Spermatogenesis is a complex, multistep process that maintains male fertility and is sustained by rare germline stem cells. Spermatogenic progression begins with spermatogonia, populations of which express distinct markers. The identity of the spermatogonial stem cell population in the undisturbed testis is controversial due to a lack of reliable and specific markers. Here we identified the transcription factor PAX7 as a specific marker of a rare subpopulation of A single spermatogonia in mice. PAX7+ cells were present in the testis at birth. Compared with the adult testis, PAX7+ cells constituted a much higher percentage of neonatal germ cells. Lineage tracing in healthy adult mice revealed that PAX7+ spermatogonia self-maintained and produced expanding clones that gave rise to mature spermatozoa. Interestingly, in mice subjected to chemotherapy and radiotherapy, both of which damage the vast majority of germ cells and can result in sterility, PAX7+ spermatogonia selectively survived, and their subsequent expansion contributed to the recovery of spermatogenesis. Finally, PAX7+ spermatogonia were present in the testes of a diverse set of mammals. Our data indicate that the PAX7+ subset of A single spermatogonia functions as robust testis stem cells that maintain fertility in normal spermatogenesis in healthy mice and mediate recovery after severe germline injury, such as occurs after cancer therapy.
PAX7 expression defines germline stem cells in the adult testis

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Spermatogenesis is a complex, multistep process that maintains male fertility and is sustained by rare germline stem cells. Spermatogenic progression begins with spermatogonia, populations of which express distinct markers. The identity of the spermatogonial stem cell population in the undisturbed testis is controversial due to a lack of reliable and specific markers. Here we identified the transcription factor PAX7 as a specific marker of a rare subpopulation of A single spermatogonia in mice. PAX7+ cells were present in the testis at birth. Compared with the adult testis, PAX7+ cells constituted a much higher percentage of neonatal germ cells. Lineage tracing in healthy adult mice revealed that PAX7+ spermatogonia self-maintained and produced expanding clones that gave rise to mature spermatozoa. Interestingly, in mice subjected to chemotherapy and radiotherapy, both of which damage the vast majority of germ cells and can result in sterility, PAX7+ spermatogonia selectively survived, and their subsequent expansion contributed to the recovery of spermatogenesis. Finally, PAX7+ spermatogonia were present in the testes of a diverse set of mammals. Our data indicate that the PAX7+ subset of A single spermatogonia functions as robust testis stem cells that maintain fertility in normal spermatogenesis in healthy mice and mediate recovery after severe germline injury, such as occurs after cancer therapy.

Introduction

The functional unit of the mammalian testis, the seminiferous tubule, is a multilayered epithelium that matures from spermatogonial precursors located at the basal layer to more advanced cell types that migrate toward the tubular lumen, where spermatozoa are released (1). Classically, type A single spermatogonia, which reside on the basement membrane (i.e., the basal layer), were thought to represent the stem cell population of the testis, as these cells were the earliest identifiable morphological progenitors (2, 3). Meticulous histological studies have shown that A single spermatogonia progress through multiple rounds of mitoses with incomplete cytokinesis to produce “chains” of A pair and “aligned” A al4, A al8, and A al16 spermatogonia, which consist of 2, 4, 8, and 16 interconnected cells, respectively (4). A single-A al16 spermatogonia are sometimes called “undifferentiated” spermatogonia, a term that is useful but also somewhat misleading, in that this population encompasses the true stem cells as well as a progressive series of differentiating, transit-amplifying intermediates. Interestingly, time-lapse imaging studies of mouse testes have clearly documented that A single-A al16 spermatogonia are highly migratory, capable of moving across large distances on the basement membrane (5). A al16 spermatogonia differentiate to give rise to type A−A, and then to type B spermatogonia, which become spermatocytes that initiate meiosis. Round haploid spermatids, the products of meiosis, initiate a dramatic cytoskeletal rearrangement to produce elongate spermatids, which at the end of this maturation sequence are released within the tubular lumina as spermatozoa (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI75943DS1; and ref. 6).

The continuous production of spermatozoa throughout adult life, as well as the multitude of cell divisions from A single spermatogonia to mature spermatozoa, clearly implies the existence of a dynamic germline stem cell capable of self-maintenance, but also differentiation into the transit-amplifying intermediates that constitute the spermatogenic series (7). The identity of this adult testis stem cell remains unknown (8). As stated above, some models have posited that all A single spermatogonia represent functional stem cells, consistent with their status as the earliest known morphological precursor. A single spermatogonia can be reliably identified by morphologic criteria (i.e., their singularity by confocal microscopy of intact tubules) but have remained largely undefined at the molecular level, although recently ID4 was described as a marker of A single spermatogonia (9). On the other hand, some studies have suggested that only a subset of A single spermatogonia are functional stem cells (10). If so, then this would suggest that A single spermatogonia encompass the true stem cells (a distinct fraction of A single spermatogonia), along with other A single subsets that serve as transit-amplifying descendants prior to their eventual differentiation to A pair spermatogonia.

Transplantation of spermatogonia from a donor mouse to a germ cell-deficient recipient testis (11) has been extensively used to explore the properties and biology of spermatogonial stem cells.
In these assays, the regeneration of complete spermatogenesis occurs via the formation of spermatogenic colonies thought to arise from a single transplanted cell. Clonogenicity is a notable strength of the assay, permitting assessment of stem cell numbers in the donor population. However, transplantation has not proven decisive in identifying the true (presumably rare) stem progenitors in the adult testis. Most strategies to enrich SSCs in transplantation assays to date have used cell surface selection markers such as THY1 (13) or α6/β10 integrins (14) that are expressed across broad subsets of spermatogonia, limiting their precision in pinpointing rarer subsets of stem progenitors (1, 8, 15). Furthermore, transplantation assays do not mirror stem cell functionality in the undisturbed testis. Donor germ cells are dissociated into single-cell suspensions, resulting in chain fragmentation, a phenomenon that occurs in vivo and has been proposed as a distinct mechanism promoting stem cell renewal, though this has not yet been conclusively demonstrated (5). Additionally, germ cells in the recipient are ablated by treatments that also damage the somatic environment, which may induce stemness by increasing the number of available niches or by eliminating negative feedback signals that emanate from other germ or somatic cells to regulate stem cell numbers within the testis (3, 16, 17).

