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REVIEW

Mechanisms regulating mammalian spermatogenesis and fertility recovery following germ cell depletion

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Abstract

Mammalian spermatogenesis is a highly complex multi-step process sustained by a population of mitotic germ cells with self-renewal potential known as spermatogonial stem cells (SSCs). The maintenance and regulation of SSC function are strictly dependent on a supportive niche that is composed of multiple cell types. A detailed appreciation of the molecular mechanisms underpinning SSC activity and fate is of fundamental importance for spermatogenesis and male fertility. However, different models of SSC identity and spermatogonial hierarchy have been proposed and recent studies indicate that cell populations supporting steady-state germline maintenance and regeneration following damage are distinct. Importantly, dynamic changes in niche properties may underlie the fate plasticity of spermatogonia evident during testis regeneration. While formation of spermatogenic colonies in germ-cell-depleted testis upon transplantation is a standard assay for SSCs, differentiation-primed spermatogonial fractions have transplantation potential and this assay provides readout of regenerative rather than steady-state stem cell capacity. The characterisation of spermatogonial populations with regenerative capacity is essential for the development of clinical applications aimed at restoring fertility in individuals following germline depletion by genotoxic treatments. This review will discuss regulatory mechanisms of SSCs in homeostatic and regenerative testis and the conservation of these mechanisms between rodent models and man.

Keywords Spermatogonial stem cells · Regeneration · Spermatogenesis · Fertility · Adult stem cells

Introduction

Maintenance of the mammalian male germline is supported by a rare population of spermatogonial stem cells or SSCs, which self-renews and differentiates for continuous production of gametes through the tightly controlled process of spermatogenesis. Male and female gametes (spermatozoa and oocytes, respectively) pass on genetic information to the next generation and are, therefore, essential for species survival [1, 2]. While the fundamental role of SSCs in male fertility is readily appreciated, contrasting models of SSC identity and cellular hierarchy in the male germline are proposed [3–6]. Moreover, the molecular mechanisms responsible for maintaining SSC function and regulating SSC-dependent germline regeneration following damage are incompletely understood.

Mammalian spermatogenesis takes place within the testis seminiferous epithelium and can be broadly divided into the following stages: mitotic divisions of spermatogonia, meiotic division of spermatocytes, and maturation of haploid spermatozoa. Spermatogenesis in mice takes 35 days to complete and is critically reliant on a population of somatic cells within the seminiferous tubules, known as Sertoli cells [7, 8]. Sertoli cells have extended cytoplasmic projections that make contact with germ cells at multiple stages of differentiation. Sertoli cells also produce key growth factors required for maintenance of both SSCs and differentiating spermatogonia [9–11]. Importantly, tight junctions between the Sertoli cells constitute the blood–testis barrier (BTB) that functionally separates basal and adluminal tubule compartments [1, 12]. Within the basal compartment, SSCs undergo mitotic divisions to produce SSCs for self-renewal and A-type progenitor spermatogonia that are destined to

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differentiate [12, 13]. As the meiotic program is initiated, spermatocytes migrate away from the basement membrane and through the BTB into the adluminal space to continue meiosis, where homologous recombination takes place, random segregation of paternal and maternal chromosomes occurs, and haploid spermatids are ultimately generated [3, 8]. These spermatids subsequently mature to become spermatozoa that are ready to be released from the seminiferous tubule lumen and transported to the epididymis. Therefore, as spermatogenesis progresses, germ cells move away from the seminiferous tubule basement membrane towards the lumen, resulting in a stratification of differentiating germ cells [1, 12] (Fig. 1).

The continuous production of spermatozoa and hence maintenance of male fertility is absolutely dependent on SSCs, which are known to be regulated through an extensive array of cell-intrinsic and extrinsic factors from the niche. Both SSCs and some progenitor populations are proposed to contribute to germline regeneration following damage, e.g., in response to systemic genotoxic therapy. This review will focus on stem cell models proposed for the male germline, the molecular mechanisms responsible for regulating SSC function, and SSC behaviour during germline regeneration.

Spermatogonial hierarchy

Spermatogenesis commences upon production of differentiation-destined daughter cells from SSC division that subsequently undergo mitotic expansion prior to entering meiosis (Fig. 2) [2, 14–16]. From studies of rodents, the traditional "A_s model" of spermatogonial hierarchy was proposed by Huckins and Oakberg and described the most primitive spermatogonia as single-isolated cells, called A_{single} spermatogonia (A_s) [17, 18]. Upon mitotic division, A_s cells can generate A_s for self-renewal or differentiation-committed A_{paired} (A_{pr}) daughters that remain connected by an intercellular bridge due to incomplete cytokinesis. Subsequent division

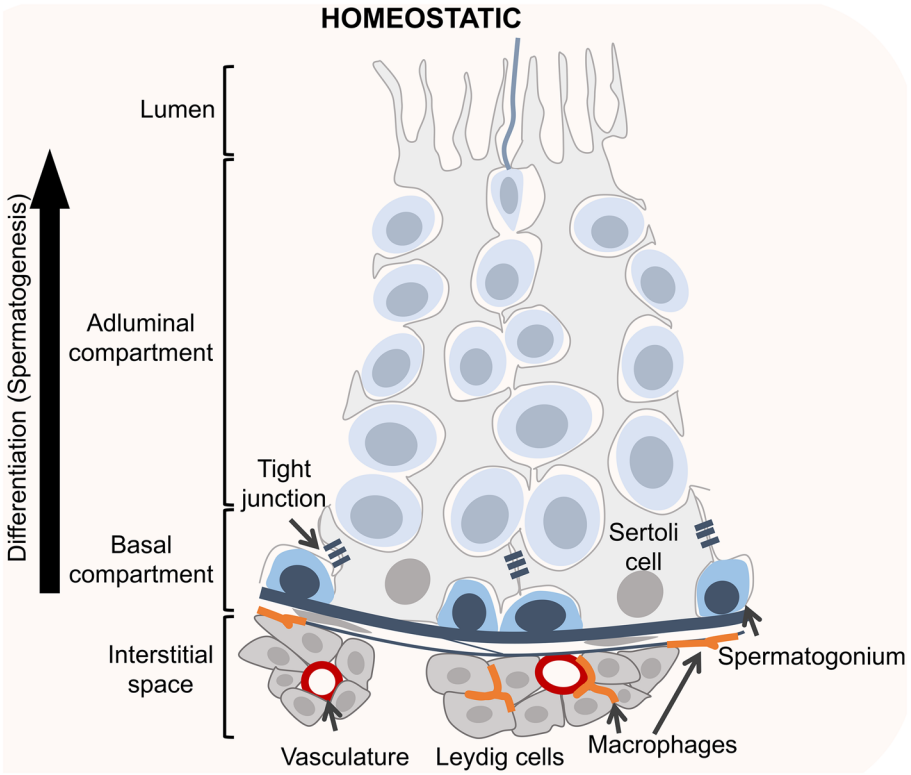


Fig. 1 Schematic cross-section of seminiferous epithelium and interstitial tissue of mouse testis. In adult seminiferous tubules germ cells at all stages of spermatogenesis are surrounded by Sertoli cells, which play crucial roles in supporting spermatogonial stem cell (SSC) function and spermatogenesis. Sertoli cells form tight junctions with neighbouring Sertoli cells that functionally separate the basal and adluminal compartments. Spermatogonia (undifferentiated and differentiating) are present in the basal compartment. As meiosis progresses, germ cells migrate across Sertoli cell tight junctions into the adluminal compartment and generate mature spermatocytes then haploid spermatozoa. Peritubular myoid cells surround the outer surface of seminiferous tubules, provide structural support and produce growth factors. The interstitial space located between seminiferous tubules contains Leydig cells, macrophages and the vasculature, which play important roles in supporting spermatogenesis. A unique population of peritubular macrophages are also found surrounding the tubule

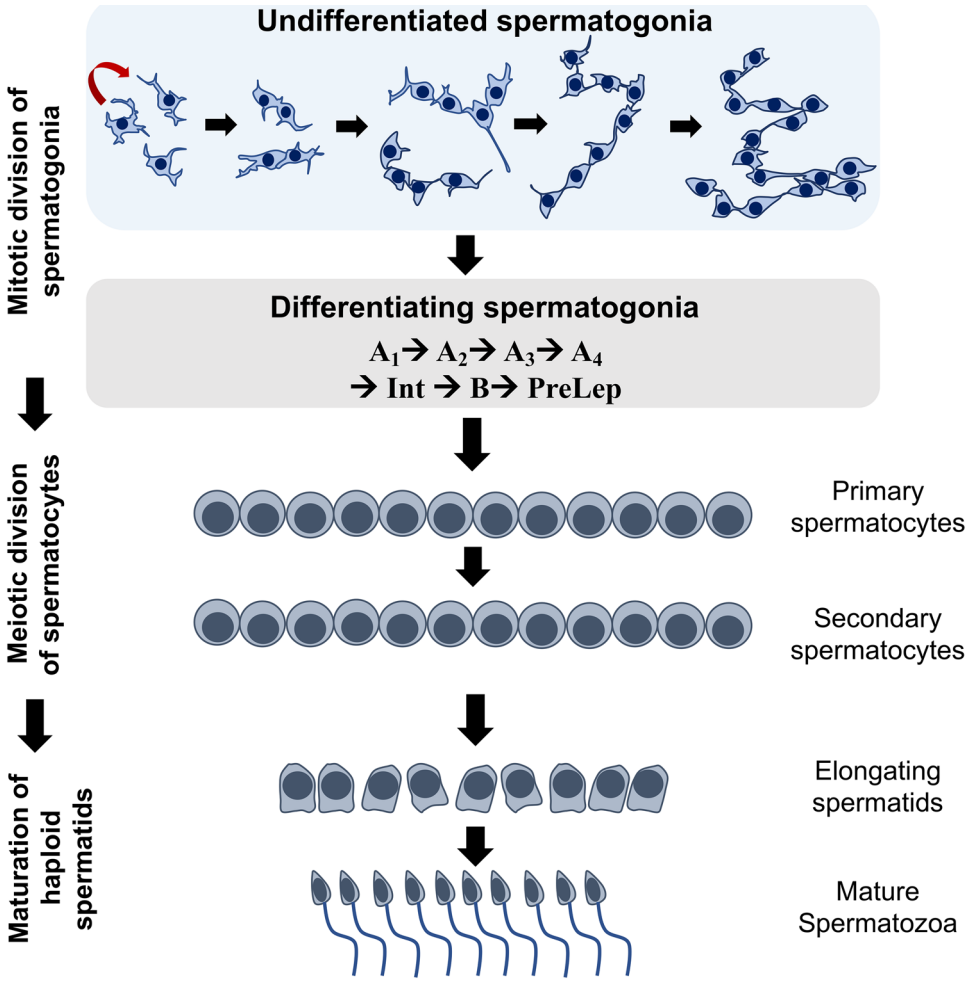


Fig. 2 Overview of spermatogenesis in the mouse. Germline maintenance is dependent on a population of undifferentiated spermatogonia (A_s, A_{pr}, and A_{al} cells) with self-renewal potential. In undisturbed testis, only a subset of undifferentiated cells self-renews, while the bulk acts as transit-amplifying progenitors. Spermatogenesis is initiated when undifferentiated spermatogonia (particularly A_{al} cells) undergo differentiation commitment and produce A_1 cells, which then undertake a series of co-ordinated mitotic divisions prior to entering meiosis and generating spermatocytes. Differentiating spermatogonia of each mitotic division have unique nomenclature (A_1, A_2, A_3, A_4, intermediate/Int, and B-type spermatogonia). Upon meiotic initiation, spermatocytes migrate into the adluminal compartment of the seminiferous tubules to complete meiosis and ultimately give rise to haploid round spermatids. These spermatids undergo a process of maturation or spermiogenesis to generate mature motile spermatozoa that are then released into the seminiferous epithelial lumen

of A_{pr} produces interconnected chains of $2n$ daughter cells of generally between 4 and 16 cells, known as $A_{aligned}$ (A_{al4}, A_{al8}, and A_{al16}). The A_s model proposes that A_s are SSCs and A_{pr} plus A_{al} are committed progenitors or transit-amplifying cells. However, A_s, A_{pr}, and A_{al} share a similar cellular morphology except for chain length and are collectively referred to as undifferentiated spermatogonia [2, 12, 19]. Spermatogenesis is a cyclic process and the seminiferous epithelium of mouse can be divided into 12 stages (I–XII) [8, 20]. A_{al} cells accumulate during early stages of the epithelium cycle (II–VI), while relatively constant numbers of A_s and A_{pr} are present throughout the cycle [15]. At mid-cycle stages (VI–VIII), essentially, all A_{al} generated from undifferentiated cell proliferation convert to A_1 differentiating spermatogonia without cell division, which then undergo a rapid series of co-ordinated mitotic divisions prior to entering meiosis [16]. Each generation of daughter spermatogonia generated by differentiating cell divisions has distinct nomenclature; A_1 cells generate A_2 spermatogonia that form A_3, A_4, Intermediate (In), and finally B-type spermatogonia (Fig. 2). Different stages of the seminiferous epithelium are characterised by the presence of distinct generations of differentiating spermatogonia that divide in a synchronized fashion at defined points in the cycle [8, 20, 21].

It was widely considered that the formation of stable intercellular bridges between spermatogonia forecasts differentiation commitment and the process of increasing chain length is strictly unidirectional and irreversible [2,

22]. However, as discussed in more detail below, with the advent of genetic tools and molecular markers, researchers in the field have developed alternative models that describe SSC identity and spermatogonial hierarchy. The generation of such models is dependent on distinct functional assays for SSC activity [23, 24].

SSC functional assessment

Transplantation assay

Development of the germ cell transplantation technique in 1994 was a critical milestone in SSC research. This assay involves the preparation of a testis cell suspension and injection into the seminiferous tubules of recipient mice depleted of endogenous germ cells through chemical or genetic means [23, 25]. Only spermatogonia with stem cell potential successfully engraft in the seminiferous tubule basal layer and generate stable spermatogenic colonies. Genetic markers such as lacZ-based reporter transgenes are used to identify donor-derived colonies post-transplant, which are then scored to provide a measure of stem cell abundance in the donor cell preparation. The transplantation technique, initially developed in mice, not only provides a gold-standard method of assessing SSC activity through restoration of spermatogenesis in infertile recipients, but also provides a unique experimental system allowing examination of the interaction between SSCs and their niche during regeneration [25, 26]. Notably, SSCs from a variety of species including rat, rabbit, pig, cow, baboon, and human can colonize seminiferous tubules of immune-deficient mice to varying extents, confirming the conserved nature of the SSC niche [26]. The transplantation assay is routinely employed in the field to confirm functional roles of candidate genes in SSC self-renewal or to compare stem cell activity in distinct fractions of sorted testis cells [27–30]. Through qualitative and quantitative analyses of transplantation, important information regarding stem cell function and niche environment can be derived. The number of colonies, referring to patches of spermatogenesis generated from individual transplanted stem cells, correlates to the number of spermatogonia capable of acting as stem cells in the donor population. In addition, the assessment of colony appearance, size, and extent of growth plus differentiation can reflect the influence of recipient testis environment on colonisation and/or functional characteristics of transplanted cells [31–33]. Therefore, the germ cell transplantation assay allows a robust quantitative assessment of stem cell numbers plus analysis of functional characteristics of stem cells in donor samples.

