

Molecular regulation of spermatogonial stem cell renewal and differentiation

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Abstract

The intricate molecular and cellular interactions between spermatogonial stem cells (SSCs) and their cognate niche form the basis for life-long sperm production. To maintain long-term fertility and sustain sufficiently high levels of spermatogenesis, a delicate balance needs to prevail between the different niche factors that control cell fate decisions of SSCs by promoting self-renewal, differentiation priming or spermatogenic commitment of undifferentiated spermatogonia (A_{undiff}). Previously the SSC niche was thought to be formed primarily by Sertoli cells. However, recent research has indicated that many distinct cell types within the testis contribute to the SSC niche including most somatic cell populations and differentiating germ cells. Moreover, postnatal testis development involves maturation of somatic supporting cell populations and onset of cyclic function of the seminiferous epithelium. The stochastic and flexible behavior of A_{undiff} further complicates the definition of the SSC niche. Unlike in invertebrate species, providing a simple anatomical description of the SSC niche in the mouse is therefore challenging. Rather, the niche needs to be understood as a dynamic system that is able to serve the long-term reproductive function and maintenance of fertility both under steady-state and during development plus regeneration. Recent data from us and others have also shown that A_{undiff} reversibly transition between differentiation-primed and self-renewing states based on availability of niche-derived cues. This review focuses on defining the current understanding of the SSC niche and the elements involved in its regulation.

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Introduction

Maintenance of adult tissues often depends on a resident stem cell population that is able to both self-renew and produce differentiating progeny in a limitless fashion. Stem cell potential in the mouse testis is restricted to a population of A-type undifferentiated spermatogonia or A_{undiff} . However, under steady-state conditions most A_{undiff} are primed for spermatogenic differentiation and only a small subset undergoes self-renewal (Fig. 1). Whether stemness in the male germline is a characteristic of a subset of isolated single cells (A_s) or a property shared by most A_{undiff} is heatedly debated (de Rooij 2017, Lord & Oatley 2017). While traditional models propose that stem cell capacity is restricted to A_s spermatogonia (Lord & Oatley 2017), the majority of current data support a dynamic stem cell model in which fate of A_{undiff} cells is context-dependent and plastic (Hara *et al.* 2014, Carrieri *et al.* 2017, Garbuzov *et al.* 2018, Hermann *et al.* 2018, La *et al.* 2018b). Lineage-tracing studies and mathematical modeling support stochastic stem cell behavior *in vivo*, while *in vitro* data demonstrate the

seminal role of niche-derived cues in the regulation of A_{undiff} fate.

The spermatogonial stem cell (SSC) niche in the mammalian testis is considered an open niche that cannot be precisely anatomically defined. Due to cyclical fluctuations in the expression of paracrine regulators, the SSC niche changes its nature over the course of the seminiferous epithelial cycle. Thus, the SSC niche is likely defined by molecular, not anatomical, criteria and a microenvironment that enables and promotes SSC self-renewal at the expense of differentiation priming, constitutes the minimal criteria for a SSC niche.

Development of an *in vitro* culture system for A_{undiff} has considerably advanced research on male germline stem cells (Kanatsu-Shinohara *et al.* 2003, Kubota *et al.* 2004). Cultured A_{undiff} can be expanded essentially indefinitely, while retaining their self-renewal capacity and the capability to give rise to long-term spermatogenic colonies when transplanted to a germ cell-depleted testis. While this has enabled the effect of a number of candidate molecules on A_{undiff} physiology to be assessed, it has also introduced an unappreciated dilemma:

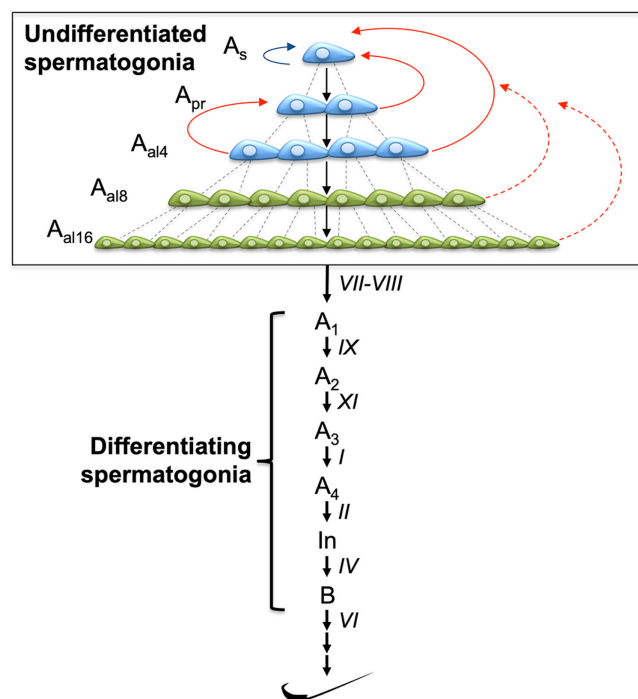


Figure 1 Germ cell expansion and kinetics in the mouse testis. The pool of undifferentiated spermatogonia, A_{undiff} , is composed of isolated single cells (A_s), and syncytia of typically 2 (A_{pr}), 4 (A_{al4}), 8 (A_{al8}) or 16 (A_{al16}) interconnected cells. There are two models to describe stemness within the male germline: a revised A_s model (blue curved arrow) proposes self-renewal capacity to lie primarily within a subset of A_s spermatogonia, while the fragmentation model suggests that cytokinesis in the male germline is typically incomplete and the population of A_s spermatogonia is maintained by fragmentation of short syncytia (red curved arrows). According to the fragmentation model, longer syncytia rarely fragment under homeostatic conditions (red curved, dashed arrows) but do so readily upon germline damage and induction of a regenerative response. A subset of A_{undiff} commits to spermatogenesis at stage VII–VIII of the seminiferous epithelial cycle. The mitoses of differentiating spermatogonia (type A1, A2, A3, A4, In and B) are dictated by the progress of the seminiferous epithelial cycle, whereas A_{undiff} divide in a random fashion, although typically only in stages X–II. Type B spermatogonia give rise to meiotic spermatocytes that ultimately produce sperm.

most A_{undiff} in standard cultures have progenitor-like characteristics, and cells, that display a transcriptomic signature typical of the *in vivo* self-renewing state, form a minority (La *et al.* 2018b). This is because robust expansion of A_{undiff} is typically preferred but the means to monitor the composition of the culture (i.e., ratio of stem vs progenitor-like cells) have been very limited until recently (La *et al.* 2018b).

According to current knowledge, multiple cell types can contribute to the regulation of SSC self-renewal and differentiation. While Sertoli cells likely represent the most important of these, recent research has expanded the components of the SSC niche to include a number of somatic cell types plus different cohorts of spermatogenic cells, whose significance for the paracrine regulation of

spermatogenesis is increasingly appreciated (Griswold 2016, Potter & DeFalco 2017, Mäkelä & Toppari 2018a). This review provides an overview of the composition and regulation of the SSC niche in mouse. To highlight its dynamic nature, the effects of somatic maturation, aging, cyclical function of the seminiferous epithelium and regenerative conditions on the niche are also discussed.

Kinetics of mouse undifferentiated spermatogonia

SSCs constitute a subset of A_{undiff} that are present on the basement membrane of the seminiferous epithelium. A_{undiff} in the mouse are found as single cells (A -single or A_s spermatogonia) or as syncytia of typically 2, 4, 8 and 16 cells interconnected by cytoplasmic bridges (A -paired, A_{pr} and A -aligned, A_{al4-16}) (Mäkelä & Toppari 2018b). Odd-numbered syncytia (mainly A_{al3}), that are observed at a low frequency, are thought to originate from fragmentation of longer syncytia, especially A_{al4} (Hara *et al.* 2014). According to the ‘dynamic SSC model’ (discussed in more detail below), this same mechanism is responsible for replenishing the pool of A_s , since the progeny of their division are normally connected by a cytoplasmic bridge (A_{pr}) (Hara *et al.* 2014), whereas a ‘revised A_s model’ proposes that A_s maintain their numbers by undergoing complete cytokinesis (Lord & Oatley 2017). A subset of A_{undiff} irreversibly commits to spermatogenesis at a specific stage of the seminiferous epithelial cycle, and the mitoses of differentiating spermatogonia, unlike those of A_{undiff} , are dictated by the progress of the spermatogenic program (Tegelenbosch & de Rooij 1993, Mäkelä & Toppari 2018a) (Fig. 1).

Stemness within the mouse undifferentiated spermatogonial population is considered inversely proportional to syncytial length. Thus, A_s cells were traditionally regarded as the actual stem cells, whereas A_{pr} and A_{al} were thought to represent transit-amplifying progenitors (de Rooij 2017). However, with the advent of new experimental tools and molecular markers, it became apparent that A_{undiff} hierarchy is more complex than originally proposed and A_s can directly commit to differentiate without prior amplification (Nakagawa *et al.* 2010, Hara *et al.* 2014). Furthermore, syncytial fragmentation has been proposed to guarantee that probably any cell within an undifferentiated syncytium can re-enter the A_s state in an appropriate environment (Hara *et al.* 2014). Therefore stemness within the A_{undiff} population is potentially a shared feature of the entire population, and the continuous cycling between equipotent single and short syncytial states is a mechanism that both maintains stemness and provides a sufficiently high number of differentiation-primed progeny to enter spermatogenesis at a specific stage of the seminiferous epithelial cycle (Hara *et al.* 2014). Notably, an alternative model to accommodate traditional views and recent progress on the field has also been proposed (Lord & Oatley 2017).

Over the years, there has been numerous efforts to further dissect the A_s population into functional categories (active stem cells, reserve stem cells, ultimate stem cells, the most primitive stem cells and so forth) and the quest to identify and isolate these cells is still ongoing. A number of molecular markers that identify these distinct A_s populations have been proposed (PAX7, ID4, BMI1, NANOG and ERBB3) but none are generally accepted to identify specific subsets of SSCs or A_s spermatogonia (Ventelä *et al.* 2012b, Abid *et al.* 2014, Aloisio *et al.* 2014, Chan *et al.* 2014, Komai *et al.* 2014).