A notable strength of the mouse testis as a model system for stem cell biology is the ability to establish cultured SSCs ex vivo. Cultured SSCs are capable of self-renewal and are truly immortal: they can be passaged and expanded indefinitely, maintaining genetic stability. The dynamics of stem cell maintenance in the murine SSC culture system is incompletely understood, but the cultures contain a much higher fraction of stem cells relative to murine SSC culture system are incompletely understood, but the cultures contain a much higher fraction of stem cells relative to adult tissue (18, 19). Cultured SSCs can be transplanted into a host testis, where they function as tissue stem cells, reestablishing functional spermatogenesis (20). The limitless expansion of SSCs in culture is dependent on the growth factor GDNF, which acts through the RET/GFRα1 receptor complex, although additional growth factors are also necessary (20–22).

Here we report on our identification of a rare subset of A singly spermatogonia characterized by expression of the paired box transcription factor PAX7. PAX7 has been previously identified and extensively used as a marker of satellite cells, which function as a normally quiescent stem cell population within adult skeletal muscle (23). In contrast, PAX7+ spermatogonia were highly proliferative during steady-state spermatogenesis. To explore the contribution of PAX7+ spermatogonia to normal spermatogenesis in the undisturbed testis, we performed a variety of cell lineage–tracing studies with an inducible Pax7-CreER T2 allele and 2 different reporters. These studies revealed that PAX7+ spermatogonia normally serve as robust stem cells that contribute to full-lineage maturation, as evidenced by the formation of clones including all stages of spermatogenesis from A singly spermatogonia to spermatozoa. Furthermore, labeled (i.e., lineage-traced) descendants including A singly spermatogonia were observed even after prolonged intervals (16 weeks), which demonstrated that PAX7+ spermatogonia function as bona fide stem cells that self-maintain and differentiate to sustain spermatogenesis, and are not merely transit-amplifying intermediates. Lineage-tracing studies with neonatal mice suggest that adult PAX7+ spermatogonia are derived from an initial cohort of PAX7+ spermatogonia present at birth. Finally, we conducted a number of investigations to explore the contribution of these rare PAX7+ spermatogonia to spermatogenic recovery after germ cell ablation, such as occurs after chemotherapy or radiotherapy. Remarkably, PAX7+ spermatogonia selectively survived cytotoxic drugs and radiation despite widespread germ cell death. Not only did their numbers not decrease in the immediate aftermath of such treatments, but instead, PAX7+ spermatogonia rapidly expanded to replenish normal spermatogenesis. Thus, our results demonstrated that PAX7+ spermatogonia are stem cells that maintain fertility in the healthy adult, and also serve particularly important roles in replenishing spermatogenesis following treatments that damage the germline.

Results

PAX7 specifically marks a small subset of A singly spermatogonia in vivo. We reasoned that a bona fide testis stem cell marker should be highly expressed in SSC cultures, but at low (perhaps undetectable) levels in the adult testis, where true stem cells are a rare subpopulation. An RNA-based approach previously used for marker discovery in ovarian cell subpopulations (24) led us to the identification of Pax7 (Figure 1A). At the mRNA level, Pax7 was highly expressed in SSC cultures, but undetectable in adult testis (>180-fold difference; Figure 1B). In vivo, Pax7+ transcripts were detectable in type A (early) spermatogonia, but not in differentiated type B spermatogonia, spermatocytes, or round spermatids. Pax7+ transcripts were absent in testes at embryonic days 11.5–18.5 (e11.5–e18.5) and first detected at postnatal day 2 (PD2). Among adult tissues, Pax7 was expressed only in skeletal muscle, consistent with Pax7’s eminence as a marker of satellite cells, the dormant tissue stem cell population that regenerates skeletal muscle after injury (23, 25). In comparison, the pan-germ cell marker Ddx4 (also known as VASA) was expressed in adult testis (26) and in all germ cell subpopulations, but not in any somatic tissues, and transcripts were markedly decreased in germ cell–deficient (Kit+/Kit−) testes, as expected (Figure 1C). Thus, in contrast to Pax7, Ddx4 did not exhibit a stem cell signature.

We sought to visualize PAX7+ cells in sections of adult testis with an anti-PAX7 monoclonal antibody. PAX7+ cells were rare: in 1 complete testis cross-section, a single PAX7+ cell might be detected within seminiferous tubules (Figure 2A). Despite this rarity, several observations confirmed that the detection of PAX7+ spermatogonia was specific, defining a novel population of spermatogonia. First, the PAX7+ cells always rested on the basement membrane and were isolated, single cells (i.e., consistent with A singly spermatogonia; see also below). Second, PAX7 protein in spermatogonia was always nuclear, as expected based on its function and nuclear localization within satellite cells (23, 25).

To further define these PAX7+ spermatogonia, we compared their abundance with that of other subsets of spermatogonia defined by well-characterized markers (Supplemental Figure 1). Kit+ differentiating spermatogonia were the most abundant (10.9 cells/tubule), with FOXO1+ and PLZF+ spermatogonia being more restricted, as expected, given that FOXO1 and PLZF are both markers of undifferentiated (A singly→A pair) spermatogonia, a less abundant population (27, 28). RET+ spermatogonia were rarer still, consistent with RET’s more restricted expression in A singly and A pair spermatogonia (29). However, PAX7+ spermatogonia were...
approximately 2 orders of magnitude rarer than RET+ spermatogonia (Figure 2B). PAX7+ spermatogonia were FOXO1+ and GFRα1+, while most FOXO1+ or GFRα1+ cells were PAX7− (Figure 2C), which demonstrated that PAX7+ spermatogonia represented a subset of undifferentiated, GFRα1+ spermatogonia.

