Transcriptional/Translational Regulation of Mammalian Spermatogenic Stem Cells

7

Cathryn A. Hogarth

Keywords
Germ cell niche • miRNA • Spermatogenesis • Spermatogonial stem cells • Testis

Abbreviations

A_s	A single
A_{pr}	A paired
A_{al}	A aligned
dpp	days post partum
E	embryonic day
Lu	luxoid
PTU	polythiouracil
PGCs	primordial germ cells
RA	retinoic acid
SSC	spermatogonial stem cell

7.1 Introduction

A fundamental feature of mammalian spermatogenesis is the continuous production of sperm within the testis throughout an animal's entire reproductive lifetime. It takes many weeks for a single spermatogonial stem cell (SSC) to become a functional sperm yet it has been estimated that the human testis produces 1,000 sperm with each heartbeat or about 37 billion sperm per year [1]. To achieve and sustain this immense level of production, the pool of SSCs and the commitment of these cells to differentiation must be carefully coordinated. Like many other organ stem cell populations, very little is known about the factors that regulate the balance between SSC self-renewal and their commitment to spermatogenesis within the testis. This chapter will review our current understanding of the characteristics of mammalian SSCs and the transcriptional and translational controls governing SSC self-renewal and differentiation. I will focus predominantly on rodent models, as they have generated the majority of data in this field, however, where possible I will also comment on the regulation of SSC pools in other species.

7.2 Spermatogonial Stem Cells

The SSC pool in an adult mouse testis originates from a small cluster of cells, known as the primordial germ cells (PGCs), that reside in the proximal epiblast at embryonic day (E) 6 – E6.5. The synergistic activity of BMP4 and BMP8,

C.A. Hogarth (✉)
School of Molecular Biosciences and the Centre for Reproductive Biology, Washington State University, Pullman, WA 99164-7520, USA
e-mail: chogarth@vetmed.wsu.edu

G. Hime and H. Abud (eds.), *Transcriptional and Translational Regulation of Stem Cells*, Advances in Experimental Medicine and Biology 786, DOI 10.1007/978-94-007-6621-1_7,

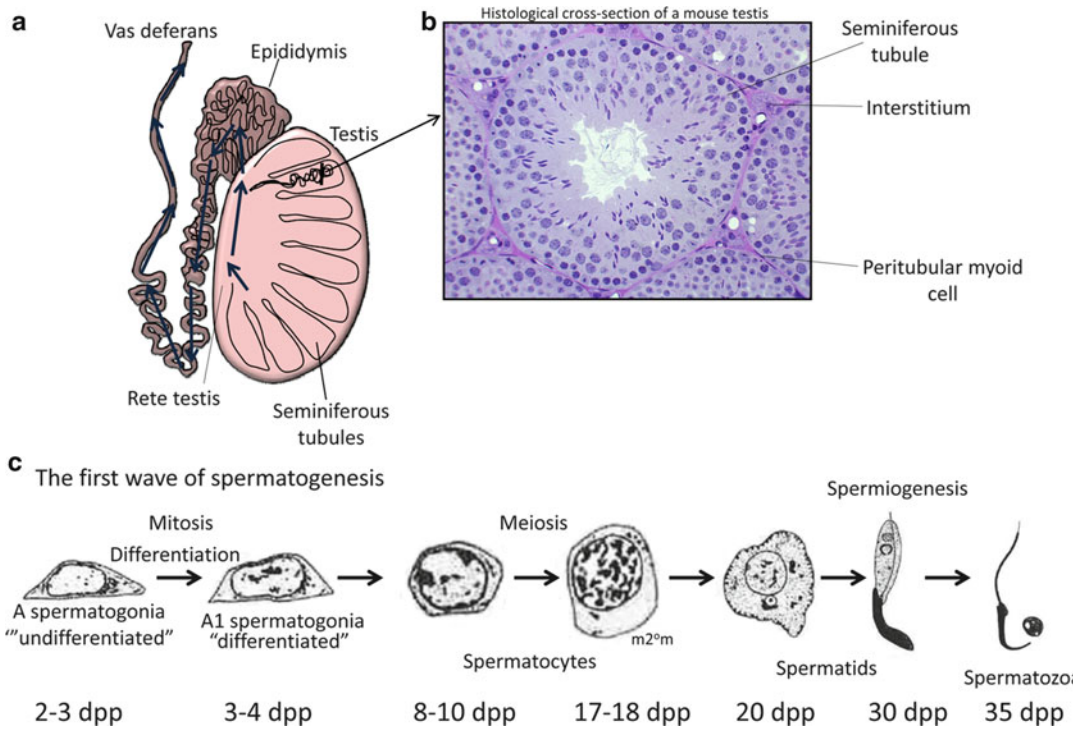

Fig. 7.1 Structure and cell types of the mouse testis. The mammalian testis is composed of seminiferous tubules intertwined so that the "start" and "end" of these tubules are both located in the rete testis. (**a**) Immotile sperm flow (*blue arrows*) from the lumen of the seminiferous tubules into the epididymis via the rete testis. During their passage through the epididymis to the vas deferens, sperm acquire their motility (Adapted from Cooke and Saunders [11]) (**b**) Histological cross-section through an adult mouse testis depicting seminiferous tubules, the peritubular myoid cells, and the interstitium (space between tubules). (**c**) The undifferentiated spermatogonia are derived from the gonocytes/prospermatogonia and are first present between 2 and 3 dpp. Expansion of both the undifferentiated, and differentiating spermatogonia, which first appear at 3–4 dpp, occurs via mitosis. Undifferentiated spermatogonia enter the differentiation pathway at the time of the A to A1 transition. The subsequent conversion of differentiating spermatogonia to spermatocytes occurs between 8 and 10 dpp and represents the initiation of meiosis. The first appearance of secondary spermatocytes (m2°m) occurs at around 18 dpp with the process of spermiogenesis initiating as soon as the first round spermatids are present. Elongating spermatids first appear at 30 dpp, with the first spermatozoa produced by at 35 dpp. Overall, these cellular events represent with first wave of spermatogenesis

produced in the extra-embryonic ectoderm, is essential for the differentiation of these cells [2] and by E7-E7.25, they become positive for the PGC marker alkaline phosphatase [3]. Over a period of around 4 days, these PGCs proliferate and then migrate, first passively as the hindgut invaginates internally during gastrulation, then actively towards the developing genital ridges [4]. It is estimated that by E12.5 approximately 3,000 PGCs have colonized the differentiating genital ridges [5] and are awaiting cues from the gonadal somatic cell environment to begin their differentiation down the male pathway [6–8]. PGCs that find themselves in a differentiating testis develop into gonocytes that continue to proliferate until approximately E16.5 when they then enter a period of quiescence until just before or shortly after birth [9, 10].