Undifferentiated male germ cells

Given a lack of definitive SSC markers, the stem cell nature of a germ cell can only be estimated retrospectively by assessing its ability to give rise to long-term spermatogenesis following transplantation to an infertile recipient (Brinster & Zimmermann 1994). It is evident that most A_{undiff} of the mouse testis are primed for differentiation and display only latent self-renewal capacity (Nakagawa *et al.* 2007, Nakagawa *et al.* 2010, Hara *et al.* 2014). Importantly, the experimental conditions for transplanted cells to demonstrate their SSC nature are somewhat unphysiological, as the transplantation procedure presumably inflicts unusual stress on the cells, and SSCs under steady state are not expected to translocate from the tubular lumen to the basement membrane of the seminiferous epithelium. Consequently, the homing efficiency of SSCs is estimated to be approx. 10%, although precise efficiencies in many contexts are undefined (Nagano *et al.* 1999, Nagano 2003). The ability of transplanted cells to engraft and generate spermatogenic colonies is thus unlikely to provide a perfect measure of stem cell capacity.

Contrasting views on SSC identity and A_{undiff} hierarchy have been proposed. The A_{undiff} compartment of the mouse testis can be envisaged as a continuum of dynamic interconvertible cell states with progressively declining self-renewal capacity or likelihood (Nakagawa *et al.* 2007, Nakagawa *et al.* 2010, Hara *et al.* 2014, La *et al.* 2018b). It is also proposed that SSCs comprise a small subset of A_s , although evidence for the existence of these ‘ultimate SSCs’ remains limited (de Rooij 2017, Lord & Oatley 2017). Potentially, the behavior of an undifferentiated spermatogonium may solely be determined by the microenvironment, i.e. the niche, where it is found (Nakagawa *et al.* 2010, La *et al.* 2018b). Self-renewal ability of male germline stem cells would thus not solely be an inherent property of the cell but profoundly affected by the microenvironment. This notion is supported by the ability of differentiation-primed A_{undiff} to generate long-lived spermatogenic colonies in infertile recipients and indicates that the number of cells capable of functioning as stem cells within the testis is considerably higher than the number of actual stem cells (Nakagawa *et al.* 2010, Carrieri *et al.*

2017, La *et al.* 2018b). Our recent data further show that cultured A_{undiff} readily interconvert between stem and progenitor states based on the availability of niche factor GDNF (glial cell line-derived neurotrophic factor), and the environmental permissiveness thus defines the state (stem/progenitor) of an A_{undiff} (La *et al.* 2018b).

To further complicate the assessment of stemness within the male germline, we have to consider the use of this term in different contexts. As previously highlighted, male germline stem cells have been assigned a number of tasks: maintenance of homeostasis, regeneration of tissue after injury and ability to restore spermatogenesis after transplantation into an infertile recipient (Yoshida 2012). Whether there are different subsets of A_{undiff} for different tasks awaits clarification. This would, however, be a rather complicated scenario. In our opinion it is more likely that in-built heterogeneity existing within the A_{undiff} population combined with their flexible and stochastic behavior safeguards male germline maintenance both under steady-state conditions and after tissue damage (Nakagawa *et al.* 2007, Nakagawa *et al.* 2010, Hara *et al.* 2014, Carrieri *et al.* 2017, Garbuzov *et al.* 2018, La *et al.* 2018b).

Evidence for a ‘revised A_s model’ comes from histological studies and more recent research performed in Jon Oatley’s group utilizing an Id4-eGFP transgene to delineate the self-renewing subset within A_{undiff} (Fig. 2) (Lord & Oatley 2017). The model proposes that stemness within the A_{undiff} population is arranged in a strict hierarchy, and only a subset of A_s spermatogonia ($SSC_{ultimate}$) is capable of self-renewal and marked by high levels of *Id4* expression. While the model supports that some plasticity may exist for A_{undiff} at the early phase of transition from $SSC_{ultimate}$ to progenitor state (i.e. $SSC_{transitory}$), it argues against reversion of cell fate from progenitor states to the stem cell pool or fragmentation of A_{undiff} syncytia – two fundamental concepts of the ‘dynamic SSC model’. Oatley and colleagues have demonstrated that the Id4-eGFP^{bright} A_{undiff} population is highly enriched for SSC activity. However, conclusions from these studies suffer some limitations due to (1) use of early postnatal mice where the niche and SSCs themselves are different from the adult counterparts (Ernst *et al.* 2019) and (2) flow cytometric-based sorting of cells for transplantation studies compared activity of Id4-eGFP^{bright} versus Id4-eGFP^{dim} populations but functional capabilities and identity of an abundant population expressing intermediate levels of the *Id4* reporter were not characterized (Chan *et al.* 2014, Helsel *et al.* 2017). Moreover, recent independent assessments of *Id4* expression by single-cell RNA sequencing, immunostainings and use of independent mouse reporter lines have demonstrated that *Id4* expression is substantially more widespread within the A_{undiff} (or male germline in general) than previously described (Hermann *et al.* 2018, La *et al.* 2018a, Kitadate *et al.* 2019, La *et al.* 2018b). Notably, in the adult testis, *Id4* expression

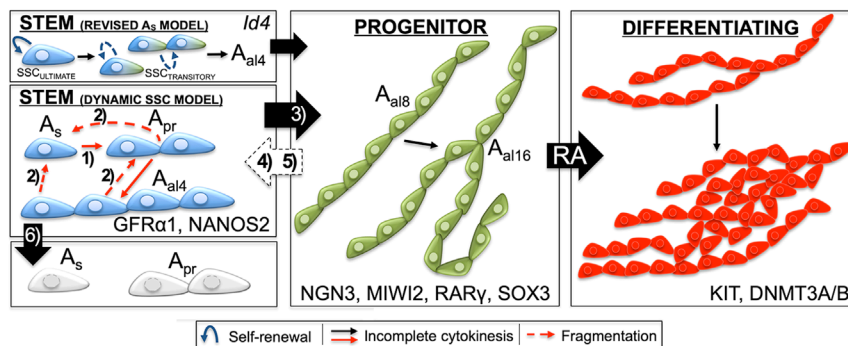


Figure 2 Dynamics of A_{undiff} in the adult mouse testis. According to a ‘revised A_s model’, stem cell capacity in the male germline is restricted to a subset of A_s spermatogonia, i.e. $SSC_{ultimate}$. These cells both maintain their own population (self-renewal; curved arrow) and give rise to cells (A_s and A_{pr}) in transit to the progenitor state, i.e. $SSC_{transitory}$ that possess limited capability for self-renewal. Reversion of cell fate from a progenitor to stem state is strictly not possible under any conditions. As an alternative, a ‘dynamic SSC model’ proposes that (1) cytokinesis (solid arrow) of male germ cells is incomplete, and also the progeny of A_s division (A_{pr}) is connected by a cytoplasmic bridge. (2) $GFR\alpha1/NANOS2$ -positive A_{undiff} continuously interconvert between equipotent single-cell and short syncytial states via fragmentation (dashed arrow) and incomplete cytokinesis (solid arrow). (3) $GFR\alpha1/NANOS2$ -positive A_{undiff} also give rise to $NGN3/MIWI2$ -positive progenitor cells that undergo differentiation priming. (4) Under steady-state conditions $NGN3$ -positive A_{undiff} do not typically revert back to the self-renewing state. (5) In regenerative conditions, however, $NGN3/MIWI2$ -positive A_{undiff} readily contribute to the long-lived stem cell population via reversion of characteristic gene expression patterns. (6) If an SSC niche is depleted of stem cell(s), cell migration from neighboring niches safeguards the long-term spermatogenic function. RA induces the irreversible differentiation commitment in the germline. The color key used for different cell types is used throughout this article.

displays limited enrichment in A_{undiff} fractions endowed with the highest SSC capacity (Garbuzov *et al.* 2018, La *et al.* 2018b). The validity of this model therefore awaits confirmation from other groups.

Support for a ‘dynamic SSC model’ is derived from studies where distinct reporter mouse lines are used in conjunction with intravital imaging, lineage tracing and computational analysis. These techniques have enabled monitoring of the fate of individual undifferentiated spermatogonia over the course of several days or months, and these experiments have given answers to many long-standing questions in the field of germline stem cell biology. These include (Fig. 2):

1. A_s division is (almost) always incomplete and results in the formation of A_{pr} (Hara *et al.* 2014)
2. The population of A_s spermatogonia is maintained by fragmentation of short syncytia, although the underlying regulatory mechanisms are thus far unknown (Nakagawa *et al.* 2010, Hara *et al.* 2014)
3. $NANOS2/GFR\alpha1$ -positive ($GDNF$ family receptor alpha 1) undifferentiated spermatogonia constitute the steady-state SSC population (Sada *et al.* 2009, Nakagawa *et al.* 2010, Hara *et al.* 2014)
4. Differentiation-primed $NGN3/MIWI2$ -positive (neurogenin 3/PIWIL4) progenitors rarely contribute to the long-term stem cell population in undisturbed tissue (Nakagawa *et al.* 2007, Nakagawa *et al.* 2010, Hara *et al.* 2014, Carrieri *et al.* 2017)
5. Under regenerative conditions $NGN3/MIWI2$ -positive cells, however, can revert back to the $GFR\alpha1$ -positive stem cell state and form long-term

spermatogenic colonies (Nakagawa *et al.* 2010, Hara *et al.* 2014, Carrieri *et al.* 2017)

6. There is an active turnover within the stem cell compartment and over the course of time, stem cells are stochastically lost via differentiation and replenished by cell migration from neighboring niches (Klein *et al.* 2010, Hara *et al.* 2014)

These findings form the basis of a ‘dynamic SSC model’ developed from the studies of Shosei Yoshida’s lab and supported by work from independent groups (Hara *et al.* 2014, Carrieri *et al.* 2017, Garbuzov *et al.* 2018, La *et al.* 2018b). According to this model A_{undiff} fate is plastic and context dependent emphasizing the role of environmental cues in defining the A_{undiff} state. Despite obvious merit, the model is far from complete, and mechanisms regulating fragmentation of A_{undiff} syncytia, an integral component of this model, are essentially undefined.

