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Functional assessment of spermatogonial stem cell purity in experimental cell populations

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Abstract

Historically, research in spermatogonial biology has been hindered by a lack of validated approaches to identify and isolate pure populations of the various spermatogonial subsets for in-depth analysis. In particular, although a number of markers of the undifferentiated spermatogonial population have now been characterized, standardized methodology for assessing their specificity to the spermatogonial stem cell (SSC) and transit amplifying progenitor pools has been lacking. To date, SSC content within an undefined population of spermatogonia has been inferred using either lineage tracing or spermatogonial transplantation analyses which generate qualitative and quantitative data, respectively. Therefore, these techniques are not directly comparable, and are subject to variable interpretations as to a readout that is representative of a 'pure' SSC population. We propose standardization across the field for determining the SSC purity of a population via use of a limiting dilution transplantation assay that would eliminate subjectivity and help to minimize the generation of inconsistent data on 'SSC' populations. In the limiting dilution transplantation assay, a population of *LacZ*-expressing spermatogonia are selected based on a putative SSC marker, and a small, defined number of cells (i.e. 10 cells) are microinjected into the testis of a germ cell-deficient recipient mouse. Using colony counts and an estimated colonization efficiency of 5%; a quantitative value can be calculated that represents SSC purity in the starting population. The utilization of this technique would not only be useful to link functional relevance to novel markers that will be identified in the future, but also for providing validation of purity for marker-selected populations of spermatogonia that are commonly considered to be SSCs by many researchers in the field of spermatogenesis and stem cell biology.

Keywords

Spermatogonial stem cell; Limiting dilution; Transplantation

1. Introduction

Continued fertility in the male is reliant on the actions of spermatogonial stem cells (SSCs). In rodent testes, the maintenance and self-renewal of the SSC pool is, for the most part, thought to adhere to the dynamics described in a revised version of the traditional A_{single}

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model (de Rooij, 2017; Helsel et al., 2017; Huckins, 1971; Lord and Oatley, 2017b; Oakberg, 1971). In this model, the SSCs are a subset of A_{single} spermatogonial cell pool that reside in a specialized niche along the basement membrane of the seminiferous tubules (Lord and Oatley, 2017a; Oatley and Brinster, 2012). Self-renewal activity sustains a reservoir of A_{single} SSCs from which transitory A_{single} will arise and begin the transition towards a transit-amplifying (TA) progenitor state in which the subsequent division will yield a pair of spermatogonia connected via an intercellular bridge, referred to as A_{paired}. The A_{paired} will then undergo further divisions with incomplete cytokinesis to yield $A_{aligned}$ spermatogonia that are poised for transition to a differentiating pathway in response to retinoic acid stimulation, ultimately resulting in the production of spermatozoa. A limiting factor in conducting research on the SSC population has been a lack of means by which to distinguish and isolate the SSCs from morphologically identical transitory and TA progenitors that reside in the same anatomical location within seminiferous tubules. Accordingly, much research effort has been directed at identifying SSC-specific markers, and further, into developing methodologies that can be used to assess the SSC content of an experimental population of spermatogonia.

Although the number of markers characterized for expression in the undifferentiated spermatogonial population is ever-increasing, standardized practices have not been established to unequivocally assess SSC content. To avoid the potential for misrepresentation of findings and the generation of conflicting data, it would clearly be beneficial to establish guidelines that the field can utilize for assessing the specificity of putative markers, before asserting their ability to identify and isolate ‘pure’ SSC populations. In this short review, we discuss the advantages and possible limitations of both lineage tracing and spermatogonial transplantation as methodologies for establishing the efficacy of potential SSC markers, and propose the use of a limiting dilution transplantation assay as a standardized tool to unequivocally determine the specificity of putative SSC markers.

