A revised A_{single} model to explain stem cell dynamics in the mouse male germline

Tessa Lord and Jon M Oatley

Center for Reproductive Biology, School of Molecular Biosciences, College of Veterinary Medicine, Washington State University, Pullman, Washington, USA

Correspondence should be addressed to J M Oatley; Email: joatley@vetmed.wsu.edu

Abstract

Spermatogonial stem cells (SSCs) and progenitor spermatogonia encompass the undifferentiated spermatogonial pool in mammalian testes. In rodents, this population is comprised of A_{single} , A_{paired} and chains of 4–16 A_{aligned} spermatogonia. Although traditional models propose that the entire A_{single} pool represents SSCs, and formation of an A_{paired} syncytium symbolizes irreversible entry to a progenitor state destined for differentiation; recent models have emerged that suggest that the A_{single} pool is heterogeneous, and $A_{\text{paired}}/A_{\text{aligned}}$ can fragment to produce new SSCs. In this review, we explore evidence from the literature for these differing models representing SSC dynamics, including the traditional ' A_{single} ' and more recently formed 'fragmentation' models. Further, based on findings using a fluorescent reporter transgene (*eGfp*) that reflects expression of the SSC-specific transcription factor 'inhibitor of DNA binding 4' (*Id4*), we propose a revised version of the traditional model in which SSCs are a subset of the A_{single} population; the ID4-eGFP bright cells (SSC_{ultimate}). From the SSC_{ultimate} pool, other A_{single} and A_{paired} cohorts arise that are ID4-eGFP dim. Although the SSC_{ultimate} possess a transcriptome profile that reflects a self-renewing state, the transcriptome of the ID4-eGFP dim population resembles that of cells in transition ($SSC_{\text{transitory}}$) to a progenitor state. Accordingly, at the next mitotic division, these $SSC_{\text{transitory}}$ are likely to join the progenitor pool and have lost stem cell capacity. This model supports the concept of a linear relationship between spermatogonial chain length and propensity for differentiation, while leaving open the possibility that the $SSC_{\text{transitory}}$ (some A_{single} and potentially some A_{paired} spermatogonia), may contribute to the self-renewing pool rather than transition to a progenitor state in response to perturbations of steady-state conditions.

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Background

The actions of the undifferentiated spermatogonial population provide the foundation for continuity and robustness of spermatogenesis. Spermatogonial stem cells (SSCs) are a rare subset of the population intermingled with an abundant pool of progenitors that transiently amplify in number before transitioning to a differentiating state at periodic intervals in response to a retinoic acid (RA) pulse. Self-renewal activity by SSCs sustains a reservoir from which the next cohort of progenitors will arise. Whether self-renewal is a symmetric or asymmetric mode remains undefined and likely varies based on circumstances (reviewed by Oatley & Brinster (2008)). Signals that communicate the changing requirements for SSC self-renewal or progenitor production are thought to originate from support cells that constitute a stem cell niche unit (reviewed by Oatley & Brinster (2012)). The stem cell niche microenvironment exists within the seminiferous tubule, in the basal compartment of the seminiferous epithelium between the basement membrane and the Sertoli cell tight junctions. Sertoli cells not only provide

architectural support, but are also thought to be the major source of growth factors that are known to be integral for maintenance of the undifferentiated population, including glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor (FGF2) (Meng *et al.* 2000, Chen & Liu 2015). Growth factor support is also thought to be provided by somatic cells in the interstitial space such as the Leydig and peritubular myoid cells (Piquet-Pellorce *et al.* 2000, Oatley *et al.* 2009, Chen *et al.* 2016). Indeed, the supplementation of media with these growth factors (GDNF and FGF2) is required for the maintenance of an SSC pool in primary cultures of rodent undifferentiated spermatogonia (Kanatsu-Shinohara *et al.* 2003, 2005, Kubota *et al.* 2004, 2011, Hamra *et al.* 2005, Ryu *et al.* 2005, Wu *et al.* 2009, Kaucher *et al.* 2012).

