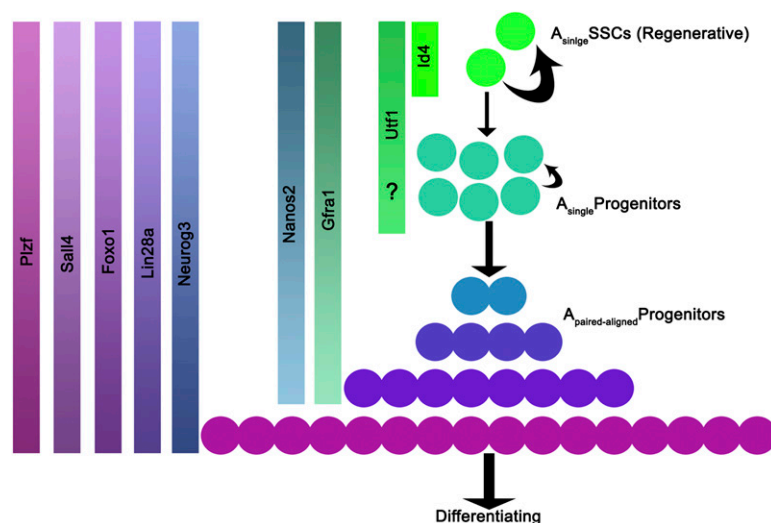


Figure 7. The hierarchy for subsets of spermatogonia in the undifferentiated population of mouse testes and expression profile for intracellular molecular markers. The A_{single} spermatogonial pool consists of SSC and transitional progenitor subtypes. The SSC subtype possesses regenerative capacity and unique self-renewal properties to remain as A_{single} following division, thereby sustaining a pool from which new transitional A_{single} progenitor spermatogonia arise. In contrast, the transitional subtype attains enhanced propensity to form initial chained spermatogonia (A_{paired}) upon the next division and potentially retains limited capacity for self-renewal but lacks regenerative capacity. The SSC and transitional A_{single} subtypes express genes that are also expressed by chained ($A_{\text{paired-aligned}}$) progenitors but are distinguished by expression of specific genes such as *Id4* and *Ulf1*. Gene expression that distinguishes the transitional A_{single} progenitor spermatogonia is undefined but may include reduced levels of some genes also expressed by A_{single} SSCs.

pools. For example, expression of *Neurog3* in spermatogonia is associated with the progenitor state (Nakagawa et al. 2007; Kaucher et al. 2012), but a subset of *Neurog3*⁺ spermatogonia possess SSC capacity (Nakagawa et al. 2007). In addition, expression of *Nanos2* and *Gfra1* appears to be heterogeneous in the *A*_{paired} and *A*_{aligned} spermatogonial pools (Suzuki et al. 2009). Furthermore, until now, a molecular marker of *A*_{single} spermatogonia only had not been described. The results of transcriptome analyses in the current study indicate that the SSC pool possesses a fixed and distinguishable molecular phenotype. This signature includes expression of many markers that are shared by progenitor spermatogonia, such as *Neurog3*, *Nanos2*, *Plzf*, *Lin28*, *Sall4*, *Foxo1*, and *Gfra1*, but also expression of distinguishing markers such as *Id4* and *Utf1* (Fig. 7). Indeed, while not yet characterized in mice, the expression of *Utf1* is restricted to a rare subset of undifferentiated spermatogonia in rat testes (van Bragt et al. 2008), similar to *Id4* expression in the spermatogonial lineage of mouse testes.

A defining feature of stem cells is the capacity for regeneration of a cycling cell lineage. For the male germline, stem cells are defined functionally by the ability to regenerate and sustain spermatogenesis. An unequivocal measure of this ability is generation of donor-derived colonies of spermatogenesis in testes of recipient mice following transplantation (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). In the present study, a major goal was to assess the regenerative capacity of *Id4-Gfp*⁺ spermatogonia to determine whether these cells fulfill the defining criteria of an SSC. Results of transplanting *Id4-Gfp*⁺ cells isolated from donor testes directly revealed this capacity. Furthermore, results of transplantation analysis with sorted *Id4-Gfp*⁺ and *Id4-Gfp*⁻ fractions from primary cultures of undifferentiated spermatogonia demonstrated that most, if not all, regenerative capacity resides within the *Id4-Gfp*⁺ cells. Considering that all cells in primary cultures express markers of undifferentiated spermatogonia, but only a subset possesses regenerative capacity, these findings indicate the *Id4-Gfp*⁺ population represents the SSC pool, whereas the *Id4-Gfp*⁻ population represents transiently amplifying progenitors. A recent study suggests that the colonizing ability of SSCs in testes of adult recipient mice but not immature pup recipients varies based on the phase of the cell cycle (Ishii et al. 2014). These findings indicate that cell cycle status does not alter the core ability of an SSC to regenerate spermatogenesis but does impact the efficiency of establishing an SSC niche unit via interaction with mature somatic support cells (i.e., Sertoli cells). It is possible that expression of the *Id4-Gfp* transgene varies by phase of the cell cycle, thereby influencing regenerative capacity following transplantation into testes of adult recipient mice. Regardless, the outcomes of transplantation analyses in the present study demonstrate that expression of *Id4-Gfp* marks spermatogonia that possess the core ability to function as an SSC.