Within 2 days of birth, the gonocytes migrate to the basement membrane of the seminiferous cords, re-enter the mitotic cell cycle and are globally termed undifferentiated spermatogonia. This migration and resumption of mitosis initiates the first wave of spermatogenesis and a defined sequence of cell cycle events and morphological changes ensues before mature spermatozoa are produced. Figure 7.1 is a schematic representation of the first wave of spermatogenesis and outlines

the cell types present at each step and the days post partum (dpp) at which they appear. The term "gonocyte" is most often used to refer to germ cells within an embryonic or newborn testis. However, it has been proposed that these cells can also be categorized into three different types of prospermatogonia [4]; (M)-prospermatogonia undergoing proliferation in the embryonic testis, T1-prospermatogonia entering mitotic arrest in the embryo, and T2-prospermatogonia representing the population of gonocytes that resume proliferation shortly after birth as they migrate to the basement membrane. In any case, it is the gonocytes/T2-prospermatogonia that colonize the basement membrane of the newborn testis tubule and ultimately have one of two fates; either they immediately become differentiating A1 spermatogonia, representing the initiation of the first wave of spermatogeneisis, or they remain as undifferentiated spermatogonia and contribute to either the SSC pool or eventually commit to spermatogenesis in a highly regulated fashion [4, 9, 12].

In rodents, undifferentiated spermatogonia, also known as Type A spermatogonia, exist as A single (A_s), A paired (A_{pr}) or A aligned (A_{al}) cells. A_{pr} cells are two cells connected by an intracellular bridge and are the result of incomplete cytokinesis after the division of an A_s cell. The A_{al} populations are generated from dividing A_{pr} cells and can consist of chains of up to 4, 8, 16, or on the rare occasion, 32 cells developing in a syncytium. Currently, it is believed that the true SSCs are a subpopulation of the A_s cells and the chains of A_{al} cells represent a more differentiated population (reviewed in [13]). However, it has recently been postulated that the A_{al} spermatogonia also retain stem cell potential and may contribute to the SSC pool [14–16]. In any case, there are Type A spermatogonia that eventually become physiologically competent to respond to environmental cues and are triggered to differentiate to Type A1 spermatogonia. This differentiation step is known as the A to A1 transition and represents the commitment to spermatogenesis. A1 spermatogonia then progress through a series of five mitotic divisions, forming the A2, A3, A4, Intermediate and B spermatogonia, before becoming the first meiotic cells, the preleptotene spermatocytes, in the absence of a cell division. Two meiotic divisions of the spermatocyte population take place over a period of about 2 weeks before haploid spermatids appear. These spermatids undergo dramatic morphological changes in a process known as spermiogenesis and ultimately become spermatozoa. This carefully controlled series of differentiation steps is orchestrated into a cycle, known as the cycle of the seminiferous epithelium, such that specific types of germ cells are always in association (known as stages) at any given point along the tubules within the testis. In addition, the A to A1 transition and release of spermatozoa occurs simultaneously each time a specific set of germ cells is present within the tubule (Stage VIII) (recently reviewed in [17]). This cyclic arrangement of spermatogonial differentiation allows for the asynchronous production of sperm. In mice, it is believed that every pair of A_{pr} spermatogonia can give rise to 8,192 spermatozoa [18]. However, given that sperm production must continue to occur for months in rodents and years in humans, the regulation of SSC numbers must be carefully regulated (Fig. 7.2).

The model for balancing SSC self-renewal with differentiation in mammals is constantly evolving and differs between rodents and humans. In contrast to the rodent $A_s/A_{pr}/A_{al}$ model, the current paradigm for spermatogonial differentiation in humans and non-human primates suggests there are two different categories of spermatogonia; the A_{dark} population that act as a "reserve" pool of SSCs and do not actively contribute to spermatogenesis, and the A_{pale} spermatogonia that actively proliferate and balance their divisions between generating new A_{pale} cells and producing differentiating B spermatogonia (reviewed in [19]). This two-category model was also once proposed in rodents [20–22] and while the $A_s/A_{pr}/A_{al}$ model is currently favored among researchers in the field, there are recent data that support the hypothesis that a quiescent "reserve" pool of SSCs is present in the mouse testis [23].

The majority of data supporting the models described above have been derived from high-resolution morphology analyses and histological staining for markers of undifferentiated spermatogonia within whole mount preparations of seminiferous tubules. While these studies