In many mammalian species, cytokinesis is incomplete following mitotic division of progenitor spermatogonia, thus leading to the formation of doublets (A_{paired}) and chains (A_{aligned}) of up to 16 interconnected cells (Huckins 1971, Oakberg 1971). Upon reception of an RA signal, the progenitor spermatogonia but not SSCs respond by

transitioning to a differentiating state. Although the vast majority of progenitors encompassed in chains of 4, 8 and 16 cells experience RA-induced differentiation, as represented by hallmark expression of the RA-responsive genes *Stra8* and *Kit* (Zhou *et al.* 2008, Busada *et al.* 2015), some paired and single spermatogonia are resistant to this signal (Tagelenbosch & de Rooij 1993, Ikami *et al.* 2015). The differentiating spermatogonia undergo several further rounds of mitosis, from A1 to A4, then transition to intermediate and type B spermatogonia; again accompanied by mitotic division, resulting in an exponential increase of cell number in relation to the 'parent' SSC. It is from type B spermatogonia that spermatocytes form, accompanied by two rounds of meiosis to generate haploid cells. From spermatocytes arise round and then elongate spermatids, and then finally spermatozoa (reviewed by Oatley & Brinster (2008)). It should be noted that the SSC pool and the mode of amplification for the progenitor spermatogonial populations in humans and non-human primates differ slightly compared to those in rodents. The SSCs in primate species are distinguished as A_{pale} or A_{dark} ; that are thought to be active and reserve SSCs respectively (Clermont 1969, Clermont & Antar 1973). However, the presumptive subsets may actually constitute a single population, and, in the human at least, progenitors produced from the SSC mitotic division transition directly into type B differentiating spermatogonia (Clermont 1966). Although the discrete details of spermatogonial activities are slightly different between primates and rodents, the overarching principles and kinetics are similar. In this review, we will focus on studies using the mouse because genetically tractable models exist to study the spermatogonial populations in detail.

Although we do have some understanding of the journey of the SSC through to formation of the spermatozoon, numerous challenges exist in attempting to study the SSC population specifically, and thus in monitoring SSC dynamics. First, the rarity of SSCs, which are thought to make up only 0.03%, possibly less, of cells in the entire testis (Tagelenbosch & de Rooij 1993), makes them notoriously difficult to monitor. In addition to this, historically, there has been a lack of markers available with which to distinguish SSCs from the undifferentiated progenitors that also reside in the same compartment of the seminiferous epithelium. This difficulty is primarily a consequence of the closely related molecular and morphological profiles of the two cell types (de Rooij & Russell 2000, Grisanti *et al.* 2009, Chan *et al.* 2014, Hermann *et al.* 2015). In addition, although putative SSC markers have been identified, the specificity of a number of these for the SSC population has been contested; in part as a result of the different methodologies utilized to assess SSC purity. Notwithstanding the aforementioned challenges, a number of models depicting the dynamics of the SSC population have arisen in the field, including a revised version of the traditional A_{single}

model, and a 'fragmentation' model that proposes fluid interchangeability between A_{single} , A_{paired} and A_{aligned} cell types. In this review, we will first discuss the tools that are currently available to assess dynamics of the SSC population; including recently identified putative SSC markers, and the spermatogonial transplantation and lineage tracing techniques that have been used to both indirectly and directly assess SSC dynamics. In explaining these tools, we will explore the experimental evidence for the more recently formulated models of SSC dynamics, as well as recap traditional models that were generated in the 1970s, and, in light of these models, speculate as to what characteristics encompass a male germline stem cell.

Approaches for studying SSC dynamics

Assessment of SSC content in spermatogonial populations using transplantation analyses

Spermatogonial transplantation provides a means not only to establish the number of regenerative cells within a selected spermatogonial population, but also to assess specificity of putative SSC markers prior to their utilization for alternative non-quantitative methodologies that can be used to assess SSC dynamics; such as lineage tracing (Helsel & Oatley 2017). The spermatogonial transplantation technique uses a donor population of testis cells that constitutively express a marker such as LacZ. After isolation using a variety of techniques including magnetic or fluorescence activated cell sorting (MACS or FACS), an isolated testis cell population is microinjected into the rete testis of a recipient mouse that lacks endogenous spermatogenesis (Brinster & Avarbock 1994, Brinster & Zimmermann 1994). From 2 months following transplantation, colonization of the recipient testes by donor SSCs can be visualized if they possess a marker transgene (e.g. X-gal staining for LacZ expressing cells). The colonies represent regeneration of continual spermatogenesis, a hallmark of stem cell capacity for the male germline. As one colony in the recipient testes is representative of regeneration by one SSC (Dobranski *et al.* 1999, Zhang *et al.* 2003, Kanatsu-Shinohara *et al.* 2006), direct quantitative comparisons can be made between different populations of interest. As such, this technique can retrospectively provide assessments of different subpopulations within the undifferentiated pool.