While the transplantation assay provides unequivocal evidence of stem cell activity in donor populations, it is becoming apparent that it suffers a critical limitation. Namely, that transplantation appears unable to distinguish whether cells function as stem cells in situ in intact tissue, simply that populations have stem cell potential. A well-studied example concerns spermatogonia expressing *Neurogenin3* (NGN3+), which primarily marks A_{al} within the undifferentiated population [24, 34]. NGN3 is a basic helix–loop–helix (bHLH) transcription factor, many of which are implicated in cellular differentiation in various mammalian tissues [35]. Lineage-tracing studies of homeostatic tissue have demonstrated that the NGN3+ population is principally differentiation-committed; descendants of NGN3+ cells typically enter the spermatogenic pathway and are ultimately lost from the testis through differentiation [24, 34]. However, lineage-marked NGN3+ spermatogonia can function much more effectively as stem cells and support multiple rounds of spermatogenesis when transplanted [24]. Similarly, a recent study identified a novel sub-population of NGN3+ spermatogonia marked by expression of *Miwi2* (also known as *piwi like RNA-mediated gene silencing 4*). MIWI2+ spermatogonia act as a transit-amplifying committed cell population in steady-state testis and make a limited contribution to the SSC pool, but exhibit robust regenerative capacity and the ability to support multiple rounds of spermatogenesis when transplanted [36, 37]. The assessment of transplantation capabilities of undifferentiated cells sorted according to expression of the cell-surface receptor glial cell line-derived neurotrophic factor family receptor α1 (GFRα1) revealed a comparable scenario [29]. GFRα1 marks most A_s and a majority of A_{pr} spermatogonia and expression is generally mutually exclusive with that of the progenitor marker NGN3 [5, 34]. Importantly, lineage-marked GFRα1+ cells make a consistent contribution to the GFRα1+ population over time plus generate persistent-labelled spermatogenic patches in steady-state tissue, suggesting that the SSC population is contained within the GFRα1+ cell pool [5, 34]. When transplanted, GFRα1+ cells effectively generate long-lived spermatogenic colonies as anticipated [29]. However, GFRα1− undifferentiated cells also produce stable colonies when transplanted albeit at a lower frequency than the GFRα1+ population [29]. These observations were also recapitulated in an independent study using an Oct4-GFP reporter mouse [30]. Within the adult undifferentiated pool, Oct4-GFP expression unexpectedly marks GFRα1− cells and is mostly undetectable in the SSC-enriched GFRα1+ fraction, indicating that Oct4-GFP marks a similar population as NGN3 and MIWI2. Importantly, both Oct4-GFP+ and GFP− undifferentiated spermatogonia produce long-lived spermatogenic colonies, although transplantation capacity is enriched within the GFP− fraction as anticipated [30]. Combined, these studies indicate that undifferentiated spermatogonia unable to contribute significantly to the stem cell pool under homeostatic conditions are still able to regenerate the germline and produce stable spermatogenic colonies upon transplantation. Such

populations can be considered "potential stem cells" and are found from studies of MIWI2+ spermatogonia to underpin efficient tissue recovery under regenerative conditions (see below) [24, 29, 30, 34, 36].

While quantitative analysis of donor-derived colonies post-transplantation allows assessment of stem cell potential, it provides more limited information on contribution of cells to the steady-state SSC pool in situ. The functional assessment of stem cell activity through transplantation alone can, therefore, be misleading. Interestingly, the "de-differentiation" of cells typically differentiation-committed in undisturbed tissue to a stem cell fate upon tissue injury is observed in many mammalian organ systems [38, 39]. The germ cell-depleted recipient testis is expected to mimic a damaged tissue and can provide appropriate cues for progenitor cells to revert back to a stem cell fate [40–42]. Such cues may involve increased production of the niche-derived growth factor GDNF, a key mediator of SSC self-renewal [40–42]. Moreover, transplanted spermatogonia are introduced directly into the lumen of the depleted seminiferous tubules and must migrate to the basal compartment through a partially intact BTB to generate a spermatogenic clone [33]. Isolated spermatogonia are, of course, not normally found in the lumen of empty seminiferous tubules in steady-state testis. The reconstruction of a spermatogenic seminiferous epithelium is not normally achieved by a single-cell suspension containing SSCs. It can, therefore, be argued that the transplantation assay, while a landmark development in the field, is of limited physiological relevance. More studies are required that definitively address the association between transplantation capabilities and homeostatic stem cell activity.

Lineage tracing

Given evident limitations of the transplantation assay, genetic labelling of cells and their progeny through lineage tracing or pulse-labelling approaches represent a powerful experimental approach to assess stem cell activity in the undisturbed tissue in vivo [43, 44]. Most lineage-tracing studies utilise the inducible CreER transgenic system, in which a cell-type specific promoter drives expression of a tamoxifen-activated Cre recombinase. The CreER line is then crossed with a Cre-dependent reporter line (commonly within the ubiquitously expressed *Rosa26* locus) carrying a cassette encoding either the LacZ enzyme or a fluorescent protein [43, 45]. Upon tamoxifen treatment, the activated Cre excises a floxed STOP cassette to allow permanent expression of the reporter gene in respective cells and all progeny. Hence, fate and behaviour of lineage-marked cells can be traced over time in intact tissue [39, 43, 44]. In renewing tissues such as the testis, long-term persistence of lineage-marked cell clones within the tissue indicates that the labelled population included homeostatic stem cells. This experimental system has been applied extensively within the male germline to help define the stem cell compartment under steady-state conditions, as summarised in Table 1 [5, 6, 24, 46–49].

Lineage-tracing studies together with mathematical modelling approaches have provided critical insight into mechanisms of SSC self-renewal (Fig. 3) [50, 51]. Specifically, tissue homeostasis is dependent on balanced division of fate amongst daughter cells from dividing stem cells, such that equal proportions of stem and differentiation-committed cells are generated. Distinct mechanisms are proposed to account for the balance of fate amongst progeny arising from stem cell division [44, 52]. The first is "invariant asymmetric cell division", in which a stem cell division strictly produces one stem cell and one differentiation-committed progenitor to maintain a constant number of stem cells in the tissue while generating differentiating progeny [52]. If this was the case in the mammalian germline system, lineage-marked cell clones derived from labelled SSCs would have a constant size when followed over time and there would be a constant number of labelled colonies within the testis, reflective of the number of SSCs initially labelled [50]. In

Table 1 Lineage tracing studies characterizing clonal behaviours in mice

Makers/transgene	Mice	Time-point of analysis	Clonal behaviour
Ngn3/CreERT2; *Rosa26*-LacZ [24]	Adult	3, 4, 6, 10 and 14m	Reduced clone number and increased clone size
Gfra1/CreERT2; *Rosa26*-LacZ [5]	Adult	2, 3, 6, 10 and 14m	Reduced clone number and increased clone size
Nanos2/CreERT2; *Rosa26*-LacZ [49]	Adult	18w, 22w and 26w	Reduced clone number and increased clone size
Bmi1/CreERT2; *Rosa26*Rbw [46]	Adult	6w, 12w, 24w and 48w	Reduced clone number and increased clone size
Axin2/CreERT2; *Rosa26*Rbw [47]	Adult	1w, 2w, 4w, 12w and 6m	Reduced clone number and increased clone size
Pax7/CreERT2; *Rosa26*-LacZ [6]	Adult	1w, 4w and 6w	Constant clone number and increased clone size
Pax7/CreERT2; tdTomato/eGFP [6]	Adult	1w, 4w, 6w, and 16w	Constant clone number and increased clone size
Id4/CreERTM-tdTomato; *Rosa26*-LacZ [48]	Adult	2d, 5d, 35d, 2, 5, and 13m	N/A
Id4/CreERT2-tdTomato; *Rosa26*-LacZ [48]	Juvenile	3w, 4w and 6w	Constant clone number and constant clone size

d days, *w* weeks, *m* months

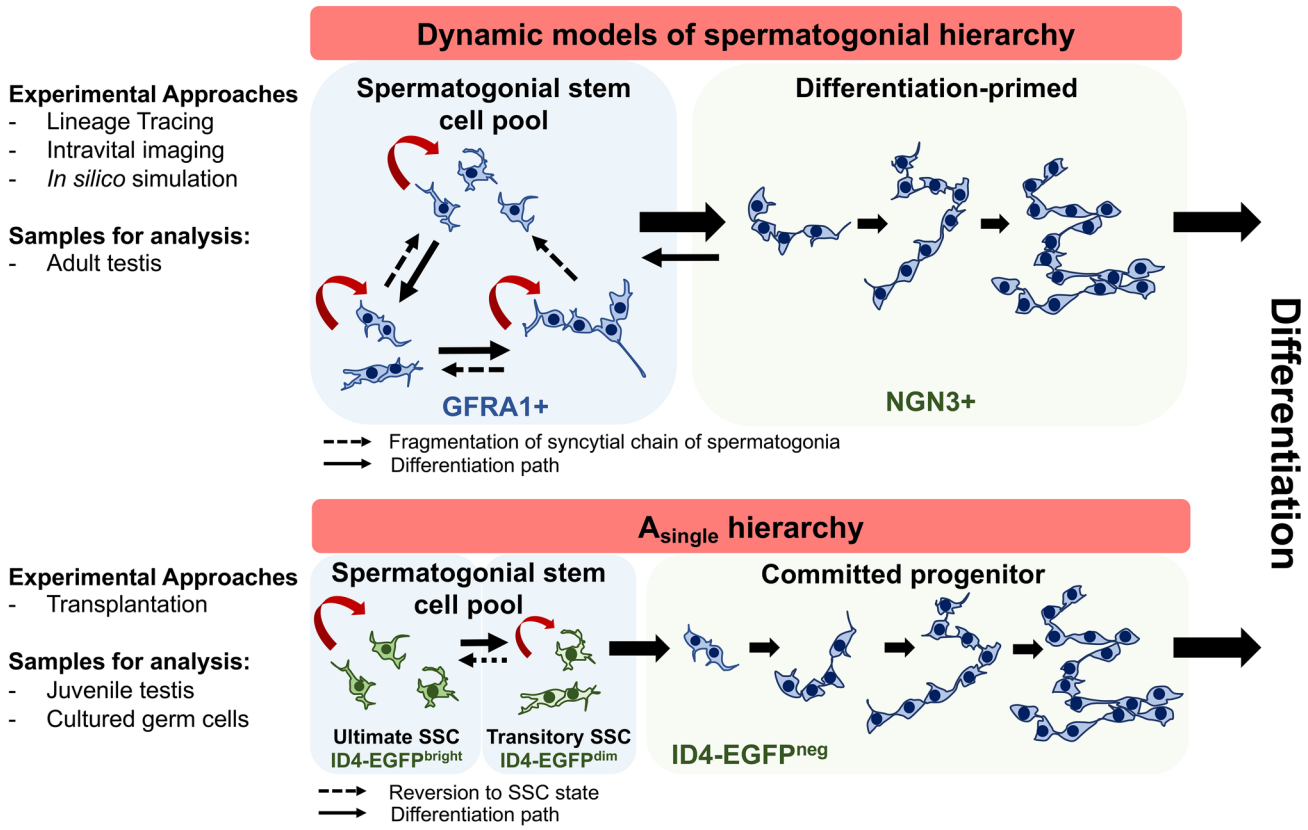


Fig. 3 Spermatogonial stem cell models and spermatogonial hierarchy. According to a dynamic SSC model, a minor fraction of undifferentiated spermatogonia marked by GFRα1+ (typically A_s, A_{pr} and A_{al4}) represents the SSC pool of homeostatic testis. Dividing GFRα1+ cells continually interconvert between isolated and syncytial states through a process of chain fragmentation. In terms of stem cell activity, GFRα1+ A_s, A_{pr} and A_{al4} spermatogonia are equipotent. This model also proposes that the bulk of the undifferentiated pool is marked by NGN3 and under steady-state conditions is mostly differentiation-committed. However, NGN3+ undifferentiated cells can also revert to a GFRα1+ stem cell state through a process of chain fragmentation and switch in gene expression pattern and, therefore, possess latent stem cell activity. The NGN3+ undifferentiated population can, therefore, be regarded as being differentiation-primed rather than differentiation-committed. Contribution of NGN3+ undifferentiated spermatogonia to the SSC compartment is minimal in undisturbed tissue but substantially enhanced following germline depletion and induction of a regenerative response. In contrast, an A_{single} hierarchical model suggests that only a subset of A_s are stem cells (referred to as ultimate SSCs or $SSC_{ultimate}$) and differentiation progresses in a linear and irreversible direction. A large fraction of A_s plus all A_{pr} and A_{al} are, therefore, irreversibly differentiation-committed although cells transiting from an SSC to progenitor state may retain the ability to revert to an SSC state under regenerative conditions. High levels of *Id4* expression mark the $SSC_{ultimate}$ population. As indicated, the dynamic and hierarchical stem cell models are based on data from distinct experimental approaches and testis samples

contrast, "populational asymmetric cell division" proposes that when averaged across the whole SSC pool, cell divisions generate balanced numbers of SSCs and committed progenitors for stem cell maintenance and generation of differentiating progeny to feed the spermatogenic pathway. This populational model predicts that daughter cells from SSC divisions have a similar probability of being stem or progenitor cells rather than invariably one SSC and one progenitor [44, 51]. In this case, lineage-marked colonies are predicted to increase in size over time, but colony numbers per testis should decrease, as some of the labelled SSCs are lost to differentiation in a stochastic fashion [44, 50, 52].

To date, lineage-tracing studies in which CreER is expressed in a variety of adult spermatogonial populations (GFRα1+, BMI1+, NANOS2+, NGN3+, and AXIN2+) support a populational asymmetric cell division model as the mechanism of SSC maintenance, in which labelled colonies reduce in number when followed over time (at least 6 months), but mean colony length increases (Table 1) [5, 24, 46, 47, 49]. However, interesting observations were made when tracing fate of spermatogonia marked by PAX7, which is expressed in rare A_s cells [6]. Specifically, a constant number of colonies were found, and colony length was also seen to increase over a 16-week period post-labelling. These observations support elements of both stem cell division models, but analysis was only performed for up to 16 week post-labelling, whereas other lineage-tracing studies were followed for more than 6 months to observe a decline in

labelled colony numbers. As discussed in more detail below, ID4 is increasingly used as a unique SSC marker [28], and a lineage-tracing study employing a knock-in *Id4* CreER line demonstrated that long-term (> 1 year post-tamoxifen) lineage-marked clones are generated following labelling of ID4+ spermatogonia [48]. While these data confirmed that ID4 marks SSCs in undisturbed adult tissue, no quantitative data over this extended time course were provided, and therefore, the mode of self-renewal of ID4+ SSCs could not be inferred [48].

From empirical evidence, the numbers of labelled spermatogenic colonies initially generated in lineage-tracing experiments and the degree of persistence of marked colonies at early timepoints post-labelling are dependent on the gene regulatory elements used to drive expression of Cre recombinase [39, 43]. Specifically, whether the corresponding genes preferentially mark stem (*Bmi1*, *Gfra1*, *Id4*, *Nanos2,* and *Pax7*) or differentiation-primed (*Ngn3* and *Axin2*) populations under homeostatic conditions. For instance, the rapid decline of marked colonies after labelling NGN3+ spermatogonia reflects the predominant expression of *Ngn3* within differentiation-destined A_{al} of the undifferentiated pool [24]. In contrast, numbers of lineage-marked colonies produced by labelling NANOS2+ or BMI1+ cells only reduce modestly over-extended periods of time, confirming predominant expression of *Nanos2* and *Bmi1* in SSC populations [46, 49].

A primary drawback of lineage-tracing approaches is that data interpretation is heavily dependent on how faithfully the cell population of interest is initially labelled, which is dependent on specificity and efficiency of transgenes used, tamoxifen dose, and knowledge of gene expression patterns [39, 43, 50]. For example, a LacZ-based reporter for the WNT-responsive gene *Axin2* indicated endogenous *Axin2* expression and hence WNT signalling in 77.5% and 53.5% of GFRα1+ and PLZF+ spermatogonia, respectively, in adult mice. This suggested that the stem-enriched GFRα1+ population is more WNT responsive when compared to the bulk undifferentiated and early differentiating PLZF+ population. However, lineage-tracing labels based on the *Axin2* promoter ineffectively marked the GFRα1+ pool (12.8%), but effectively labelled PLZF+ cells (95.5%) [47]. In this instance, the lineage-marked cell population may not be reflective of endogenous *Axin2* expression, with reporter gene recombination instead occurring more efficiently in the progenitor pool. Regardless, the persistence of lineage-marked colonies in this model up to 6 months after tamoxifen confirmed that despite preferential labelling of progenitor cells rather than SSCs, *Axin2* was expressed by cells with long-term stem cell potential and, therefore, that this population is WNT-responsive [47]. These findings are also consistent with observations made in NGN3+ lineage-tracing models, in which a small subset of NGN3+ progenitor cells can function as stem cells that persist and support spermatogenesis for up to 14 months [24].