Confocal microscopy of intact seminiferous tubules further showed that PAX7+ spermatogonia were a subset of Aingle spermatogonia. PAX7+ spermatogonia were singular, and larger chains of undifferentiated spermatogonia (i.e., Aal4–Aal16) never contained PAX7+ spermatogonia (Figure 2D and Supplemental Video 1). Additional confocal microscopy studies confirmed that PAX7+ spermatogonia were always KIT−; no KIT+PAX7+ spermatogonia were ever observed (Figure 2E). Thus, PAX7 defined a rare but specific subset of Aingle spermatogonia, revealing striking heterogeneity within Aingle spermatogonia in vivo.

PAX7+ spermatogonia are rare in the adult testis, but constitute a much higher fraction of germ cells in the neonatal testis. Interestingly, a much higher percentage of germ cells (defined by the pan–germ cell marker germ cell nuclear antigen [GCNA]) were PAX7+ at birth (28% in neonates); however, this fraction steadily decreased postnatally, stabilizing at 6 weeks of age (Figure 3A). The much higher fraction of PAX7+ germ cells at birth further underscores their rarity in adults, and also demonstrated that PAX7+ spermatogonia can be reliably identified in tissue sections; analyses of conditional knockout testes also confirmed antibody specificity (see below).

This age-dependent decrease in the proportion of PAX7+ cells per total GCNA+ cells could reflect decreased absolute numbers of PAX7+ spermatogonia, versus their dilution due to the massive expansion of spermatogenic cells that normally occurs during postnatal life (e.g., testes weights increase from ~1 mg at birth to ~60 mg in adult males) (30). To distinguish between these possibilities, we serially sectioned and immunostained entire PD1 and adult testes and documented similar numbers of PAX7+ spermatogonia per testis (504 ± 29 and 402 ± 33, respectively; mean ± SEM; Figure 3B). Thus, the dramatic age-dependent decrease in the fraction of PAX7+ germ cells reflects mainly the rapid expansion of spermatogenesis, and not a large decrease in absolute numbers of PAX7+ cells. These results also strongly suggest that the postnatal PAX7+ spermatogonia represent the initial founder population for PAX7+ spermatogonia in adult testes.
PAX7+ spermatogonia are rapidly cycling during normal spermatogenesis and function as robust stem cells that give rise to all stages of spermatogenesis. We considered the possibility that (by analogy with satellite cells) adult PAX7+ spermatogonia might represent a quiescent subset of A\textsubscript{single} spermatogonia. To our surprise, however, EdU labeling showed that PAX7+ cells, like other subsets of spermatogonia, were rapidly cycling (Figure 3C).

We then sought to explore the contribution of PAX7+ spermatogonia and their descendants to normal, steady-state spermatogenesis in the undisturbed adult testis through lineage tracing. We used a Pax7-Cre\textsuperscript{ERT2} allele, in which the tamoxifen-inducible recombinase Cre\textsuperscript{ERT2} was knocked into the Pax7 locus, driving Cre\textsuperscript{ERT2} expression in cells that express Pax7, such as satellite cells (Supplemental Figure 2A and refs. 31, 32). We generated mice harboring Pax7-Cre\textsuperscript{ERT2} and the Rosa26 β-galactosidase lox-stop-lox reporter, R26R (33). 6-week-old adult males were treated with tamoxifen to activate Cre in PAX7+ cells. Untreated Pax7-Cre\textsuperscript{ERT2};R26R males exhibited no Cre-mediated recombination in testis or skeletal muscle, demonstrating tight control of Cre. Expression of Pax7 in labeled clones confirmed faithful Pax7-Cre\textsuperscript{ERT2} expression in PAX7+ spermatogonia (Supplemental Figure 2, B–D). To characterize PAX7+ descendants within the tes-
Lineage-tracing experiments with Pax7-CreERT2 and a double-fluorescent tdTomato/eGFP reporter (mT/mG) (35) gave nearly identical results. Clones began as single cells. At 1 week after Cre induction, labeled single, paired, and al4–Aal8 chains were identified. At 6 weeks, larger clones were visualized, and elongate spermatid tails were first identified in tubular lumina. By 16 weeks, clones were even larger (Figure 5A), and motile labeled sperm were present in epididymides (Supplemental Video 2). Labeled single spermatogonia were observed at all time points (Figure 5A and Supplemental Videos 3 and 4). To more clearly delineate clonal architecture, we also analyzed frozen tissue sections of intact testes, which permitted better visualization of spermatogenic layers and cell types. In some clones, all of the germ cells in the entire tubular cross-section were clearly labeled (green; Figure 5B), which demonstrated that all the germ cells (spermatogonia, spermatids, and spermatocytes) were derived from a PAX7+ progenitor. These results indicated that PAX7+ spermatogonia give rise to full-lineage maturation (Figure 5C), thereby fulfilling a key criterion of an adult testis stem cell. As with R26R-based lineage tracing, clone numbers did not decrease, even when the analyses were extended to 16 weeks after tamoxifen treatment (Figure 5, D and E). We concluded from these analyses with 2 distinct reporters that PAX7+ spermatogonia are rare but robust tissue stem cells. They give rise to other single spermatogonia that persist even after very long intervals of 16 weeks, and also give rise to all stages of spermatogenesis, including motile sperm.

