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

Supplemental material is available for this article.

Received February 21, 2014; revised version accepted May 14, 2014.

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.
(Huckins 1971; de Rooij and Russell 2000; Oatley and Brinster 2012). Proliferation of A\textsubscript{paired} generates A\textsubscript{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\textsubscript{single} spermatogonia have the capacity for two different fates. The cells can separate and form two A\textsubscript{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\textsubscript{paired} spermatogonia (Oakberg 1971; de Rooij and Russell 2000; Oatley and Brinster 2012). In this model, formation of A\textsubscript{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\textsubscript{paired} and A\textsubscript{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\textsubscript{single} model implies. However, whether the newly formed putative A\textsubscript{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 measure 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 \(\sim 3000\) cells with regenerative capacity (Nagano 2003), yet the number of A\textsubscript{single} spermatogonia has been calculated to be \(\sim 35,000\) (Tegelenbosch and de Rooij 1993); thus, SSCs likely represent a subset of A\textsubscript{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 (\(~\sim 3000\)) 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\textsubscript{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\textsubscript{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\textsubscript{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\textprime\ 7512-base-pair (bp)] and 3\textprime\ 9214-bp] flanking regions, was captured from a BAC clone. A yeast recombinering 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,
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, 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.
Asingle, A paired, and short chains (i.e., four cell cohorts) population, which has been reported to be restricted to the Gfra1-expressing undifferentiated spermatogonia. Relative abundance and distribution of Id4-Gfp of Aaligned spermatogonial subsets (Suzuki et al. 2009; Bars, 50 μm). (Fig. 3C). Taken together, these findings demonstrate that the more abundant Gfra1 population represents a subset of individual cells within the Gfra1-expressing undifferentiated 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% ± 1.1% and 88.1% ± 1.8%, respectively. During advancing postnatal development, the frequency decreased to 70.8% ± 4.8%, 61.6% ± 9.6%, 29.9% ± 7.8%, and 6.8% ± 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 clearly 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% ± 0.9% and 46.9% ± 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% ± 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.
Stem cell capacity of the undifferentiated spermatogonial population resides in the Id4-Gfp\(^+\) fraction

While expression of Id4-Gfp by a subset of A\(\text{single}\) 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 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 \(\mu\)m.

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 \(\mu\)m; right images, 50 \(\mu\)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 ± 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 \(\mu\)m.

Defining features of male germ line stem cells

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 [\(\sim 20\%\) 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 ± 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
The 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 spermatogenisis 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 ± 98.1 colonies per 10^5 cells transplanted, whereas, the Id4-Gfp− population produced 35.6 ± 7.9 colonies per 10^5 cells transplanted [Fig. 5D]. Thus, ~95% of the SSC pool was captured in the FACS-isolated Id4-Gfp+ fraction, and ~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...
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 \(\text{(Trapnell et al. 2012)}\). 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 \(\text{(FPKM)}\) values were calculated to provide a quantitative value of abundance using the Cuffdiff program \(\text{(Trapnell et al. 2012)}\). 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 \(\text{(Supplemental Data Set S1)}\). Examination of genes known to be essential for primordial germ cell specification and germ cell licensing \(\text{(Lesch and Page 2012)}\) revealed no difference in expression between the two cell populations \(\text{(Fig. 6A)}\). 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 Utfl was significantly different between the two populations \(\text{(Fig. 6A)}\). 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 \(\text{(Fig. 6B)}\). Also, the abundance of 25 transcripts was significantly greater in the Id4-Gfp\(^*\) population compared with the Id4-Gfp\(^*\) population \(\text{(Fig. 6B)}\). Last, we used quantitative RT–PCR \(\text{(qRT–PCR)}\) analysis to validate differential abundance of the 11 transcripts in the Id4-Gfp\(^*\) cell population \(\text{(Supplemental Fig. S5)}\). 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 \(\text{(de Rooij and Russell 2000; Oatley and Brinster 2012; Griswold and Oatley 2013)}\). For decades, the only means to unequivocally identify the SSC component has been retrospective determination of regenerative capacity following transplantation \(\text{(Brinster and Zimmermann 1994; Brinster and Nagano 1998; Brinster 2002, 2007; Oatley and Brinster 2008, 2012)}\). 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
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 spermatocytes.
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 spermagonia possess regenerative capacity, thereby classifying the population as bona fide male germ cell stem cells.