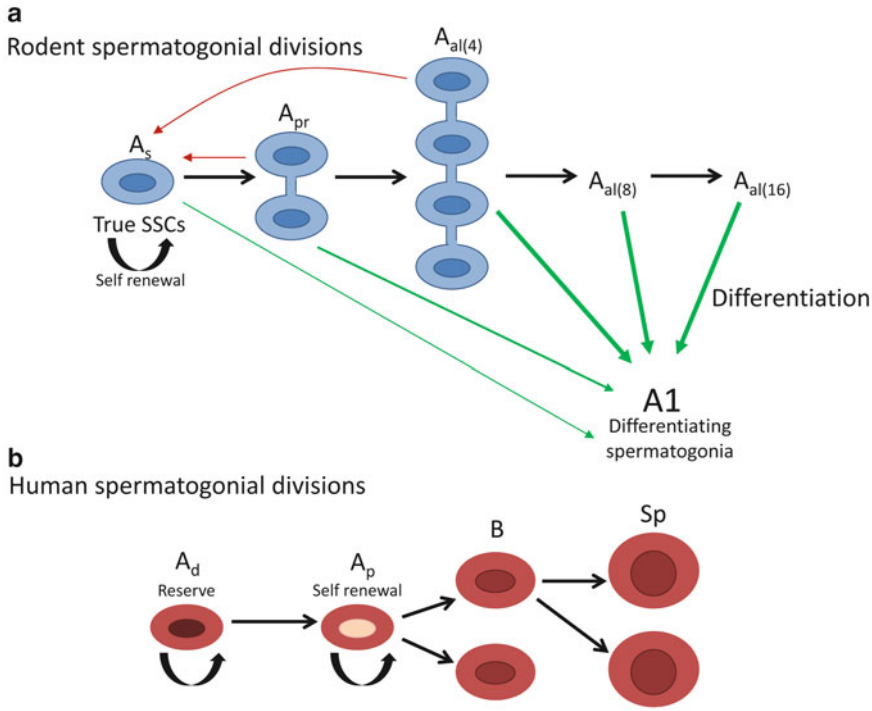

Fig. 7.2 Spermatogonial divisions in rodents and humans. (**a**) The rodent undifferentiated spermatogonial population consists of the A_{single} (A_s), A_{paired} (A_{pr}) and $A_{aligned}$ (A_{al}) spermatogonia. It is currently thought that the true SSCs are a subpopulation of the A_s cells and these cells balance their divisions between self-renewal and differentiation to progenitor A_{pr} spermatogonia connected by an intracellular bridge. Usually, A_{pr} spermatogonia would then divide simultaneously to generate chains of A_{al} spermatogonia consisting of 4, 8 or 16 connected cells. The A_{al} population is the most likely spermatogonial subtype to transition to differentiating A1 spermatogonia (*thick green arrows*). However, there is recent evidence to suggest that, if required, a small percentage of A_s and A_{pr} cells can also differentiate directly to A1 spermatogonia (*thin green arrows*), skipping the A_{al} stage of development [12, 14]. It has also been observed that A_{pr} and A_{al} cells can revert back to the A_s state (*red arrows*), implying that although there are more differentiated subpopulations of undifferentiated spermatogonia, their stem cell potential is only completely lost after the A to A1 transition. (**b**) In contrast to the rodent, the human undifferentiated spermatogonial population is thought to consist of A_{dark} (A_d) and A_{pale} (A_p) spermatogonia, which are named for differences in their nuclear morphology. The current model suggests that the A_d spermatogonia act as a quiescent reserve pool of stem cells and only divide when the pool of A_p cells is depleted. The A_p cells actively divide to self-renew or differentiate to form the B spermatogonia (B), which then divide and enter meiosis as spermatocytes (Sp)

have been very informative with regards to investigating how spermatogonial differentiation proceeds, there are still large gaps in our understanding of the molecular and morphological characteristics of the true SSCs. Currently, the only test that determines whether an SSC is present in a cell population is to transplant undifferentiated spermatogonia into the germ cell-depleted testis of a recipient mouse (first described in [24]. If fertility is restored in the recipient then the donated cell population contained SSCs. There are known protein markers of undifferentiated spermatogonia that disappear after the A to A1 transition, however, there has yet to be a gene product identified as SSC-specific. This chapter will review our current understanding of the genes expressed by undifferentiated spermatogonia and SSCs within the rodent testis, assess how close we are to identifying the elusive SSC marker, describe how the expression of these genes is regulated by exogenous signaling factors, and discuss the specific small RNAs that have been shown to regulate translation in undifferentiated spermatogonia.

7.3 Transcriptional Profiling of Undifferentiated Spermatogonia and SSCs

There has been a large research effort by the male reproductive biology community to identify gene products only present in SSCs and/or undifferentiated spermatogonia. Techniques used to define the molecular characteristics of these cells include sorting spermatogonia based on specific antigens (utilizing either fluorescence- or magnetic-activated cell sorting), characterizing GFP-expressing transgenic and knockout mouse models, and gene expression profiling arrays. A list of the most often utilized markers of SSCs and undifferentiated spermatogonia is given in Table 7.1. This section will overview published gene expression arrays that have provided insight into the molecular characteristics of SSCs as well as focus on those gene products that are most commonly used as markers of undifferentiated spermatogonia, those that show variations in expression between the A_s, A_{pr} and A_{al} populations and describe one that has recently been identified as a potential marker of only the true SSC population.

7.3.1 The Global Expression Profile of SSCs

The rapid improvement of technologies associated with transcriptome profiling of small samples and the isolation and culture of SSCs has lead to significant progress toward understanding the molecular characteristics of SSCs. It is estimated that only one in every 3,000–4,000 cells within an adult mouse testis is a true SSC [39]. The rarity of these cells and the lack of an unequivocal SSC marker has made isolation of a pure population of SSCs impossible. However, the use of gene knockout mice and localization studies has advanced our knowledge of markers of different populations of undifferentiated spermatogonia. In addition, an ex vivo system has been devised to support the self-renewal of SSCs, enabling the expansion of SSCs within a population of cultured undifferentiated spermatogonia [40]. Several laboratories have made use of these markers, culture systems and gene knockouts to generate global expression profiles of SSCs as well as identify new gene products essential for their self-renewal and differentiation. Table 7.2 outlines the current published array analyses of undifferentiated spermatogonia enriched for SSCs and outlines their major findings.

Culturing undifferentiated spermatogonia in a medium which supports the self-renewal of SSCs has been useful for identifying new gene products that participate in this process. Two studies examined the effect of GDNF, a growth factor produced by Sertoli cells that is essential for SSC self-renewal (discussed in detail below), on gene expression in cultured spermatogonia. Oatley et al., first starved SSC cultures of GDNF overnight, and then collected cell samples at various timepoints with and without GDNF replenishment [41]. As expected, removing GDNF from the culture medium increased the expression of genes associated with spermatogonial differentiation and downregulated the expression of genes known to play a role in SSC self-renewal. In order to select novel candidate genes that may regulate the pool of SSCs, Oatley et al., screened the data for those transcripts whose expression was first decreased by GNDF withdrawal and then increased after GDNF replacement. Only six transcripts emerged; *Bcl6b*, *Egr2*, *Egr3*, *Etv5*, *Lhx1* and *Tspan8*. Further investigation of *Bcl6b* function using siRNA knockdown in SSC cultures and analysis of a knockout mouse model suggested that BCL6B does play a role in SSC self-renewal [41]. A second study that cultured rat SSCs in the presence and absence of GDNF also saw an upregulation of *Bcl6b*, *Egr2*, *Egr3*, and *Etv5* in GDNF-treated cultures and identified three additional novel genes, *Bhlhe40*, *Hoxc4* and *Tec* [46]. Knockdown of *Bcl6b*, *Etv5*, *Bhlhe40*, *Hoxc4* and *Tec* in SSC cultures resulted in a reduction in SSC self-renewal as determined by transplantation experiments post-culture, without altering total cell numbers. Taken together, these two studies indicate that the selection of novel candidates via global expression profiling and the use of transplantation experiments after siRNA