Lineage-tracing studies of neonatal animals show that neonatal PAX7+ spermatogonia have long-term stem cell potential in vivo and also have stem cell activity in transplantation assays. Lineage-tracing studies initiated with neonatal animals (PD1–PD3) confirmed that PAX7+ spermatogonia were rapidly expanding by PD3 and...
were progenitors of subsequent single spermatogonia and spermatogenesis; clones grew in size over time and persisted into adulthood (Figure 6, A and B). Concordantly, PAX7+ spermatogonia were rapidly proliferating by PD3, as shown by EdU incorporation rate, without significant cell death (Figure 6C). Finally, although flow sorting of live PAX7+ spermatogonia was not possible with available reagents, transplantations were conducted with unsorted cells from tamoxifen-treated Pax7-CreERT2;tdTomato reporter mice (36). Labeled, lineage-traced germ cells were observed in every host (n = 3; Figure 6D), which indicated that PAX7+ spermatogonia and their descendants have stem cell activity in transplantation assays.

PAX7+ spermatogonia are selectively resistant to anticancer therapies that kill other germ cells in the adult testis (radiotherapy and chemotherapy) and also contribute to spermatogenic recovery after ablation of most germ cells. Spermatogenesis is highly sensitive to systemic genotoxic stresses, such as cytotoxic chemotherapy. Chemotherapy-induced ablation of germ cells has been studied in rodent models. After treatment with the alkylating agent busulfan (also known as Myleran; used to treat hematopoietic malignancies), germ cells undergo massive cell death in a dose-dependent manner, with higher doses leading to near-total germ cell depletion. This results in an interval of azoospermia and infertility, followed by a gradual recovery of spermatogenesis and, in most
animals, restoration of fertility even after high doses (37). Such spermatogenic recovery poses a paradox: the germline is almost entirely ablated, yet the restoration of spermatogenesis implies the existence of rare stem cells that not only survive, but replenish spermatogenesis during the recovery period (38).

To study the contribution of PAX7+ spermatogonia to spermatogenic recovery, adult mice (6 weeks of age) were treated with busulfan. As expected, testes underwent massive germ cell death with dose- and time-dependent germ cell loss, as visualized by immunohistochemistry (IHC) with the pan–germ cell...
Demonstrating that busulfan treatment stimulated PAX7+ cell division acutely and suggesting that cell division is one mechanism underlying the formation of PAX7+ cell clusters. In contrast to PAX7+ spermatogonia, FOXO1+ undifferentiated spermatogonia counts fell more than 15-fold 8 days after 40 mg/kg busulfan administration, but then recovered coincident with the peak of PAX7+ expansion (Figure 8A). These data indicate that spermatogonia are sensitive to genotoxic stress as previously reported (40), emphasizing the unique properties and survival of PAX7+ spermatogonia after treatments that ablate the vast majority of germline cells. That the increase in FOXO1+ spermatogonia coincided with the decrease of PAX7+ spermatogonia (Figure 8, B and C) is further evidence that PAX7+ spermatogonia eventually differentiate. In control experiments, neither tamoxifen nor the DMSO solvent had a significant effect on testis weight or morphology or PAX7+ spermatogonia (Supplemental Figure 3, A–D).

We then analyzed the response of PAX7+ spermatogonia to ionizing radiation and a second chemotherapeutic agent commonly used in the clinic, cyclophosphamide. Cyclophosphamide marker GCNA (39). In striking contrast, both relative and absolute numbers of PAX7+ spermatogonia increased several-fold, also in a time- and dose-dependent manner (Figure 7A). Absolute numbers of PAX7+ cells peaked (>5-fold higher than untreated mice) 16 days after treatment with the highest dose of busulfan (40 mg/kg). PAX7+ cell counts then decreased between 16 and 32 days, most likely a consequence of differentiation (see below). Thus, while germ cells as a whole were largely ablated by busulfan, PAX7+ cells not only survived, but expanded in number.

Histology and immunostaining confirmed massive loss of germ cells. Whereas in untreated animals, virtually all PAX7+ cells were single, isolated cells (with only extremely rare cells being present as pairs, and never in clusters of ≥3), larger PAX7+ clusters of 2 to ≥4 cells were observed after busulfan treatment (Figure 7, B and C). This difference in cluster sizes (1 versus ≥2) was highly statistically significant in untreated animals versus those 32 days after treatment with 40 mg/kg busulfan (P = 2 × 10⁻⁹). The PAX7+ fraction undergoing DNA replication, based on EdU incorporation, increased 4 days after busulfan treatment (Figure 7D), demonstrating that busulfan treatment stimulated PAX7+ cell division acutely and suggesting that cell division is one mechanism underlying the formation of PAX7+ cell clusters. In contrast to PAX7+ spermatogonia, FOXO1+ undifferentiated spermatogonia counts fell more than 15-fold 8 days after 40 mg/kg busulfan administration, but then recovered coincident with the peak of PAX7+ expansion (Figure 8A). These data indicate that spermatogonia are sensitive to genotoxic stress as previously reported (40), emphasizing the unique properties and survival of PAX7+ spermatogonia after treatments that ablate the vast majority of germline cells. That the increase in FOXO1+ spermatogonia coincided with the decrease of PAX7+ spermatogonia (Figure 8, B and C) is further evidence that PAX7+ spermatogonia eventually differentiate. In control experiments, neither tamoxifen nor the DMSO solvent had a significant effect on testis weight or morphology or PAX7+ spermatogonia (Supplemental Figure 3, A–D).