As already mentioned, spermatogonial transplantation is also valuable for assessing the purity of populations selected with putative SSC markers, prior to utilization of these markers for alternative methodologies such as lineage tracing. Specifically, populations of cells selected with the marker of interest (using MACS or FACS) can be transplanted alongside an unselected cell population, or alternatively alongside the opposing population within the undifferentiated pool that does not

express the marker of interest (i.e. positive and negative populations). Although the capture of SSCs within a marker-expressing population depicts enrichment of SSCs, it is not necessarily representative of SSC purity. More recently, spermatogonial transplantation has been used in a limiting dilution (LD) manner to provide a means to directly calculate SSC purity in the donor population (Helsel *et al.* 2017). In this paradigm, spermatogonia that are selected for transplantation are diluted into populations of 1000, 100 and 10 cells; with the rationale that only highly pure populations of SSCs will generate colonies following transplantation of such restricted numbers of spermatogonia. The colonization efficiency of SSCs following transplantation is estimated to be between 5 and 12% (Nagano *et al.* 1999, Nagano 2003, Ogawa *et al.* 2003); with the capacity for colonization likely being limited by niche availability and saturation (Oatley *et al.* 2011b) as well as successful homing of SSCs through the blood-testis barrier to the available niches (Nagano 2003). Using an estimated colonization efficiency of 5%, a pure population of SSCs would produce only one colony in every second LD10 transplanted testis. Using colony counts from the LD10 testes, the 'purity' of the donor SSC population can be assessed using the calculation: ($\# \text{ cells transplanted} \div (\# \text{ colonies of donor-derived spermatogenesis} / 5\% \text{ colonization efficiency}) = X$), where 1 in every X cells is an SSC.

Monitoring SSC dynamics using reporter transgenes and lineage tracing

In contrast to the quantitative readout of SSC content that can be made by transplantation, current approaches for lineage tracing provide only a qualitative assessment of whether self-renewing cells are contained within the labeled population. However, lineage tracing has the advantage of facilitating observation of *in vivo* dynamics during steady-state conditions. Lineage tracing involves the utilization of animal models containing a transgene reporter system that is drug inducible. For example, a tamoxifen-induced Cre transgene in which expression is driven by the regulatory elements of a putative SSC-specific marker will lead to irreversible activation of a reporter transgene in cells expressing the marker, and all daughter cells forming from the induced cell also inherit expression of the label. As such, this technique has been used in conjunction with live imaging to monitor the formation of chains of cells from a single spermatogonia, and further, the presumptive fragmentation of single cells from spermatogonial chains (Nakagawa *et al.* 2007, 2010, Hara *et al.* 2014). This technique has also been used to infer specificity of a marker of interest to the SSC population by tracking the presence of fluorescent cells in the testes at long-term intervals (months to a year) following tamoxifen labeling (Hara *et al.* 2014, Sun *et al.*

2015). However, only a portion of a marker expressing cell population appears to be amenable to labeling after systemic treatment with a drug inducer (Nakagawa *et al.* 2007, Sun *et al.* 2015), thus quantitative assessment of the portion of the population in question that possess SSC capacity is challenging. Also, a potentially unrealized nuance of tamoxifen-induced labeling is the effect that the drug may have as a stressor to the system (Verma & Krishna 2017), which could actually lead to a non-steady-state condition after administration.

Markers of the SSC pool

In order to study the dynamics of the SSC population; either via direct monitoring using lineage tracing or retrospective assessment via transplantation analyses, it is clearly integral to have markers that can accurately identify and distinguish these stem cells from their immediate undifferentiated progenitor counterparts. Unfortunately, a consensus has not been reached in the field as to the specificity of purported SSC markers that have been used to study the population dynamics. Certainly, the development of contesting models across the field may have arisen from the assessment of different undifferentiated subpopulations that were considered at the time to be pure SSCs but later found to be heterogeneous populations. The primary markers used to formulate the 'fragmentation' and 'revised A_{single} ' models of SSC dynamics are GDNF family receptor alpha 1 (*Gfra1*) and neurogenin3 (*Ngn3*) and inhibitor of DNA binding 4 (*Id4*) respectively. The evidence for specificity of these markers specifically is discussed below.