Under steady-state conditions it can be argued that the differences between these two models are rather insignificant and primarily dispute the mechanism for maintenance of the A_s population, i.e. complete cytokinesis vs syncytial fragmentation, while both models claim that all (Oatley) or most (Yoshida) SSC capacity is restricted to A_s and A_{pr} spermatogonia. However, under regenerative conditions, the differences become more fundamental in nature as the Oatley model argues against the possibility of differentiation-primed progenitors being able to contribute to the long-lived stem cell pool in contrast to the Yoshida model.

SSCs niche

SSC density

Similar to stem cells present in other adult tissues, SSCs might be predicted to localize preferentially to restricted regions within the tubules that contain an environment supportive of self-renewal (the niche). Despite displaying a preferential localization to tubular areas bordering the interstitial tissue and vasculature (Chiarini-Garcia *et al.* 2001, Chiarini-Garcia *et al.* 2003, Yoshida *et al.* 2007, Hara *et al.* 2014), A_{undiff} are rather uniformly distributed on the basement membrane of mouse seminiferous epithelium and have not been found to accumulate to any substantial degree in specific regions in undisturbed WT testis (see below). As recently demonstrated by Kitadate *et al.* (2019), competition for limited levels of FGFs secreted by lymphatic endothelial cells (LECs) associated with vasculature and the interstitium regulates density and size of the SSC population within the tubule basal layer (Kitadate *et al.* 2019). Specifically, SSC self-renewal and proliferation are favored at areas of high FGF concentration, that is vasculature- and interstitium-proximal regions, and SSCs need to be exposed to a sufficiently high FGF stimulus in order to maintain the self-renewing state. Spatially restricted availability of FGFs forces – and innate motile behavior enables – SSCs to compete with each other for consumption of FGF. SSCs that receive more FGF become biased toward self-renewal over differentiation, and a mechanism based on limited availability and competition for FGFs thus plays a key role in regulation of SSC density (Kitadate *et al.* 2019).

Vasculature-associated niche

Unlike in many invertebrate species, the mouse SSCs niche cannot be precisely defined solely by anatomical criteria. Undifferentiated spermatogonia are, however, typically found on the basement membrane at an area that is adjacent to interstitial tissue and vasculature (Chiarini-Garcia *et al.* 2001, Chiarini-Garcia *et al.* 2003, Yoshida *et al.* 2007). Moreover, live-cell imaging studies have demonstrated that GFR α 1-positive spermatogonia actively move within or between these vasculature-proximal regions, and alteration of the vasculature pattern around the tubule perimeter results in rearrangement of undifferentiated spermatogonia to the proximity of blood vessels (Yoshida *et al.* 2007, Hara *et al.* 2014). The movement of GFR α 1-positive spermatogonia within the basal compartment is arguably important for quantitatively normal spermatogenesis because stochastic emptying of the niche is a common feature of mouse spermatogenesis (Klein *et al.* 2010, Hara *et al.* 2014). Were the empty niches not occupied by new stem cells, the number of spermatogenic units

would inevitably decrease resulting in reduced sperm production over time. It is not known if the movement of GFR α 1-positive A_{undiff} follows a chemotactic gradient (such as GDNF) or if some other mechanism drives their displacement (Hara *et al.* 2014). Interestingly, GDNF has been shown to function as a chemoattractant for freshly isolated A_{undiff} , and it could therefore stimulate chemotactic movement of SSCs to areas of high GDNF concentration, i.e. an SSC niche (Kanatsu-Shinohara *et al.* 2012, Dovey *et al.* 2013). Upon differentiation, spermatogonia lose preference for these areas and become dispersed throughout the basal compartment of the seminiferous epithelium.

There are at least three obvious explanations for the preferred localization of GFR α 1-positive A_{undiff} to vasculature-proximal regions:

- SSCs depend on (a) blood-borne compound(s)
- SSCs depend on (a) factor(s) that is/are derived from vasculature-associated somatic cells (primarily vascular endothelial cells or Leydig cells)
- SSCs depend on the somatic paracrine milieu near the vasculature, that is influenced by endocrine factors carried to the testis by blood stream, and thus found at the highest level at these areas

There is evidence to support the latter two, and while many factors found in blood plasma are crucial for spermatogenesis (such as, FSH and LH (for a review see Mäkelä & Toppari 2017; Mäkelä & Toppari 2017)), they may not directly act on SSCs. According to recent data the biased localization of SSCs toward the vasculature and surrounding interstitium can be explained by the unique paracrine milieu found within these testicular zones. Bhang *et al.* (2018) and Kitadate *et al.* (2019) identified testicular endothelial cells (TECs) and LECs, respectively, as critical sources of factors supporting SSCs, thus providing an explanation for the enrichment of SSCs at longitudinal areas in proximity of the vasculature (Bhang *et al.* 2018, Kitadate *et al.* 2019). The SSC niche may be understood as an entity to which numerous different somatic and germ cell types contribute (Box 1 and Fig. 3). Since postnatal testis development encompasses maturation of somatic cell types and appearance of meiotic and post-meiotic germ cells, the SSC niche in the adult mouse is understandably different from the one found in juvenile mice. For instance, Sertoli cells of juvenile mice are functionally immature and during the first month of postnatal life they undergo maturation that encompasses profound changes in their transcriptome, function and structure (Mäkelä & Toppari 2017). Furthermore, due to cyclical fluctuations in the expression of paracrine regulators, the SSC niche changes its nature in the adult over the course of the seminiferous epithelial cycle (Mäkelä & Toppari 2018a).

Box 1: Cell types contributing to the SSC niche

Sertoli cells

Sertoli cells are the guardians of the germline, and support, nurture and protect germ cells in numerous ways (for a review see [Franca et al. 2016](#); [Franca et al. 2016](#) and references therein). SSCs (like all other germ cells) are in direct contact with Sertoli cells, and lack of a report describing a germ cell-only tubular phenotype implies that SSCs and more advanced germ cells cannot exist without Sertoli cells *in vivo*. Sertoli cells secrete numerous paracrine factors that act specifically on A_{undiff} , most notably GDNF ([Meng et al. 2000](#)).

Peritubular myoid cells

Seminiferous tubules are encased by contractile smooth muscle cells called peritubular myoid cells (PMCs). Besides providing structural support and propelling the flow of luminal fluid toward the *rete testis*, PMCs also secrete paracrine factors important for SSCs, including GDNF ([Chen et al. 2014](#)).

Peritubular macrophages

Tissue-resident macrophages are often neglected in everyday testis research. This is especially true for peritubular macrophages, a cell population that went completely unnoticed until 2015 ([DeFalco et al. 2015](#)). While the specific physiological role for these cells still warrants future studies, the available data suggest that they may take part in control of SSC maintenance and differentiation (for a review see [Potter & DeFalco \(2017\)](#); [Potter & DeFalco 2017](#)).

Testicular endothelial cells

TECs are a rich source of cytokines implicated in stem cell biology, including GDNF. As recently demonstrated by [Bhang et al. \(2018\)](#), TECs are able to support SSCs *in vitro* without exogenous GDNF, and TEC-derived factors significantly promote the repopulation of the seminiferous epithelium after a cytotoxic insult ([Bhang et al. 2018](#)). These data suggest that TECs are a key component of the SSC niche.

Leydig cells

Leydig cell-derived testosterone is a master paracrine factor in the testis. While testosterone is strictly indispensable for spermatogenesis, under normal conditions, it regulates the expression of thousands of genes in different somatic cell populations in the testis ([O'Hara & Smith 2015](#), [Oduwole et al. 2018](#)). Some of these then act on SSCs. Besides testosterone, Leydig cells also produce factors that directly target SSCs ([Huang et al. 2009](#), [Oatley et al. 2009](#), [Wang et al. 2015](#)).

Lymphatic endothelial cells

LECs are found at the border of seminiferous tubules and testicular interstitium and cover the surface of the lymphatic space. LECs in proximity to vasculature express a number of FGFs (FGF4, 5 and 8), which were shown to regulate the density of GFR α 1-positive A_{undiff} ([Kitadate et al. 2019](#)). Through production of FGFs, LECs act as key regulators of SSC population size.

Germ cells

The onset of spermatogenesis soon after birth brings another layer of complexity to the paracrine milieu of the testis, because germ cells, besides expressing receptors for soma-derived factors, can also generate soluble factors. Cyclical progression of the seminiferous epithelium guarantees appearance of specific germ cell subpopulations after fixed intervals providing a coordinated and efficient control mechanism for cell fate decisions within the seminiferous epithelium, such as onset of differentiation ([Mäkelä & Toppari 2018a](#)).

Establishment of the SSC niche

SSCs are specified perinatally as the population of fetal germ cells known as gonocytes or prospermatogonia migrates from the lumen of testis cords to the basement membrane. A subset of gonocytes contributes to the first wave of spermatogenesis, whereas the rest form the pool of SSCs ([Mäkelä et al. 2019](#)). SSCs then actively proliferate to expand the population ([Nagano et al. 2000](#)). This coincides with a period of high *Gdnf* expression, and GDNF has been suggested to stimulate SSC proliferation in the early postnatal testis ([Naughton et al. 2006](#), [Pui & Saga 2017](#)). Besides maintaining active divisions of SSCs, the specific microenvironment of the developing juvenile seminiferous tubule also provides a relatively high number of niches for the mitotic progeny of SSCs ([Shinohara et al. 2001](#), [Kitadate et al. 2019](#)).