Stem cells in most, if not all, tissues are thought to reside within a specialized microenvironment that influences their activities (Oatley and Brinster 2012). A

stem cell niche in mammalian testes has been difficult to define due to the tubular configuration and lack of a polarized nature for the seminiferous epithelium in which SSCs reside as well as undefined markers that distinguish SSCs from other undifferentiated germ cell types. Results of several previous studies suggest that SSCs reside within regions of seminiferous tubules juxtaposed to interstitial tissue and blood vessels (Chiarini-Garcia et al. 2001, 2003; Yoshida et al. 2007). These observations have led to the postulation that the SSC niche is associated with vascular architecture (Yoshida et al. 2007). However, it is important to note that these observations were made by investigating the heterogeneous undifferentiated spermatogonial population as a whole (Chiarini-Garcia et al. 2001, 2003) or examining a subpopulation marked by expression of a transgene predominantly associated with progenitor spermatogonia (Yoshida et al. 2007). Results of our previous studies revealed that the overall number of a particular testis somatic cell population, Sertoli cells, is correlated to the number of niches accessible for colonization by transplanted SSCs (Oatley et al. 2011b). Interestingly, we found that increased niche accessibility did not coincide with a change in seminiferous tubular area associated with the vasculature or interstitial tissue (Oatley et al. 2011b). In the present study, we aimed to address this discrepancy by determining whether *Id4-Gfp*⁺ spermatogonia are localized preferentially in certain regions of seminiferous tubules. Results from scoring of these cells within cross-sections from testes of adult mice indicated no bias for regions adjacent to other tubules or juxtaposed to interstitial tissue. However, assessment of the *Id4-Gfp*⁺ spermatogonia residing in tubule regions juxtaposed to interstitial tissue specifically revealed limited association with areas containing blood vessels. Based on these observations, we postulate that the SSC niche is likely avascular.

Many putative markers of SSCs have been described in recent years, but all are expressed by multiple subsets of spermatogonia, and some are even expressed by testicular somatic cells. Until now, a definitive marker that is expressed selectively by *A*_{single} and labels a spermatogonial population that contains most, if not all, of the germline regenerative cells has been lacking. At present, the LT-11B6 mouse model developed in this study allows for arguably the most accurate investigation of SSCs. Using this model, we made seminal observations of defining features for the regenerative spermatogonial population. These include labeling by *Id4-Gfp* expression, existing predominantly as single spermatogonia, comprising a rare subset of the undifferentiated spermatogonial population in adulthood, localizing in avascular regions of seminiferous tubules, and possessing a unique transcriptome defined by differential expression of a small subset of genes. In future studies, utilization of the LT-11B6 mouse model will undoubtedly reveal many more features of the SSC population in mammalian testes. In addition, *Id4* is known to be expressed by stem cells of other lineages (Yun et al. 2004; Jeon et al. 2008; Du and Yip 2011). Thus, comparative analysis to define commonalities among various tissue-specific stem cell popula-

tions and differences that dictate lineage specificity is likely possible using the LT-11B6 mouse model.

Materials and methods

RNA-seq analyses

Total cellular RNA from FACS-isolated Id4-Gfp⁺ and Id4-Gfp⁻ spermatogonial populations of three different primary cultures was isolated using a combination of Trizol reagent (Invitrogen) and RNeasy columns (Qiagen) as described previously (Oatley et al. 2006, 2009). Libraries of cDNA were then generated using oligo d(T) priming. The samples were subjected to Illumina HiSeq2000 analysis (Otogenetics, Inc.), which generated ~24 million to 30 million paired-end reads of 100 bp in length for each sample. Sequencing reads were mapped against the mouse genome (mm9 build) using TopHat version 2.0.5. Confidently mapped reads for each sample were analyzed by Cufflinks. Cuffdiff for transcript assembly and generation of a FPKM value for each transcript that was directly proportional to abundance. Significant differences in FPKM values for individual transcripts between Id4-Gfp⁺ and Id4-Gfp⁻ populations were determined statistically using Cuffdiff. A Q-value of <0.05 was considered significantly different. The entire data set can be accessed through the NCBI-supported Sequence Read Archive (SRA) database, accession SRR1291311.

Transgenic mouse production

A mouse *Id4-Gfp* reporter gene construct was generated using a yeast-based recombineering strategy described previously (Bentley et al. 2010). Briefly, a sequence comparison of the human and mouse *Id4* locus was carried out using VISTA plot analysis to identify conserved regions surrounding the *Id4* coding region. A 17-kb genomic fragment containing all exons, introns, and 5' (7512-bp) and 3' (9412-bp) flanking regions of high similarity (~80%) was identified. The similarity tapered off on either side of the locus, suggesting that major regulatory elements controlling *Id4* expression were present within this genomic fragment. A 192-kb BAC clone (RP32 344L21) containing the mouse *Id4* locus was purchased from the BACPAC consortium. Two 100-bp double-stranded oligonucleotides were designed to capture 17 kb of the *Id4* genomic fragment (Supplemental Table S3). The oligonucleotides contained regions of homology with linearized pClasperA vector and 5' and 3' regions of the 17-kb *Id4* genomic fragment to be captured. The clone pClasper-*Id4* was further modified to insert an *eGfp-Ura3* cassette in-frame of the *Id4* coding region by recombineering in yeast. The resulting *Id4-Gfp* genomic fragment was used to generate transgenic mice in the FVB strain as described previously (Shashikant et al. 1995). Mice carrying the *Id4-Gfp* transgene were identified by Southern hybridization performed on genomic DNA isolated from tail biopsies. Five founder lines carrying transgenes were identified, and males from F1 generation were examined for Gfp expression in the testis. All five lines showed clear, bright Gfp activity, and one was chosen for crossing with C57BL/6J females. Backcrossing of hybrid animals containing the *Id4-Gfp* transgene with C57BL/6J mice was then carried out for another six generations to produce a line denoted as LT-11B6. A PCR-based strategy was then designed to more readily detect transgenic mice.

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