Collectively, the use of both transplantation and lineage-tracing approaches are essential to definitively assess SSC activity. While the lineage-tracing approach provides a more qualitative analysis of stem cell function under homeostatic conditions over-extended periods of time, the transplantation assay allows a detailed quantitative analysis of stem cell activity in donor populations. Combined, these approaches can comprehensively define SSC activity of cells marked by a gene of interest.

Spermatogonial stem cell models

Dynamic models of spermatogonial hierarchy and SSC identity

The traditional "A_s model" was based on meticulous histological analysis of cell populations in the rodent testis throughout the seminiferous epithelium cycle [17, 18]. This model proposed that A_s are the most primitive cells within the testis and form a homogenous self-renewing population, while A_{pr} and A_{al} are committed progenitors/transit-amplifying cells without self-renewal capabilities. The A_s model has been challenged by a number of studies over the last decade upon technological advances [5, 24, 30, 34]. Definitive markers distinguishing SSCs from differentiation-committed cells within the adult undifferentiated population are not universally agreed upon in the field [5, 24, 28, 34, 36, 53]. However, recent SSC studies have been based on expression of a variety of genes proposed to mark functional SSCs in transplantation and/or lineage-tracing assays [5, 6, 28, 30, 46, 48]. The niche-derived growth factor GDNF is established to be essential for SSC self-renewal and the co-receptor for GDNF, GFRα1, marks the large majority of A_s plus most A_{pr} cells, suggesting that SSCs are marked by GFRα1, in agreement with lineage-tracing data [5]. In contrast, the expression of *Ngn3* predominantly marks A_{al} within the undifferentiated population is only detected in a minority of A_{pr} plus rare A_s and is suppressed by GDNF stimulation [24, 34, 54]. As predicted, *Ngn3* expression is also largely, albeit not entirely, mutually exclusive with that of *Gfra1*, together supportive of the conclusion that NGN3 marks the earliest stages of differentiation commitment [55]. Utilising *Ngn3* as a gene of interest to study undifferentiated spermatogonial dynamics, Nakagawa et al. [24] demonstrated that under steady-state conditions, *Ngn3* marks undifferentiated spermatogonia destined to differentiate to A_1 and enter the spermatogenic pathway. However, under conditions of tissue regeneration such as transplantation or busulfan-induced germ cell depletion, NGN3+ spermatogonia could act relatively efficiently as stem cells and contributed to regeneration of spermatogenesis [24]. These observations

lead to classification of NGN3+ cells as "potential stem cells" due to the ability of NGN3+ spermatogonia to revert from a differentiation-committed or differentiation-primed state under homeostatic conditions to an SSC state during germline repair after damage [24]. Interestingly, however, lineage-tracing studies of NGN3+ cells in undisturbed testis demonstrated that rare-persistent-labelled colonies were also present up to 14-month post-labelling, suggesting that a small fraction of the NGN3+ population can also function as stem cells in homeostatic conditions and support long-term spermatogenesis [24, 51]. This study was the first to provide evidence of plasticity of cell fate within the undifferentiated population and that stem cell capabilities are likely not limited to A_s cells as originally proposed.

According to the traditional A_s model, formation of a stable intercellular bridge upon SSC division and formation of A_{pr} are the first and irreversible step in the spermatogenic pathway. Increasing length of the syncytial chains of spermatogonia is reflective of progression along the differentiation pathway [1–3]. Thus, chain length is one of the key morphological features to distinguish SSCs from their differentiated-committed daughters [2, 16]. Transplantation studies confirm that stem cell potential is strongly enriched in but not exclusive to the GFRα1+ undifferentiated fraction, which represents the majority of A_s and most A_{pr} [29]. However, lineage-tracing data demonstrate that under homeostatic conditions, essentially, all SSC activities are contained within the GFRα1+ population [5, 29]. GFRα1+ cells will generate NGN3+ A_{pr} and A_{al} cells, which then differentiate to become A_1 [5, 24, 34]. Importantly, the heterogeneous expression of marker genes among A_s, A_{pr}, and A_{al} suggests that differentiation does not necessarily follow a strict linear process in which gene expression coupled with chain length provides an accurate indicator of cell fate. Namely, A_s can express markers of differentiation priming and commitment including both *Ngn3* and *Kit*, meaning that not all A_s are equivalent and not all A_s function as stem cells [34].

Studies based on lineage-tracing data have provided evidence to challenge the notion of a definitive, long-lived stem cell compartment and instead support "neutral drift" or "neutral competition" stem cell models within the mammalian germline [44, 50]. By applying mathematical and in silico simulation approaches, Klein et al. [51] concluded that the continuous depletion of lineage-marked colonies plus increased heterogeneity of colony size as observed using *Gfra1* and *Ngn3* CreER reporters, and is due to rapid turnover of SSCs in a stochastic manner and replenishment by neighbouring stem cells over time [50, 51]. Replacement of dying or differentiating SSCs requires migration of neighbouring SSCs, either GFRα1+ cells or cells that revert from the NGN3+ to GFRα1+ state, to occupy the vacant niche and maintain constant SSC numbers [5, 34, 51]. Indeed, GFRα1+ spermatogonia are evidently highly mobile when compared to NGN3+ spermatogonia, supporting the role of GFRα1+ spermatogonia as stem cells [5, 51]. Interestingly, by intravital live imaging, GFRα1+ A_s spermatogonia were found to predominantly undergo division to produce A_{pr} then A_{al4}, and complete division of A_s to produce 2 A_s is very rare (once per 5–6 months). This observation raises a critical question of how such a rare event of A_s symmetrical renewal can maintain a constant number of SSCs over time? Strikingly, these live-imaging studies also showed that the replenishment of A_s and A_{pr} is accomplished by chain "fragmentation" when intercellular bridges in syncytial chains of GFRα1+ A_{pr} and A_{al4} break down to produce shorter chain fragments [5]. The dynamics of GFRα1+ spermatogonia reversibly transitioning between A_s, A_{pr}, and A_{al} states through division and chain fragmentation is thought to be important for maintaining a constant number of GFRα1+ SSCs while allowing continuous transition of GFRα1+ to NGN3+ spermatogonia for differentiation (Fig. 3). Importantly, chain fragmentation appears to occur most frequently amongst GFRα1+ spermatogonia, whereas NGN3+ differentiation-primed spermatogonia have limited chain fragmentation and also self-renewal tendencies in the undisturbed testis. These observations re-enforce the functional hierarchy within the undifferentiated pool [5, 34].

Collectively, these studies support the notion that a pool of GFRα1+ self-renewing spermatogonia with similar potential to self-renewal and differentiate, maintain steady-state spermatogenesis. However, given that NGN3+ differentiation-primed cells within the undifferentiated pool are still capable of contributing to long-term spermatogenesis, particularly upon tissue damage, SSCs are not strictly marked by GFRα1 and undifferentiated cells can be considered equipotent in terms of stem cell capability [51]. While empirical evidence strongly supports a model of neutral competition to explain SSC homeostasis [5, 50, 51], the concept of chain fragmentation is still not widely accepted within the spermatogonial field. Furthermore, it remains unclear whether following fragmentation of A_{al}, A_s and A_{pr} generated function as stem cells, a limitation of live imaging. It may also be difficult to unequivocally distinguish fragmenting A_{al} from multiple actively migrating A_s and A_{pr} cells present at high density in the same area of tubule. Development of transgenic reporter mice expressing a fluorescently tagged intercellular bridge component, e.g., TEX14 or CEP55 [56, 57], within the spermatogonial pool would help to confirm the physical interrelationship of cells in live-imaging approaches.

Revised A_s model

While substantial evidence supports a model of SSC maintenance through chain fragmentation and fate reversibility,

studies from other groups have suggested the existence of a definitive SSC compartment within the undifferentiated pool in which stem cell activity is context-independent. Specifically, the SSC identity and function are determined by expression of inhibitor of DNA binding 4 (*Id4*) [4, 28, 48, 58]. The initial characterisation of ID4 as a novel SSC marker demonstrated that *Id4* expression mostly labelled a subset of GFRα1+/PLZF+ A_s spermatogonia in adult mouse testis [28, 48, 58]. ID4 expression in spermatogonia was found to be abundant in early postnatal development (93% of PLZF+ cells are ID4+ at PND3) but to decline substantially during testis maturation (2% of PLZF+ cells are ID4+ in 3-month-old adult testis) [58]. *Id4* expression is significantly enriched within the THY1+ cell fraction of pup and adult testis that contains transplantable spermatogonia [28]. Furthermore, culture-based assays demonstrate that *Id4* expression is strikingly responsive to levels of self-renewal factor GDNF, implying that ID4+ cells are responding to self-renewal stimuli [28]. Importantly, *Id4* expression in cultured spermatogonia is heterogeneous and transplantation activity essentially limited to the ID4+ population, indicating that SSCs are ID4+ and remaining ID4− undifferentiated cells committed progenitors [28, 58]. An independent study using an *Id4*-tdTomato-CreER line indicated a similarly restricted expression pattern of ID4 in A_s plus A_{pr} and ability of ID4+ spermatogonia to function as homeostatic stem cells by lineage-tracing studies that followed labelled colonies for more than 1 year [48]. Quantitative assessment of labelled progeny up to 6 weeks after tamoxifen treatment of juvenile mice found a constant number of marked colonies, suggesting that all ID4+ labelled cells are SSCs [48]. However, long-term dynamics of labelled colonies was not studied.

From whole-mount confocal analysis of ID4-GFP neonatal testis (postnatal day 8), populations of ID4-EGFP-^{bright} and ID4-EGFP^{dim} spermatogonia were identified [4]. Importantly, those cells with the highest *Id4* expression were mostly A_s plus some A_{pr}, while cells with low *Id4* expression were present as both A_s and A_{pr}. This gradient in ID4-GFP signal was also observed by flow cytometry of neonatal testis cells and ID4-EGFP^{bright} and ID4-EGFP^{dim} populations subsequently isolated for stem cell assay by transplantation. Importantly, the ID4-EGFP^{bright} population was substantially enriched in transplantable cells when compared to the ID4-EGFP^{dim} fraction, consistent with use of ID4 as SSC marker [4]. Furthermore, limiting-dilution analysis indicated that all ID4-EGFP^{bright} cells could function as SSCs in a transplantation setting. Combined, these data suggest that stem cell activity within the undifferentiated pool is primarily restricted to a subset of A_s cells marked by high ID4 levels [4]. However, due to likely differences in sensitivity of GFP detection by confocal microscopy of whole-mount tubules and flow cytometry of testis cell suspensions, it remains ambiguous whether ID4-EGFP^{bright} and ID4-EGFP^{dim} cells observed by confocal microscopy equate to the ID4-EGFP-^{bright} and ID4-EGFP^{dim} populations gated in flow sorting experiments. Furthermore, functional capabilities and biological relevance of ID4-EGFP^{intermediate} cells as detected by flow cytometry were not explored yet comprised a large proportion of the total ID4+ population (~40%) [4].

Besides use as an SSC marker, gain- and loss-of-function models of *Id4* have provided evidence for specific roles of ID4 in the male germline. *Id4*$^{-/-}$ adults are sub-fertile and display progressive loss of germline cells and PLZF+ spermatogonia with age, consistent with a role of ID4 in SSC maintenance and function [28]. *Id4* knockdown studies in cultured spermatogonia followed by transplantation confirm this role [28]. In contrast, constitutive overexpression of *Id4* within the mouse germline inhibited postnatal expansion of the undifferentiated spermatogonial pool and formation of committed progenitors [4]. Together, these data indicate that ID4 levels dictate the self-renewing state of spermatogonia.

From analysis of *Id4* expression pattern, observed transplantable activity of ID4+ cells and ID4 function in the stem to progenitor transition, a revised A_s model of spermatogonial hierarchy has been proposed [4, 58]. Specifically, a subset of A_s cells marked by high levels of *Id4* expression represents the SSC population in testis (referred to as $SSC_{ultimate}$) and the remaining undifferentiated cells (a substantial fraction of A_s plus A_{pr} and A_{al}) are differentiation-committed. However, under regenerative conditions, ID4+ A_s cells that are transiting out of the SSC pool and have lower expression of self-renewal genes ($SSC_{transitory}$) may be capable of reverting to a stem cell state rather than progressing to a progenitor state (Fig. 3) [4]. This revised A_s model has stimulated a lot of debate in the SSC field and highlights the role played by ID4 in SSC function. However, a recent single-cell sequencing study found more extensive expression of *Id4* within the adult undifferentiated population than suggested by this model and provides evidence to suggest that all undifferentiated cells possess transplantation activity, i.e., both SSC and progenitor fractions [30]. Furthermore, from analysis of an independent *Id4* knock-in reporter line, the majority (> 85%) of GFRα1+ spermatogonia are ID4+ in adults, while less (< 15%) of SOX3+ progenitors are ID4+ [59], confirming that ID4 preferentially marks self-renewing populations, but expression is more common within the undifferentiated pool than suggested [30, 59]. The validity of this revised ("ultimate") A_s model, therefore, remains unclear.

Insights from the proposed SSC models

Regardless of the contrasting stem cell models proposed, these studies have provided valuable insight into SSC function and behaviour in the male germline throughout development.

1. Consistent with the original A_s model, an association between undifferentiated cell morphology and stem cell activity is still apparent. A_s and A_{pr} are more likely to retain self-renewal activity regardless of markers being proposed. Longer chains of A_{al} are typically differentiation primed and have a high tendency to undergo differentiation rather than self-renewal [6, 28, 30, 34, 49].
2. While it remains controversial that GFRα1+ cells represent the SSC pool, this population is enriched in transplantable stem cell activity compared to the GFRα1− undifferentiated fraction by at least 2.5 fold [29]. Furthermore, expression of the novel stem cell markers *Pdx1* and *Eomes* are found in a subset of the GFRα1+ population [30, 60]. PDX1+ spermatogonia exhibit approx. threefold higher transplantation activity than PDX1− undifferentiated cells [30]. "Ultimate" SSCs (as marked by ID4 and PAX7) are also primarily confined within the GFRα1+ population under homeostatic conditions [4–6, 48, 58]. GFRα1, therefore, remains a useful marker of an SSC-enriched population.
3. Neutral drift as a mechanism to describe long-term SSC behaviour is also described in other stem cell systems [5, 44, 51]. Stochastic stem cell fate is observed in intestine and epidermis [50, 61]. Evidence from lineage-tracing studies using reporters for *Bmi1*, *Nanos2*, *Gfra1*, *Ngn3*, and *Axin2* strongly support the neutral drift model and stochastic stem cell turnover in testis [5, 24, 46, 47, 49].
4. Stem cell plasticity is revealed during tissue repair and regeneration. Long chains of undifferentiated spermatogonia are predominantly marked by NGN3, MIWI2 plus Oct4-GFP and are generally GFRα1− RARγ+ and show a high tendency to differentiate rather than self-renew under steady-state conditions. However, NGN3/MIWI2/Oct4-GFP+ spermatogonia can exhibit stem cell activity during tissue regeneration and/or upon transplantation. Stem cell activity is thus not strictly limited to the homeostatic SSC pool, but can extend to other undifferentiated spermatogonial fractions under conditions of tissue regeneration [24, 29, 30, 34, 36].
5. SSC behaviour and fate are context dependent and substantially influenced by the niche environment. Importantly, evidence suggests that the SSC niche is distinct during postnatal development, in homeostatic conditions and when germ cell depleted as in recipient testes of transplantation assays [30, 32, 41, 42]. These dynamic niche characteristics can affect interpretation of studies. We recently identified a subset of GFRα1+ spermatogonia in the adult homeostatic testis marked by co-expression of developmental regulators *Pdx1*, *Eomes*, and *Lhx1*, which have potent transplantation capacity [30]. However, *Pdx1* is downregulated, while *Lhx1* and *Eomes* expression upregulated during short-term SSC expansion in regenerative and developing testis. Changes in status of the niche during development, regeneration, and upon attainment of homeostasis can change gene expression patterns and properties of SSCs [30]. Therefore, ID4+ SSC behaviour in pre-pubertal testis may not be directly equivalent to ID4+ SSC behaviour in adults [4]. Similarly, progenitor (NGN3+ or MIWI2+) behaviour is distinct under homeostatic (differentiation-primed) vs. regenerative (stem cell reversion) conditions [24, 36].