2. Identification of SSCs within a heterogeneous pool of spermatogonia

In rodent species, identification and/or isolation of live spermatogonial populations enriched for SSCs can be facilitated either by antibody based methodologies, or via development of a mouse line containing a reporter transgene. If the goal is to isolate a pure population of SSCs, then the efficiency of both strategies relies on utilization of a marker or markers that can delineate the SSC and TA progenitor populations. Antibody based enrichment of live SSC populations is only feasible when the targeted marker is expressed on the surface of the cell; with antibody labelled cells then being separated from the heterogeneous spermatogonial pool using fluorescence- or magnetic-activated cell sorting (FACS or MACS, respectively). While a number of cell surface markers have been characterized that can be used in this manner to enrich SSC content when compared to an unselected population of spermatogonia; including THY1 (Kubota et al., 2004), α6 and β1 integrin (Shinohara et al., 1999), and Cadherin 1 (Tokuda et al., 2007); surface proteins with true SSC specificity are yet to be identified. This is an unfortunate limitation, particularly for translating techniques commonly utilized in rodent models to higher order mammals

including humans, as the utilization of reporter transgenes with human spermatogonia is not possible, and thus identification of an SSC-specific cell surface phenotype is a necessity.

As the bulk of factors identified as potential SSC-specific molecules in rodent spermatogonia are intrinsically expressed, the field has largely relied upon the development of mouse lines containing a fluorescent reporter transgene driven by the factor of interest, again facilitating cell sorting techniques. This strategy also circumvents issues with antibody quality and specificity and thus has been adopted to explore the role of a number of markers expressed in the TA progenitor/SSC populations; including Inhibitor of DNA binding 4 (ID4) (Chan et al., 2014), Neurogenin 3 (NGN3) (Yoshida et al., 2004), GDNF receptor α1 (GFRA1) (Hara et al., 2014), and POU Class 5 Homeobox 1 (POU5F1/OCT4) (Youn et al., 2013). The capacity for these markers to enrich the SSC population is inevitably variable, depending on the degree of overlapping expression with the TA progenitor pool.

3. Techniques for assessing SSC purity in selected spermatogonial populations

In the quest for identifying SSC-specific factors that can be used to study this rare population of cells, two techniques have regularly been adopted; lineage tracing and spermatogonial transplantation. While both techniques are advantageous for addressing different experimental questions, downfalls to these methodologies do exist when attempting to assess SSC ‘purity’ in an experimental population. Lineage tracing relies on transient induction of transgene expression to irreversibly label a cell population and then trace all of the decedents. In mice, this technique has relied upon inducing the activity of Cre Recombinase that is expressed as a transgene under the control of regulatory elements for a putative SSC marker. The Cre activity will subsequently act on a second transgene to cause LoxP recombination to excise a stop sequence for constitutive expression of a fluorescent reporter. Following this, all cells that arise from mitotic division of the labelled cell (i.e. the putative SSC) will also possess the reporter tag. As only SSCs will persist and continue to generate the spermatogenic lineage over time; while TA progenitors and more mature spermatogenic cells will be eliminated from the testes in the subsequent rounds of spermatogenesis, the persistence of labelled cells after several rounds of spermatogenesis post-activation of Cre is an indication that the marker of interest is expressed by SSCs (Aloisio et al., 2014; Sun et al., 2015). The state-of-the-art for inducing conditional activation of a Cre for which expression is reflective of a putative SSC marker is the estrogen receptor antagonist tamoxifen. Because tamoxifen treatment is achieved via systemic injections or the diet, ensuring that all putatively marked cells within any tissue are exposed at a high enough level to induce Cre activation is challenging. Thus, the ‘readout’ provided by lineage tracing is purely qualitative, as the presence of labelled cells does not necessarily indicate that expression of the reporter transgene is occurring in the entirety of the SSC population (nor that the SSC pool was the sole population to be labelled). Further, this experimental approach does not allow for quantitative comparisons between multiple populations of spermatogonia, and previous studies have suggested that tamoxifen induced labelling may not be successful in capturing the entirety of the target population (Nakagawa et al., 2007; Sun et al., 2015). Finally, although lineage tracing is theoretically valuable for

assessing SSC activity during homeostatic spermatogenesis, the possibility that tamoxifen acts as a stressor on the system thereby subsequently disrupting steady-state conditions makes interpretation of findings challenging (Verma, 2001). Indeed, tamoxifen is known to alter pituitary gonadotropin output, and luteinizing hormone was recently shown to stimulate Wnt expression in the testes which influences SSC activities (Tanaka et al., 2016; Tokue et al., 2017). Thus, while lineage tracing is a valuable technique for establishing the presence of spermatogonia with long-term regenerative capacity within a selected population, and for monitoring the dynamics of these cells in an in vivo environment, care needs to be taken in interpreting these results in the context of assessing SSC 'purity'.