GFRA1 is part of the receptor complex for the growth factor GDNF that is well known to influence SSC maintenance (Meng *et al.* 2000, Kubota *et al.* 2004, Chen *et al.* 2016). However, whether expression is SSC-specific is arguable because the outcomes of multiple studies suggest that expression traverses into the progenitor population. Outcomes of immunohistochemical and gene expression analyses suggest that GFRA1 expression is present in all single and paired, and even some aligned undifferentiated spermatogonia (Ebata *et al.* 2005, Grasso *et al.* 2012). Although this does not conclusively suggest that GFRA1 is a pan-undifferentiated marker, transplantation analysis of the sorted GFRA1+ spermatogonial population has revealed little to no enrichment in the number of regenerative cells when compared to an unsorted spermatogonial population (~2.5-fold increase in pups, and a decrease in adults) (Buageaw *et al.* 2005, Ebata *et al.* 2005, Grisanti *et al.* 2009). Alternatively, lineage tracing analyses have demonstrated that derivatives of GFRA1+ labeled cells do persist in the mouse testis for up to a year following tamoxifen injection, suggesting that at least a portion of this population are true SSCs (Hara *et al.* 2014),

but labeling of the entire population for quantitative assessment is not possible using this approach. Although it has been suggested that the inconsistencies between transplantation and lineage tracing data could be a consequence of sensitivity of the GFRA1 surface receptor to the enzyme treatments used prior to transplantation, primary cultures of undifferentiated spermatogonia are regularly exposed to trypsin at the time of sub-culturing but remain responsive to exogenous GDNF (Oatley *et al.* 2006), thus suggesting that this is not the case. Moreover, GFRA1+ spermatogonia isolated from testes using enzymatic treatments respond to GDNF exposure, thus demonstrating that at least some of the cells still possess an intact receptor complex (Hofmann *et al.* 2005). Together, these data infer that although a percentage of the GFRA1+ population is indeed SSCs, this population also consists of progenitors that lack regenerative (i.e. stem cell) capacity. In contrast to GFRA1, NGN3 is thought to be expressed primarily by progenitors, where it interacts with the transcription factor STAT3 to regulate differentiation (Kaucher *et al.* 2012). Indeed, it has been demonstrated that NGN3+ spermatogonia have a greater propensity for RA-induced differentiation than their NGN3- counterparts (Nakagawa *et al.* 2010). Despite this, it appears that at least a subset of NGN3 expressing cells are SSCs, as previous studies demonstrated self-renewing properties in NGN3-expressing spermatogonia using lineage tracing analyses (Yoshida *et al.* 2004). Analysis of SSC dynamics by a single research group using these markers has led to the formulation of the so-called 'fragmentation model' to explain SSC dynamics (Hara *et al.* 2014). At present, unequivocal functional evidence that GFRA1 and NGN3 are specific for SSCs and progenitors respectively is lacking. Thus, it is important to factor in this nuance when deriving independent interpretations of whether the findings support a model in which fragmentation of chained spermatogonia produces cells with a functional capacity to behave as SSCs.

The basis for development of the revised A_{single} model of SSC dynamics is the monitoring of the ID4-expressing spermatogonial population. ID4 is a transcription factor that our laboratory has identified to be heterogeneously expressed within the undifferentiated spermatogonial population, primarily within A_{single} cells (Oatley *et al.* 2011a, Chan *et al.* 2014, Sun *et al.* 2015, Helsel *et al.* 2017). Using a mouse line containing an *Id4-eGfp* transgene, along with spermatogonial transplantation, we have demonstrated that the ID4+ population of primary spermatogonial cultures encompasses the veritable entirety (>95%) of the SSC population (Chan *et al.* 2014). Further, Sun and coworkers (Sun *et al.* 2015) used lineage tracing techniques to independently establish that at least a portion of the ID4-expressing spermatogonial population possess long-term self-renewal capacity *in vivo*, in both neonatal and adult testes. Although the ID4+ spermatogonial population as a whole is highly

enriched for SSCs, it is important to note that heterogeneity exists even within this pool of cells; as depicted by a spectrum of ID4-eGFP fluorescence (bright to dim) (Chan *et al.* 2014, Helsel *et al.* 2017), and the differential expression of factors such as TSPAN8 (Mutoji *et al.* 2016). The significance of heterogeneity in terms of levels of ID4 expression will be discussed briefly in the context of the A_{single} model. However, it is important to note that in using a LD spermatogonial transplantation assay on post-natal day 6–8 spermatogonia, we have established that 1 in 0.94 cells within the subset of the population that contains the highest levels of ID4 (i.e. ID4-eGFP^{Bright}) is an SSC. Thus, the population is highly enriched for, if not pure, stem cells (Helsel *et al.* 2017). Although lineage tracing data are suggestive that the ID4-expressing spermatogonial population also encompasses SSCs in the adult testes (Sun *et al.* 2015), similar assessments of population purity using transplantation analyses have not yet been conducted.