Results of quantitative analyses using cross-sections of seminiferous tubules and imaging of whole-mount preparations indicate that Id4-Gfp spermagonia 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 spermagonia 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 + spermatagonia] 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 + spermatagonia] 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 spermatogonia. The molecular phenotypes that distinguish SSC and transitional progenitor subtypes express genes that are also expressed by chain [A paired-aligned] progenitors but are distinguished by expression of specific genes such as Id4 and Utf1. Gene expression that distinguishes the transitional A single progenitor spermatagonia is undefined but may include reduced levels of some genes also expressed by A single SSCs.
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 Apaired and Aaligned 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 predominately 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 Asingle 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 will undoubtedly reveal many more subsets of genes. In future studies, utilization of 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 (Otogethens, 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 pClasper-A 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 testes. In all five lines showed clear, bright Gfp activity, and one was chosen for crossing with C57BL/6 females. Backcrossing of hybrid animals containing the Id4-Gfp transgene with C57BL/6 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.

Acknowledgments

This research was supported by a grant awarded to J.M.O. from the National Institutes of Child Health and Human Development (HD061665).

References

Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, Seandel M, Geijser J, Hchedhlinger K. 2011. Sox2(+)-adult stem and progenitor cells are important for tissue regeneration and survival of mice. Cell Stem Cell 9: 317–329.

Barker N, van Es JH, Kuipers J, Kuja P, van den Born M, Cozijnsen M, Hagegarth A, Korving J, Begthel H, Peters PJ, et al. 2007. Identification of stem cells in small intestine and colon by marker gene Lgr5. Nature 449: 1003–1007.

Bentley KL, Shashikant CS, Wang W, Rudd NH, Ruddle FH. 2010. A yeast-based recombinogenic targeting toolset for transgenic analysis of human disease genes. Ann N Y Acad Sci 1207: E58–E68.

Brinster RL. 2002. Germline stem cell transplantation and transgenesis. Science 296: 2174–2176.

Brinster RL. 2007. Male germline stem cells: from mice to men. Science 316: 404–405.

Brinster RL, Avarbock MR. 1994. Germine transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci 91: 11303–11307.

Brinster RL, Nagano M. 1998. Spermatogonial stem cell transplantation, cryopreservation and culture. Semin Cell Dev Biol 9: 401–409.

Brinster RL, Zimmermann JW. 1994. Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci 91: 11298–11302.

Chiarini-Garcia H, Hornick JR, Griswold MD, Russell LD. 2001. Distribution of type A spermatogonia in the mouse is not random. Biol Reprod 65: 1179–1185.

Chiarini-Garcia H, Raymer AM, Russell LD. 2003. Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. Reproduction 126: 669–680.

de Rooij DG. 1998. Stem cells in the testis. Int J Exp Pathol 79: 67–80.

de Rooij DG, Russell LD. 2000. All you wanted to know about spermatogonia but were afraid to ask. J Androl 21: 776–798.

Drumond AL, Meistrich ML, Chiarini-Garcia H. 2011. Spermatogonial morphology and kinetics during testis development in mice: a high-resolution light microscopy approach. Reproduction 142: 145–155.

Du Y, Yip HK. 2011. The expression and roles of inhibitor of DNA binding helix-loop-helix proteins in the developing and adult mouse retina. Neuroscience 175: 367–379.

Gassei K, Orwig KE. 2013. SALL4 expression in gonocytes and spermatogonial clones of postnatal mouse testes. PLoS ONE 8: e53976.