In contrast to lineage tracing, the spermatogonial transplantation technique that was developed by Ralph Brinster and colleagues in 1994 (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994), provides a means to quantitatively compare the SSC content of experimental testis cell populations. In applying this method, use of cells from donor males harboring a *LacZ* transgene in the *Rosa26* locus allows for colonies of donor spermatogenesis that are regenerated from transplanted SSCs to be clearly identified and quantified. Spermatogonia selected based on a putative SSC-specific marker are microinjected into the rete testis of a recipient mouse lacking endogenous germline via pre-treatment with a chemotherapeutic alkylating agent (e.g. busulfan) or due to a genetic deficiency (e.g. W/Wv mutants). Any SSCs present in the selected donor cell population are capable of engrafting in seminiferous tubules of recipient testes to establish stem cell-niche units and initiate regeneration of spermatogenesis. Typically, colonies of donor-derived spermatogenesis in recipient testes are then assessed at two to four months following transplantation using X-gal staining (if donor cells contained a constitutively expressed *LacZ* transgene). This approach results in a dense blue staining for donor-derived colonies that consist of complete spermatogenesis. As each colony is clonally derived from a single SSC (Dobrinski et al., 1999; Kanatsu-Shinohara et al., 2006; Zhang et al., 2003), a relative 'SSC number' can be calculated for the transplanted donor cell populations. By applying a standard means of expressing colony numbers as per 10^5 cells injected, direct quantitative comparison of SSC content in different experimental cell populations can be made. Although this approach provides a measure of SSC content that allows for comparing relative enrichment between cell populations, the purity cannot be clearly discerned because of limitations in the efficiency of colonization and accessible niches. Thus, to assess SSC purity of a population, a limiting number of cells must be transplanted.

4. Standardization of a limiting dilution transplantation assay to determine SSC purity

Here, we propose a methodology to assess SSC purity of selected testis cell populations for mouse studies that yields quantitative data for direct comparisons. In particular, we propose the utilization of an adapted limiting dilution transplantation approach, variations on which are commonly employed to evaluate stem cell populations in other tissue types (Illa-Bochaca et al., 2010). Using this methodology, experimental testis cell populations (e.g. cells expressing a marker of interest) are isolated using cell sorting strategies, and then serially diluted for transplantation; generating populations of 1000 (LD1000), 100 (LD100) and 10

(LD10) cells (Fig. 1). The entirety of each population is then microinjected into the testis of a recipient mouse that is depleted of endogenous germline. Suitable experimental replication would be cells isolated from at least three different donor mice as biological replicates, with each dilution factor from each donor being transplanted into at least four recipient testes as technical replicates. The utilization of low cell numbers for transplantation means that saturation of available SSC niches in the recipient testes should not occur, thus, the number of colonies formed will accurately reflect the number of cells with regenerative capacity in the donor population. Using a serial transplantation approach, the colonization efficiency for transplanted SSCs has been estimated to be 5–12% (Nagano et al., 1999; Ogawa et al., 2003); thus, 'pure' populations of SSCs would theoretically generate one colony in every second LD10 injected recipient testis. Contrastingly, heterogeneous populations with a large number of contaminating progenitor spermatogonia would be expected to produce only very rare colonies, if any, in LD10 injected testes, however colonization would still be expected in LD1000 and LD100 testes; albeit at a lower frequency than that seen with pure SSC populations. Using colony counts from testes transplanted with LD10 populations, the theoretical frequency of SSCs in the donor population can be calculated using the formula:

[# cells transplanted (i.e. 10 cells × 4 testes = 40) ÷ (# colonies of donor-derived spermatogenesis/5% colonization efficiency) = x], where 1 in every x cells is an SSC.