Models of SSC dynamics

Traditional A_{single} model

For several decades, the ' A_{single} model' that was devised from experimentation in both mice and rats has served as the predominant explanation of SSC dynamics in mammalian testes (Fig. 1) (de Rooij 1969, Huckins 1971, Oakberg 1971, de Rooij 1973). These experiments used morphological assessment of whole mount seminiferous tubules and ³H-thymidine tracing to propose that stem cell activity is restricted to the A_{single} spermatogonia in the niche (Oakberg 1971). As mentioned previously, these A_{single} spermatogonia can undergo mitotic division to produce new single cells, or an A_{paired} syncytium in which the cells remain connected by an intercellular bridge. The A_{single} model predicts that the incomplete abscission of an intercellular bridge following mitotic division is an indication of irreversible transition to a progenitor state. Accordingly, these studies stated that there was no evidence for reversion of chains of spermatogonia back to the A_{single} cell type. The A_{single} model of SSC dynamics has provided the basis for several decades of research, however the capacity for experimentation on the SSC population has now far exceeded dependence on morphological assessments. As such, more modern models of SSC dynamics build upon, and in some cases contradict the original hypothesis. As will be discussed below, both of the recently developed models of SSC dynamics have used fluorescent reporter transgenes under the control of putative SSC-specific markers to challenge the notion that the entirety of the A_{single} population is synonymous with the SSC population, and further, that reversion into a self-renewing cell type does not occur once steps toward differentiation have been initiated.

Figure 1 Different models to explain the dynamics of the SSC pool. The traditional model proposes that all A_{single} spermatogonia comprise the SSC pool. These A_{single} cells can either self-renew, or transition to a progenitor state; as represented by the formation of an A_{paired} syncytium. A_{paired} progenitors undergo further mitotic divisions to produce longer chains of spermatogonia (A_{aligned}) that will respond to retinoic acid (RA) by transitioning to a differentiating state. In the traditional A_{single} model, formation of A_{paired} signifies irreversible commitment to a differentiating pathway. In contrast to the A_{single} model, the fragmentation model proposes that the self-renewing SSC pool is comprised of interchanging A_{single} , A_{paired} and A_{aligned} spermatogonia, all of which express GFRA1. In this model, the transition to progenitor is signified by loss of GFRA1 expression and gain of NGN3 expression. Finally, the revised A_{single} model proposes that a specified subset of the A_{single} pool (i.e. cells with high levels of ID4 expression) represent the SSC_{ultimate} population that serve as the self-renewing reservoir in steady-state conditions. From the SSC_{ultimate} pool, transitory cells (i.e. spermatogonia with lower levels of ID4) arise. The continuum of transition to a progenitor state involves declining expression of key self-renewal genes in $SSC_{\text{transitory}}$ that will allow for the next mitotic division to result in a true A_{paired} syncytium. In all models, RA drives A_{paired} and A_{aligned} progenitors into a differentiating state at periodic intervals, characterized by the attainment of Kit expression. Following this, spermatogonia transition through A1, A2, A3, A4, intermediate and type B stages, before entering into meiosis as spermatocytes, then producing haploid spermatids and finally spermatozoa.

Fragmentation model

Based on the outcomes of lineage tracing and live imaging analyses using mice containing the aforementioned GFRA1 and NGN3 reporter transgenes, the 'fragmentation model' of SSC dynamics has emerged over the last 10 years (Fig. 1) (Nakagawa *et al.* 2007, 2010, Hara *et al.* 2014). This model predicts that

spermatogonia are constantly 'breaking off' from A_{paired} and A_{aligned} chains to replenish the self-renewing SSC pool (Hara *et al.* 2014); a direct contradiction to traditional schools of thought that depicted these chains of cells as progenitors that were irreversibly committed to a differentiating fate (de Rooij & Kramer 1968, de Rooij 1969, 1973, Huckins 1971, Oakberg 1971, De Rooij