Regulation of spermatogenesis

SSC niche microenvironment

Sustained spermatogenesis and continuous production of spermatozoa are dependent on the activity of germline stem cells [12, 16, 23, 62]. Therefore, an appreciation of the molecular mechanisms underlying SSC function is fundamental to our understanding of spermatogenesis and male fertility. SSC maintenance is dependent on a complex interplay between multiple extrinsic niche-derived signals and cell-intrinsic factors. While the exact nature of the testicular niche that maintains SSC function remains poorly understood [10], it is appreciated that a variety of somatic cells, including Sertoli, Leydig, and peritubular myoid cells play crucial roles in supporting SSCs [10, 11, 63–65]. Evidence indicates that SSCs preferentially reside in tubule regions adjacent to tissue vasculature and resident macrophages also contribute to the niche [66–68]. Sertoli cells form a key component of the SSC niche and are in close proximity with both undifferentiated plus differentiating germ cells and support multiple stages of spermatogenesis [10]. Importantly, SSC activity is regulated through the production of specific growth factors by Sertoli cells including glial cell-derived neurotropic factor (GDNF), basic fibroblast growth factor (bFGF or FGF2), and WNT ligands [47, 69]. It should be noted, however, that Sertoli cells are not the sole cell type that generates these growth factors in testis. For instance, production of GDNF by peritubular myoid cells is implicated in SSC maintenance [65]. Interestingly, the reduction in male mouse fertility upon aging is associated with a decline in the ability of Sertoli cells to produce GDNF and, therefore, maintenance of SSC function [40]. Accordingly, serial transplantation of SSCs into young recipients supports SSC self-renewal well beyond the natural lifespan [40]. Germ cell depletion upon treatment with the genotoxic alkylating agent busulfan increases *Gdnf* expression in the testis, indicating dynamic changes in the niche during tissue regeneration [40–42]. Elevation of key niche factors upon tissue damage that promote SSC self-renewal may underlie the ability of differentiation-primed progenitors

to revert to a stem cell fate under these conditions [24, 40–42].

Although SSC function is highly dependent on Sertoli cells [9], somatic cells in the interstitial space can also directly and/or indirectly regulate SSCs [10]. For instance, Leydig cells produce testosterone that Sertoli cells are responsive to and indirectly regulate germ cells [10]. Likewise, peritubular myoid cells surrounding the seminiferous tubule provide structural support and produce a variety of growth factors (GDNF, bFGF, and IGF1) and extracellular matrix (laminin, fibronectin, and collagen) that influence germ and Sertoli cell function [63, 64]. As mentioned, distribution of undifferentiated spermatogonia on the basement membrane is not random and this population is preferentially localised adjacent to interstitial areas with vasculature [68]. As differentiation progresses, spermatogonia migrate away from the interstitial areas, suggesting that interstitial tissues attract and maintain spermatogonia in the undifferentiated state [68, 70]. Importantly, endothelial cells within the interstitial space produce growth factors (GDNF and FGFs) that support SSC self-renewal [71, 72]. In particular, lymphatic endothelial cells surrounding the tubules produce FGF5 and preferentially localise towards interstitial regions. Evidence indicates that FGF5 and other FGFs promote spermatogonial proliferation and maintenance of an undifferentiated state [72]. Competition of SSCs for the limited supply of FGFs is proposed to regulate SSC density within the seminiferous epithelium [72]. Furthermore, the biased positioning of SSCs towards interstitial regions of the tubule is reflective of the location of the FGF source [72].

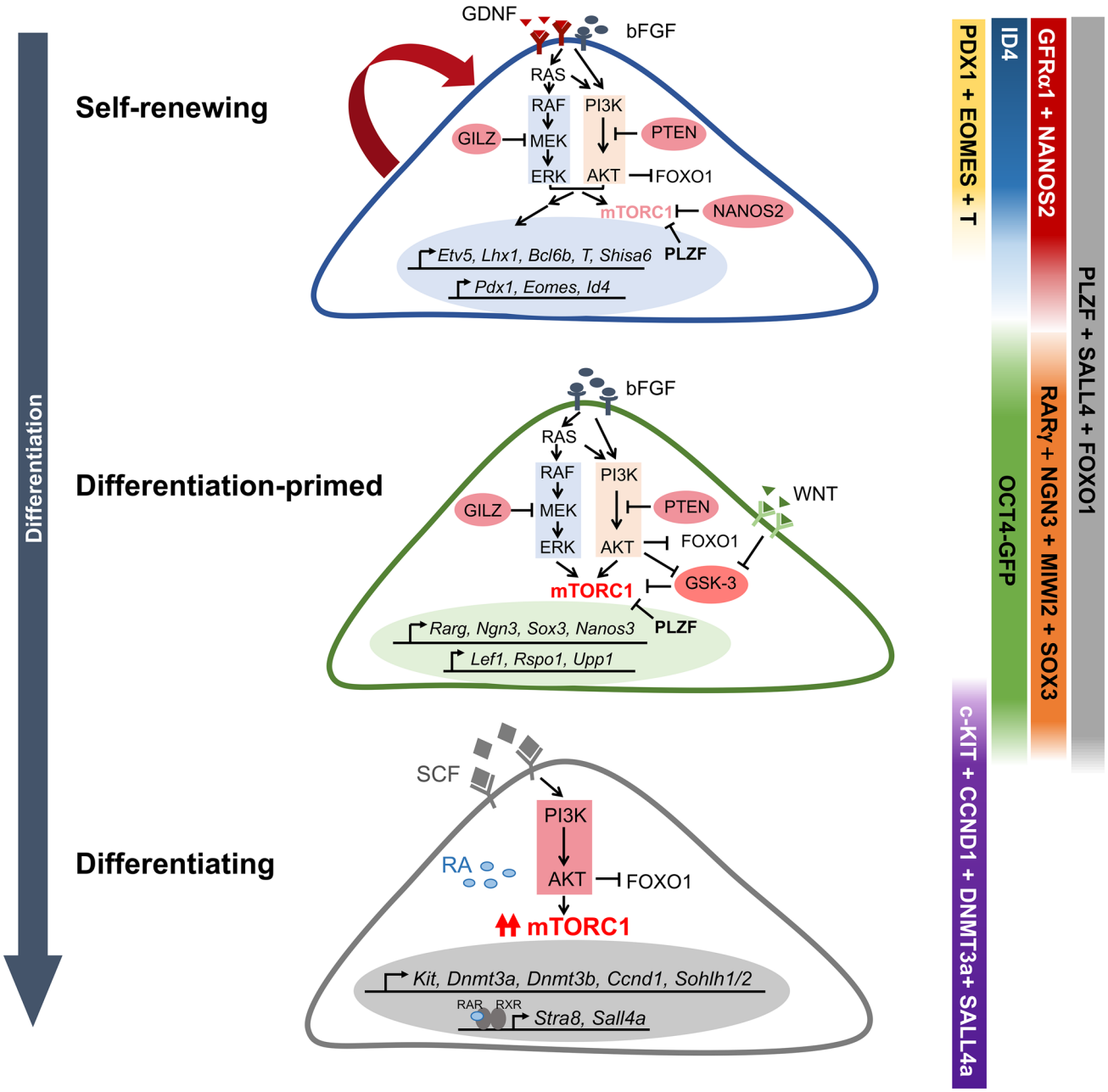
Macrophages are found in close proximity with vasculature and Leydig cells and are also present along the seminiferous tubules, where they preferentially associate with regions containing high densities of undifferentiated spermatogonia [66]. Macrophages produce colony-stimulating factor 1 (CSF1), which has been found to promote proliferation and self-renewal of undifferentiated spermatogonia in vitro [66, 67]. Macrophages also express retinoic acid synthesis enzymes, implying important roles in regulating spermatogonial differentiation and meiotic initiation [66]. Although the exact identity of undifferentiated spermatogonia that are attracted to the interstitial niche remains unclear, the testis niche microenvironment plays an essential role in maintaining testis homeostasis through regulation of SSC function, spermatogenesis, and germline regeneration [10].

Cellular pathways regulating SSC maintenance

The key cellular pathway that governs SSC self-renewal is GDNF receptor signalling (Fig. 4) [69, 73–75]. GDNF from Sertoli cells simulates SSCs in a paracrine fashion by activating the GFRα1/c-RET receptor complex and downstream signalling pathways. Through genetic manipulation of *Gdnf*, it was demonstrated that tight regulation of GDNF signalling is crucial in controlling SSC fate decisions and testis homeostasis. Specifically, the reduction of GDNF levels in *Gdnf* $^{+/-}$ mice resulted in progressive germ cell loss and a reduced spermatogonial proliferation rate [69]. In contrast, hyper-activation of GDNF signalling in *Gdnf*-overexpressing transgenic mice leads to accumulation of undifferentiated A_s and A_{pr} spermatogonia due to a block in differentiation and germ tumour formation in aged mice [73]. Interestingly, when treated with retinol to induce differentiation, the accumulated undifferentiated spermatogonia in *Gdnf*-overexpressing mice underwent apoptosis rather than differentiation, indicating defective response to differentiation-inducing stimuli [69].

Identification of GDNF as the key factor mediating SSC self-renewal has enabled development of a long-term culture system for SSCs. The culture of germ cells isolated from neonatal testis in medium supplemented with key growth factors GDNF, bFGF, and epidermal growth factor (EGF) sustains SSC self-renewal and expansion in vitro for more than 2 years [76]. Stem cell activity in long-term cultures is established through transplantation. Importantly, cultured spermatogonia derived from both pre-pubertal and adult testis expresses markers of the undifferentiated state and maintains stem cell activity in transplantation assays [76–78]. However, it should be noted that the majority of cells present in these cultures are thought to represent progenitors rather than SSCs and transplantation efficiency can be relatively low. Culture of undifferentiated spermatogonia in vitro typically requires both GDNF and bFGF, which act synergistically to promote cell growth [79, 80]. However, functional roles of bFGF in SSC maintenance in vivo remain poorly defined [80].

The ability to culture undifferentiated spermatogonia for extended periods of time in vitro provides a valuable experimental system to characterise molecular mechanisms underlying SSC function and regulation [76, 81–83]. For example, in vitro studies have identified key downstream effectors of the niche factor GDNF, which was found to signal through SRC family kinases and activate PI3K/AKT to promote SSC proliferation and maintenance [74, 84]. Introduction of an activated AKT construct into cultured undifferentiated spermatogonia can support long-term cell expansion in the absence of GDNF [85]. However, while GDNF-dependent PI3K/AKT activation is critical for SSC function in vitro, a balanced level of PI3K/AKT activity is evidently required for SSC maintenance in vivo. Conditional inactivation of *Pten* in the germline, which encodes a lipid phosphatase and inhibitor of PI3K/AKT signalling, drives rapid and complete germline exhaustion [86]. In contrast, loss of the kinase PDK1 in the germline that is required for AKT activation blocks differentiation of the undifferentiated spermatogonia



without affecting SSC maintenance [86]. Notably, the transcription factor FOXO1 is inactivated by AKT-dependent phosphorylation and is required for spermatogonial maintenance and differentiation in vivo [86].

Self-renewal activity of cultured spermatogonia is also found to be dependent on activation of the ERK MAPK pathway downstream bFGF (Fig. 4) [30, 79]. However, other studies based on in vivo models suggest that GDNF-dependent ERK MAPK activation is essential for SSC maintenance [75, 80, 87]. Despite the ability of both GDNF and bFGF to activate ERK MAPK in undifferentiated spermatogonia, the central role of the ERK MAPK pathway in SSC self-renewal, and routine supplementation of SSC media with both GDNF and bFGF, culture-based studies suggest that there are differences in SSC behaviour when grown in media with GDNF or bFGF alone. Spermatogonia cultured with GDNF or bFGF are similarly positive for SSC surface markers (CDH1, EPCAM, CD9, ITGA6, and ITGB1). However, cells grown with bFGF but not GDNF express significantly higher levels of *Kit* plus genes associated with committed progenitors (*Ngn3*, *Nanos3*), while cultured cells maintained with GDNF, but not bFGF show higher expression of SSC-associated markers (*Nanos2*) [80]. Transplantation assays confirmed enhanced stem cell activity of cells cultured with GDNF alone vs. bFGF alone [80]. These results suggest that the modulation of culture condition can alter the cellular

◂**Fig. 4** Regulation of spermatogonial fate and function by cell-intrinsic and extrinsic factors. GDNF produced by Sertoli cells activates the GFRα1/RET receptor complex present on SSCs and is essential for SSC self-renewal. bFGF is generated from a variety of cell sources within the testis and can promote SSC self-renewal independently from GDNF. In response to stimulation with GDNF and bFGF, PI3K/AKT and ERK MAPK pathways are activated and promote expression of genes involved in SSC function and maintenance, e.g., *Bcl6b*, *Etv5, Lhx1* and *Id4*. Other genes associated with the SSC state and responsive to GDNF stimulation are indicated (*T, Shisa6, Pdx1* and *Eomes*). The mTORC1 pathway is also activated downstream PI3K/AKT and ERK MAPK signalling and normally promotes SSC differentiation commitment. Intrinsic factors such as PLZF, NANOS2 and GILZ act to suppress mTORC1 activity in SSCs through a variety of mechanisms to maintain self-renewal capacity. AKT inhibits the FOXO1 transcription factor through phosphorylation and FOXO1 activity is involved in SSC function. The PTEN tumour suppressor gene antagonizes activity of AKT and is essential for SSC maintenance. SHISA6 suppresses canonical WNT signalling, which promotes transition from an SSC to differentiation-primed/committed progenitor state. Downstream targets of WNT signalling in spermatogonia are poorly defined but WNT can promote activation of the differentiation-promoting mTORC1 pathway in other systems by inhibiting GSK3. When present as primary niche factor, bFGF stimulation promotes adoption of a differentiation-primed cell state. Differentiation-primed/committed progenitor spermatogonia are marked by NGN3, MIWI2, RARγ, SOX3 plus other indicated factors and in contrast to GFRα1+ SSCs are highly sensitive to retinoic acid (RA) that drives formation of A_1 differentiating spermatogonia. Acting through nuclear receptors, RA directly promotes expression of the *Sall4a* isoform and *Stra8* that are both involved in differentiation. The PI3K/AKT pathway is also strongly activated during spermatogonial differentiation (via RA stimulation and SCF/c-KIT-mediated signalling) as indicated by cytosolic (inactive) FOXO1 and promotes further increases in mTORC1 signalling, which enhances translation of mRNAs encoding differentiation-associated factors including *Kit* and *Sohlh1/2*. Expression of genes that predominantly mark the distinct spermatogonial populations are indicated on the right

fate decisions of SSCs, such that GDNF-alone conditions maintain cultured cells towards "stemness", whereas bFGF-alone conditions promote spermatogonia to become more differentiation-primed [80]. Importantly, undifferentiated cells are found to dynamically interconvert between stem and progenitor states when cultured in vitro [30, 80]. The propensity of cultured spermatogonia to adopt either of these two states, as indicated by variation in Oct4-GFP expression, is highly dependent on the culture conditions. Increased density of spermatogonial cultures correlated with more rapid depletion of GDNF from the media and adoption of an Oct4-GFP+ PDX1− progenitor state [30]. Culture of cells with GDNF but without bFGF caused them to transition preferentially into an Oct4-GFP− PDX1+ primitive stem cell state, while culture with bFGF without GDNF triggered adoption of the Oct4-GFP+ PDX1− progenitor state, confirming that GDNF rather than bFGF promotes stemness [30]. In addition, the positivity of cultured spermatogonia for markers associated with the stem cell state (PDX1, EOMES) was dramatically reduced by treatment with MEK inhibitors but not PI3K or mTOR inhibitors, implying that MEK/ERK is more critical than other signalling pathways for maintaining the self-renewing state of SSCs [30, 75, 80, 87].