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Figure 6. PAX7+ spermatogonia have long-term stem potential in vivo, and their descendants function as stem cells in transplantation assays. For PD3 time points, tamoxifen injections were performed at PD1 and PD2; for later time points, tamoxifen administration was performed for 3 consecutive days starting at PD3. (A) Clone size. Each column represents 1 testis from separate animals (total n = 15); red bars denote means. Note that many labeled A<sub>cre</sub> spermatogonia were present at PD21, demonstrating that PD21 PAX7+ spermatogonia are derived from neonatal PAX7+ spermatogonia. Clones grew over time and persisted in aged animals (12 weeks). (B) Representative clone morphologies by confocal microscopy (n denotes number of cells in labeled chain shown); z stacks confirmed cell counts and A<sub>cre</sub> status. (C) Mitotic and apoptotic indices of PAX7+ cells at PD3 (n = 3 animals) demonstrated that early PAX7+ cells were highly proliferative and not characterized by significant apoptosis. Error bars denote SEM. (D) Transplantation assay. A Pax7<sup>Cre<sub>ERT2</sub></sup>;tdTomato donor was treated with tamoxifen at PD3. Testes were disaggregated at PD14 and transplanted into germ cell–deficient Kit<sup>W/Kit<sup>W</sup></sup> hosts, which were sacrificed after 4 weeks (n = 3). All hosts (but no controls) showed multiple labeled clones (i.e., 15–20); representative examples are shown. Scale bars: 25 μm (B); 100 μm (D).
is less toxic to the germline, necessitating a longer treatment protocol (150 mg/kg i.p. every 5 days for 25 days) than busulfan, which was administered as a single dose. Radiation was administered in a single (nonfractionated) dose of 5 Gy. Selective survival and clustering of PAX7+ spermatogonia (P < 10⁻⁵) similar to that observed after busulfan were also observed after either external irradiation or cyclophosphamide (Supplemental Figures 4 and 5). After cessation of each treatment, the number of PAX7+ spermatogonia increased, then subsequently declined, as was observed with busulfan. These results are significant in that they demonstrate that in the mouse, PAX7+ spermatogonia selectively survive first-line cancer therapies that often result in reversible or permanent sterility in men and boys (41). These findings make PAX7+ spermatogonia strong candidates as the “spermatogenic recovery” cells postulated to be responsible for the restoration of fertility after cytotoxic/genotoxic treatments in rodent models (42).

To further explore this possibility, lineage-tracing studies were performed with adult mice treated with 20 mg/kg busulfan. In the first experiment, lineage tracing was initiated by tamoxifen (usual 3-day regimen) followed by busulfan (referred to herein as the tam→bu protocol). In a second experiment, the order of treatments was reversed, and tamoxifen was administered 15–17 days after busulfan administration, the time point coinciding with the peak of PAX7+ cells (bu→tam protocol; Figure 9A). In each experiment, PAX7+ spermatogonia contributed to spermatogenic recovery, as evidenced by the presence of labeled clones 8 weeks after the last treatment. With the tam→bu protocol, the number of clones was fewer than in untreated control mice (P = 0.002), whereas the number of clones was greater than controls in the bu→tam protocol (P = 0.021). These results were consistent with the expansion of PAX7+ cells observed at 16 days after busulfan (Figure 7A and Figure 9, B and C). The somewhat smaller mean clone sizes in the bu→tam versus tam→bu experiments (161 vs. 378 clones) could be explained by the administration of tamoxifen 15–17 days after busulfan, whereas in the tam→bu protocol, busulfan was administered only 8 days after tamoxifen. This 7- to 9-day difference might permit 1 or more additional cell doublings to occur in the tam→bu protocol, thus accounting for the modestly increased clone size (~2-fold difference). Clone morphology was similar to that observed in the prior lineage-tracing experiments; an example of a 4-cell group is shown (insets; enlarged ×4).

Preliminary observations demonstrate that PAX7 is dispensable for spermatogenesis. To study the genetic requirements for Pax7 in spermatogenesis, we performed conditional genetic knock-
out (cKO) with the germline-specific VASA-Cre (VC), which we had previously generated and characterized (43), and a conditional (floxed) Pax7flox allele (32). The resulting Pax7 germline cKO mice (VC;Pax7flox) had testes that were morphologically normal and exhibited normal spermatogenesis, as evidenced by normal weights and histological analyses. Furthermore, all males (n = 3) were fertile, with normal litter sizes (Figure 10, A–D), which indicated that Pax7 is dispensable for male fertility in mice. Pax7 cKO males treated with busulfan (n = 3) showed a significant lag in spermatogenic recovery 8 weeks after treatment (Supplemental Figure 6), although this lag was somewhat variable. In Pax7 cKO males, testes showed a trend toward smaller size (P = 0.25), but many tubules lacked complete spermatogenesis, while in control animals, practically all tubules had recovered (P = 0.016).

The availability of Pax7 cKO testes permitted us to confirm the specificity of Pax7 immunodetection. Whereas intratubular germ cells were readily detectable by GCNA immunostaining of control and Pax7 cKO testes, Pax7 expression was abolished in Pax7 cKO testes compared with wild-type controls (Figure 10E), confirming the specificity of Pax7 immunodetection. Thus, Pax7 appears to be dispensable for spermatogenesis, at least in the laboratory setting, but may make a functional contribution to the recovery of spermatogenesis under conditions of germline stress (see Discussion).

PAX7+ spermatogonia are present across mammalian species. We sought to determine whether PAX7+ spermatogonia are phylogenetically conserved in spermatogenesis, as many aspects of spermatogenesis are shared by diverse species (44–47). The monoclonal antibody we used to detect PAX7+ spermatogonia was generated against chicken Pax7 (Gallus gallus; amino acid [aa] 300–523), which suggests that the epitope might be broadly conserved (48). However, there was no a priori guarantee that this would be the case. We epitope-mapped the anti-PAX7 monoclonal antibody with a tiled peptide array of both the chicken and corresponding mouse aa sequences at 1-aa resolution. This identified a distinct 10-aa peak at identical positions in the chicken and mouse polypeptides (Figure 11A). Western blotting with a 22-aa blocking peptide spanning this epitope effectively eliminated the PAX7 signals (but not nonspecific background bands; Figure 11B), which confirmed that this was the epitope detected by the PAX7 monoclonal antibody. Alignment of corresponding aa sequences from diverse species revealed that the 10-aa PAX7 epitope is conserved across all 11 mammalian Pax7 homologs evaluated, but not in the zebrafish (Danio rerio) or the fruit fly (Drosophila melanogaster) (Table 1). We then performed immunolocalization of PAX7 in tissue sections of paraaffin-embedded, formalin-fixed testes from diverse mammalian species, including companion and domestic animals, nonhuman primates, and humans. Rare basal Pax7+ spermatogonia were present in these species (Figure 11C). Interestingly, Pax7+ cells were more abundant in juvenile testes (which were available for cat and baboon), with multiple cells in some tubules, similar to our observations in mice. These results suggest that Pax7+ spermatogonia serve important roles as adult testis stem cells and contribute to spermatogenesis in a wide range of species.