1988, De Rooij *et al.* 1989, de Rooij & Grootegoed 1998, de Rooij & Russell 2000). Thus, the fragmentation model proposes that the primary stem cell pool, believed to be encompassed by all GFRA1 expressing spermatogonia, is not only comprised solely of A_{single} cells, but also of pairs and short spermatogonial chains. In this paradigm, the GFRA1+ spermatogonia can alternatively transition into a GFRA1-/NGN3+ progenitor state, in which the propensity for differentiation is increased. Furthermore, it has been proposed that in times when regeneration is pertinent; the NGN3+ progenitor population can also experience fragmentation, with single progenitor spermatogonia breaking off from chains and reverting to a GFRA1+ state to re-join the self-renewing pool (Nakagawa *et al.* 2010). Indeed, recent independent research has suggested a capacity for NGN3+ cells to presumably revert to a GFRA1+ state (Zhang *et al.* 2016), a process that was only observed during circumstances of regeneration, and appeared to be reliant on the presence of Doublesex and Mab-3-related transcription factor 1 (DMRT1). Considering that NGN3 is expressed by subsets of A_{single} , A_{paired} and A_{aligned} spermatogonia, these findings do not prove unequivocally that fragmentation is the mode by which new GFRA1+ cells are generated. The possibility that NGN3+/GFRA1- A_{single} cells revert to a GFRA1+ state has not been ruled out.

Although the propensity for alteration or reversion of spermatogonial expression profiles in response to different requirements within the niche is a concept that is becoming more readily accepted; the notion that these reverting cells are 'fragments' broken off from spermatogonial chains is controversial. Currently, evidence for these phenomena are based primarily on observations of fragmentation using live imaging of testes in mice that are maintained in a stress condition of long-term anesthesia, and computer generated biophysical models (Nakagawa *et al.* 2007, 2010, Hara *et al.* 2014). However, as yet, no direct functional evidence has been provided for re-established self-renewal capacity in the fragmented cells. Further qualms underlying the fragmentation model rest with the aforementioned uncertainty surrounding the specificity of GFRA1 and NGN3 as markers for the SSC and progenitor populations respectively. Moreover, this model of SSC dynamics is not easily harmonized with previously published data that has demonstrated that the propensity for RA-induced differentiation is correlated with spermatogonial chain length; i.e. 100% of A_{aligned} chains consisting of 8–16 spermatogonia, 94% of A_{aligned} chains containing four spermatogonia, 64% of A_{paired} spermatogonia and 0% of A_{single} spermatogonia differentiate in response to RA (Tagelbosch & de Rooij 1993); findings that suggest a more linear relationship between the stem cell state and spermatogonial chain length. Furthermore, the computational models generated based on live imaging and lineage tracing analysis do not take into account the stages of the seminiferous cycle in which

the undifferentiated-to-differentiating transition occur and different cohorts of spermatogonia are known to randomly divide.

Revised A_{single} model to explain SSC dynamics

Based on the analysis of the ID4+ spermatogonial population as well as recent findings using other markers for a subset of A_{single} with stem cell properties, we propose a revised version of the traditional model to describe SSC dynamics in rodents (Fig. 1). This paradigm posits that a subset of the A_{single} population are functionally true SSCs, and further suggests that some plasticity may exist between the self-renewing SSC pool and spermatogonia that are in a transitioning state between SSC and progenitor. Early evidence for heterogeneity within the A_{single} population came from transplantation analyses of single cell suspensions from whole testes. Although the number of A_{single} cells in the adult mouse testis is estimated to be approximately 35,000 (Tagelbosch & de Rooij 1993), the number of regenerated spermatogenic colonies in recipient testes following transplantation of an unselected total donor testis cell population is less than 10% of this value, at approximately 3000 cells (Nagano 2003). Further supporting the notion that not all A_{single} cells are SSCs, a number of research groups have reported heterogeneity in expression of various genes within the A_{single} population; including *Id4* (Oatley *et al.* 2011a, Chan *et al.* 2014, Hermann *et al.* 2015), Paired box 7 (*Pax7*) (Aloisio *et al.* 2014), *Gfra1* (Grisanti *et al.* 2009), *Nanos2* (Suzuki *et al.* 2009), *Ngn3* (Yoshida *et al.* 2004, Kaucher *et al.* 2012) and *Oct4* (Ohmura *et al.* 2004).