Table 7.1 Common markers of undifferentiated spermatogonia

Full name	Abbreviation	Cell type	Function	References
Zinc finger and BTB domain containing 16	*Zbtb16* (also known previously as *Plzf*)	Most undifferentiated spermatogonia, small group of As cells not positive	Transcription factor	Buaas et al. [25], Costoya et al. [26], Oatley et al. [27]
Glial cell line derived neurotrophic factor family receptor alpha 1	*Gfrα1*	As and Apr spermatogonia. Longer Aal chains rarely positive	Receptor for GDNF signaling	Viglietto et al. [28], Dettin et al. [29], Ebata et al. [30], He et al. [31], Nakagawa et al. [14]
Thymus cell antigen 1, theta	*Thy1*	All undifferentiated spermatogonia	Glycoprotein cell surface marker	Kubota et al. [32]
POU domain, class 5, transcription factor 1	*Pou5f1* (also known previously as *Oct4*)	All undifferentiated spermatogonia	Transcription factor	Pesce et al. [33]
Neurogenin 3	*Ngn3*	Most Apr and Aal chains. As cells rarely positive	Helix-loop-helix transcription factor	Yoshida et al. [34], Yoshida et al. [12], Nakagawa et al. [14]
Nanos homolog 2	*Nanos2*	Most As and Apr spermatogonia. Longer Aal chains rarely positive	Zinc-finger RNA binding protein	Sada et al. [35], Suzuki et al. [36]
Spermatogenesis and oogenesis specific basic helix-loop-helix 1	*Sohlh1*	Present in all Aal spermatogonia and differentiating spermatogonia	Helix-loop-helix transcription factor	Ballow et al. [37, 38]
Inhibitor of DNA binding 4	*Id4*	Some As spermatogonia	Helix-loop-helix transcription factor	Oatley et al. [27]

Table 7.2 Published array analyses profiling SSCs

Experiment	Major findings	Reference
Moue SSCs starved of GDNF followed by GDNF replacement	6 transcripts decreased after GDNF withdrawl, increased after replacement: *Bclb6*, *Egr2*, *Egr3*, *Etv5*, *Lhx1*, *Tspan8*; *Bclb6* involved in SSC self renewal.	Oatley et al. [41]
Young versus aging GFRα1-positive mouse spermatogonia	Genes overexpressed at 6 dpp but not 8 months: *Gpr107*, *Tyrobp*, *Smad4*, *Ms4a7*, *Mrc1*; Known progenitor markers such as *Gfrα1* not affected.	Kokkinaki et al. [42]
Compared profiles in mouse THY1-positive, THY1-depleted and cultured THY1-postive spermatogonia	202 genes expressed 10-fold or higher in THY1-positive compared to THY1-depleted fraction, most notably *Bcl6b*, *Gfrα1*, *Lhx1*, *Csf1r*; CSF1R and its ligand, CSF1, important for stem cell self renewal.	Oatley et al. [43]
Compared profiles in mouse GFRα1-positive versus GFRα1-negative spermatogonia	639 transcripts differentially expressed, most notably *Lhx1*, *Bcl6b*, *Tyrobp*, *Csf1r*. Ligand of CSF1R, CSF1, promoted spermatogonial proliferation.	Kokkinaki et al. [44]
Arrays of wild-type, busulfan-treated and cryptorchid mouse testes	88 genes overexpressed in cryptorchid testes therefore may be enriched in SSCs; most notably *Crabp1*, *Dnmt3a*, *Sall4*, *Ccl7*, *Tyrobp*, *Oct4*.	Orwig et al. [45]
Rat SSCs cultured with and without GDNF	Identified *Bcl6b*, *Etv5*, *Egr2* and *Egr3*; 3 novel genes identified: *Bhlhe40*, *Hoxc4*, *Tec*. siRNA knockdown of *Bclb6*, *Etv5*, *Bhlhe40*, *Hoxc4* and *Tec* reduced SSC numbers in culture.	Schmidt et al. [46]
Compared profiles in human testes containing spermatogonia and Sertoli cells versus Sertoli cell-only	239 best candidates of human spermatogonially expressed genes, most notably *FGFR3*, *DSG2*, *c-CBL*, *UTF1*, *SNAP91*, *CTAG1A/B*.	Von Kopylow et al. [47]
Compared miRNA profiles in THY1-positive versus THY1-depleted spermatogonia	Identified miRNAs miR-21, miR-34c, miR-182, miR-183, miR-146, miR-465a-3p, miR-465b-3p, miR-465c-3p and miR-465c-5p as being enriched in THY1-positive cells. ETV5 can potentially upregulate miR-21 expression. miR-21 potentially important for SSC self-renewal.	Niu et al. [48]
Analyzed miRNA profiles in THY1-positive spermatogonia cultured with and without RA	Identified miR-17-92 and miR-106b-25 as being downregulated during spermatogonial differentiation. miR-17-92 knockout mice have smaller testes but only a mild spermatogenic defect.	Tong et al. [49]

knockdown of candidates in SSC cultures have been useful for identifying new gene products important for SSC self-renewal.