Thus, a value of 1 would be expected if a donor population were isolated by a marker that was truly SSC-specific.

5. Previously characterized markers of the 'SSC' population

To our knowledge, the only marker for the SSC population that has been validated using all three of the aforementioned techniques; lineage tracing, standard spermatogonial transplantation, and limiting dilution transplantation, is ID4. Using a mouse line with an *Id4-eGfp* transgene, the ID4-eGFP+ cell population, which is comprised of approximately 6000 cells in the adult testis (Chan et al., 2014), was shown to encompass 95% of the SSC pool in primary cultures of spermatogonia, whereas the ID4-eGFP- cells were the progenitors that had lost the capacity for regeneration of the spermatogenic lineage (Chan et al., 2014). In addition, lineage tracing analyses using a tamoxifen inducible Cre recombinase under the control of nascent *Id4* regulatory elements confirmed that the spermatogenic lineage stems directly from at least a subset of ID4 expressing spermatogonia in vivo, in both neonatal and adult mice (Sun et al., 2015). Finally, in recent studies, limiting dilution transplantation analyses were performed using the population of spermatogonia in the testes that exhibit the highest levels of ID4 expression (the ID4-eGFP^{Bright} population) (Helsel et al., 2017). Using the formula described in Section 4, 1 in every 0.94 ID4-eGFP^{Bright} cells was found to possess regenerative capacity, thus demonstrating SSC purity. Importantly, the ID4-eGFP^{Bright} population was also determined to be primarily comprised of A_{single} spermatogonia, as well as a small number of A_{paired} structures (Helsel et al., 2017). Thus, the correlation between ID4 expression and A_{single} status in this manuscript supported the 'revised A_{single} model' of SSC dynamics (de Rooij, 2017; Lord and Oatley, 2017b), in which SSCs are primarily the A_{single} cells, while the ID4-eGFP^{Bright} A_{paired} structures are likely to

be ‘false pairs’; i.e. A_{single} SSCs that have recently completed mitosis in which abscission of the cytoplasmic bridge had not yet occurred.

Beyond ID4, a number of other potential markers of the SSC population have been put forth; including, Paired Box 7 (PAX7) (Aloisio et al., 2014), BMI1 proto-oncogene, polycomb ring finger (BMI1) (Komai et al., 2014), Shisa family member 6 (SHISA6) (Tokue et al., 2017), GFRA1 (Nakagawa et al., 2010), and OCT4 (Dann et al., 2008; Li et al., 2015; Tana, 2013), as well as markers of the undifferentiated population as a whole including PLZF (ZBTB16) (Costoya et al., 2004), LIN28 (Zheng et al., 2009), THY1 (Kubota et al., 2004) and Spalt-like transcription factor 4 (SALL4) (Gassei and Orwig, 2013; Hobbs et al., 2012). Several of these markers show promise for being selective for the SSC population, however, based on our proposal for more stringent analyses, the purity of these populations remains undefined. Although an exhaustive investigation into evidence for SSC specificity of each molecular marker is beyond the scope of this review, brief selected examples will be provided below; specifically focusing on PAX7 and GFRA1.

Interest in PAX7 as a potential marker for the SSC population was sparked by the observation of expression by a rare subset of the A_{single} spermatogonia; estimated at only 400 per mouse testis (Aloisio et al., 2014). In addition, lineage tracing analyses showed that PAX7 is indeed expressed by at least some SSCs in mice, but the percentage of the population that are PAX7+ is undefined. Thus, limiting dilution transplantation analyses are needed to truly assess whether PAX7 is a specific marker of the SSC pool or transiently expressed by a subset of the population, possibly at certain stages of the cell cycle during the self-renewal process. Indeed, nearly all PAX7+ spermatogonia were found to be rapidly transiting through the cell cycle, so to maintain rarity within the heterogeneous spermatogonial population, there would need to be high levels of apoptosis or rapid transition to a differentiating state, or the expression of PAX7 would need to be transient.