A recent in vivo study used growth factor-soaked gelatin microspheres injected into the testis interstitium to examine differential effects of GDNF and bFGF stimulation on the SSC-enriched GFRα1+ population [88]. Interestingly, while both GDNF and bFGF-adsorbed microspheres promoted the expansion of GFRα1+ spermatogonia, bFGF primed the GFRα1+ population towards differentiation as demonstrated by increased expression of RARγ, a key transcription factor required for differentiation commitment. In contrast, GDNF primarily promoted expansion of GFRα1+ RARγ− spermatogonia that would be predicted to have higher self-renewal tendencies [5, 29]. Besides growth factors such as GDNF and bFGF, spermatogonial function is regulated by canonical WNT ligands such as WNT3A, which promotes proliferation of cultured undifferentiated spermatogonia. Based on in vitro studies, a high level of Wnt signalling was concluded to be associated with SSC differentiation commitment [89]. However, the functional relevance of Wnt/β-catenin signalling in SSCs in vivo has only recently been studied in detail. From analysis of an Axin2-LacZ reporter mouse line that marks cells with active Wnt/β-catenin signalling, a majority of GFRα1+ (77.5%) and PLZF+ spermatogonia (53.5%) were LacZ+ , suggesting that a large proportion of the undifferentiated pool in adults in vivo are responding to WNT ligands [47]. Sertoli cells are the likely source of WNT ligand for undifferentiated cells and express *Wnt6* [47]. Further analysis of GFRα1+ and NGN3+ spermatogonia in vitro revealed similar levels of expression of multiple transcripts associated with Wnt/β-catenin signalling [90]. However, the expression of a direct target of the Wnt/β-catenin pathway (*Lef1*) was higher in NGN3+ spermatogonia, suggesting that the NGN3+ differentiation-primed population is more responsive to WNT than the GFRα1+ SSC-enriched pool [90]. Accordingly, the expression of *Ngn3* in cultured undifferentiated spermatogonia was increased when recombinant WNT3A was added indicating that the initial differentiation commitment from GFRα1+ to NGN3+ spermatogonial states is promoted by WNT signalling [90]. Interestingly, reduction in WNT signalling in adult testis did not evidently affect the GFRα1+ population, but decreased the relative proportion of RARγ+ progenitors [47, 90]. Hyper-activation of WNT (through modification of *Ctnnb1* to stabilise β-catenin) resulted in GFRα1+ spermatogonial exhaustion [90]. These results imply that GFRα1+ spermatogonia are intolerant of high levels of Wnt/β-catenin stimulation [90]. Inhibition of canonical WNT signalling through β-catenin deletion does, however, suppress proliferation of the bulk PLZF+ spermatogonial pool, indicating that Wnt/β-catenin signalling drives proliferation of committed progenitors (Fig. 4) [47]. Importantly, a subset of GFRα1+ spermatogonia is known to express high levels of *Shisa6*, a

cell-autonomous inhibitor of WNT signalling, and reduction of *Shisa6* expression synergized with enhanced Wnt/β-catenin signalling to deplete the GFRα1+ spermatogonial population [90]. SHISA6+ GFRα1+ cells can be concluded to represent a WNT-resistant self-renewing population with minimal propensity for differentiation. However, downstream effectors of WNT signalling responsible for driving the initial commitment step of undifferentiated spermatogonia remain unknown [90].

Intrinsic regulators of undifferentiated spermatogonia

One of the first intrinsic regulators of mammalian germline stem cell maintenance identified was the transcription factor promyelocytic leukaemia zinc finger (PLZF, also known as ZBTB16). In testis, *Plzf* expression marks undifferentiated spermatogonia and spermatogonia at early stages of differentiation [21, 78, 91–93]. *Plzf*-null mice are viable, but males exhibit a progressive germ cell loss with age associated with SSC failure and produce few functional spermatozoa [91, 92]. PLZF was found to promote SSC self-renewal in a cell-autonomous manner through indirect inhibition of mTORC1 signalling (Fig. 4) [78]. mTORC1 is a highly conserved and critical regulator of cell growth plus metabolic status [94]. Through a negative feedback effect, aberrant mTORC1 activation upon PLZF loss was associated with reduced response of undifferentiated spermatogonia to niche factor GDNF, increased differentiation commitment and SSC exhaustion [78]. Although PLZF plays important roles in SSC maintenance, its broad expression within the undifferentiated compartment suggests diverse roles in both stem and progenitor cells [21, 78, 91].

In undifferentiated spermatogonia, PLZF has been demonstrated to interact with and modulate activity of Spalt-like 4 (SALL4), a transcription factor essential for development that plays key roles in embryonic stem cell function [95]. *Sall4* is broadly expressed in both undifferentiated and differentiating spermatogonia and essential for spermatogonial differentiation [21, 93, 96]. The functional relationship between PLZF and SALL4 is evidently complex and requires additional clarification [21, 97]. PLZF is capable of suppressing transcriptional activity of SALL4 and preventing its association with specific chromatin domains. Conversely, increasing levels of SALL4 sequester PLZF to heterochromatic regions within the nucleus and potentially inhibit PLZF transcriptional function [93]. A balance of PLZF and SALL4 activities may, therefore, regulate the switch in spermatogonial activity between self-renewal and differentiation [21, 93, 97]. However, PLZF and SALL4 are co-recruited to many target promoters in cultured spermatogonia and can co-regulate genes associated with spermatogonial function [21, 97]. While PLZF may be required for SALL4 recruitment to cohorts of target genes [21, 93, 97], acute loss of SALL4 in adult spermatogonia in vivo resulted in mis-localisation of PLZF to the cytosol, suggesting that SALL4 is also required for PLZF transcriptional function [21]. Besides an essential role in spermatogonial differentiation, SALL4 is required for long-term maintenance of SSC regenerative capacity and via recruitment of the NuRD co-repressor complex can suppress activity of genes that inhibit SSC survival, proliferation and response to niche factors [21]. Interestingly, SALL4 also associates with polycomb repressor complex (PRC) components in spermatogonia and both are recruited to genes activated during spermatogenesis [96].

Other cell-intrinsic factors have been identified to play important roles in SSC function. One such factor is the zinc-finger RNA-binding protein NANOS2 [49]. Expression of *Nanos2* within the adult male germline is primarily restricted to A_s and A_{pr} undifferentiated cells, suggesting a specific role in the self-renewing compartment. Accordingly, conditional deletion of *Nanos2* resulted in abrupt loss of the GFRα1+ SSC-containing population and subsequent germline degeneration, while *Nanos2*-overexpression in the germline results in accumulation of undifferentiated spermatogonia due to a block in differentiation [49]. Mechanistically, NANOS2 was demonstrated to interact with other messenger ribonucleoprotein (mRNPs) components and inhibit translation of gene transcripts associated with differentiation, e.g., *Sohlh2*, *Dmrt1*, *Dazl*, and *Taf7l*, plus to suppress mTORC1 activity by sequestration of the mTOR kinase itself to mRNPs [98]. Combined, these important post-transcriptional mechanisms account for the ability of NANOS2 to maintain SSCs in a primitive, undifferentiated state [49, 98]. In general, post-transcriptional mechanisms of gene regulation play essential roles throughout spermatogenesis and have been reviewed in detail elsewhere [99].

The spermatogonial culture system has been used to screen for genes responsive to self-renewal stimuli such as GDNF and that have potential roles in SSC function. For instance, expression of *Bcl6b*, *Egr2*, *Egr3*, *Etv5*, *Lhx1*, *Id4*, and *Tspan8* was substantially enriched in cultured spermatogonia stimulated with GDNF [27, 28, 74, 79]. While reduced expression of *Bcl6b* and *Id4* impairs SSC activity in vitro, functional analysis of the role of these factors in SSCs in vivo through knockout approaches is more limited [27, 28]. *Bcl6b* null mice are fertile, although testis cross sections show mild spermatogenic defects [27], while *Id4* null mice are sub-fertile and exhibit germ cell loss [28]. While novel genes have been identified to mark SSC populations, they may not play any obvious functional role. The PAX7 transcription factor was found to mark a rare subset of GFRα1+ A_s spermatogonia that through lineage-tracing studies were demonstrated to have stem cell function. In addition, PAX7+ spermatogonia are highly resistant to

radiotherapy and chemotherapy and are evidently important in germline regeneration following these genotoxic insults [6]. While PAX7 represents a marker of an SSC population, *Pax7* deletion is suggested to have little effect on germline maintenance [6]. However, SSC maintenance clearly involves a complex network of cell-intrinsic transcriptional regulators, RNA-binding proteins, and signalling modulators plus multiple extrinsic stimuli from the niche, indicating that loss of any one component might be compensated for by activity of other factors.

Role of the mTORC1 pathway in regulating SSC fate

The balanced self-renewal and differentiation of SSCs is essential for germline maintenance and fertility [78, 100]. Appropriate regulation of SSC fate decisions requires functional interaction and crosstalk between cell extrinsic and intrinsic factors that converge onto intracellular signalling systems to dictate SSC function [47, 54, 79, 87, 90, 98]. One such central signalling pathway is mTORC1, a well-characterised master regulator of cellular homeostasis that integrates multiple upstream signalling pathways, including PI3K/AKT, ERK MAPK, WNT, plus nutrient availability and cellular stress to control cell growth, proliferation, differentiation, metabolism, and the autophagy response [94]. Insights into functional roles of mTORC1 in adult stem cells have been extensively explored in the haematopoietic system in which it is evident that the activity of mTORC1 within the stem cell compartment must be tightly regulated to maintain stem cell function [101–104]. Involvement of mTORC1 signalling in stem cell-mediated tissue regeneration has also been characterised [105].

Within the male germline, GFRα1+ A_s and A_{pr} spermatogonia have relatively low levels of mTORC1 activity, while longer chains of progenitor and differentiating spermatogonia exhibit increasing levels of mTORC1 signalling [98, 100, 106]. Increased levels of mTORC1 activity in spermatogonia were found to be essential for differentiation progression through enhanced translation of differentiation-associated transcripts, e.g., *Kit*, *Sohlh1/2* [107, 108]. Inhibition of mTORC1 signalling by rapamycin treatment or through genetic deletion of *Mtor* in the male germline results in block of spermatogonial differentiation and accumulation of GFRα1+ undifferentiated spermatogonia [107, 109]. Importantly, aberrant activation of mTORC1 in the germline via deletion of upstream negative regulators, e.g., *Tsc1/Tsc2*, promotes SSC differentiation commitment at the expense of self-renewal and results in SSC exhaustion [78, 100, 106]. PLZF and NANOS2, key intrinsic regulators of SSC maintenance and self-renewal, suppress mTORC1 activity through diverse mechanisms (see above). In addition, we have recently defined a role for the pleiotropic cell regulator GILZ in SSC maintenance through inhibition of mTORC1 via modulation of upstream MAPK/ERK signalling [59]. Acute loss of *Gilz* in adults results in hyperactive mTORC1 signalling that drives rapid exhaustion of the GFRα1+ SSC-enriched population plus increased proliferation of committed progenitors. Importantly, treatment with an mTOR inhibitor (Torin1) rescues GFRα1+ spermatogonial failure upon GILZ loss, indicating that GILZ-dependent mTORC1 inhibition is essential for SSC maintenance [59].

Both PI3K/AKT and MAPK/ERK pathways are important positive regulators of mTORC1 signalling in undifferentiated spermatogonia and are activated in response to growth factor stimulation [78]. While niche growth factors promote self-renewal, proliferation and survival of SSCs through PI3K/AKT and MEK/ERK pathways, these pathways also stimulate mTORC1 that promotes SSC differentiation. Intrinsic factors GILZ, NANOS2, and PLZF act as critical rheostats of cellular signalling pathways and limit the ability of growth factors to activate mTORC1, thus preserving the undifferentiated state (Fig. 4) [59, 78, 98].

Epigenetic regulation of spermatogenesis and male fertility

Epigenetic regulatory mechanisms are appreciated to play essential roles in spermatogenesis and male fertility [37, 110, 111]. The term "epigenetics" refers to heritable changes in gene expression and resulting phenotype without changes in the DNA sequence. During embryonic development, there are two key epigenetic reprogramming events. The first occurs after fertilisation, in which the paternal epigenome is remodelled through chromatin repackaging and DNA methylation. The addition of methyl groups to cytosine residues of DNA (DNAme) is associated with gene repression [112, 113]. The second event takes place in primordial germ cells (PGCs) in which the genome is demethylated during migration to the genital ridge (between E6.5 and E13.5). Subsequently, the male PGC genome regains DNAme (E13.5–E16.5) and sex-specific imprints are established by the time of birth [111–113]. Gonocytes are foetal precursors of the SSC pool responsible for continuous spermatogenesis in adulthood [111, 114]. Multiple association studies in humans and rodents have linked abnormal epigenetic marks in the male genome with infertility and detrimental effects on offspring health [111]. The exact causes of these epigenetic abnormalities in the germline are unclear. However, evidence indicates that epigenome maintenance in the male germline through appropriate DNA methylation and chromatin remodelling is crucial for fertility.

DNA de novo methylation and demethylation

DNAme is one of the best studied epigenetic marks and is important for male germline development [112, 113]. Whole-genome bisulfite sequencing comparing A_{undiff} (THY1+) and A_{diff} (c-KIT+) spermatogonia of adult mice uncovered a similar DNAme pattern between these two populations, indicating that alterations in DNAme do not underlie the transition from self-renewing to differentiating cell states [110]. These observations were recapitulated in adult human spermatogonia comparing the DNAme profile of stem-enriched SSEA4+ and c-KIT+ differentiating cells [115, 116]. While the spermatogonial compartment does not appear to exhibit dynamic changes in DNAme during differentiation in both humans and mice, abnormal DNAme profiles have been associated with poor quality of spermatozoa in infertile males (reviewed in Ref. [111]).

A critical regulator of epigenetic marks is DNA methyltransferase 3 like (DNTM3L), which lacks catalytic activity, but co-operates with other DNA methyltransferases including DNMT3A and DNMT3B to promote de novo DNA methylation [117, 118]. *Dnmt3l* knockout mice are viable but infertile due to progressive germ cell loss and a defective meiotic program in juvenile testis [117, 119]. While DNMT3L is not abundantly expressed within PLZF+ spermatogonia [37], DNMT3L was reported to negatively regulate CDK2 and control PLZF protein stability in spermatogonia [120]. Functional characterisation of DNMT3L demonstrated a key role in epigenetic regulation through negative regulation of transposable elements (intracisternal A particle retrotransposons or IAPs) through DNA methylation [119, 121]. In depth characterisation of a common phenotype observed in $Dnmt3l^{-/-}$ and $Miwi2^{--/--}$ mutant males (see below) showed a reduction in undifferentiated spermatogonia plus abnormal spermatogonial proliferation and differentiation [37]. Further analyses combining RNA-seq and whole-genome bisulfite sequencing approaches revealed that in the absence of *Dnmt3l* or *Miwi2*, IAPs were de-repressed, resulting in abnormal DNAme status and perturbed patterns of gene expression [37]. This study confirmed and highlighted the importance of epigenome maintenance by DNMT3L and MIWI2.