Isolating different subsets of undifferentiated spermatogonia based on cell surface markers has also been effective in obtaining enriched populations of SSCs for global expression analysis. Two different studies made use of markers known to be present on SSCs, THY1 and GRFα1, and collected cells from testes at 6 dpp, when spermatogonia are the prominent germ cell type within the testis [43, 44]. Both studies found *Bcl6b* and *Lhx1*, two transcripts that were also upregulated in undifferentiated spermatogonia cultured with GDNF [41, 46], to be enriched in SSCs. In addition, *Csf1r* exhibited the highest fold change in the SSC-enriched population by both analyses. Therefore, there is significant overlap in the global expression profiles of SSC-enriched cell populations even though different cell surface markers were being used to isolate the SSCs in these studies. Further investigation of

the function of CSF1R revealed that addition of its ligand, CSF1, to culture medium enhanced SSC self-renewal [43, 44], indicative of a role for CSF1R in signaling to the cell to maintain stemness. Also in support of a role in SSC self-renewal, Oatley et al. reported CSF1R protein to only be present on individual spermatogonia in very few testis tubules of 10 dpp testis cross sections [43]. Given that the true SSCs are thought to exist as single cells and are few in number, this staining pattern fits with CSF1R only marking the SSC population. However, these findings are in contrast to those of Kokkinaki et al. who were able to detect three to four CSF1R-positive spermatogonia per tubule in 6 dpp testis cross sections [44]; presumably too many positive cells for CSF1R to be thought of as a true SSC marker. Whether this discrepancy is due to a difference in sample age or is a function of reagents utilized to assess CSF1R localization has yet to be resolved. Transplantation analyses with near pure populations of CSFR1-positive germ cells will be important for determining whether that population contains a higher number of SSCs in comparison to isolations performed with the markers currently used for SSC isolation.

Microarray analyses have also been performed in order to compare SSC gene expression profiles in young versus old mice [42], in human testes containing spermatogonia and Sertoli cells versus Sertoli cell-only [47], and in mouse models that are highly divergent with respect to stem/progenitor germ cell content [45]. Interestingly, even though the starting cell populations in these studies were very different from the ones described above, there was overlap in the identification of SSC-enriched genes. For example, the BCL6 signaling network was found to be over-represented in human spermatogonia [47] and two transcripts that were identified in the GFRα1-positive population in 6 dpp mice [44], *Tyrobp* and *Ccl7*, were also present in the stem/progenitor spermatogonia fraction of cryptorchid testes [45], which are known to contain significantly higher numbers of SSCs [50]. These studies also revealed that the SSC population appears to contain an over-representation of proteins associated with RNA binding, DNA metabolism and protein biosynthesis, suggesting that post-transcriptional mechanisms play a role in regulating SSC function.

Global expression arrays have proven to be very useful for analyzing the molecular characteristics of undifferentiated spermatogonia, and in some analyses, identifying novel regulators of SSCs. However, they have provided limited information specific for SSCs due to the lack of a specific marker of this population. Technical advances in our ability to visualize cells within live tissue and continued identification of markers of subpopulations of undifferentiated spermatogonia has enabled researchers to combine multiple lines of evidence in order to keep narrowing down the list of required molecular characteristics of a true SSC. This next section will discuss the genes and proteins most often utilized as markers of undifferentiated spermatogonia and how integrating their expression patterns and functions is broadening our understanding of the SSC population.

7.3.2 Zinc Finger and BTB Domain Containing 16 (*Zbtb16*)

Zbtb16, also known as *Plzf*, was the first gene discovered to be essential for stem cell self-renewal in the mouse testis [25, 26]. It was identified as the defective gene in the naturally occurring *luxoid* (*lu*) mutant mouseline that first arose in the 1950s. These mutants showed limb abnormalities, impaired skeletal differentiation and a progressive loss of male fertility. The testis of an 8 month old *lu* mutant contained seminiferous tubules with highly variable phenotypes ranging from normal to devoid of all germ cells. Interestingly, tubules could be found that contained elongating spermatids but no other differentiating germ cell types, suggesting that normal spermatogenesis was initiated but the store of stem cells was depleted and further initiation and spermatogonial differentiation was blocked [25, 26]. In addition, transplantation experiments demonstrated that *lu* mutant spermatogiona cannot be maintained in an undifferentiated state and differentiate in an unregulated fashion [25, 26].

While it is clear that ZBTB16 is important for maintaining the SSC pool, there is still much to be learned regarding its function in the testis. ZBTB16 is expressed by most stem cell pools and has been shown to act as a transcriptional repressor by recruiting polycomb group proteins and histone deacetylases to chromatin [51]. A direct link between the repression of *C-kit*, a marker of differentiated spermatogonia, and ZBTB16 has been established both in vitro and in vivo [52]. In addition, ZBTB16 appears to be directly responsible for the downregualtion of mTORC1, a complex of proteins that promote cellular differentiation and cell growth by phosphorylating components of the translational machinery in SSCs [53]. It is also possible that ZBTB16 functions by regulating the epigenetic repression of chromatin in undifferentiated spermatogonia as histone methylation patterns are perturbed in *Zbtb16*-null germ cells [54].

Further analysis of ZBTB16 function in undifferentiated spermatogonia is required before fully understanding its role in the self-renewal of these cells but at present, ZBTB16 has surfaced as the gold standard marker of SSCs and undifferentiated spermatogonia. Tools for detecting *Zbtb16* transcript and/or protein are widely used to demonstrate the presence of undifferentiated spermatogonia in tissue/cell samples and histological sections. It is also often used to localize other proteins to SSCs and undifferentiated spermatogonia in multiple mammalian species (sheep, bulls, pigs, humans and non-human primates) and has recently been reported as being a useful marker in fish [55, 56].

7.3.3 Glial Cell-Derived Neurotrophic Factor Family Membrane Receptor Alpha-1 (GFRα1)

GFRα1 was first shown to be uniquely expressed within the testis in Type A undifferentiated spermatogonia in 2000 [28, 31]. This protein is the membrane bound receptor for GDNF and regulates cellular differentiation through activation of RET tyrosine kinase. Because GFRα1 sits on the outside of the cell, it can be exploited as a target of antibodies attached to magnetic beads, allowing the isolation of undifferentiated spermatogonia from testis tissue (MACS). Buageaw et al. demonstrated that when MACS was used to collect GFRα1-positive cells from rodent testes, the resulting cell population was enriched for known markers of SSCs (ITGA6 and CD9), depleted of C-KIT-positive cells, and could repopulate a germ cell-deficient testis after transplantation, suggesting that a subset of GFRα1-positive cells are also true SSCs [57]. MACS directed against GFRα1 is now common practice in laboratories attempting to isolate undifferentiated spermatogonia from whole testes.