Probably as an outcome of somatic cell maturation and establishment of hypothalamus–pituitary–testis axis, this microenvironment changes and the number of accessible niches diminishes during the course of development ([Shinohara et al. 2001](#)). Importantly, not only is the niche different, but also the SSCs of pup and adult mice differ and SSCs from pup testis lean toward differentiation at the expense of self-renewal ([Ebata et al. 2007](#)). The adult-type SSC niche is characterized by considerably lower, yet still readily detectable, levels of GDNF which is partially under endocrine control, and also regulated by the cycle of the seminiferous epithelium ([Tadokoro et al. 2002](#), [Grasso et al. 2012](#), [Ventelä et al. 2012a](#), [Tokue et al. 2017](#), [Sharma & Braun 2018](#)). Interestingly, *Gdnf* is upregulated in regenerating testis after loss of most differentiating spermatogonia and a substantial subset of A_{undiff} suggesting that a juvenile-

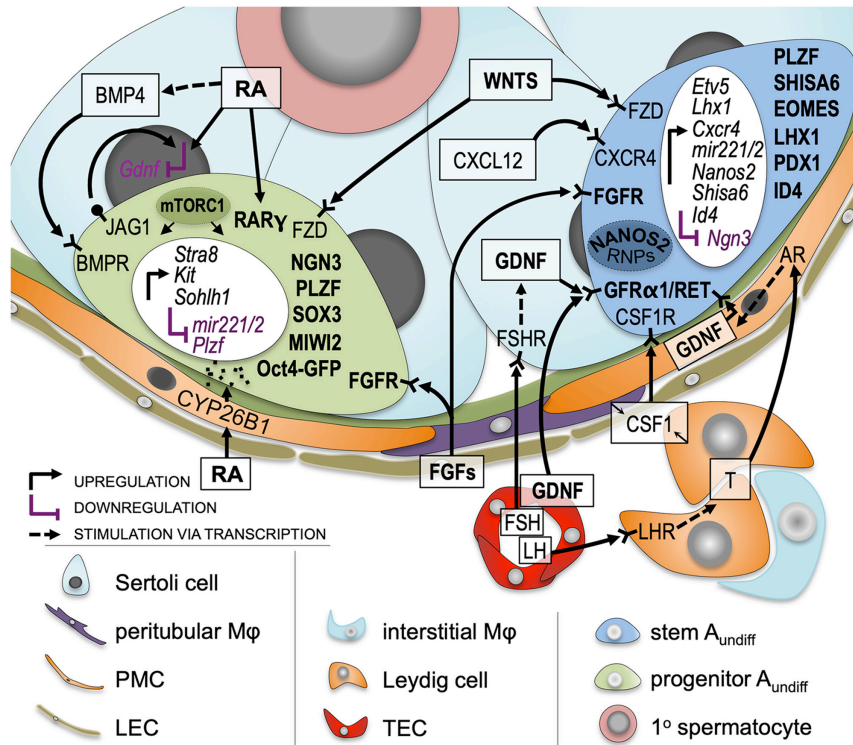


Figure 3 Regulation of A_{undiff} in the adult mouse. The adult mouse SSC niche is formed by contributions from different somatic cell types and germ cells. The response of A_{undiff} to niche-derived cues is determined by receptors and other proteins that they express. $GFR\alpha 1$ -positive stem- A_{undiff} (blue; PLZF/SHISA6/PDX1+) respond to GDNF and other factors by upregulating genes that are needed to maintain the self-renewing state, including *Etv5*, *Lhx1*, *Cxcr4*, *Nanos2* and *Id4*. SHISA6 is a WNT inhibitor and makes these cells refractory to WNT-mediated differentiation priming. Differentiation-primed progenitor A_{undiff} (green; NGN3/SOX3/PLZF+) are derived from $GFR\alpha 1$ -positive cells in response to WNT stimulation. They have adopted a gene expression signature (NGN3, SOX3) that clearly separates them from the self-renewing cells and confers on them a capability to respond to differentiation-inducing RA via expression of RAR γ (retinoic acid receptor gamma). RA stimulus evokes a differentiation commitment in these cells leading to upregulation of *Stra8*, *Kit* and *Sohlh1*, and downregulation of *Plzf* and *Kit*-degrading *mir221/2*. RA also acts on Sertoli cells and stimulates the expression of differentiation and cell survival-promoting agents, such as BMP4 (dashed arrow) while simultaneously down-regulating *Gdnf*. JAG1 expressed by germ cells also suppresses *Gdnf* expression in Sertoli cell via Notch signaling. RA is synthesized by Sertoli cells and primary spermatocytes. CYP26B1 enzyme in PMCs degrades extratubular RA. GDNF secretion is under endocrine regulation (dashed arrows) both in Sertoli cells (FSH) and PMCs via LH-stimulated testosterone (T) synthesis in Leydig cells. CSF1 is likely derived from multiple sources, at least Leydig cells and peritubular macrophages (M ϕ), potentially also from select PMCs. Testicular interstitium, with both vascular and lymphatic endothelial cells (TECs and LECs), is a rich source of factors implicated in SSC self-renewal.

like microenvironment is recreated upon genotoxic stress (Zohni *et al.* 2012).

Niche factors

GDNF

GDNF is produced by testicular somatic cells. While Sertoli cells have been considered the primary source of GDNF during steady-state spermatogenesis, TECs express *Gdnf* at a higher level than Sertoli cells and might be the major GDNF-producing population in the testis (Meng *et al.* 2000, Chen *et al.* 2016, Bhang *et al.* 2018). Interestingly, peritubular myoid cells secrete GDNF under androgen stimulation, and studies conducted using PMC-specific conditional *Gdnf*-knockout mice indicated that long-term maintenance of male fertility

depended on PMC-derived GDNF (Chen *et al.* 2016). However, the validity of these data have subsequently been questioned since the Cre model that was used is not specific for PMCs but is also expressed by TECs (Chen *et al.* 2016, Chen & Liu 2016). Future investigations are warranted to elucidate the role of distinct sources of GDNF for the maintenance of SSCs and normal cyclic function of the seminiferous epithelium.

GDNF is indispensable for maintenance of SSCs both *in vivo* and *in vitro* (Meng *et al.* 2000, Kubota *et al.* 2004). Conversely, overexpression or increased availability of GDNF results in the accumulation of A_{undiff} spermatogonia (Meng *et al.* 2000, Uchida *et al.* 2016, Masaki *et al.* 2018a, Sharma & Braun 2018, Faisal *et al.* 2019). GDNF acts on A_{undiff} via binding to the $GFR\alpha 1$ /RET receptor complex on their cell surface and subsequent activation of PI3K/AKT, RAS/ERK MAPK

and SRC family kinase pathways (Airaksinen & Saarma 2002, Oatley *et al.* 2007, Lee *et al.* 2007, He *et al.* 2008). **SHP2** protein tyrosine phosphatase encoded by the *Ptpn11* (protein tyrosine phosphatase, non-receptor type 11) gene is a key regulator of GDNF signaling within SSCs (Puri *et al.* 2014). Deletion of GDNF receptor components triggers rapid SSC depletion resulting in a Sertoli-cell-only phenotype (Meng *et al.* 2000, Naughton *et al.* 2006). The expression of GFR α 1 within the A_{undiff} is reduced as the syncytial length is increased. While approx. 90% of A_s spermatogonia express GFR α 1, approx. 75% of A_{pr} , 40% of A_{al4} and 15% of A_{al8} are positive for GFR α 1, whereas A_{al16} lack GFR α 1 expression altogether (Nakagawa *et al.* 2010). In addition, the expression level per cell is typically lower in aligned syncytia than single cells or pairs (Grasso *et al.* 2012). Interestingly, GDNF has been shown to regulate the expression of *Gfra1* in A_{undiff} through a long non-coding RNA which is a partial anti-sense transcript of *Gfra1* (Li *et al.* 2016).

Gdnf itself is regulated in Sertoli cells by a combination of endocrine, paracrine and autocrine mechanisms (Tadokoro *et al.* 2002, Simon *et al.* 2007, Garcia *et al.* 2014, 2017, Mäkelä *et al.* 2014). Interestingly, Notch signaling, activated by germ cell-expressed ligand JAG1 (jagged 1), has been implicated in negative regulation of *Gdnf* expression in Sertoli cells (Garcia *et al.* 2014, 2017). *Gdnf* expression in Sertoli cells is also downregulated by FGF2 and RA (see below), which thus function to oppose GDNF (Hasegawa *et al.* 2013, Masaki *et al.* 2018a). The role of pituitary-derived follicle-stimulating hormone (FSH) in *Gdnf* regulation is often emphasized. The evidence for *in vivo* stimulation of *Gdnf* expression by FSH is, however, rather limited and contradictory (Tanaka *et al.* 2016, Sakai *et al.* 2018). Moreover, FSH is a general regulator of the Sertoli cell transcriptome (McLean *et al.* 2002). Thus, underlining its putative stimulatory role on *Gdnf* might be misleading if we do not know how FSH affects the balance of self-renewal vs differentiation-promoting factors as a whole.

GDNF regulates gene expression in A_{undiff} and its target genes, including **Nanos2**, **Etv5**, **Lhx1**, **T(Brachyury)**, **Bcl6b**, **Id4** and **Cxcr4**, have been implicated in the maintenance of the self-renewing state and/or prevention of differentiation (Chen *et al.* 2005, Oatley *et al.* 2006, 2007, 2011, Sada *et al.* 2012, Yang *et al.* 2013a). Moreover, GDNF downregulates **Ngn3** to promote the self-renewing state (Kaucher *et al.* 2012). In addition to these, a number of GDNF-independent proteins (including PLZF, FOXO1, GILZ and TAF4B) working in SSCs in a cell-autonomous fashion to promote SSC survival and self-renewal have been identified (Buaas *et al.* 2004, Costoya *et al.* 2004, Falender *et al.* 2005, Goertz *et al.* 2011, La *et al.* 2018a). While the role of extrinsic factors in the regulation of SSCs is often highlighted, SSC-intrinsic factors are just

as relevant in the maintenance of stem cell function. Other paracrine factors involved in SSC maintenance in synergy with GDNF include CXCL12 (see below) and different isoforms of **VEGFA** (vascular endothelial growth factor A) (Caires *et al.* 2012, Yang *et al.* 2013a).