Discussion

Our data revealed surprising heterogeneity in cells previously identified through morphologic criteria as A single spermatogonia. This finding also suggests the existence of further A single subtypes, which may be characterized by the expression of other distinct markers, such as ID4 or ERBB3 (9, 49, 50). Pax7 defined an unexpectedly small subset of A single spermatogonia. Pax7+ spermatogonia were highly proliferative in steady-state spermatogenesis and fulfilled criteria of self-renewal and complete lineage differentiation in the adult testis. That Pax7 is a marker of germline stem cells in the testis is notable in light of extensive studies of Pax7 as a marker of satellite cells (23, 25). Our work shows some commonalities between Pax7+ stem cells in the testis and skeletal muscle, but also some important differences. Pax7+ cells were rarer in the testis, making them difficult to detect. In skeletal muscle, a tissue characterized by little cellular proliferation, Pax7+ cells are normally quiescent, only to become reactivated after injury. In contrast, in the testis, Pax7+ spermatogonia were highly proliferative and continually replenish spermatogenesis.
Some have argued that SSC transplantation represents a gold standard and is the only reliable assay for studying testis stem cell activity. Reconstitution of a self-maintaining cellular clone in a host organ is indisputable evidence that the cell of origin functioned as a stem cell in the assay. However, other investigators in the stem cell field have challenged the assumption that transplantation assays recapitulate stem cell function in native, undisturbed organs, and have pointed out limitations inherent in transplantation assays (51). These concerns are valid for SSC transplantations (5, 8, 16, 17), particularly since transplantation requires treatments (cell dissociation in the donor, near-complete germ cell ablation in the host) that may strongly stimulate regenerative potential in ways that are not fully understood. There is an important distinction to be made between actual stemness and the potential for stemness. Transplantation assays are clearly useful for studying the latter, but do not necessarily accurately reflect the former (51). Future investigations are needed to define plasticity with respect to actual stemness versus stemness potential (52) in the adult testis.

**PAX7 as a testis stem cell marker.** Our data are consistent with a model whereby PAX7<sup>+</sup> A<sub>single</sub> spermatogonia function as stem cells in the adult testis. The fact that only a minority of A<sub>single</sub> spermatogonia were PAX7<sup>+</sup> indicated that the A<sub>single</sub> population is more heterogenous than some models propose, although elegant studies previously suggested that only a subset of A<sub>single</sub> spermatogonia function as true stem cells (8, 10). Our present findings indicate that the fraction of A<sub>single</sub> spermatogonia that are PAX7<sup>+</sup> is in the range of 1% to 10% (3). We speculate that PAX7<sup>+</sup> spermatogonia sit at the top of the differentiation hierarchy, further suggesting that there are other subsets of A<sub>single</sub> spermatogonia — perhaps defined by currently unknown markers — that function as transit-amplifying intermediates prior to differentiating to A<sub>pair</sub> spermatogonia (Figure 12). However, other models are possible (5, 17), necessitating future investigations to gain a complete understanding of the cellular hierarchies underlying stem cell maintenance and differentiation in the mammalian testis.

Some have argued that SSC transplantation represents a gold standard and is the only reliable assay for studying testis stem cell activity. Reconstitution of a self-maintaining cellular clone in a host organ is indisputable evidence that the cell of origin functioned as a stem cell in the assay. However, other investigators in the stem cell field have challenged the assumption that transplantation assays recapitulate stem cell function in native, undisturbed organs, and have pointed out limitations inherent in transplantation assays (51). These concerns are valid for SSC transplantations (5, 8, 16, 17), particularly since transplantation requires treatments (cell dissociation in the donor, near-complete germ cell ablation in the host) that may strongly stimulate regenerative potential in ways that are not fully understood. There is an important distinction to be made between actual stemness and the potential for stemness. Transplantation assays are clearly useful for studying the latter, but do not necessarily accurately reflect the former (51). Future investigations are needed to define plasticity with respect to actual stemness versus stemness potential (52) in the adult testis.

**Figure 9. Lineage tracing of PAX7<sup>+</sup> spermatogonia following busulfan (20 mg/kg) treatment of Pax7-Cre<sup>ERT2</sup>;mT/mG males at 6 weeks of age.** (A) Schematic showing both busulfan lineage-tracing experiments. Testes were harvested 8 weeks after the last drug dose for each experiment. (B) Number of clones 8 weeks after busulfan administration. Each point represents 1 testis from 1 animal; red bars denote means; P values were determined by unpaired t test. (C) Clone size 8 weeks after tamoxifen administration. Red bars denote means; P values were determined by unpaired t test. (D) Composite image of representative large clone from tam→bu experiment. Tubule borders are highlighted with dashed lines. Sp, elongate spermatids (arrows denote individual cells or small groupings forming a "trail" of cells). (E) Cryosection of testis from tam→bu experiment showing germ cell clone spanning the entire tubule. ST, seminiferous tubule; LC, Leydig cells. Scale bar: 200 μm (D); 25 μm (E).
Lack of genetic requirement for Pax7 in normal spermatogenesis in the mouse. In preliminary genetic studies, we did not find evidence for a functional requirement for Pax7 in spermatogenesis. Germ cell–specific Pax7 inactivation (confirmed by the apparent lack of PAX7 protein in germ cells) did not result in male infertility or have a discernible effect on spermatogenesis. It will be interesting to study the effect of Pax7 inactivation in SSC cultures, which may exhibit phenotypes not apparent in vivo. Challenging these Pax7 cKO mice with busulfan, however, demonstrated that lack of PAX7 delayed spermatogenic recovery, a finding that should be further explored, particularly as the number of animals analyzed was relatively small and busulfan was tested at only 1 concentration.