Griswold MD, Oatley JM. 2013. Concise review: defining characteristics of mammalian spermatogenic stem cells. Stem Cells 31: 8–11.

Handel MA, Eppig JJ. 1979. Sertoli cell differentiation in the testes of mice genetically deficient in germ cells. Biol Reprod 20: 1031–1038.

Huckins C. 1971. The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec 170: 533–557.

Ishii K, Kanatsu-Shinohara M, Shinohara T. 2014. Cell-cycle-driven cyclin E and notch signaling. Differentiation 84: 17–29.

J Androl 31: 776–798.

Jeon HM, Jin X, Lee JS, Oh SY, Sohn YW, Park HJ, Joo KM, Park WY, Nam DH, DePinho RA, et al. 2008. Inhibitor of differentiation 4 drives brain tumor-initiating cell genesis through cyclin E and notch signaling. Genes Dev 22: 2028–2033.
Kaucher AV, Oatley MJ, Oatley JM. 2012. NEUROG3 is a critical downstream effector for STAT3-regulated differentiation of mammalian stem and progenitor spermatogonia. *Biol Reprod* 86: 164.

Kubota H, Avarbock MR, Brinster RL. 2004. Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. *Proc Natl Acad Sci* 101: 16489–16494.

Kuijk EW, de Gier J, Lopes SM, Chambers I, van Pelt AM, Colenbrander B, Roelen BA. 2010. A distinct expression pattern in mammalian testes indicates a conserved role for NANOG in spermatogenesis. *PLoS ONE* 5: e10987.

Lesch BJ, Page DC. 2012. Genetics of germ cell development. *Nat Rev Genet* 13: 781–794.

Lok D, Jansen MT, de Rooij DG. 1983. Spermatogonial multiplication in the Chinese hamster. II. Cell cycle properties of undifferentiated spermatogonia. *Cell Tissue Kinet* 16: 19–29.

Nagano MC. 2003. Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. *Biol Reprod* 69: 701–707.

Nakagawa T, Nabeshima Y, Yoshida S. 2007. Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. *Dev Cell* 12: 195–206.

Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S. 2010. Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. *Science* 328: 62–67.

Oakberg EF. 1956. Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. *Am J Anat* 99: 507–516.

Oakberg EF. 1971. Spermatogonial stem-cell renewal in the mouse. *Anat Rec* 169: 515–531.

Oatley JM, Brinster RL. 2008. Regulation of spermatogonial stem cell self-renewal in mammals. *Annu Rev Cell Dev Biol* 24: 263–286.

Oatley JM, Brinster RL. 2012. The germline stem cell niche unit in mammalian testes. *Physiol Rev* 92: 577–595.

Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL. 2006. Identifying genes important for spermatogonial stem cell self-renewal and survival. *Proc Natl Acad Sci* 103: 9524–9529.

Oatley JM, Oatley MJ, Avarbock MR, Tobias JW, Brinster RL. 2009. Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. *Development* 136: 1191–1199.

Oatley MJ, Kaucher AV, Racicot KE, Oatley JM. 2011a. Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. *Biol Reprod* 85: 347–356.

Oatley MJ, Racicot KE, Oatley JM. 2011b. Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. *Biol Reprod* 84: 639–645.

Shashikant CS, Bieberich CJ, Belting HG, Wang JC, Borbely MA, Ruddle FH. 1995. Regulation of Hoxc-8 during mouse embryonic development: identification and characterization of critical elements involved in early neural tube expression. *Development* 121: 4339–4347.

Suzuki H, Sada A, Yoshida S, Saga Y. 2009. The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. *Dev Biol* 336: 222–231.

Tegelenbosch RA, de Rooij DG. 1993. A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. *Mutat Res* 290: 193–200.

Trapnell C, Roberts A, Goff L, Pertea G, Kim D, Kelley DR, Pimentel H, Salzberg SL, Rinn JL, Pachter L. 2012. Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat Protoc* 7: 562–578.