FGFs

FGFs (at least FGF2, 4, 5 and 8) exert a mitogenic effect on A_{undiff} (Kubota *et al.* 2004, Kitadate *et al.* 2019). **FGF2** and GDNF work in synergy to promote the robust growth of A_{undiff} *in vitro* (Kubota *et al.* 2004, Kanatsu-Shinohara *et al.* 2005, Ishii *et al.* 2012, La *et al.* 2018b). However, the exact significance of FGF2 for maintenance of SSCs *in vivo* remains an area of active study. Interestingly, prolonged stimulation with FGF2 *in vivo* results in the accumulation of progenitor A_{undiff} (Masaki *et al.* 2018). These data support our *in vitro* findings indicating a differentiation-promoting effect for FGF2 on A_{undiff} (Masaki *et al.* 2018, La *et al.* 2018b). FGF2 also regulates the availability of RA by suppressing the expression of RA-degrading enzyme *Cyp26b1* (Masaki *et al.* 2018a). Interestingly, GDNF expression in TECs is stimulated by FGF2 providing a mechanism for observed synergy between these two factors (Bhang *et al.* 2018). The origin of testicular FGF2 remains contentious (Mullaney & Skinner 1992, Masaki *et al.* 2018, Kitadate *et al.* 2019). Apparently, A_{undiff} can be maintained *in vitro* in GDNF-free conditions in the presence of FGF2 but they display poor growth and low stemness (as judged by transplantation assay) in these conditions (Takashima *et al.* 2015, La *et al.* 2018b).

Vasculature-associated LECs plus select interstitial cells secrete **FGF4**, **5** and **8**. Both stem and progenitor A_{undiff} express the molecules needed to bind and internalize FGF signals, and in the presence of GDNF, FGFs were proposed to promote SSC proliferation plus self-renewal (over differentiation), and thus regulate SSC density, i.e. number of SSC niches (Kitadate *et al.* 2019). Notably, LECs provide a constant supply of FGFs over the course of the seminiferous epithelial cycle, which contrasts with GDNF, WNT and RA signals. It is therefore unclear how FGF action is tethered into stage-specific regulation of SSCs, such as proliferation. Kitadate *et al.* (2019) propose a minimal model in which SSCs compete for a limited supply of FGFs whose availability on the basement membrane is inversely proportional to the distance from the source (vasculature-proximal LECs and interstitium) and number of A_{undiff} (the FGF sink) (Kitadate *et al.* 2019). It is unclear why the effects of distinct FGF ligands (FGF2 vs FGF4/5/8) on SSCs are so different. However, much can be explained by different study settings (*in vitro* vs *in vivo*), dosage and dependency on physiological levels of GDNF signaling.

The **WNT** pathway has recently been implicated in differentiation priming in the male germline

(Takase & Nusse 2016, Tokue *et al.* 2017). Tokue *et al.* (2017) demonstrated that transition from stem (GFR α 1+) to progenitor (NGN3+) state is driven by WNT/ β -catenin signaling (Tokue *et al.* 2017). Moreover, they identified **SHISA6**, a cell-autonomous WNT inhibitor, as a novel marker for a subset of GFR α 1-expressing A_{undiff} . SHISA6 might thus act as a WNT trap in this subset of A_{undiff} to maintain the self-renewing state and prevent premature entry into the differentiation-primed state.

Interestingly, the availability of GDNF and WNT6, a WNT family member that is abundantly expressed by Sertoli cells, during the seminiferous epithelial cycle differs, suggesting that they have distinct windows of action (Takase & Nusse 2016, Tokue *et al.* 2017). Androgen-regulated Sertoli cell gene *WNT5A* has also been implicated in control of SSC self-renewal, but the available data indicate that WNT5A is an A_{undiff} mitogen (Tanaka *et al.* 2016). Whether it supports adoption of either the stem or progenitor state is unclear. Moreover, *Wnt5a* expression is downregulated in the testis soon after birth, and in the adult mouse *Wnt5a* levels are relatively low suggesting that it might be a developmental regulator of the SSC niche (Tanaka *et al.* 2016, Faisal *et al.* 2019).

Retinoic acid

Retinoic acid (RA) is a potent hormone that plays an indispensable role in the induction of differentiation in the male germline. Lack of RA or vitamin A (RA is a vitamin A metabolite) results in the accumulation of A_{undiff} that are cleared through differentiation once normal RA metabolism is re-established in the seminiferous epithelium (Morales & Griswold 1987, van Pelt & de Rooij 1990). Exposure of differentiation-primed A_{undiff} to RA results in their commitment to spermatogenesis (transition to type A1 differentiating spermatogonia) and makes them refractory to niche-derived factors (Endo *et al.* 2015).

Notably, RA opposes GDNF function at two different levels: it downregulates the expression of *Gdnf* in Sertoli cells (and stimulates the expression of differentiation-supporting factors, such as *Bmp4* and *Scf*) and antagonizes the effect of GDNF in A_{undiff} (Pellegrini *et al.* 2008, Carlomagno *et al.* 2010, Barrios *et al.* 2012, Yang *et al.* 2013b). RA is primarily produced by Sertoli cells within the testis but recent research suggests that meiotic and post-meiotic germ cells are intimately involved in the regulation of RA availability during the seminiferous epithelial cycle (Raverdeau *et al.* 2012, Sugimoto *et al.* 2012, Endo *et al.* 2017, Mäkelä & Toppari 2018a, Teletin *et al.* 2019). Peritubular macrophages have also been implicated in RA-mediated control of spermatogonial differentiation but the physiological significance of these findings remains unclear (DeFalco *et al.* 2015).

CXCL12

CXCL12 (C-X-C motif chemokine ligand 12) and its cognate receptor **CXCR4** (C-X-C motif chemokine receptor 4) play significant roles during male germline development (for a review see Loveland *et al.* 2017) and have also been implicated in the maintenance of SSCs (Kanatsu-Shinohara *et al.* 2012). CXCL12/CXCR4 signaling is crucial for proper homing of SSCs to their cognate niche at the basement membrane and might also keep SSCs from leaving the niche (Kanatsu-Shinohara *et al.* 2012, Yang *et al.* 2013a). Notably, expression of *Cxcr4* is also stimulated by GDNF in A_{undiff} (Kanatsu-Shinohara *et al.* 2012, Yang *et al.* 2013a). CXCL12 is potentially produced by Sertoli cells but definitive data are lacking. The data concerning the mitogenic effect of CXCL12/CXCR4 signaling on A_{undiff} are contradictory but it has been suggested to promote the self-renewing state and prevent transition into the progenitor state in cultured A_{undiff} (Kanatsu-Shinohara *et al.* 2012, Yang *et al.* 2013a).

CSF1

CSF1 (colony-stimulating factor 1) does not affect the proliferation of cultured A_{undiff} but increases their stemness, that is, the ability to give rise to spermatogenic colonies after transplantation (Oatley *et al.* 2009). The role of CSF1 *in vivo* is not clear due to endocrine effects of *Csf1* deficiency on fertility (*op/op*) (Cohen *et al.* 1996). Notably, A_{undiff} display a highly enriched expression for *Csf1r*, the receptor for CSF1 (Oatley *et al.* 2009). Oatley *et al.* (2009) showed the expression of CSF1 in interstitial Leydig cells and select peritubular cells (Oatley *et al.* 2009). Further, a recent report by DeFalco *et al.* (2015) suggests that these rare CSF1-positive peritubular cells might actually be peritubular macrophages (DeFalco *et al.* 2015). CSF1 expression was also demonstrated in interstitial macrophages and vascular smooth muscle cells (DeFalco *et al.* 2015). The issue, however, warrants further investigation (Fig. 3).

Regulation of cell fate decisions in the SSC niche

Maintenance of the self-renewing state

GDNF is indispensable for maintenance of the GFR α 1-expressing A_{undiff} subset, that is thought to contain or possibly form (through active interconversion between equipotent singly isolated and short syncytial states, Figs 1 and 2) the steady-state stem cell population of the adult mouse testis (Meng *et al.* 2000, Hara *et al.* 2014). However, it does not achieve this alone but in collaboration with SSC-autonomous factors, including PLZF, FOXO1, GILZ and TAF4B (Buaas *et al.* 2004, Costoya *et al.* 2004, Falender *et al.* 2005, Goertz *et al.* 2011, La *et al.* 2018a).

PLZF (promyelocytic leukemia zinc finger) is expressed throughout the A_{undiff} population plus spermatogonia at early differentiation stages and plays a key cell-autonomous role in promoting SSC self-renewal. Accordingly, loss of functional PLZF results in progressive germ cell loss, testicular hypoplasia and infertility (Buaas *et al.* 2004, Costoya *et al.* 2004, Fischer *et al.* 2008). PLZF is a transcriptional regulator that can both stimulate and repress the expression of its target genes (David *et al.* 1998). In mouse SSCs, PLZF has been suggested to work in at least three different ways to ensure SSC maintenance: firstly, by modulating activity of SALL4, whose action is associated with spermatogonial differentiation; secondly, by directly and indirectly (via *Foxo1* and *Etv5*, e.g.) repressing differentiation genes (including *Kit*) and stimulating spermatogonial stemness genes (many of which are also GDNF targets); and thirdly, by indirectly opposing the mTORC1 pathway through upregulation of *Ddit4* (Filipponi *et al.* 2007, Hobbs *et al.* 2010, 2012, 2015, Lovelace *et al.* 2016, Chan *et al.* 2017). The fact that GDNF signaling and PLZF share a number of important target genes (*Bcl6b*, *Etv5* and *Lhx1*) strongly supports a model in which PLZF operates in a molecular circuit that amplifies the responsiveness to GDNF as a means to maintain SSCs (Song & Wilkinson 2014, Lovelace *et al.* 2016).

FOXO1 (Forkhead box protein O1) belongs to the family of forkhead transcription factors that have pleiotropic cell regulatory functions. In the mouse testis, loss of *Foxo1* results in spermatogenic failure due to defective SSC maintenance and a block in spermatogenesis (Goertz *et al.* 2011). FOXO1 exerts its effect on spermatogenesis through regulation of several genes specifically or highly expressed by the SSCs (including *Ret*, *Lhx1*, *Egr2* and *Sall4*) and needed for their maintenance (Goertz *et al.* 2011). Whether FOXO1 directly regulates these genes in A_{undiff} awaits further study.