Here, we took advantage of lineage tracing as a method to explore the stem behavior of a novel population of spermatogonia defined by PAX7+ expression. We propose some criteria by which stem cell lineage–tracing studies should be evaluated in the context of the adult testis, in the addition to the requirement for full-lineage maturation. First, we believe one important criterion is that the labeled germ cell clones begin as single cells. For example, lineage tracing initiated with a Cre driver expressed in broad subsets of spermatogonia (e.g., FOXO1+ or PLZF+ spermatogonia) would initiate labeling in several spermatogonia, including larger chains of spermatogonia, only a few of which represent actual stem cells. Extending this logic to our present study, it is possible that only a subset of PAX7+ spermatogonia function as stem cells, although the remarkable rarity of PAX7+ spermatogonia is one argument against this possibility. Another criterion we believe should be considered is the long-term perdurance of labeled clones. Such perdurance excludes the possibility that the labeled cells represent transit-amplifying intermediates, which would be diluted out and thus disappear with time. In the lineage-tracing studies with the mT/mG reporter, we studied clones for up to 16 weeks (112 days) and observed no decrease in clone numbers, while the duration of spermatogenesis in mice is approximately 40 days (34).
also very sensitive to radiation-induced damage. Doses of 1.2 Gy and higher are associated with an increased risk of infertility (58).

Remarkably, murine PAX7+ spermatogonia proved resistant to both radiotherapy and chemotherapy. They not only survived the immediate aftermath of these genotoxic insults, but also rapidly expanded, forming clusters of PAX7+ spermatogonia never observed in normal, untreated mice. Lineage-tracing studies confirmed that PAX7+ spermatogonia contribute to the restoration of spermatogenesis. Future studies will be needed to more fully define the extent of the role of PAX7+ spermatogonia as spermaticogenic recovery cells and to determine the relative contributions to spermatogenic recovery of PAX7+ spermatogonia versus other spermatogonial subtypes. It is also interesting to consider the possibility that PAX7+ spermatogonia might contribute to the recovery of fertility in cancer patients, or that their failure to recover might account for permanent sterilization after chemotherapy or radiotherapy. If so, improved understanding of the biological pathways regulating the behavior of PAX7+ spermatogonia might someday lead to strategies to protect the male germline in cancer patients. It will also be interesting to explore the biological mechanisms that render PAX7+ spermatogonia resistant to genotoxic stresses.

Implications for iatrogenic male infertility. Infertility is a common and well-known complication of cancer treatment that profoundly affects men and boys (41). Virtually all standard therapies (e.g., cytotoxic chemotherapies and radiotherapy) are highly toxic to the male germline. The likelihood of infertility with chemotherapy is drug-specific and dose-related. Alkylating agents pose the highest risk of infertility, with platinum analogs, anthracyclines, and nitrosoureas posing an intermediate level of risk (58). The germline is also very sensitive to radiation-induced damage. Doses of 1.2 Gy and higher are associated with an increased risk of infertility (58).

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The resistance of PAX7+ spermatogonia to both radiation and chemotherapy argues against models in which resistance is medi-
In closing, PAX7+ spermatogonia represent a rare but functionally important stem cell population in the healthy adult testis, and also serve an important role in spermatogenic recovery following injury to the germline, such as occurs after chemotherapy and radiotherapy. That PAX7+ spermatogonia are rapidly cycling and yet resistant to such stress is a notable aspect of their biology.

**Methods**

**mRNA analysis and PAX7 discovery.** RNA preparation, microarray hybridization, normalization, quality control, and digital Northern analysis was performed as described previously (24). Additional data sets included in this analysis were intact PD2 testes and cultured SSCs analysis was performed as described previously (27). Additional data sets were downloaded from GEO (accession nos. GSE4193, GSE4308, and GSE6916 (62–64)). Probe sets were ranked based on signal strength in SSCs as a proportion of that in the intact adult testis.

**Mouse strains and procedures.** Mice harboring the Pax7-CreERT2 [B6;129-Pax7tm1(cre/ERT2)Fan/J] and Pax7fl [B6;129-Pax7fltm2.1(cre/ERT2)Fan/J] alleles as well as the R26R [FVB;129S4(B6)-Gt(Rosa)26Sortm1(cre)Flvd/J], mT;mG (tdTomato/eGFP) reporter [Gt(Rosa)26Sortm1(cre)Flvd/J], and nuclear tdTomato reporter [B6.Cg-Gt(Rosa)26Sortm1(cre)Flvd-Tdtm1(cre)Flvd/J] alleles were purchased from Jackson Laboratories (31–33, 35). Busulfan (CAS no. 55–98-1, TCI America) was dissolved in DMSO and administered as a single i.p. dose. EdU (catalog no. CI0338, Invitrogen) was dissolved in water and injected i.p. (50 mg/kg). Cyclophosphamide was dissolved in PBS and administered at 150 mg/kg i.p. every 5 days for 25 days. Tamoxifen (catalog no. T5648, Sigma-Aldrich) was dissolved at 100 mg/ml in 100% ethanol, then resuspended at 20 mg/ml in corn oil. 2 mg tamoxifen was delivered i.p. to each adult mouse daily for 3 days. Neonatal mice (PDS or earlier) were injected i.p. with 0.2 mg tamoxifen daily for 3 days. Whole-body irradiation was administered (single dose) while the mice were restrained in acrylic boxes at a dose rate of 1.44 Gy/min. No specific method for randomization for animal studies was used; investigators were not blinded.