A cell surface marker that has been lauded as being specific for SSCs is GFRA1, which is part of the receptor complex for glial cell line-derived neurotrophic factor (GDNF); a growth factor important for maintenance of SSC and TA progenitor spermatogonia. Studies using immunostaining analyses have reported that A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia are GFRA1+, albeit at varying staining intensities, thus indicating expression by SSCs and TA progenitor spermatogonia (Grisanti et al., 2009). Evidence for SSC specificity of GFRA1 has been provided mainly from lineage tracing analyses, which demonstrated that continued long-term spermatogenesis does indeed arise from a subset of the GFRA1+ spermatogonial population (Hara et al., 2014). Although these findings demonstrate that at least some GFRA1+ cells are SSCs, as is suggested by the expression of GFRA1 within the ID4 population (Helsel et al., 2017; Lord et al., 2018), the outcomes of transplantation analyses argue that selection with GFRA1 results in acquisition of population that is also largely comprised of TA progenitor spermatogonia. Studies of Ebata et al. (2005) found that the GFRA1+ population in mouse pup testes is enriched by only a 2.5-fold for SSCs compared to the total testis cell population; whereas the GFRA1+ population in adult mouse testes was measured to be depleted of SSCs. Confirming these findings, studies of Grisanti et al. (2009) also depicted the isolated GFRA1+ population of mouse testes as being depleted of SSCs, using transplantation analyses. Thus, while the GFRA1+ population does encompass a

subset of spermatogonia with regenerative capacity, it certainly does not appear to be representative of a ‘pure’ SSC population.

In evaluating SSC purity of spermatogonial populations such as those marked by ID4, PAX7, and GFRA1 expression, studies conducted using different tools, or the same tool but in a different manner, can result in contradictory outcomes and alternative interpretations. Indeed, lineage tracing versus spermatogonial transplantation analyses can facilitate interpretation of two very different conclusions for the same molecular marker. Further, even when lineage tracing and spermatogonial transplantation outcomes do agree that a selected population is highly enriched for SSCs, such as in the case of ID4, the possibility that progenitor spermatogonia are also selected by a marker can only be ruled out using a limiting dilution transplantation approach. Thus, although it is certainly possible that markers such as PAX7 and ID4 select for different subsets of a heterogeneous population of SSCs, more comparable analyses are required in order to make this determination.

6. Conclusions

There is much ambiguity within the field of germline stem cell research as to markers that are expressed specifically by SSCs and not by undifferentiated progenitors. These inconsistencies are the consequence of using alternative methodologies for the assessment of SSC purity of selected populations; namely lineage tracing versus spermatogonial transplantation, along with the variable interpretations of outcomes from these assays. To avoid the generation of conflicting data within the field, we suggest that it would be beneficial to standardize a limiting dilution transplantation assay as the methodology by which SSC purity is determined within a population expressing a marker of interest. Quantitative values generated from this assay reflect SSC purity in the donor population, as has been demonstrated with ID4-eGFP^{Bright} spermatogonia. This means that comparisons can be made between different research groups, while also facilitating direct comparisons of effectiveness of SSC isolation by different markers. We propose that adoption of this technique across the field would be useful for unequivocal validation of specificity of markers that are commonly considered to be SSC specific, as well as for assessing emerging putative markers of SSCs.