Deletion of **Gilz** results in rapid exhaustion of A_{undiff} and degeneration of the germline through aberrant activation of the mTORC1 pathway in SSCs (La *et al.* 2018a). In WT SSCs, GILZ negatively regulates mTORC1 activity through a number of potential mechanisms, including suppression of ERK MAPK signaling and maintenance of expression of deubiquitinase USP9X. GILZ also plays a key role in spermatogenesis and promotes the expression of factors such as ZMYM3, which are required for spermatogenesis, in an mTORC1-independent fashion (Romero *et al.* 2012, La *et al.* 2018a).

TAF4B (TATA-box binding protein associated factor 4b) is a gonad-specific subunit of transcription initiation factor TFIID. *Taf4b*-null mice recapitulate the phenotype that is shared by many genes with a role in SSC maintenance: progressive loss of germ cells eventually leading to Sertoli-cell-only phenotype in most, if not all, seminiferous tubules (Falender *et al.* 2005). *Taf4b*-deficient mice become infertile by 3 months of

age, but the phenotype may not solely originate from SSC maintenance defects since perinatal germ cell development (gonocyte-to-spermatogonia transition, e.g.) is disrupted in these mice (Falender *et al.* 2005, Lovasco *et al.* 2015).

Data also demonstrate that maintenance of SSCs relies on sequestering differentiation-promoting factors, including components of the mTORC1 pathway, in ribonucleoprotein (RNP) complexes by the RNA-binding protein **NANOS2**, and the self-renewal promoting cellular transcriptome is thus partially indirectly achieved (Zhou *et al.* 2015). This likely provides SSCs with a fail-safe mechanism and buffers against undesirable effects of stochastic changes in transcriptional activity. Moreover, proteins and mRNAs trapped in RNP complexes might have an essential role in efficient and synchronous differentiation commitment once RNPs dissociate and the sequestered molecules are released into the cytoplasm. Recently, it was shown that **NEDD4** (neural precursor cell expressed developmentally downregulated protein 4-1), an E3 ubiquitin ligase, targets NANOS2 for degradation, and thus promotes differentiation (Zhou *et al.* 2017). NANOS2 also associates with **DND1** (Dead end protein homolog 1) in A_s and A_{pr} , and deletion of either results in gradual depletion of SSCs (Sada *et al.* 2009, Niimi *et al.* 2019).

Of the numerous GDNF target genes, the role of **ID4** in A_{undiff} has been most extensively studied. A_{undiff} display a heterogeneous expression for *Id4*, with the highest levels in stem populations (Oatley *et al.* 2011, Helsen *et al.* 2017, La *et al.* 2018b). Importantly, overexpression of ID4 blocks the stem-to-progenitor transition indicating that ID4 is a key regulator of the undifferentiated state (Helsen *et al.* 2017). Key regulators of A_{undiff} are summarized in Table 1.

In addition to these transcription factors and other proteins, a variety of **epigenetic and post-transcriptional mechanisms** have been implicated in the regulation of SSC maintenance and cell fate decisions within A_{undiff} . The latter include a number of non-coding RNAs (both short (miRNAs, e.g.) and long non-coding RNAs (lncRNAs)) with a proposed role in SSC regulation as reviewed recently elsewhere (van den Driesche *et al.* 2014, Hilz *et al.* 2016, Bie *et al.* 2018). Moreover, MIWI2, a protein associated with piRNA-mediated genome silencing and DNA methylation displays a restricted expression within progenitor A_{undiff} , the significance of which is yet to be defined (Carriero *et al.* 2017, Vasiliauskaite *et al.* 2018). Interestingly, the epigenome (DNA methylation at CpG sites plus histone modifications) of male germ cells undergoes profound changes during fetal development, whereas in postnatal germ cells, the epigenetic marks are more stable (Mäkelä *et al.* 2019).

It has been shown that the epigenetic landscape of SSCs is plastic and, similar to pluripotent cell types, characterized by bivalent (both activating H3K4me3

Table 1 Extrinsic and intrinsic factors with well-defined regulatory roles in maintenance or differentiation of SSCs in the adult mouse testis.

Factor	Expressed in	Significance	Key references
GDNF	SCs, TECs, PTMs	Critical for maintenance of SSCs <i>in vivo</i> and <i>in vitro</i>	Meng <i>et al.</i> 2000, Kubota <i>et al.</i> 2004, Bhang <i>et al.</i> 2018
FGF4/5/8 FGF2	LECs Likely many testis cell types	Regulates the number of SSCs/their niches Promotes SSC proliferation in synergy with GDNF (<i>in vitro</i>)	Kitadate <i>et al.</i> 2019 Kubota <i>et al.</i> 2004, Kanatsu-Shinohara <i>et al.</i> 2005, Ishii <i>et al.</i> 2012, La <i>et al.</i> 2018a
WNT6	SCs and interstitium	Promotes entry into the progenitor state	Takase & Nusse 2016, Tokue <i>et al.</i> 2017
RA	SCs and meiotic germ cells	Induces differentiation in the germline	van Pelt & de Rooij 1990, Sugimoto <i>et al.</i> 2012, Raverdeau <i>et al.</i> 2012, Endo <i>et al.</i> 2017
GFR α 1/RET	On the cell surface of self-renewing A _{undiff}	GDNF receptor complex, deletion results in rapid SSC depletion	Meng <i>et al.</i> 2000, Naughton <i>et al.</i> 2006
SHISA6	A subset of GFR α 1+ A _{undiff}	Confers resistance to differentiation-promoting WNT/ β -catenin signaling	Tokue <i>et al.</i> 2017
RAR γ	Differentiation-primed A _{undiff}	Required for A _{undiff} to A ₁ transition, i.e. differentiation	Gely-Pernot <i>et al.</i> 2012, Ikami <i>et al.</i> 2015
PLZF	A _{undiff} plus early differentiating spermatogonia	Promotes SSC self-renewal cell-autonomously by several mechanisms	Costoya <i>et al.</i> 2004, Filipponi <i>et al.</i> 2007, Hobbs <i>et al.</i> 2010, 2012
NANOS2	GFR α 1+ A _s and A _{pr}	Maintains the self-renewing state by sequestering differentiation-associated mRNAs in RNP complexes	Sada <i>et al.</i> 2009, 2012, Zhou <i>et al.</i> 2015
ID4	GFR α 1+ A _{undiff} differentiating male germ cells	Promotes the self-renewing state	Helsel <i>et al.</i> 2017
SALL4	A _{undiff} and differentiating spermatogonia	Required for spermatogenic differentiation and long-term maintenance of SSCs	Hobbs <i>et al.</i> 2012, Chan <i>et al.</i> 2017

A₁, type A₁ differentiating spermatogonia; A_{undiff}, undifferentiated type A spermatogonia; LECs, lymphatic endothelial cells; PTMs, peritubular myoid cells; SC, Sertoli cells; TECs, testicular endothelial cells.

and repressing H3K27me3) histone modifications placing promoters in a poised state capable of dynamic activation (Liu *et al.* 2016). Despite this potential, resolving bivalency is rarely accompanied with gene activation during early developmental transitions within the male germline leaving the significance of histone modifications for early cell fate decisions an open question (Hammoud *et al.* 2014). Although the global CpG methylation levels remain relatively stable in postnatal germ cells, locus-specific differential methylation in and around genes important for maintenance of a specific state, or transition into the following one, might still play an essential role in cell fate decisions within A_{undiff} (Kubo *et al.* 2015). Despite substantial research into epigenetic regulation of SSCs – summarized in Box 2 – the question as to whether dynamic changes in the epigenome regulate cell fate decisions within the A_{undiff} population warrants further investigation.

Differentiation priming

To become sensitive to the differentiation-inducing stimulus (RA), A_{undiff} need to exit the self-renewing state and undergo differentiation priming (Ikami *et al.* 2015, Tokue *et al.* 2017). This transition involves the activation of the mTORC1 pathway that plays a critical role in maintenance of SSCs, and aberrant mTORC1 activation promotes stem cell exhaustion (Hobbs *et al.* 2010, 2015, Zhou *et al.* 2017, La *et al.* 2018a). Exit from the GFR α 1-positive state entails cell size growth and induction of a

transcriptional program typical of differentiation-primed undifferentiated spermatogonia or progenitors (Hobbs *et al.* 2010, 2015, Ikami *et al.* 2015). These genes, whose expression is strongly upregulated or induced, include *Ngn3*, *Sox3*, *Lin28a* and *Rarg*, whereas *Gfra1*, *Ret*, *Lhx1*, *Eomes* and *Pdx1* are downregulated (La *et al.* 2018b). WNT/ β -catenin signaling plays an important role in the differentiation priming of A_{undiff} by promoting the transition from self-renewing to RA-responsive state (Yeh *et al.* 2012, Takase & Nusse 2016, Chassot *et al.* 2017, Tokue *et al.* 2017). Interestingly, Tokue *et al.* (2017) identified SHISA6 as a novel marker for a specific subset of GFR α 1-expressing A_{undiff} (Tokue *et al.* 2017). SHISA6 is suggested to act as WNT signaling inhibitor and thus confer resistance to the differentiation-priming program.

Differentiation commitment

It is widely considered that induction of RAR γ in a subset of A_{undiff} gives the cells a capacity to respond to RA, although alternative explanations have also recently been proposed (Gely-Pernot *et al.* 2012, Ikami *et al.* 2015, Lord *et al.* 2018) (Fig. 3). RA is the inducer of differentiation in the germline and to prevent premature exit from the progenitor state (that displays latent self-renewal capacity), its local availability and RAR γ expression need to be tightly regulated within the seminiferous epithelium (Mäkelä & Toppari 2018a). Extratubular RA that might interfere with proper timing of spermatogenic onset (from circulation or testicular

Box 2: Epigenetic regulation of A_{undiff}

KDM1A (lysine (K)-specific demethylase 1A) is a histone H3 lysine demethylase with gene-regulating activities including but not limited to removal of mono- and di-methylation at lysine 4 on histone H3 (H3K4). KDM1A is needed for postnatal maintenance of the germline, and its loss results in rapid depletion of all germ cells potentially due to destabilization of gene expression and the consequent inability to maintain functional SSCs or the spermatogenic process (Lambrot *et al.* 2015, Myrick *et al.* 2017). However, available data do not allow definitive conclusions to be drawn and further studies are needed to elucidate the functional role of KDM1A in A_{undiff} .