Undifferentiated spermatogonia in the mouse lack detectable DNMT3A and DNMT3B, but these DNA methyltransferases are strikingly upregulated in c-KIT+ differentiating spermatogonia [122]. Ectopic expression of *Dnmt3b* in undifferentiated spermatogonia in vivo triggered induction of differentiation marker c-KIT, while inducible knockout of *Np95*, which is involved in maintenance of DNAme, resulted in differentiation defects [122]. It was concluded that de novo DNAme is essential for the transition from an undifferentiated to differentiating spermatogonial state, although relevant mechanisms remain incompletely understood [122]. Therefore, in contrast to data obtained from bisulfite sequencing of spermatogonial populations, these observations suggest that dynamic changes in DNAme may be required during germ cell differentiation.

Chromatin remodelling

A unique chromatin remodelling event occurs during the last stage of spermatogenesis, in which a large proportion of core histone proteins (H2A, H2B, H3, and H4) are replaced with protamines [123]. This histone-to-protamine transition is essential for tight packaging of DNA in the haploid spermatozoa. Inefficient histone-to-protamine transition is associated with reduced fertility, highlighting the importance of chromatin architecture in maturation of spermatozoa [111]. More broadly, appropriate chromatin modifications are required for maintenance of SSC fate [124], spermatogenesis, and male fertility [110, 125–127].

Chromatin remodelling through posttranslational modification of DNA-associated histones by methylation, acetylation, and phosphorylation is essential for regulating transcriptional activity during mitotic and meiotic phases of spermatogenesis [110, 111, 113]. Within the adult human testis, chromatin accessibility, an indicator of chromatin remodelling through histone modification, is largely comparable between SSEA4+ undifferentiated spermatogonia and c-KIT+ differentiating spermatogonia, as demonstrated by ATAC-Seq analysis [115]. In contrast, significant changes in chromatin accessibility are evident during the transition from mitotic spermatogonia to meiotic spermatocytes in mouse [127]. Importantly, SCML2 (a germline-specific Polycomb protein) has been identified as a key factor controlling chromatin closure in autosomes for gene suppression at later spermatogenic stages while facilitating de novo formation of accessible chromatin in sex chromosomes during meiosis [127].

Chromatin accessibility is determined by acetylation status of histones which in turn regulates expression of neighbouring genes [128]. Histone acetyltransferases (HATs) add acetyl groups to histones and are associated with transcriptional activation, while histone deacetylases (HDACs) remove acetyl groups from histones and are associated with transcriptional repression [128]. Deregulation of histone acetylation in germ cells has been implicated in impaired spermatogenesis and infertility. For instance, human CDY and mouse CDYL are testis-specific acetyltransferase enzymes involved in H4 hyperacetylation during spermatid elongation. Lack of CDY expression is linked with infertility in man, signifying the importance of chromatin structure in spermatozoa maturation [129].

Core components within HDAC-containing complexes have been identified to play vital roles in spermatogenesis and fertility, for example, SIN3A [125, 130]. Conditional

germline deletion of *Sin3a* results in germ cell apoptosis during postnatal development and leads to a Sertoli cell-only phenotype in adulthood [125, 130]. Similarly, roles for enzymes that methylate or demethylate histone H3K4 in gene regulation processes essential for germline maintenance and spermatogenesis have been defined [131, 132]. Methylation of histone H3K4 is linked with gene activation [131]. Loss of H3K4 demethylase KDM1A in spermatogonia results in aberrant expression of genes associated with SSC differentiation, meiotic initiation, and epigenetic regulation [132]. Conversely, germline deletion of H3K4 methyltransferase KMT2B disrupts spermatogonial differentiation [131, 133]. KMT2B is proposed to prime genes in undifferentiated spermatogonia for expression at later stages of spermatogenesis [131].

Chromatin remodelling is also regulated by polycomb group proteins that exist in two distinct complexes; polycomb-repressive complexes 1 and 2 (PRC1 and PRC2), which contain multiple distinct subunits [134]. PRC1 and PRC2 act to compact chromatin for gene silencing through monoubiquitylation of histone H2A and methylation of H3K27, respectively [134]. RNF2 is a key component within PRC1 and conditional knockout of *Rnf2* in the germline disrupts PRC1 function resulting in infertility [96, 127, 134]. Transcriptome analysis of THY1+ undifferentiated spermatogonia and c-KIT+ differentiating spermatogonia upon *Rnf2* deletion revealed changes in expression of genes associated with spermatogonial differentiation plus survival and meiotic progression [96]. The PRC2 complex is comprised of EED, SUZ12, EZH1/2, and RbBP4/7 and catalyses di- and trimethylation of histone H3K27 [134, 135]. Disrupting PRC2 function through genetic loss of core components *Eed* or *Suz12* depletes the H3K27me3 epigenetic mark in spermatocytes and results in defects in double-strand break repair plus chromosome synapsis, leading to meiotic arrest [135]. These data demonstrate the importance of PRC2 during meiosis. Similarly, PRC2 component EZH2 is highly expressed in adult testis and germline deletion of *Ezh2* resulted in increased spermatid apoptosis and declining sperm count with age. Evidence also indicates that EZH2 is required for balanced self-renewal and differentiation of undifferentiated spermatogonia [136]. However, the exact mechanisms by which EZH2 regulates spermatogonial function remain incompletely understood.

Roles of additional epigenetic regulators in the male germline including the JUMNOJI (JMJ) family of histone demethylase proteins have been reported. Surprisingly, conditional knockout of the histone H3K27 demethylase *Jmjd3* had little apparent effect on H3K27me marks and fertility. Instead, *Jmjd3* deletion disrupted cytoplasmic bridge formation among syncytial chains of spermatogonia [137]. Similarly, loss of the H3K9 demethylase JMJD1C did not change levels of H3K9me in spermatogonia and meiotic spermatocytes [138]. The unaffected chromatin status in these mutants might suggest diverse roles played by JMJ proteins in the germline besides epigenetic regulation or functional redundancy [137, 138].

Together, these studies highlight the requirement for dynamic chromatin remodelling during spermatogenesis and demonstrate the importance of epigenetic regulation in germline maintenance and spermatogonial function.

Non-coding RNAs in spermatogenesis

It is appreciated that a large fraction of transcripts does not encode for protein, i.e., are non-coding RNAs (ncRNA) [123, 139]. Most species of ncRNA are small, between 20 and 35 nucleotides, and play important roles in regulating gene expression. These ncRNAs are divided into microRNA (miRNA), small interfering RNA (siRNA), Piwi-interacting RNA (piRNA), and circular RNA (circRNA) [123, 139–141]. Small ncRNAs have been profiled extensively in mouse testis [110, 142–145]. Testis-specific circRNAs were identified in germ cells at multiple stages of spermatogenesis [145]. CircRNAs lack 5′ and 3′ ends and are produced by a distinct type of alternative splicing [145]. However, not much is known about this new class of RNAs and their functional relevance in the germline [140, 145].

In contrast, miRNAs are abundant in the germline, especially within Type A spermatogonia of the juvenile testis [142]. Characterisation of these miRNAs has provided key insight into regulatory mechanisms of spermatogenesis and has been summarised in detail elsewhere [139]. Here, we highlight a few miRNAs known to be involved in SSC maintenance and differentiation. For instance, the expression of miRNA-21, 34c, 182, 183, and 146a was found to be selectively enriched in THY1+ undifferentiated spermatogonia. Transient inhibition of miRNA-21 in cultured undifferentiated spermatogonia disrupted cell growth [146], consistent with described roles as an anti-apoptotic factor [147]. Transplantation of cultured undifferentiated spermatogonia transfected with miRNA-21 inhibitor showed a reduction in colony-forming efficiency, supporting a role for miRNA-21 in maintenance of SSC activity [146]. Similarly, in vitro analysis of cultured spermatogonia identified miRNA-221, miRNA-222 and miRNA-202 as being responsive to the self-renewal factor GDNF [148, 149]. Reduced expression of these miRNAs results in upregulation of KIT expression and reduced self-renewal activity as assessed by transplantation [148, 149]. Accordingly, overexpression of miRNA-221/222 suppressed *Kit* mRNA translation and blocked spermatogonial differentiation [148]. miRNA-202 plays key roles in spermatogenesis through direct binding and inhibition of translation of transcripts encoding for RNA-binding proteins involved in meiotic initiation including *Rbfox2* and *Cpeb1* [149]. miRNA-26b is expressed at a substantially higher level within c-KIT+ differentiating spermatogonia than

undifferentiated spermatogonia and regulates differentiation through repressing *Plzf* expression and allowing *Kit* induction [150, 151]. Interestingly, miRNA-26b binds the *Tet3* transcript in cultured germ cells, encoding a critical enzyme for converting 5mC to 5hmC for removal of DNAme, suggesting that miRNA-26b regulates spermatogonial differentiation through modulation of 5hmC [151]. Together, these studies indicate the diverse mechanisms by which miRNAs regulate spermatogenesis.

piRNAs are between 21-32 nucleotides and directly bind PIWI family proteins (MIWI, MILI, and MIWI2) to mediate regulatory functions [141]. piRNA and PIWI are well-known for their role in repressing mobile transposable elements (TEs) to preserve germ cell genomic integrity [152–154]. Importantly, mouse spermatogenesis is dynamically regulated by piRNAs and PIWI. Pre-pachytene piRNAs are produced before birth plus during early postnatal development and bind MIWI2 and MILI for TE suppression [123, 141]. Deletion of *Piwi* family members *Miwi2* or *Mili* resulted in male sterility due to increased germ cell apoptosis, defective meiotic initiation, and enhanced TE expression [155–157]. Pachytene piRNAs are expressed by meiotic spermatocytes and bind MIWI and MILI to regulate spermiogenesis, e.g., *Miwi* mutants display spermatogenesis arrest at the round spermatid stage [158]. Subsequently, it has been demonstrated that during late spermatogenesis, MIWI is required for development and maturation of spermatids [159, 160]. Therefore, tight regulation of the piRNA pathway is crucial for sustaining spermatogenesis. Mechanistic insights to the modes of piRNA regulation of germline maintenance and development have been reviewed previously [123, 141].

Long ncRNAs (lncRNA) are transcribed by RNA polymerase II, typically > 200 bp in length and polyadenylated. lncRNAs are known to regulate RNA processing, X-chromosome inactivation, and gene imprinting through distinct mechanisms [144]. In contrast to small ncRNAs that are appreciated to play vital roles in mammalian spermatogenesis, little is known regarding the functional roles of lncRNA in germline maintenance and spermatogenesis. Interestingly, comparative analyses suggest that while lncRNA is less abundant than mRNA in germ cells at all stages of spermatogenesis, lncRNA expression is highly testis-specific compared to mRNAs [110, 145, 161]. A small number of lncRNAs have characterised roles in murine spermatogenesis. *Mrhl*-lncRNA is involved in meiotic initiation and regulation of Wnt signalling in spermatogonia through modulation of DDX5, an RNA helicase that plays essential roles in germline maintenance and fertility [162–164]. *Tsx*-lncRNA regulates meiotic progression [165], *Spga*-lncRNA is involved in spermatogonial maintenance [143], and *HongrES2* is required for maturation of spermatozoa [166]. In addition, studies from *Drosophila* testis confirmed functional importance of testis-specific lncRNAs in regulating global gene expression during spermatogenesis [161]. However, the molecular mechanisms by which lncRNAs regulate spermatogenesis and fertility awaits further investigation. Interestingly, high-throughput sequencing approaches have uncovered a large number of poorly characterised testis-specific and spermatogenesis-stage specific lncRNAs [110, 145], demonstrating the need for further studies in this area.

Spermatogonial differentiation

Mechanisms regulating SSC self-renewal are essential for germline maintenance and the focus of many studies. However, the ability of undifferentiated spermatogonia to differentiate successfully is of critical importance for the generation of large cohorts of cells to enter meiosis and the process of spermatogenesis [3, 167]. Spermatogonial differentiation specifically refers to the irreversible transition of undifferentiated spermatogonia (A_s, A_{pr}, A_{al}) to differentiating A_1-type spermatogonia. Only a subset of undifferentiated spermatogonia is sensitive to differentiation stimuli and induced to convert into A_1 spermatogonia each cycle of the seminiferous epithelium [53]. Transition from the undifferentiated to differentiating state is irreversible and regulated by retinoic acid (RA). Spermatogonial differentiation is marked by cell-surface expression of c-KIT and induction of DNA methyltransferases that may silence genes involved in undifferentiated cell function [122, 168].

Spermatogonial differentiation involves cyclical regulation by Sertoli cells, which change their gene expression pattern and function to support A_{al} differentiation and subsequent steps of spermatogenesis [169]. The critical regulator of cyclical spermatogonial differentiation and meiotic initiation is RA, an active metabolite of vitamin A. When mice and rats are placed on a vitamin A deficient (VAD) diet, insufficient RA is produced and the differentiation of A_{al}–A_1 cells is blocked [170, 171]. Importantly, re-administration of vitamin A into VAD mice resets the seminiferous epithelial cycle to stage VII, suggesting that RA signalling peaks at this stage to regulate multiple phases of spermatogenesis, particularly A_{al}–A_1 transition and meiotic initiation [53, 167, 170].

The pulse of RA production and RA response of germ cells between stages VII and IX is regulated by dynamic expression of genes associated with RA metabolism and differential expression of RARs [53, 170, 172, 173]. RA signalling is suppressed in early stages of the cycle (II–VI) due to increased expression of RA storage enzymes (*Lrat* and *Adfp*). In contrast, during the later stages of the cycle (VII–I), co-ordinated expression of *Stra6* in Sertoli cells and *Aldh1a2* in spermatocytes promotes RA synthesis [170]. In addition, the cytochrome P450 family 26 (CYP26) enzymes, which are involved in degradation of RA, are detected in germ and Sertoli cells in the

seminiferous tubules [174]. Knockout of CYP26 isoforms *Cyp26a1* and *Cyp26b1* in both Sertoli cells and germ cells results in subfertility [174]. Combined, these studies support the importance of periodic activation of RA signalling in germ cells between stages VII–VIII for the regulation of multiple stages of spermatogenesis.

The A_{al}–A_1 spermatogonial transition

The initial step in the spermatogenic pathway involves the fate decision of SSCs to adopt a differentiation-primed progenitor cell state marked by NGN3 (see above) [55]. *Ngn3* expression within the undifferentiated pool is largely, although not entirely, mutually exclusive with that of the SSC-associated marker *Gfra1* and A_{al} cells are typically NGN3+ , while the majority of A_s and A_{pr} are GFRα1+ [24, 34]. The differentiation-primed nature of NGN3+ spermatogonia is associated with enhanced sensitivity of this population to RA compared to the GFRα1+ undifferentiated fraction [53]. Increased sensitivity of NGN3+ vs. GFRα1+ cells to the differentiation-inducing stimulus RA is found to be dependent on differential expression of the retinoic acid receptor gamma (RARγ), which is predominantly expressed within the NGN3+ undifferentiated fraction [53]. Given the differentiation-destined fate of NGN3+ spermatogonia in steady-state testis, but their ability to contribute to the stem cell pool under regenerative conditions, the NGN3+ fraction can be viewed as a stem cell population that has gained differentiation competence rather than a transit-amplifying pool [24, 34, 53]. GFRα1+ cells have limited RARγ expression and hence are insensitive to the key differentiation signal [53]. Importantly, overexpression of RARγ within GFRα1+ cells in vivo promotes direct differentiation of GFRα1+ to c-KIT+ spermatogonia, which rarely occurs under homeostatic conditions [53]. These findings highlight key differences between GFRα1+ and NGN3+ spermatogonia. Accordingly, RARγ functions as an intrinsic factor to regulate the response of undifferentiated spermatogonia to differentiation stimuli, and RARγ represents an additional marker for progenitor/differentiation-primed spermatogonia in vivo [53].