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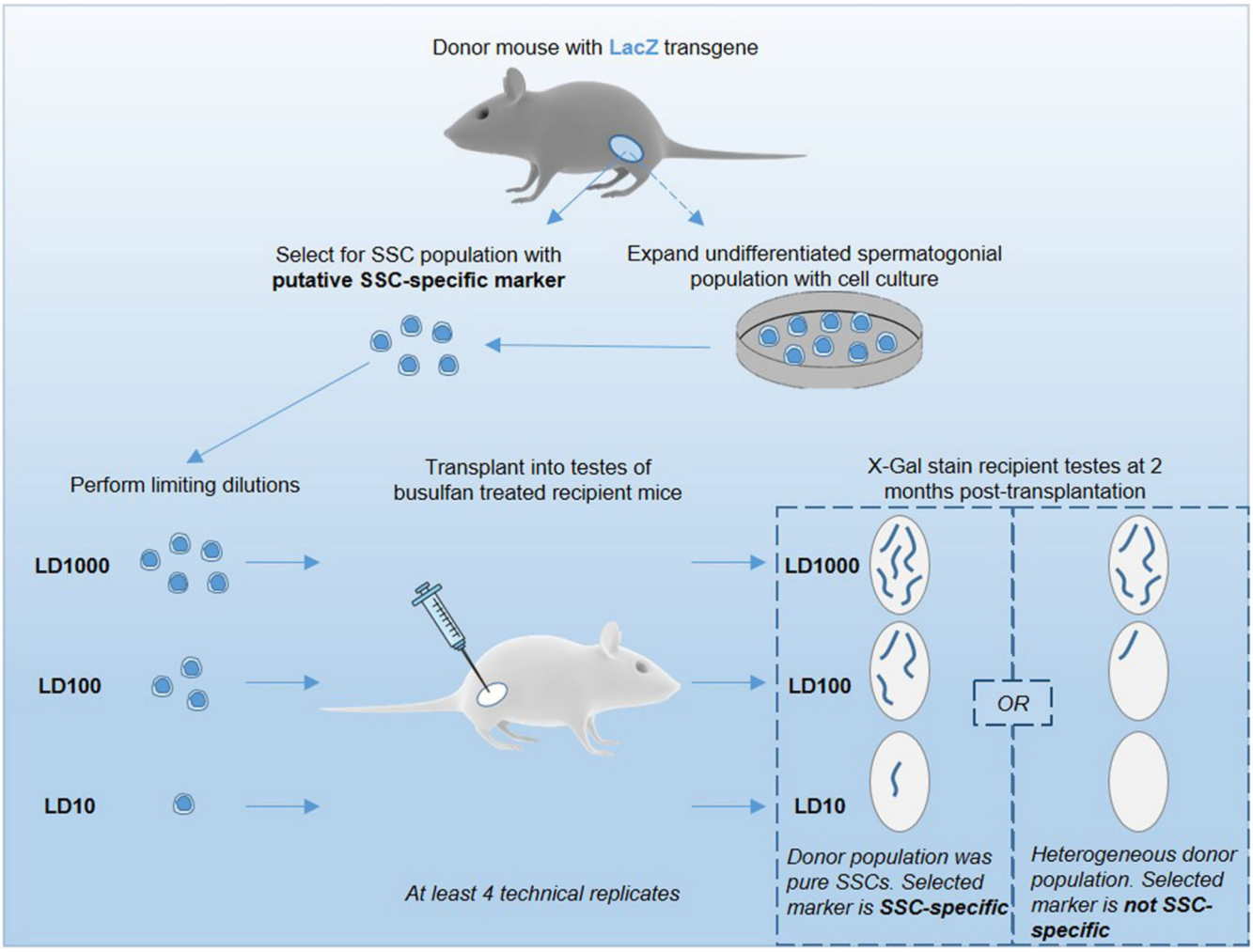

Fig. 1.

A limiting dilution transplantation assay to determine SSC content in undefined testis cell populations. Testis cells are collected from a donor mouse harboring a ubiquitously expressed *LacZ* transgene. A sub-population of cells are then isolated based on expression of a marker of interest using cell sorting strategies. Selected cells are subjected to a limiting dilution series, creating populations of 1000 (LD1000), 100 (LD100) and 10 (LD10) cells. These populations are transplanted into the testes of busulfan treated mice. At >2 months post-transplantation, *LacZ* expressing donor-derived colonies are visualized in recipient testes following X-gal staining. By factoring in a colonization efficiency of 5%, it is expected that donor populations containing pure SSCs would produce colonies not only in LD1000 and LD100 transplanted testes, but also in a subset of LD10 treated testes. Contrastingly, colony number would be expected to be reduced in LD100 and absent in LD10 transplanted testes if the donor population was a heterogeneous mix of SSC and transit amplifying progenitor spermatogonia. A quantitative value reflecting SSC purity in the donor population can be calculated using the formula: [# cells transplanted / (# colonies

of donor-derived spermatogenesis/5% colonization efficiency)] = *x*, where 1 in every *x* cells is an SSC.