Further evidence to support a role for GFRα1 in the maintenance of the SSC pool has stemmed from studies of the *Gfrα1*-null mouse and knockdown studies. Mice deficient in *Gfrα1* die shortly after birth due to defects in the development of their enteric nervous and renal systems. To study this model, Naughton et al. dissected the testes from either *Gfrα1*-null male mice or their wild-type siblings on the day of birth and explanted them subcutaneously into nude mice in order to investigate whether postnatal germ cell development could proceed normally in these testes [58]. Normal spermatogenesis was observed in the explanted wild-type testes after 8 weeks. However, there was a complete absence of germ cells in the mutant testes at 8 weeks post-surgery, with a significant decrease in germ cell numbers seen after 7 days. Immunohistochemical studies were used to determine that the numbers of SSCs in the *Gfrα1*-deficient testes were comparable to those in the wild-type animal at birth, indicating that the reduction in germ cell numbers after 7 days was not due to unequal germ cell numbers to begin with [58]. Whether germ cell loss was a result of decreased proliferation, apoptosis or premature differentiation was not investigated in the explanted *Gfrα1*-null testes. However, studies on *Gdnf*- and *Ret*-deficient testes suggested that loss of this signaling pathway resulted in a loss of proliferation of the SSCs and in their advanced differentiation [58, 59]. This conclusion is also supported by siRNA knockdown studies of *Gfrα1* in cultures of undifferentiated spermatogonia [31]. Transfecting *Gfrα1*-specific siRNAs into

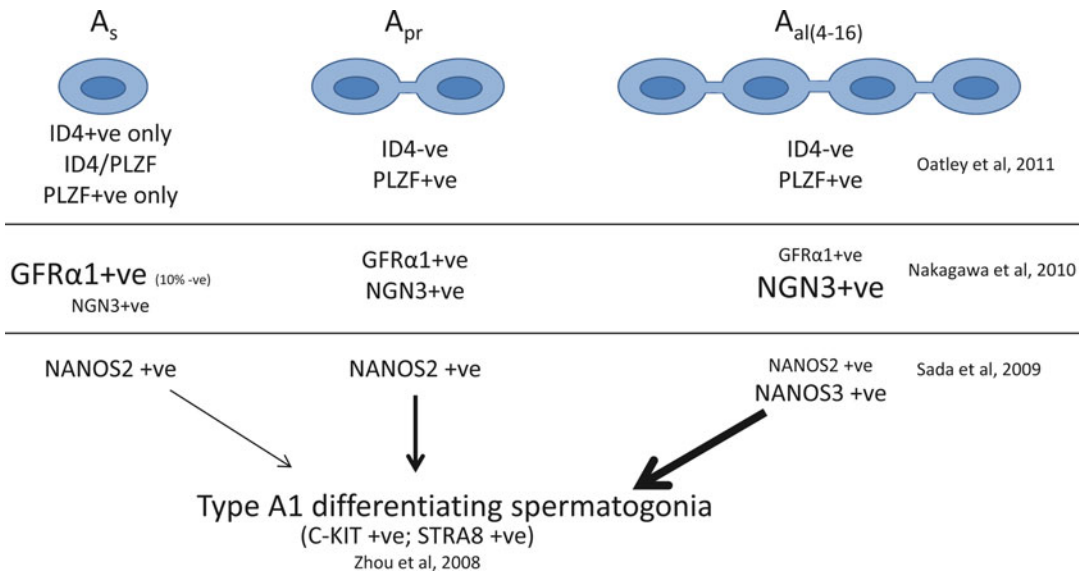

Fig. 7.3 Undifferentiated Type A spermatogonial patterning. Various markers can be used to distinguish between the different subpopulations of Type A spermatogonia (A_s, A_{pr}, $A_{al(4–16)}$). Listed below the schematics of each subpopulation are the markers that can be used to differentiate between them. The articles from which these data were derived are given. Size of lettering for each protein represents its relative expression level across the different subpopulations, i.e. small lettering equals weak expression and/or very few positive cells; larger lettering equals strong expression and/or lots of positive cells. The *black arrows* indicate that Type A1 differentiating spermatogonia can be derived from all three subpopulations but the *thickness of the arrows* indicates that this differentiation step is more likely to occur from the A_{al} cells over the A_{pr} or A_s

cultures of undifferentiated spermatogonia resulted in a reduction in both the numbers and size of germ cell clusters due to a decrease in proliferation and excessive differentiation of these cells. In addition, the phosphorylation of RET was significantly reduced in cultured cells, demonstrating a block in the GDNF signaling pathway. Taken together, these data show a requirement for GFRα1 in maintaining the pool of SSCs yet very little work has been performed to ascertain the downstream targets of signaling through GFRα1. Use of the Cre-Lox gene targeting system will be important for further investigation of the downstream effects of GFRα1, specifically in germ cells.

The precise localization pattern for GFRα1 in the testis has evolved over the years. GFRα1 was thought to be a marker of undifferentiated spermatogonia, yet its expression appears to be non-uniform among the A_s, A_{pr} and A_{al} cell populations and displays stage-specificity across the cycle of the seminiferous epithelium. Grisanti et al. noted a heterogeneous pattern of GFRα1 localization in A_s and A_{pr} cells, with approximately 10 % of A_s spermatogonia being negative for GFRα1 and approximately 5 % of A_{pr} chains displaying asymmetric GFRα1 staining (one cell positive and not the other) [60]. The asymmetric staining of GFRα1 in A_{pr} and A_{al} chains was also observed in a separate study [36], although the question of whether asymmetric staining represents functionally distinct cells within one cyst or is the result of capturing a moment in time when the expression pattern is changing throughout the whole cyst in a wave-like manner remains unanswered.

Another study has postulated that GFRα1 localization, in tandem with the expression pattern of NGN3, another marker of undifferentiated spermatogonia, can be used to differentiate between Type A spermatogonia (Fig. 7.3). Whole mount immunohistochemistry and live tissue imaging were used to investigate expression within, and the behavior of, the Type A spermatogonial chains [14]. GFRα1 was only found in a

small percentage of undifferentiated spermatogonia, with the A_s and A_{pr} cells being the predominant positive cell types. GFRα1-positive A_{al} cysts were rare [14]. Less than 20 % of the 140 8-cell cysts and none of the 54 16-cell cysts examined were positive for GFRα1 and in contrast to the studies that reported asymmetric staining, asymmetric staining was not evident in A_{al} cysts that were positive [14]. This study also visualized the fragmentation of long A_{al} cysts into smaller chains and also single cells, suggesting that cells within an A_{al} cyst maybe be able to recover their stem cell potential. The numbers of spermatogonial chains found to be positive for GFRα1 also appears to vary across the cycle of seminiferous epithelium [61]. In all stages of the cycle in the mouse testis, Grasso et al. reported that GFRα1-positive chains of A_{al} cells were more prevalent than either A_s or A_{pr}. However, significantly fewer GFRα1-positive A_{al} chains were observed in Stages VII and VIII, the point in the cycle during which the A to A1 transition is known to occur. Taken together, these data suggest that GFRα1 is an important marker of the undifferentiated spermatogonia and may be lost from these cells as they prepare to differentiate. Continued advances in live imaging techniques will resolve whether A_{pr} and A_{al} chains are truly asymmetric for GFRα1 in addition to contributing to our understanding of whether chained cells retain stem cell potential.