Based on the use of ID4 as an SSC marker, an alternative mechanism has been proposed to account for differing response of stem and progenitor fractions within the undifferentiated pool to RA-induced differentiation. Namely, the distinct niche microenvironments of these populations dictate RA responsiveness [175]. Both ID4+ and ID4− fractions of Id4-GFP spermatogonial cultures expressed detectable levels of RA nuclear receptors including RARγ (although RARγ levels were substantially higher in ID4− progenitor vs. ID4+ stem fractions) and were induced to differentiate when treated with RA, arguing that both stem and progenitor spermatogonia are intrinsically sensitive to RA stimulation. Consistent with this observation, Id4-GFP^{bright} (stem) and Id4-GFP^{dim} (progenitor) spermatogonial fractions isolated directly from testis responded equivalently to RA ex vivo when in single-cell suspension. In contrast, when detunicated but otherwise intact Id4-GFP testis was incubated with RA ex vivo, Id4-GFP^{bright} stem cells did not differentiate, while Id4-GFP^{dim} progenitors activated differentiation markers as efficiently as when treated in a single-cell suspension [175]. Together, these data indicate that SSCs are inherently sensitive to RA, but are selectively protected from this differentiation-inducing stimulus by the testicular architecture and the microenvironment/niche.

These contrasting observations that cell-intrinsic or extrinsic mechanisms dictate the insensitivity of SSCs to RA are most likely due to the distinct experimental approaches, sources of samples for analysis and the stem/progenitor markers used. Ikami et al. primarily used lineage-tracing approaches in adults plus a VAD model of RA deprivation and vitamin A restimulation, which provides data relevant to in vivo homeostatic testis and presumably involved more physiological levels of RA stimulation [53]. Regardless, these studies provide evidence that the distinct fates of stem and progenitor spermatogonia are dictated by their differential sensitivity to RA, which is likely controlled by cell-intrinsic and extrinsic mechanisms.

In addition to RARγ, a number of cell-intrinsic transcription factors have also been implicated in spermatogonial differentiation. For instance, besides marking the differentiation-primed undifferentiated fraction, NGN3 itself has been directly linked with SSC differentiation commitment downstream STAT3 signalling [54]. The bHLH transcription factors SOHLH1 and SOHLH2 are primarily expressed in A_{al} chains and type A differentiating spermatogonia and are required for spermatogonial differentiation [176, 177]. Genetic loss of both *Sohlh1* and *Sohlh2* disrupts the transition from the undifferentiated to differentiating spermatogonial states and leads to an accumulation of abnormally long chains of GFRα1+ undifferentiated spermatogonia plus absence of differentiating c-KIT+ spermatogonia for meiotic progression, resulting in infertility [176, 177]. Interestingly, studies in neonatal mice demonstrate that endogenous RA signalling promotes translation of *Sohlh1*, *Sohlh2,* and *Kit* mRNA via activation of the PI3K/AKT/mTORC1 pathway [107, 178]. RA also induces expression of *Sall4*, encoding a transcription factor essential for spermatogonial differentiation [179]. Therefore, RA is responsible for inducing multiple distinct intrinsic regulators of differentiation.

Meiotic initiation

Besides promoting spermatogonial differentiation, RA is required for meiotic initiation during spermatogenesis [180, 181]. In mice lacking the RA-responsive gene *stimulated by retinoic acid 8* (*Stra8*), germ cell differentiation is blocked

at the meiotic prophase, indicating a critical role for RA-dependent *Stra8* expression in induction of meiosis [180]. Within adult mice, STRA8 is detected at low levels within the spermatogonia compartment (both PLZF+ and c-KIT+ cells), but the highest levels of STRA8 are found in preleptotene spermatocytes [100, 181]. Interestingly, maximal *Stra8* expression is observed during stages VI–VIII, which coincides with both induction of spermatogonial differentiation and meiotic initiation [182, 183]. While the strong *Stra8* expression in pre-meiotic spermatocytes is required for meiotic initiation, detection of *Stra8* within spermatogonia suggests additional roles in promotion of spermatogonial differentiation [100, 180, 181]. Accordingly, in *Stra8*-deficient mice, undifferentiated spermatogonia accumulate and differentiating spermatogonia are depleted [184].

While RA-induced *Stra8* expression promotes both the induction of spermatogonial differentiation and meiotic initiation in stages VI–VIII, RA induces substantially lower levels of *Stra8* in spermatogonia compared to pre-meiotic spermatocytes. As undifferentiated and B-type differentiating spermatogonia are presumably exposed to equivalent levels of RA during these seminiferous cycle stages, intrinsically expressed factors in these populations likely modulate their differential RA response [173, 181]. One such identified factor is doublesex and mab-3-related transcription factor 1 (DMRT1), which is prominently expressed in PLZF+ spermatogonia, but downregulated during spermatogonial differentiation [183]. Mechanistically, DMRT1 suppresses RA signalling and directly inhibits *Stra8* expression in undifferentiated spermatogonia while promoting *Sohlh1* expression to drive spermatogonial proliferation and differentiation. In contrast, reduced DMRT1 expression in pre-meiotic spermatocytes would allow maximal activation of RA signalling and *Stra8* expression for meiotic initiation. Loss of *Dmrt1* results in ectopic *Stra8* expression in spermatogonia, which leads to skipping of the A_{al}–A_1 transition and uncontrolled meiotic entry [183]. Therefore, DMRT1 functions as a master regulator of pre-meiotic cell transitions and a novel intrinsic inhibitor of the response to RA [183].

Germline regeneration

The mammalian male germline is highly sensitive to irradiation and chemical alkylating agents such as busulfan. These cytotoxic treatments are commonly used in anti-cancer therapy and place male patients at high risk of infertility. Effects of busulfan on the germline have mostly been characterised in rodent studies, where it causes rapid apoptosis of SSCs plus differentiating germ cells and disrupts spermatogenesis. The likelihood of fertility restoration following busulfan treatment is heavily dependent on dose of the drug and on numbers of surviving SSCs [41, 185]. In mice, very few SSCs persist following treatment with the highest non-lethal dose of busulfan (approx. 40 mg/kg) and animals essentially become infertile [6, 185]. Spermatogenic properties of the few SSCs capable of surviving high-dose busulfan are reportedly compromised and germ cell transplantation is an alternative and effective approach to restore spermatogenesis [25, 185]. Transplant studies have unequivocally confirmed that germline regeneration in this setting requires functional donor SSCs and a supportive recipient niche environment to re-establish spermatogenesis and restore fertility [186].

Germline regeneration from endogenous germ cells

Studies focused on the molecular mechanisms responsible for SSC-mediated germline regeneration following treatment with germ-cell-depleting agents are somewhat limited but an area of increasing attention in the field. Regenerative studies have primarily used a low dose of busulfan (10 mg/kg) to induce depletion of a majority of germ cells, including SSCs while leaving a small population of undifferentiated spermatogonia to restore the germline (Fig. 5) [6, 24, 34, 36, 41, 185]. One of the first studies to examine in detail the dynamics and contribution of undifferentiated spermatogonia during regeneration focused on the NGN3+ progenitor population [24]. Under homeostatic conditions, lineage-marked NGN3+ spermatogonia generate long-lived spermatogenic clones rarely given their differentiation-primed nature (see above). However, when mice were treated with busulfan following lineage marking NGN3+ cells, the number of long-lived spermatogenic clones increased substantially, indicating that the NGN3+ population contributes much more effectively to the SSC pool under regenerative vs. homeostatic conditions [24]. Subsequent studies provided further evidence that NGN3+ differentiation-primed progenitors can revert back to a stem cell state following busulfan treatment and that this population makes important contributions to the regenerative response [24, 34]. While the direct reversion of NGN3+ to GFRα1+ SSCs has been inferred from lineage-tracing data, direct observation of this cellular transition from intravital imaging remains to be demonstrated [34].

Collectively, these studies demonstrate the fate plasticity of progenitors and their ability to regain stem cell activity following tissue damage [24, 34]. More recently, a subset of NGN3+ spermatogonia were characterised that were marked by piRNA-binding protein MIWI2 and comprised a large fraction of the GFRα1− undifferentiated cell population [36]. Acute diphtheria-toxin (DTx)-mediated depletion of MIWI2+ cells in adult knock-in mice expressing the DTx receptor from the *Miwi2* locus resulted in dramatic loss of c-KIT+ spermatogonia without affecting the GFRα1+ SSC-enriched fraction. Accordingly, spermatogenesis was transiently disrupted following DTx treatment but ultimately recovered [36]. Combined, these data indicate that MIWI2+

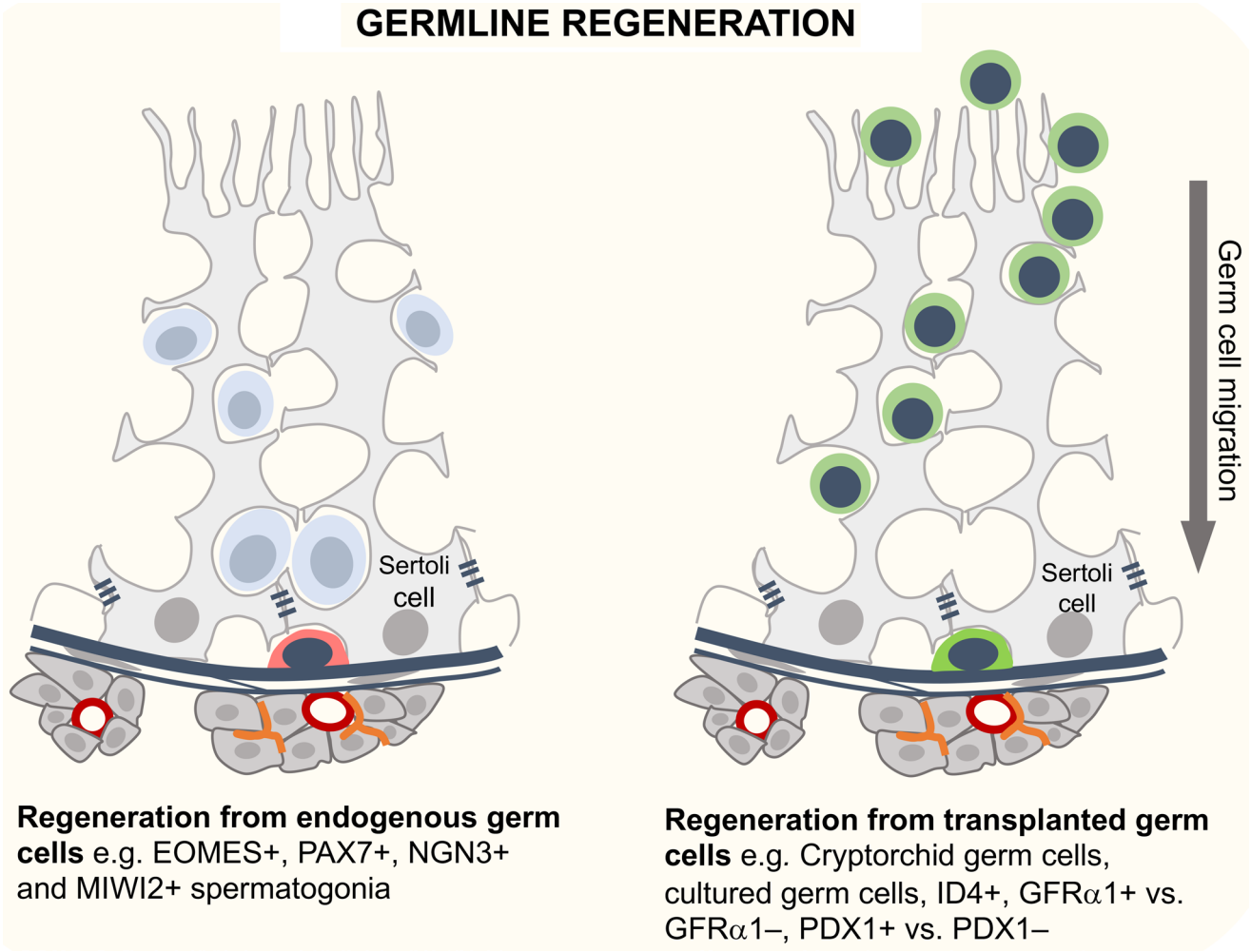


Fig. 5 Germline regeneration following germ cell depletion. The male germline is highly sensitive to radiation, chemotherapeutic agents and other genotoxic insults. Busulfan is an alkylating agent commonly used in cancer therapy and depletes germline cells including SSCs in a dose-dependent manner. In adult male mice, a low dose of busulfan (10 mg/kg) depletes the majority of differentiating spermatogonia and a large proportion of undifferentiated cells. Germline regeneration and restoration of fertility are driven by undifferentiated spermatogonia that persist following busulfan treatment. Fertility recovery upon extensive SSC depletion can be achieved by microinjection of undifferentiated spermatogonia into the seminiferous tubules. Donor SSCs migrate through Sertoli cell tight junctions to the niche and can generate long-lived spermatogenic colonies. Primitive fractions of undifferentiated spermatogonia, such as those marked by ID4, PDX1 and GFRα1 have potent transplantation capabilities. However, differentiation-primed/committed undifferentiated fractions marked by NGN3, MIWI2 and an Oct4-GFP transgene also generate persistent spermatogenic colonies when transplanted, albeit at lower frequency than fractions enriched in steady-state stem cells. Cell-intrinsic pathways that regulate SSC-mediated germline regeneration are poorly understood. Following germ cell depletion by busulfan, production of niche factor GDNF is substantially enhanced and is considered critical for effective germline recovery. Spermatogonial populations capable of germline regeneration following low dose busulfan from endogenous cells (left) and from transplanted donor cells after extensive SSC depletion with high-dose busulfan (right) are indicated

cells are not required for long-term germline maintenance and represent a transit-amplifying population of spermatogonia in homeostatic testis [36]. In contrast, depletion of MIWI2+ spermatogonia substantially delayed spermatogenic recovery following busulfan treatment, indicating that the MIWI2+ population is required for effective germline regeneration upon damage. Specifically, while the seminiferous epithelium of controls had recovered by 12 weeks post-busulfan, minimal recovery was observed in mice in which MIWI2+ cells had been acutely depleted even at 24 week post-injury [36]. This elegant study supports conclusions from lineage-tracing studies of NGN3+ spermatogonia that undifferentiated cells destined to differentiate in undisturbed testis make key contributions to the stem cell compartment and germline regeneration following tissue damage [36]. While the exact mechanisms by which committed progenitors contribute to testis regeneration remains unclear, it is

proposed that progenitor-to-stem reversion occurs under these conditions [24, 34, 36, 187].

Other studies of relevance focused on the role of PAX7+ and EOMES+ undifferentiated spermatogonia in germline recovery following chemotherapy (busulfan) [6, 60]. Strikingly, PAX7+ and EOMES+ cells appeared selectively resistant to genotoxic damage and the population actively proliferated and transiently expanded after injury. Importantly, lineage-tracing analysis confirmed the contribution of PAX7+ and EOMES+ spermatogonia to spermatogenic recovery following busulfan [6, 60]. Combined, these data suggest that PAX7 and EOMES mark "repopulating stem cells" that have been proposed to mediate tissue recovery following genotoxic damage [188]. While there are limited studies focused on SSC dynamics during regeneration, it is evident that both stem and progenitor populations resistant to genotoxic damage contribute to germline recovery and that spermatogonial behaviour and fate can be dramatically distinct under homeostatic vs. regenerative conditions. Importantly, injury-induced plasticity in committed progenitor populations, reversion to a stem cell state and contribution to tissue regeneration are commonly observed in multiple epithelial tissues of the skin, gastrointestinal tract, lung, and stomach (detailed review in [38]).