**Transplantation procedure.** 2 testes from 1 Pax7-CreERT2;tdTomato (PD14) donor (mixed genetic background) were enzymatically digested with dispase (catalog no. 354253, BD) to obtain single cells that were resuspended in DMEM with 10% FBS plus 0.02% Trypan Blue (25). 5–10 μl of 9 × 104 cells/μl were transplanted by the effluent duct method into testes of 3 Kit+Kit+ mice (4–6 weeks old, mixed genetic background; stock no. 100410, Jackson Laboratories). Testis filling periphery was confirmed by blue dye 50%–90% in each testis. To deplete T cells and promote engraftment, 50 μg anti-CD4 antibody (catalog no. MAB554, R&D Systems) was injected i.p. 3 times every other day starting on the day of transplantation. Testes were analyzed 4 weeks after transplantation.

**Tissue processing, IHC, and immunofluorescence (IF).** For IHC, tissues were fixed in 10% buffered formalin overnight, embedded in paraffin, and cut into 5-μm sections (except for the serial analysis of an entire testis and PAX7 cluster analyses, where 20-μm sections were used), with indirect detection performed as described previously (27).

![Figure 12. Models of stemness in mouse spermatogenesis.](image)

**Figure 12. Models of stemness in mouse spermatogenesis.** Spermatogonial subsets proposed as the bona fide stem cells are shown above each of the 3 models. In the classic Ahop model (A), Ahoop spermatogonia are homogeneous and share stem cell identity (green), having the capacity for self-maintenance (circular arrows; refs. 3, 4, 66). More recently, models have been proposed arguing for greater plasticity among undifferentiated (Ahoop−→Ahp) spermatogonia, with chain fragmentation representing one possible mechanism by which stemness is maintained or regenerated (5). Although fragmentation has been shown to occur in vivo, its contributions to stem cell maintenance under normal conditions or after chemotherapy or radiotherapy have not been formally established. Our findings that only a subset of Ahoop spermatogonia expressed PAX7 and that these spermatogonia functioning as stem cells suggests a new Ahoop subset model, whereby PAX7+ spermatogonia are self-maintaining and may sit atop the hierarchy of spermatogenic differentiation. That Ahoop spermatogonia were heterogeneous and that only a subset functioned as stem cells was also suggested by previous studies (10, 67). If so, then this would suggest that some subset of Ahoop spermatogonia represent transit-amplifying (TA) intermediates. The number of such transit-amplifying steps between PAX7+ Ahoop and Ahoop spermatogonia is unknown. It will be interesting to determine whether ID4 and ERBB3, expressed in Ahoop spermatogonia, are expressed in overlapping or nonoverlapping subsets of spermatogonia relative to PAX7 (9, 49, 50). Other models are possible, such as ones combining different aspects of these models (i.e., fragmentation with the presence of PAX7+ spermatogonia, if fragmentation is confirmed as a functionally significant biological process).
For whole-mount IF, seminiferous tubules were mechanically dissociated in PBS on ice and fixed overnight in 4% paraformaldehyde (PFA). Tubules were dehydrated in a series of methanol washes and stored at -20°C. To rehydrate and permeabilize, tubules were put through a series of washes with methanol and PBS plus 0.1% Tween-20, followed by incubation with 0.2% NP-40 for IF of nuclear proteins. Tubules were blocked in 2% BSA and PBS (catalog no. 37525, Thermo Scientific Blocker) for 2 hours, then in MOM block (catalog no. MKB-2213, Vector Labs), and primary antibody was added in 0.5% BSA and PBS with 0.02% sodium azide, followed by incubation at 4°C overnight. Tubules were washed 3 times for 10 minutes each in PBS at RT. Secondary antibody (Alexa Fluor 555 anti-rabbit, Alexa Fluor 488 anti-mouse, Alexa Fluor 555 anti-goat; catalog nos. A-21428, A-21121, and A-21432, respectively, Invitrogen) was added at 1:1,000 in 0.5% BSA and PBS for 2 hours at RT followed by DAPI staining (1:10,000 in PBS; catalog no. 46290, Pierce). Tubules were placed on glass slides and mounted in Vectorshield (Vector Laboratories). For visualization of mT/mG clones in tissue sections, testes were embedded in OCT; sectioned; fixed for 30 minutes in 4% formalin, 7% picric acid, 20% sucrose at 4°C; and then cryosectioned. Microscopy was performed with a Leica TCS SP5 confocal microscope.

**Antibodies for IF and IHC.** Antibodies and titers used were as follows: PAX7 (1:200 for IHC, 1:25 for IF; Developmental Studies Hybridoma Bank), FOXO1 (1:200 for IHC, 1:50 for IF; catalog no. 2880, Cell Signaling Technology), PLZF (1:10,000 for IHC; catalog no. AF2944, R&D Systems), caspase-3 (1:250 for IF; catalog no. 559565, BD Biosciences — Pharmingen), DSRed (detects tdTomato; 1:100 for IF; catalog no. 632496, Clontech), GCNA (1:200 for IHC; provided by G.C. Enders, University of Kansas, Kansas City, Kansas, USA; ref. 39), RET (1:20 for IHC; catalog no. 18121, IBL America), GFRa1 (1:100 for IF; catalog no. AF560, R&D Systems).

**X-gal staining.** Whole-mount X-gal staining was performed by manually dissociating tubules, fixing in 4% PFA and PBS for 30 minutes at RT, and staining as previously described (43) for 6 hours to overnight, followed by refixing in 4% PFA and PBS overnight.

**Epitope mapping and phylogenetic analysis.** An arrayed microchip was designed by LC Sciences as described previously (65). The chip included 12-mer tiling peptides sequences with 1-aa resolution corresponding to the entire chicken polypeptide immunogen (aa 300–523; Genbank NP_990396.1). The corresponding mouse aa sequence was included in 12-mer tiling peptides sequences with 1-aa resolution corresponding to the entire chicken polypeptide immunogen (aa 300–523; Genbank NP_990396.1). The corresponding mouse aa sequence was included in the 10-aa immunogen (aa 300–523; Genbank NP_990396.1). The corresponding mouse aa sequence was designed by LC Sciences as described previously (65).

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