The revised A_{single} model proposes that in steady-state conditions, a subset of the A_{single} population that expresses high levels of self-renewal factors (e.g. ID4) are the foundation for continuity of the spermatogenic lineage, essentially serving as ultimate SSCs (SSC_{ultimate}). In addition to self-renewal, these SSC_{ultimate} can also give rise to $SSC_{\text{transitory}}$ (A_{single} and A_{paired}) that possess lower levels of expression for self-renewal factors and are set on a continuum of transition to a progenitor state as true A_{paired} and A_{aligned} . Thus, the differentiation from SSC to progenitor is not a 'light-switch' phenomenon, but rather a gradual transition between two functionally distinct extremes. Evidence supporting this model was generated first using whole mount imaging of live seminiferous tubules to show that spermatogonia with high levels of ID4 expression (i.e. ID4-eGFP^{Bright} cells) are primarily A_{single} spermatogonia within the testes, and further using transplantation analysis to demonstrate that these ID4-eGFP bright cells encompass over 85% of the SSC population. In contrast, ID4-eGFP 'dim' cells were identified primarily as A_{paired} and some A_{single} cells, and encompassed less than 15% of the self-renewing population when assessed via transplantation analyses (Helsel *et al.* 2017). Although a small percentage of

A_{paired} syncytium were identified with bright ID4-eGFP epifluorescence, in our opinion these are likely ‘false pairs’ that arise temporarily in the process of self-renewal when abscission of the intercellular bridge has not been resolved or abscission has completed but migration apart has not occurred (de Rooij & Russell 2000). Importantly, A_{aligned} cohorts appear to possess low to undetectable levels of ID4-eGFP (Helsel *et al.* 2017), and we have demonstrated previously that the ID4-eGFP negative population obtained from primary spermatogonial culture has little capacity for regeneration. These findings suggest that A_{paired} and A_{aligned} , with low to no expression of specific self-renewal factors such as ID4, represent the pool of undifferentiated progenitors. These experiments not only demonstrated a direct correlation between ID4 expression, spermatogonial ‘chain identity’ and self-renewal capacity; but have used transcriptome profiling to demonstrate that distinct gene expression signatures exist between the $\text{SSC}_{\text{ultimate}}$, $\text{SSC}_{\text{transitory}}$ and progenitor populations (Chan *et al.* 2014, Helsel *et al.* 2017). Notably, independent evidence for the revised A_{single} model has also recently come to light following characterization of expression of the Wnt/ β -catenin signaling inhibitor ‘SHISA6’ in the spermatogonial population. As demonstrated by the research group of Dr Yoshida (Tokue *et al.* 2017), SHISA6 acts to maintain the self-renewing pool by impeding the differentiating transition, however its expression is restricted to all but a small subset of the GFRA1+ population. Indeed, SHISA6-expressing cells were primarily identified to be A_{single} . We propose that these SHISA6 expressing cells are likely to be the $\text{SSC}_{\text{ultimate}}$ that also express high levels of ID4, while the GFRA1+/SHISA6– cells represent the $\text{SSC}_{\text{transitory}}$ and progenitor populations. While further investigation into the association between ID4 and SHISA6 is warranted, our recently published transcriptome profiling studies suggest that *Shisa6* is uniquely expressed by the ID4-eGFP^{Bright} population that is highly enriched, if not pure, SSCs with the transcript levels being 8-fold higher compared to the ID4-eGFP^{Dim} population that is in transition to become progenitors that lack stem cell capacity (Helsel *et al.* 2017).

The revised A_{single} model agrees with the traditional model in that reversion to a stem cell state from the progenitor pool does not occur in steady-state conditions or during situations of regeneration caused by external insults. However, this model does support the notion that some plasticity may exist for cells in the beginning stages of transition from the ultimate SSC state to the progenitor state (i.e. $\text{SSC}_{\text{transitory}}$ spermatogonia). Indeed, a recent independent study demonstrated that NGN3 expressing spermatogonia that are rarely found to express high levels of ID4 in steady-state conditions may be able to re-establish the stem cell expression profile and contribute to the self-renewing pool when regeneration of the germline is required, such as following cytotoxic insult (Zhang *et al.* 2016). Based

on the lack of regenerative capacity in the ID4-eGFP negative population (as assessed by transplantation analysis using populations derived from primary spermatogonial culture), and their transcriptome profile (Chan *et al.* 2014), along with the relationship between spermatogonial chain identity and RA-induced differentiation (Tagelenbosch & de Rooij 1993); we propose that the source of these reverted regenerative cells are likely A_{single} but potentially also the A_{paired} that are in the $\text{SSC}_{\text{transitory}}$ state.