Germline regeneration from transplanted germ cells

The transplantation assay is regularly used to define SSC function and is, in essence, a test of the regenerative capacity of exogenous germ cell populations. Following successful development of germ cell transplantation in mice [23, 25], studies have taken advantage of this assay to characterise SSC activity and interactions of SSCs with the niche [33, 189]. Due to a lack of available markers to purify SSCs when the transplantation assay was initially developed, germ cells from cryptorchid testis or pups were typically used as an effective donor cell source given relative enrichment in undifferentiated spermatogonia [33, 190, 191]. Successful germline regeneration following transplantation involves migration of microinjected donor SSCs from the lumen of the seminiferous tubules, across Sertoli cell tight junctions to the basement membrane, where self-renewal and differentiation are subsequently initiated. This process of SSC homing to the niche microenvironment is highly inefficient and it is estimated that only approx. 12% of transplanted cells with SSC activity successfully home and generate donor spermatogenic colonies [33]. During the first week after transplant, approx. 75% of transplanted SSCs are lost, potentially through apoptosis. Furthermore, SSCs have to migrate through tight junctions between Sertoli cells in a direction opposite to that of germ cells in intact testis to arrive at the niche, an entirely non-physiological process. When SSCs successfully arrive at the basement membrane, they actively proliferate and expand laterally and repopulate the basal layer by 1-month post-transplant. Depending on the niche environment, transplanted SSCs could be short lived or function as long-term stem cells [189]. By 2–3 months following transplantation, established colonies have increased in length and successfully completed spermatogenesis [33].

Due to the strong dependence of SSCs on a testis niche and the key role played by Sertoli cells in forming the niche, the functional maturation of Sertoli cells that occurs during postnatal testis development has a great impact on colonisation efficiency of transplanted stem cells. Studies have shown that early postnatal testis (5–12 days) provides an optimal niche environment for stem cell colonisation. Regardless of whether donor cells originated from pup or adult testis, when pups rather than adults were used as recipients, SSC colonisation efficiency and colony growth were substantially enhanced. Specifically, approx. ninefold more colonies were formed in pup vs. adult recipient testis and resultant colonies were fourfold larger [32]. This striking difference in transplantation efficiency was ascribed to the lack of tight junctions between immature Sertoli cells in pup testis, which form between 10 and 12 days postnatally and in adults impede SSC homing to the niche. In addition, expansion of the Sertoli cell population during postnatal development generates a larger niche environment for stem cell colonisation [31, 32]. Interestingly, transplanted germ cells derived from neonatal (PND0-2), pup (PND5-12), and cryptorchid adult (14–20 weeks) demonstrated a dramatic stepwise increase in the number of functional SSCs during development (a 39-fold increase from neonate to adult), suggesting that adult testes are more enriched in SSCs [32]. Collectively, these data indicate that during postnatal testis development, accessibility of the niche for transplantation purposes decreases, but SSC numbers expand and reach a peak in adulthood [32]. It is important to note that successful depletion of endogenous germ cells in the recipient testis is critical for efficient SSC colonisation as donor SSCs compete with endogenous SSCs for access to the niche [31, 32]. Moreover, levels of the key niche factor GDNF are significantly elevated upon busulfan-mediated germ cell depletion, which will drive homing and expansion of donor SSCs [40, 41, 82].

As discussed above, effective germline regeneration is dependent on the ability of transplanted SSCs to home to a suitable niche in the seminiferous epithelium basal layer. The concept of stem cell homing upon transplantation is studied extensively in the haematopoietic system, where transplanted haematopoietic stem cells (HSCs) find their way to the appropriate niche in bone marrow of irradiated recipients to restore the whole haematopoietic lineage [192, 193]. Successful HSC homing involves a variety of adhesion molecules (e.g., CD44, VCAM, and integrin VLA-4), extracellular matrix components (e.g., hyaluronic acid), and chemokines (e.g., CXCL12) [193]. Based on cell sorting and

transplantation studies, SSCs are known to express surface markers with roles in adhesion to extracellular matrix and cell–cell adhesion including α6-integrin, β1-integrin, and E-Cadherin [190, 194]. Both α6-integrin and β1-integrin are involved in binding of cultured SSCs to laminin, a core component of the seminiferous tubule basement membrane [190, 194, 195]. Transplantation analysis confirmed essential roles of β1-integrin in attachment of exogenous SSCs to the basement membrane in recipient testis. SSCs lacking β1-integrin can migrate to the basal lamina, but rapidly disappear from the basement membrane [195]. In contrast, the absence of E-cadherin expression in transplanted SSCs did not affect SSC homing and generation of spermatogenic colonies [195]. Following identification of a role for β1-integrin in retention of transplanted SSCs at the niche in vivo, involvement of the small GTPase RAC in migration of SSCs across Sertoli cell tight junctions and homing was characterised [196]. RAC is activated in response to integrin-mediated adhesion and is required for HSC homing to the bone marrow niche [197]. SSCs lacking RAC were unable to colonize adult recipients; however, the defects in SSC homing can be rescued by transplantation into immature pup testis (PND5-10) lacking Sertoli cell tight junctions [196]. A subsequent study that reconstructed the SSC niche in vitro identified essential roles for CXCL12-CXCR4 and GDNF-GFRα1/Ret signalling in SSC homing [82]. PLZF+ A_s and A_{pr} express the CXCR4 receptor, while Sertoli cells express ligand CXCL12. Interestingly, while SSCs lacking *Cxcr4* or expressing dominant negative *Ret* proliferate at a similar rate to WT SSCs in vitro, they produce significantly fewer colonies when transplanted, supporting key roles of CXCL12 and GDNF in promoting SSC homing. Importantly, transducing Sertoli cells with lentiviral vectors expressing CXCL12 provide an improved supportive niche for transplanted SSCs, resulting in a twofold increase in colony formation compared to control recipient testis. Interestingly, however, transduction of Sertoli cells with a *Gdnf*-expressing vector did not enhance colony formation. Together, these studies indicate that GDNF and CXCL12 from Sertoli cells are involved in SSC homing [82]. Furthermore, the RAC1 is required for migration of transplanted SSCs across Sertoli cell tight junctions in adults, while β1-integrin is important for SSC attachment to the tubule basement membrane. These data support the critical influence of the recipient niche environment in determining efficiency of SSC colonisation upon transplantation [82, 195, 196].

SSCs in humans and other primates

Our current understanding of mammalian spermatogenesis and SSC regulation are mainly derived from studies in rodents. Similar to the mouse system, type A undifferentiated spermatogonia are present on the basement membrane of seminiferous tubules in primate testes and spermatogenesis operates in a synchronized and cyclic pattern [198]. Undifferentiated spermatogonia in primates are referred to as A_{dark} and A_{pale} cells based on nuclear architecture and staining intensity for haematoxylin [199–201]. Spermatogonial differentiation in rhesus monkeys produces type B_1 spermatogonia that undergo further rounds of division to produce B_2, B_3, and B_4 cells prior to initiating meiosis. Each seminiferous epithelial cycle is approximately 10-14 days in non-human primates and 16 days in man; complete spermatogenesis takes 37–47 days and 64 days in non-human primates and man, respectively [198]. Furthermore, in comparison to the 12 stages in mice, the seminiferous epithelium of primates can be divided into 9–12 stages in most non-human primates, while only six stages have been identified in chimpanzees and men [198]. In contrast to the highly organised spermatogenic layers that are specific to a given stage in regions of rodent seminiferous epithelium, the epithelium of primates displays multiple stages within a single-tubule cross section [198].

Spermatogonial hierarchy and germline regeneration in primates

A_{dark} spermatogonia have been proposed to function as reserve stem cells due to their low mitotic activity, whereas A_{pale} are actively proliferating and thought to function as "active stem cells" or "renewing progenitors" [199]. Both A_{dark} and A_{pale} were frequently observed as chains of 2 (20.7%) or 4 (40.7%) cells based on *camera lucida* drawings by Clermont in 1969 [199]. Similar to the GFRα1+ SSC-enriched undifferentiated population in mice, the number of A_{dark} spermatogonia is constant throughout the seminiferous cycle in primates, while the number of A_{pale} cells is cycle-dependent and peaks at stages VI–VIII, coinciding with the onset of spermatogonial differentiation. Importantly, A_{pale} spermatogonia were found to undergo two rounds of division per seminiferous cycle, one to renew the A_{pale} population around stage VII and one to generate differentiating B_1 around stage IX [200]. Interestingly, A_{dark} of rhesus testis appeared to be resistant to irradiation (X-irradiation), while the A_{pale} population was almost entirely depleted by this DNA-damaging insult. Regeneration of the seminiferous epithelium following irradiation evidently involved differentiation/conversion of A_{dark} to A_{pale} as a dramatic reduction in numbers of A_{dark} was observed together with a concomitant increase in numbers of A_{pale} cells [202]. Together, these preliminary studies in primates indicated that A_{dark} spermatogonia are reserve stem cells as they are resistant to irradiation and activated only under regenerative conditions. In contrast, current data suggests that A_{pale} spermatogonia support steady-state spermatogenesis and are sensitive to

irradiation. It should be noted that A_{dark} and A_{pale} may not necessarily represent distinct subpopulations of undifferentiated spermatogonia but could also simply reflect distinct mitotic or cellular states [199, 200]. Considering the above experiments were primarily observational studies with limited functional assessment, roles of A_{dark} and A_{pale} under steady-state conditions remain poorly understood. Furthermore, a lack of unique molecular markers to distinguish A_{dark} and A_{pale} cells hinders analysis of the topological arrangement and heterogeneity of primate stem and progenitor spermatogonia.

Functional analysis of primate SSCs

Primate SSCs can be identified retrospectively by xenotransplantation, in which primate testis cells are transplanted into immune-deficient nude mice that have been treated with busulfan to deplete endogenous germ cells [203]. A combination of fluorescence activated cell sorting (FACS) and xenotransplantation assays have been used to identify SSC-enriched rhesus germ cell populations. Interestingly, a subset of juvenile rhesus spermatogonia expresses THY1, a cell-surface marker used to isolate mouse SSCs [77, 204]. Expression of genes associated with a primitive germ cell state including *GFRA1* and *PLZF* are enriched in the THY1+ cell fraction from rhesus testis [204]. Importantly, when transplanted into immune-deficient mice, THY1+ spermatogonia from juvenile rhesus monkeys colonize the seminiferous tubule basal layer and form interconnected chains of spermatogonia [204]. Similarly, a subset of adult rhesus spermatogonia is positive for stage specific embryonic antigen 4 (SSEA4) and transplanted SSEA4+ cells successfully colonize the basal layer of recipient mouse testis [205, 206]. While transplanted rhesus germ cells could not complete spermatogenesis in mouse testis presumably due to the large evolutionary distance, it is remarkable that primate stem cells are still able to successfully migrate to the basement membrane in mouse tubules and that mouse Sertoli cells are able to support initial colonisation [203, 204, 206, 207].

Characterisation of primate stem and progenitor spermatogonia

Taking advantage of makers associated with stem, progenitor and differentiating cells identified from mouse testis, populations of adult rhesus spermatogonia were found to express *GFRA1*, *PLZF*, *NGN3* and *KIT* [204]. As might be expected, the degree of overlap in expression between markers varies between rodent and primate spermatogonia [204, 207]. In adult rhesus testis, approx. 80% of GFRα1+ spermatogonia express PLZF, while *NGN3* exhibits limited expression within adult PLZF+ spermatogonia but shows a high degree of overlap in expression with differentiation marker c-KIT (67% of NGN3+ cells are c-KIT +) [204]. Importantly, almost all A_{dark} spermatogonia and approx. 50% of A_{pale} in adult rhesus testis are GFRα1+ PLZF+ NGN3− and c-KIT−; a marker combination associated with an SSC-enriched population in mice [198, 204]. Assuming these genes mark a similar population of spermatogonia in primates as in mice, it is proposed that SSCs in primates are more abundant than in rodents, possibly due to a more limited number of transit-amplifying progenitor spermatogonia in primates [204]. Several other markers identified in the mouse germline as being associated with SSC fractions were confirmed to be expressed in primate undifferentiated (A_{dark} and A_{pale}) spermatogonia, including α6-integrin, OCT4, SALL4 and THY1 [204–209].

Xenotransplantation assays demonstrated that stem cell activity is enriched in α6-INTEGRIN+ human testis cell populations [209]. Similar to non-human primates, SSEA4 is also expressed in undifferentiated spermatogonia with transplantation activity in humans [206, 210]. A previous report utilised SSEA4 to isolate and characterise molecular features of human SSCs by chromatin and single-cell RNA sequencing approaches [116]. However, while SSEA4+ spermatogonia represent a subset of undifferentiated spermatogonia with enriched stem cell activity, it remains unclear whether the entire SSC pool in humans is identified by SSEA4 expression (see below). One of the advantages of single-cell RNA-Seq analysis is the ability to use algorithms such as Monocle to order cells into a predicted developmental pathway (pseudotime) that indicates progression through the differentiation pathway [211]. This approach allows mapping of testis cells with similar gene expression profiles into discrete clusters, representing distinct somatic cell types and differentiation stages of germ cells. Accordingly, novel gene expression signatures associated with each cell cluster or population can be identified [212].

From a recent detailed single-cell study of unselected human testis cells, clustering analysis of identified spermatogonia (including SSCs and differentiating spermatogonia) revealed five distinct transcriptional states. State 0 and State 1 are predicted to represent human SSCs and express *ID4*, *FGFR3*, *TCF3* plus *UTF1*, while States 2, 3 and 4 represent progenitor and differentiating spermatogonia and express *MKI67* plus *KIT* [212]. Interestingly, genes associated with differentiation-primed spermatogonia in mouse such as *Utf1* and *Piwil4*, are highly upregulated in the State 0 population, which is predicted to be a primitive and quiescent stem cell state in humans [30, 36, 209, 212]. State 0 cells have strong transcriptional similarities with infant germ cells, confirming their primitive nature [212]. State 0 cells also express low levels of *GFRA1* and *SSEA4*, previously used to identify stem cell-enriched fractions in primates and expression of these markers is selectively

enriched in State 1. RNA velocity analysis and chromatin profiling suggest the ability of spermatogonia in States 0, 1 and 2 to dynamically interconvert, consistent with previous studies of mouse undifferentiated cell fractions [5, 24, 30, 34]. Immunostaining analysis confirmed the existence of State 0 and State 1 populations marked by GFRα1^{low}/UTF1^{high} and GFRα1^{high}/UTF1^{low}, respectively. Notably, State 2 cells upregulate cell cycle related genes including *MKI67* indicating they are actively proliferating [115]. Therefore, despite some apparent discrepancies in expression patterns of stem and progenitor cell markers between mouse and human male germlines, single-cell RNA-Seq is proving a powerful approach to understand transcriptomic landscapes in mammalian spermatogenesis, cellular hierarchy and germ cell dynamics. However, functional studies are still required to validate these computational predictions and place them in a biological context.

Conclusions

Sustained spermatogenesis and adult male fertility are critically dependent on the maintenance of SSC function. Evidence indicates that SSC activity and fate are tightly regulated by a complex array of cell-intrinsic and niche-derived factors. Advances in molecular analysis techniques plus the development of lineage-tracing models and mathematical simulation have provided unique and unprecedented insight into the behaviour and dynamics of the SSC population. In homeostatic conditions, a strict hierarchy appears to operate within the spermatogonial population with a small subset of undifferentiated cells acting as the self-renewing compartment. However, upon activation of an injury-induced regenerative response, progenitor spermatogonia that are differentiation-committed in undisturbed tissue can revert back to an SSC state and help restore the seminiferous epithelium. The ability of committed progenitors to dedifferentiate to a stem cell state and contribute to tissue repair and regeneration is observed in multiple adult tissues including the epidermis, liver and epithelia of gastrointestinal tissue and lung [38]. While intrinsic pathways and/or extrinsic niche factors that regulate fate plasticity of committed progenitors in the male germline remain only partially understood, such mechanisms ensure that both injury-resistant stem cells and progenitors mediate germline regeneration and the efficient restoration of fertility. Manipulation of the cellular pathways underpinning these dramatic switches in spermatogonial fate could provide the basis for therapies aimed at enhancing fertility recovery in males following treatment with genotoxic drugs.

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