7.3.4 Neurogenin 3 (NGN3)

NGN3 is a class B basic helix-loop-helix transcription factor that was first identified in undifferentiated spermatogonia using a yeast-2-hybrid screen for transcription factors in OCT4-positive germ cells [34]. Transplantation experiments demonstrated that true SSC cells are present within populations of *Ngn3*-positive cells [12] and a transgenic mouseline expressing GFP under the control of the *Ngn3* promoter has been used to demonstrate that *Ngn3* expression can delineate between the first wave and subsequent waves of spermatogenesis. *Ngn3* is expressed by A_s, A_{pr} and A_{al} spermatogonia, yet unlike other markers, is not present in gonocytes [34]. A physical separation of *Ngn3*-positive and *C-kit*-positive cells within cross sections of juvenile testes was also observed [12], supporting the theory that differentiating spermatogonial populations are spatially separate from the undifferentiated germ cells [62]. Yoshida et al. were also able to detect gonocytes that never become *Ngn3*-positive and differentiate directly into *C-kit*-positive spermatogonia [12]. Dual-labeling transgenic mouselines were utilized to illustrate that the cells which skip being *Ngn3*-positive (the *Ngn3*-negative lineage) are the cells that generate the first wave of spermatogenesis and give rise to the very first spermatozoa [12]. The gonocytes that did transition into *Ngn3*-positive undifferentiated spermatogonia (the NGN3-positive lineage) were those responsible for producing all subsequent rounds of spermatogenesis [12]. As a result, in the testis of a fully mature male mouse, the NGN3-negative lineage is absent and all spermatozoa originate from the NGN3-positive cells.

In addition to *Ngn3* marking different populations of germ cells in the neonatal testis, its localization pattern in association with GFRα1 is now thought to represent how likely a Type A spermatogonia is to differentiate in the mature testis (Fig. 7.3). In the adult, *Ngn3* can be detected in A_s, A_{pr} and A_{al} populations, however, only 10 % of A_s cells are *Ngn3*-positive, with this percentage increasing as the length of the chain increases [14]. In this study, of the 54 16-cell A_{al} chains analyzed, all were *Ngn3*-positive. Pulse-chase and live imaging experiments demonstrated that the small percentage of *Ngn3*-positive A_s cells typically divided to become A_{pr} and A_{al} chains rather than generating two new A_s cells, suggesting that *Ngn3* may mark the more advanced undifferentiated spermatogonia [14]. This study also hypothesized that the very small number of *Ngn3*-positive A_s cells that do divide and generate two new single cells are actually transiting directly into differentiating A1 spermatogonia and not undergoing self-renewal. This conclusion was based on the presence of single C-KIT-positive cells only 2 days into their pulse chase experiments [14]. These data, in combination with the GFRα1 localization data discussed

above, have generated a new model for markers that distinguish the first wave and subsequent waves of spermatogenesis and the developmental transition of A_s, A_{pr} and A_{al} cells to becoming A1 spermatogonia (Fig. 7.3). This new model suggests that Type A spermatogonia do not always arise from a linear pathway. A_s, A_{pr} and A_{al} cells can all transition directly into A1 spermatogonia. Based on the *Ngn3* and GFRα1 studies, it would appear the majority of cells in the adult testis do follow a linear pathway of differentiation and transition from being GFRα1-positive to NGN3-positive to A1 spermatogonia. However, A_s cells can differentiate directly to A1 spermatogonia and cells within a cyst still retain the ability to self-renew. Further investigation of the transcriptional profile of these different subpopulations of undifferentiated spermatogonia and the cross-talk that occurs between individual cells and cysts is required in order to determine how these cells balance between self-renewal and differentiation.

7.3.5 Nanos Homolog 2/Nanos Homolog 3 (NANOS2/NANOS3)

The NANOS family of genes was first identified as being important for germ cell development from studies of maternal effect genes in *Drosophila* [63]. While only one *Nanos* gene is present in the *Drosophila* genome and its expression is essential for the formation of functional gametes, three homologs are expressed in mammals, *Nanos 1*, *Nanos2* and *Nanos3*. Only *Nanos2* and *Nanos3* are known to be essential for normal mammalian spermatogenesis [64]. Both NANOS2 and NANOS3 are zinc-finger RNA binding proteins that appear to be able to repress protein production either by physically blocking translation or facilitating the degradation of target mRNAs [65–67].

A deletion of *Nanos2* only affects spermatogenesis and results in a loss, followed by the complete absence of germ cells within the testes of mutant animals by 4 weeks of age [64]. The decrease in germ cell numbers is first observed in null male gonads at E15.5 and NANOS2 appears to promote germ cell differentiation down the male pathway while simultaneously inhibiting the female program (recently reviewed in [68]) in the normal embryonic testis. Expression analysis in wild-type testes revealed that NANOS2 was present in undifferentiated spermatogonia in the postnatal testis [35], but its function in these cells was difficult to study given the requirement for NANOS2 in the embryonic testis. To counteract this issue, Sada et al. utilized a tamoxifen-inducible Cre-Lox system to delete *Nanos2* in the adult mouse testis [35]. A progressive loss of differentiating germ cells was observed in the testes of the tamoxifen-induced mutant animals, with all germ cells lost within only a few cycles of the seminiferous epithelium. Staining for PLZF expression in the mutant testes revealed a decrease in the numbers of undifferentiated spermatogonia by 2 weeks post-tamoxifen treatment, and GFRα1-positive cells were lost almost immediately after *Nanos2* deletion [35]. Sada et al. concluded that the germ cell loss within the conditional *Nanos2*-null testes was the result of a loss of SSCs.