KMT2B is a H3K4 methyltransferase whose action in A_{undiff} has been proposed to epigenetically prime two sets of promoters, one activated during late spermatogenesis and the other after fertilization (Tomizawa *et al.* 2018). *Kmt2b* deletion in the adult testis results in an early block in differentiation. However, poor growth of *Kmt2b*-deleted SSCs *in vitro* suggests that KMT2B is important for SSC maintenance/expansion (Tomizawa *et al.* 2018). H3K4me2/3 established by KMT2B and related proteins, function as docking sites for transcriptional co-regulators, such as **PHF13** (PHD finger protein 13), an epigenetic modifier also associated with long-term maintenance of SSCs (Bordlein *et al.* 2011, Chung *et al.* 2016).

PRC1 (Polycomb repressive complex 1) has been suggested to coordinate timely activation of gene expression during spermatogenesis (Maezawa *et al.* 2017). PRC1 component **RNF2** (Ring finger protein 2) is an E3 ubiquitin ligase for histone H2A, and induces expression of plus forms complexes with **SALL4**, a transcription factor required for both spermatogenic differentiation and long-term maintenance of SSCs (Hobbs *et al.* 2012, Chan *et al.* 2017, Maezawa *et al.* 2017). Germ cell-specific ablation of RNF2 results in the deregulation of spermatogonial gene expression and a consequent early block in spermatogenesis (Maezawa *et al.* 2017). The list of dysregulated genes includes factors with previously characterized roles in SSC maintenance, like *Plzf* and *Rb* (retinoblastoma protein) (Buaas *et al.* 2004, Costoya *et al.* 2004, Hu *et al.* 2013, Maezawa *et al.* 2017). At least some of the genomic effects of RNF2 seem to be unrelated to its histone H2A E3 ubiquitin ligase activity and association with PRC1.

An additional factor associated with PRC1, **SCML2** (Scm Polycomb Group Protein Like 2), is a germline-specific Polycomb protein, and a potential epigenetic regulator of distinct A_{undiff} states due to its active role in establishing gene-silencing epigenetic marks H3K27me3 and H2AK119ub in the male germline (Hasegawa *et al.* 2015, Maezawa *et al.* 2018). SCML2 is recruited to epigenetically active loci in A_{undiff} and mediates gene silencing by forming a complex with **PRC2**. SCML2 thus complexes with both PRC1 and PRC2 to repress and coordinate timely expression of genes in the male germline. A similar process might regulate gene expression in distinct subsets of A_{undiff} . Despite relatively high expression of SCML2 in A_{undiff} , the effects of its depletion on these cells are rather modest.

An invaluable insight into the significance of epigenetic regulators for A_{undiff} is provided by ***Kdm6b***-deficient (lysine demethylase 6B) mice (Iwamori *et al.* 2013). KDM6B removes methyl groups from H3K27 and hence promotes gene activation. Loss of *Kdm6b* destabilizes intercellular bridges (ICBs) (Mäkelä & Toppari 2018b) in A_{undiff} and results in higher incidence of A_2 spermatogonia (Iwamori *et al.* 2013). As a likely consequence of enrichment of the self-renewing population, *Kdm6b*-deficient mice show larger testis size and improved lifetime fertility compared to controls. These data suggest that KDM6B activity is involved in exit from the self-renewing state characterized by instability of ICBs (Mäkelä & Toppari 2018b).

SETDB1 (SET domain, bifurcated 1) is a histone methyltransferase that represses gene expression through establishment of H3K9me3. *Setdb1* knockdown in cultured SSCs results in the upregulation of genes associated with apoptotic cell death plus differentiation and impinges on their regenerative capacity (An *et al.* 2014). SETDB1 has recently been implicated in promoting SSC survival via PTEN/AKT/FOXO1 signaling, a previously characterized pathway involved in SSC maintenance (Goertz *et al.* 2011) and suppression of pro-apoptotic gene expression (Liu *et al.* 2017). Besides establishing H3K9me3 to silence target loci, SETDB1 may also associate with DNA methyltransferases in A_{undiff} to increase DNA methylation (An *et al.* 2014).

Methylated DNA is considered a sign of transcriptionally repressed chromatin, and activation of gene expression is typically preceded by demethylation in and around the transcribed locus. Interestingly, differentiation commitment in the male germ line is accompanied by substantial upregulation of *de novo* DNA methyltransferases DNMT3A and DNMT3B, and destabilization of the DNA methylation machinery interferes with spermatogenic differentiation (Shirakawa *et al.* 2013). Further, entry of peripubertal undifferentiated spermatogonia to a differentiating state involves considerable demethylation in specific regions within the genome (Kubo *et al.* 2015). These regions harbor key genes associated with and indispensable for spermatogonial self-renewal and differentiation (Kubo *et al.* 2015). Moreover, male mice lacking *Dnmt3l*, which lacks enzymatic activity but acts as a processive catalyst and cooperates with DNMTs, lose all their germ cells by early adulthood (Hata *et al.* 2002, 2006). DNMT3L is proposed to control A_{undiff} proliferation and differentiation commitment, although other groups have reported that DNMT3L is essentially absent from spermatogonia, casting doubt on a direct role in A_{undiff} regulation (Sada *et al.* 2009, van Pelt & de Rooij 1990).

interstitium, including macrophages) is thought to be degraded by the CYP26B1 enzyme expressed in the PMCs (Vernet *et al.* 2006, MacLean *et al.* 2007, DeFalco *et al.* 2015). As reviewed by us elsewhere, an ingenious system that probably involves the action of all the different cell types of the seminiferous tubule (PMC, Sertoli cells, 4-5 generations of germ cells) ensures that RA-induced differentiation of spermatogonia takes place specifically at stages VII–VIII of the mouse seminiferous epithelial cycle (Mäkelä & Toppari 2018a).

Sertoli cell-derived RA is considered to induce the developmental onset of spermatogenesis in an asynchronous manner over the length of the seminiferous tubule resulting in the formation of the spermatogenic wave (Raverdeau *et al.* 2012, Tong *et al.* 2013). Meiotic germ cells have also been shown to take part in RA metabolism within the seminiferous epithelium, and the seminiferous cycle has been proposed to be maintained by RA produced by preleptotene and late pachytene spermatocytes (Vernet *et al.* 2006, Raverdeau *et al.* 2012, Davis *et al.* 2013). This system would ensure that a

new cohort of germ cells is recruited into spermatogenic differentiation after every 8.6-day interval (the duration of the seminiferous cycle in mouse) at stages VII–VIII of the seminiferous epithelial cycle (Mäkelä & Toppari 2018a). However, in the light of recent studies, it is difficult to draw conclusions about the significance of RA from different sources (Sertoli and germ cells) for the onset and completion of distinct RA-dependent events during spermatogenesis, and it is possible that these two intratubular sources of RA are functionally redundant (Endo *et al.* 2017, Teletin *et al.* 2019). Sequestration and storage of RA precursors by round spermatids at stages II–VI has been proposed as a mechanism to prevent the premature entry of differentiation-primed RAR γ -expressing A_{undiff} into spermatogenesis (Sugimoto *et al.* 2012). Then, specifically at stages VII–VIII as a result of RA action, the RAR γ -positive subset of A_{undiff} transits into type A1 differentiating spermatogonia and starts to express early markers of spermatogenic differentiation, including **KIT** and **STRAB** (Schrans-Stassen *et al.* 1999, Pellegrini *et al.* 2008, Zhou *et al.* 2008, Ikami *et al.* 2015).

Stage dependency of the SSC niche

This seemingly complex interplay of **GDNF**, **WNT** and **RA** signaling becomes more understandable when we consider the temporal aspect (Fig. 4A). Mouse spermatogenesis can be divided into stages (I–XII) (Leblond & Clermont 1952). The stages form segments and follow each other in a logical order along the length of seminiferous tubule to establish the wave of the seminiferous epithelium. *Gdnf* mRNA and reporter activity is found at the highest level in stages XII–IV, whereas *Wnt6* is most highly expressed at stages I–VIII (Grasso *et al.* 2012, Tokue *et al.* 2017). The level of RA is strictly regulated and the seminiferous epithelium is exposed to an RA pulse commencing at late stage VII (Hogarth *et al.* 2015, Endo *et al.* 2017). The highest levels of RA are recorded at stage VIII–IX, but RA is present at a relatively high concentration throughout stages VII–XII (Fig. 4A). GDNF/WNT6 and RA levels thus mirror each other suggesting that RA availability might regulate the expression of both *Gdnf* and *Wnt6* during the course of mouse seminiferous epithelial cycle. Moreover, the availability of mitogenic, self-renewal-promoting FGFs is possibly highest at late stages due to their inverse dependence on the number of proposed FGF sinks, i.e. A_{undiff} (Kitadate *et al.* 2019).

Based on available data, a following model for the regulation of SSC niche in mouse is proposed: GDNF plus FGFs synergistically induce self-renewal of SSCs at stages X–II (Tegelenbosch & de Rooij 1993, Sharma & Braun 2018). WNT6 then acts on the SHISA6-negative subset of A_{undiff} to prime the cells for differentiation. As a part of that program, RAR γ is induced and the progenitors become sensitive to RA between stages II and VI (Endo *et al.* 2015, Ikami *et al.* 2015). A pulse of RA at stages

VII–IX results in the differentiation commitment of these cells. Reducing levels of RA and a sharp decline in the number of FGF-consuming cells (due to A_{undiff} -to-A1 transition) at late stages allow GDNF and FGF levels, respectively, to rise resulting in the next wave of A_{undiff} proliferation at stages X–II and so on (Fig. 4B).