What makes an SSC an SSC? – speculations based on the revised A_{single} model

Based on transcriptome comparisons between subpopulations of SSCs and progenitors, one can begin to form a picture as to how the self-renewing reservoir is maintained in the testes during steady-state conditions, and the changes that occur upon transition to, and formation of, a progenitor state. First, the $\text{SSC}_{\text{ultimate}}$ possess high expression levels of key self-renewal factors including GFRA1, and, likely as a direct consequence of this, are also characterized by high levels of expression of a number of GDNF responsive genes that are known to be involved in self-renewal; including *Bcl6b*, *Lhx1*, *Etv5* and *Id4* itself (Chan *et al.* 2014, Helsel *et al.* 2017). Although genes involved in self-renewal are upregulated in these $\text{SSC}_{\text{ultimate}}$ cells, expression of genes that are associated with a progenitor state and elevated propensity for differentiation such as *Sohlh1*, *Ngn3* and *Kit*, are repressed (Chan *et al.* 2014, Helsel *et al.* 2017). In addition to promoting self-renewal and preventing differentiation, the gene expression profile of the $\text{SSC}_{\text{ultimate}}$ also appears to be integral for maintaining a ‘slow-cycling’ state; suggested by the rare incorporation of EdU (Zhang *et al.* 2016) that is typical of stem cells in many lineages (Fuchs 2009).

As the transition to progenitor begins at the $\text{SSC}_{\text{transitory}}$ state, levels of self-renewal genes (e.g. *Id4* and *Gfra1*) are reduced, concomitant with increased expression of genes controlling the progenitor state (e.g. *Sohlh1* and *Ngn3*) (Helsel *et al.* 2017). Such findings suggest that GFRA1 expression, like ID4, may exist as a spectrum in which $\text{SSC}_{\text{ultimate}}$ are GFRA1-high and $\text{SSC}_{\text{transitory}}$ are GFRA1-low. The heterogeneity in GFRA1 expression between these two populations would again lend to the notion that results produced from studies using GFRA1-based reporter transgenes to follow SSC dynamics may be confounded by combining two functionally distinct subpopulations (Hara *et al.* 2014). In conjunction with the decreased levels of ID4 expression, EdU incorporation begins to become more frequent upon initiation of the progenitor transition, indicating more rapid cycling of these cells (Zhang *et al.* 2016). Although we have demonstrated that progenitor spermatogonia, which have lost ID4-eGFP expression, exist as chains

of spermatogonia (A_{aligned}) with expression profiles distinct from that of the SSC population (Chan *et al.* 2014, Helsel *et al.* 2017); factors that act specifically to regulate the maintenance of intercellular bridges, or abscission following a self-renewing division in SSCs, remain uncharacterized. Testis expressed 14 (TEX14) has been identified as indispensable for the formation and maintenance of intercellular bridges in mice (Greenbaum *et al.* 2006, 2011), however, any differential expression between SSC and progenitor populations is yet to be reported.

In conclusion, although progress in characterizing SSC dynamics has certainly been achieved since derivation of the A_{single} model in the 1970s, the field has yet to come to agreement on a single paradigm for this process. These inconsistencies are at least in part a result of utilizing different markers for the SSC population, as well as the different strategies used to assess SSC specificity. Despite opposing theories on which cells within the undifferentiated population actually encompass the self-renewing pool, the revised A_{single} and fragmentation models do agree that simply being a 'single' undifferentiated spermatogonia is not equivalent to being an SSC; in other words, the A_{single} population is heterogeneous in terms of marker gene expression and functional capacity. We propose that in steady-state conditions, a subset of the A_{single} population; distinguished by high levels of ID4 expression, act as the self-renewing reservoir that maintains continual sperm production (SSC_{ultimate}). In the continuum of adopting a true progenitor state, A_{single} pass through a transition state ($SSC_{\text{transitory}}$) where expression of self-renewal driving genes declines, and expression of genes driving differentiation increases. Thus, at the next cell division, we envision that the $SSC_{\text{transitory}}$ adopt a true progenitor state at which they are responsive to RA signaling. However, in circumstances where germline regeneration is pertinent, it is possible that these transitioning cells can respond to uncharacterized extrinsic signals to revert to the self-renewing pool rather than transition to a progenitor state at the next mitotic division, thereby providing a means for rapid restoration of the spermatogenic lineage.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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