Functional dissection of adult SSC niche

We have recently defined the molecular signatures of self-renewing and differentiation-primed A_{undiff} subsets (La *et al.* 2018b). Generation of a compound reporter mouse line based on distinct expression of fluorescent proteins under *Plzf* and *Oct4* promoters enabled us to functionally dissect the A_{undiff} pool and shed light on the heterogeneity within the GFR α 1-positive population.

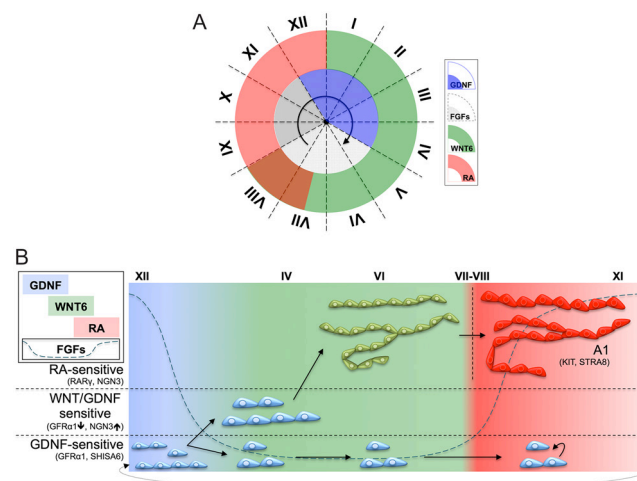


Figure 4 Regulation of the mouse SSC niche. (A) SSC niche clock. The availability of all three key factors (GDNF, WNT6 and RA) is tightly regulated during the cycle of the seminiferous epithelium. The highest level of GDNF and WNT6 is supposedly present at stages XII–IV and I–VIII, respectively, whereas a peak of RA has been measured at stages VIII–IX, but RA levels stay at a relatively high level throughout stages VII–XII. WNT6 and RA act on a common A_{undiff} subset, whereas GDNF is considered to exert its effect specifically on the self-renewing A_{undiff} subset (that is insensitive to WNT6/RA) under homeostatic conditions. Based on cyclical oscillations in the size of the proposed FGF sink (A_{undiff}), we hypothesize that the availability of LEC-derived FGFs is highest in stages IX–II. (B) Expansion of A_{undiff} and cell fate decisions within the SSC niche during the course of the seminiferous epithelial cycle. GDNF-sensitive A_{undiff} are exposed to increasing levels of GDNF and FGFs and respond to it by undergoing mitosis in stages X–II. A subset of the progeny becomes sensitive to differentiation-priming WNT6, that is upregulated at stage I on. This developmental program prepares the cells for differentiation and encompasses a shift in their transcriptome characterized by upregulation of *Rarg* and *Ngn3*, and downregulation of *Cfra1*. Differentiation-primed A_{undiff} thus become insensitive to physiological steady-state levels of GDNF and acquire competence to respond to differentiation-inducing RA by stage VII–VIII when they irreversibly transit to type A1 differentiating spermatogonia. RA-insensitive A_{undiff} are unaffected by the RA pulse and they are ready respond to the following wave of high GDNF/FGFs.

We further identified an unappreciated, adult testis-specific subset of GFR α 1-positive spermatogonia that displays a unique co-expression of *Pdx1*, *Brachyury*, *Eomes* and *Lhx1*. This A_{undiff} subset constitutes less than 0.2% of testicular cells in the adult mouse (La *et al.* 2018b).

Our data suggest that GFR α 1+ spermatogonia adopt different self-renewing states based on availability of niche factors. The self-renewing state marked by PDX1, EOMES and LHX1 prevails under tissue homeostasis. During development and under regenerative conditions, that is when temporary expansion of the SSC population is required, *Eomes* and *Lhx1* are upregulated, whereas *Pdx1* is downregulated, likely due to niche-derived cues (La *et al.* 2018b). These data suggest that the state marked by PDX1, EOMES and LHX1 might be optimized for long-term maintenance of SSCs under steady-state spermatogenesis.

The PDX1-positive A_{undiff} subset displays enriched stem cell activity in transplantation studies, although transplantation activity was not limited to this population. *In vitro* culture of A_{undiff} demonstrated that the number of PDX1-positive A_{undiff} declines upon passaging, at high cell density and in conditions where GDNF availability becomes limiting. Surprisingly, we also found that Oct4-GFP, classically considered to mark the stem populations, is primarily expressed in progenitors in the adult alongside *Ngn3*, *RAR γ* and *SOX3*, that is in cells that are destined to differentiate under homeostatic conditions. Interestingly, Oct4-GFP becomes differentially expressed in the male germline soon after birth and delineates A_{undiff} into stem and differentiation-primed subsets (Liao *et al.* 2019, La *et al.* 2018b). The extent to which the Oct4-GFP transgene faithfully marks endogenous *Oct4/Pou5f1* expression in adults remains unclear. PDX1 displays a mutually exclusive expression with Oct4-GFP both *in vivo* and *in vitro*. Importantly, late passage and dense A_{undiff} cultures are dominated by Oct4-GFP-expressing cells, that is progenitor A_{undiff} , and are nearly devoid of PDX1-positive cells (La *et al.* 2018b).

We also show that the proportion of these A_{undiff} subsets can be manipulated by regulating the availability of GDNF in the culture medium: when GDNF is readily available, cultured A_{undiff} tend to exist in a PDX1+/Oct4-GFP- (stem) state, whereas low availability of GDNF favors the PDX1-/Oct4-GFP+ (progenitor) state (La *et al.* 2018b). We propose that a similar mechanism controls A_{undiff} *in vivo* under steady state in a homeostatic tissue. However, in regenerative conditions or during development, when self-renewal stimuli are excessively expressed, (re)population of the seminiferous epithelium is mediated by A_{undiff} of a distinct self-renewing state characterized by high expression of *Eomes* and *Lhx1*. PDX1 expression in A_{undiff} is primarily established under homeostatic conditions. These data demonstrate that the niche status, i.e. niche-derived signals, differentially

support self-renewing states. Due to the dynamic nature of the niche, different states predominate during postnatal testis development, homeostasis and regeneration after tissue damage (Fig. 5).

Concluding remarks

Our understanding concerning the regulation of the SSC niche in mice has improved considerably during the past 10 years. While Sertoli cells can still be regarded as the most important somatic cell type of the SSC niche, it is increasingly appreciated that Leydig cells, PMCs, LECs and TECs also act as key niche components. The contribution of other cell types to the niche, such as peritubular macrophages and germ cells, has been insufficiently addressed and warrants further investigation. Despite substantial progress made in the field of SSC biology, the seminal findings by Meng *et al.* (2000) still remain the cornerstone, and much of SSC behavior can be explained solely based on the availability of GDNF (Meng *et al.* 2000).

As highlighted by Potter and DeFalco (2017), the complex and compartmentalized architecture of the testicular tissue, and the intricate molecular and contact-dependent interactions between the different cell populations complicates research in general, and in particular on regulation of the SSC niche. This is also due to the fact that most factors implicated in the control

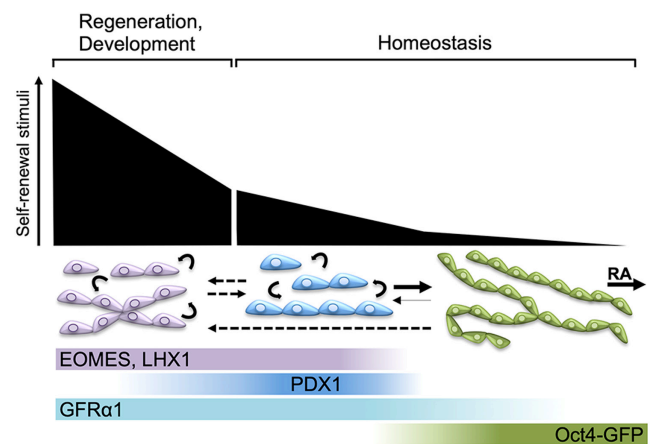


Figure 5 A simplified model describing the functional interconvertible states of A_{undiff} in the mouse SSC niche. GFR α 1-positive A_{undiff} adopt distinct self-renewing states based on the availability of self-renewal-promoting stimuli expressed by the niche. When exposed to high levels of these factors, as during regeneration or development, the state marked EOMES and LHX1 (purple cells) predominates. In a homeostatic tissue, A_{undiff} exist in a distinct PDX1/EOMES/LHX1-positive state (blue cells), potentially optimized for maintenance of life-long SSC function. If short-term expansion of A_{undiff} is required (regeneration), *Pdx1* is downregulated, whereas *Eomes* and *Lhx1* are upregulated. Importantly, Oct4-GFP+ A_{undiff} that are destined to differentiation under steady-state can revert back to the GFR α 1+ self-renewing state if exposed to sufficiently high levels of self-renewal stimuli, thus displaying latent stem cell capacity.

of SSC maintenance are likely derived from multiple sources within the testis, thus probably providing a buffer mechanism against adverse environmental effects on a particular cell population. Hence, cell type-specific ablation of a given factor, for example *Gdnf* or *Csf1*, fails to provide a comprehensive answer to the question concerning its role in SSC maintenance because SSC niche-associated factors are secreted by more than one cell type within the testis.

Thanks to robust growth of A_{undiff} *in vitro*, the effect of a candidate factor is rather easy to study in culture. Novel genetic tools that enable monitoring of the composition of the A_{undiff} culture (ratio: stem/progenitor) allow us to judge whether a molecule promotes the self-renewing state or if it merely acts to expand the population of differentiation-primed progenitors (La *et al.* 2018b). While novel molecules have been and continue to be linked with the regulation of the SSC niche, it has also been shown that the characteristics and functionality of the niche itself changes over the course of postnatal testis development and during tissue regeneration. One of the goals of future research will be to further dissect the molecular mechanisms controlling proliferation, quiescence and stem-to-progenitor transition within mouse SSCs.

Declaration of interest

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

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