Pulse-chase labeling experiments using a *LacZ* reporter gene also demonstrated that stem cells were present in the *Nanos2*-expressing population of undifferentiated spermatogonia and that by comparison to a separate but similar study, the *Nanos2*-expressing undifferentiated spermatogonial population contained a higher proportion of stem cells than the *Ngn3*-expressing population [12, 35]. Overexpression of NANOS2 in male germ cells also supports a role for this protein in the maintenance of the SSC pool [35]. In *Nanos2*-overexpressing testes, an increase in the numbers of PLZF- and GFRα1-positive undifferentiated spermatogonia was detected and these cells demonstrated a slower rate of proliferation. In addition, these testes contained a higher proportion of A_s and A_{pr} cells and a low percentage of longer chains of A_{al} spermatogonia. Therefore, *Nanos2*-overexpressing male germ cells display properties similar to what is currently believed to be the most primitive set of undifferentiated spermatogonia.

Less is known about the role of NANOS3 in the postnatal testis. The global deletion of *Nanos3*

in mice results in defects during both oogenesis and spermatogenesis, with all germs cell absent from both gonads by E15.5 [64]. Expression analyses have revealed that NANOS3 is present in undifferentiated spermatogonia and can also be detected in some differentiating spermatogonia [36]. The role that NANOS3 plays in these cells is still unclear. Knockdown of *Nanos3* in cultures of differentiating human embryonic stem cells decreased the expression of germ cell genes responsible for the maintenance of pluripotency, meiotic initiation and progression [69], suggesting a role for NANOS3 in the differentiation of spermatogonia. The observation that the overexpression of *Nanos3* in the postnatal testis leads to a delay in spermatogonial cell cycle progression also supports this premise [67]. The investigation of NANOS3 expression across the cycle of the seminiferous epithelium revealed a stage-specific localization within the A_{al} chains and differentiating spermatogonia [35]. During Stages VII and VIII, when the A to A1 transition is taking place, the NANOS3-positive A_{al} spermatogonia strongly co-expressed C-KIT, and this was not the case during other stages of the cycle. In addition, NANOS3 could be detected, albeit weakly, in the differentiating A1 and A2 spermatogonia from Stage IX through Stage XII yet this signal was lost from the A3 through B differentiating spermatogonia present in Stages I through IV. In contrast, NANOS3-positive undifferentiated spermatogonia were present throughout all stages. These expression data imply that NANOS3 could be important for the A to A1 spermatogonial transition, however, conditional knockout mouse studies will be required to gain a more detailed understanding of how NANOS3 functions in the postnatal testis.

The localization pattern of NANOS2 and NANOS3 aids in extending the evolving theory of functionally distinct populations of undifferentiated spermatogonia. Using reporter gene-expressing transgenic mice, Suzuki et al. investigated the localization of both NANOS2 and NANOS3 in all types of undifferentiated spermatogonia and colocalized the expression of these proteins with GFRα1 and NGN3 [36]. The resulting localization patterns reinforced the heterogeneity observed within the undifferentiated spermatogonia and are summarized in Fig. 7.3. As mentioned above, NANOS2 was found to be expressed almost exclusively in A_s and A_{pr} spermatogonia, with only very weak signal observed in the A_{al} chains. NANOS3 localized to undifferentiated spermatogonia of all chain lengths and some differentiating spermatogonia [32]. When compared to GFRα1 and *Ngn3* localization, NANOS2 was more likely to be detected in A_s and A_{pr} cells that were GFRα1-positive/*Ngn3*-negative, whereas NANOS3 was more strongly expressed in A_{al} chains that were GFRα1-negative/NGN3-positive. In addition, there was a small population of Type A spermatogonia that were only positive for GFRα1. These data lead to the conclusion that the undifferentiated spermatogonia can be classified into 3 basic categories: (1) GFRα1-positive/NANOS2-positive/NANOS3-negative/NGN3-negative; (2) GFRα1-positive/NANOS2-positive/NANOS3-positive/NGN3-negative; and (3) GFRα1-negative/NANOS2-negative/NANOS3-positive/NGN3-positive. Clearly, the molecular factors that uniquely equip undifferentiated spermatogonia for the A to A1 transition are complex in nature and as more markers are identified, the model of spermatogonial differentiation will continue to evolve.

The regulation of expression of *Nanos2* and *Nanos3* RNAs and the targets of their gene products are beginning to be elucidated. GDNF signaling is essential to maintain the expression of *Nanos2* and ectopic expression of this gene can help to restore the loss of SSCs seen after *Gfrα1*-depletion [70]. RA is an important regulator of spermatogonial differentiation and meiotic initiation (reviewed in [17]) and there is evidence to suggest that it represses the expression of both *Nanos2* and *Nanos3* [67, 71]. *Nanos2*-deficient gonocytes have been shown to precociously enter meiosis and overexpression of *Nanos2* in fetal female germ cells prevents meiotic entry, leading to the conclusion that NANOS2 may act as a meiotic inhibitor through a post-transcriptional regulatory mechanism. Studies showing NANOS2 can associate with ribonucleoparticles and polysomes in both fetal and postnatal male germ cells and that it can bind two RNA transcripts

that code for proteins known to be important for spermatogonial differentiation, *Gata2* and *Taf7l*, also support this conclusion [71].

7.3.6 Inhibitor of DNA Binding 4 (ID4)

The inhibitor of DNA binding (ID) protein family consists of four helix-hoop-helix transcriptional repressors that are often expressed in populations of undifferentiated cells [72, 73]. It has been known for nearly 15 years that all members of this protein family are present in either germ or Sertoli cells within the testis [74]. However, it has only recently been demonstrated that ID4 localizes to a specific subset of A_s spermatogonia and may be considered as a possible marker of the true SSC population [23]. Whole mount immunofluorescence and analysis of seminiferous tubules isolated from transgenic mice expressing GFP under the control of the *Id4* promoter demonstrated that only A_s spermatogonia expressed ID4. Colocalization of the GFP signal with ZBTB16 revealed that the majority of ZBTB16-positive cells were negative for ID4 and about 50 % of ID4-positive cells were ZBTB16-positive [23]. These results indicate that ZBTB16 may not be a global marker of all undifferentiated spermatogonia and imply that ID4 may mark a previously unidentified subpopulation of these cells. Analysis of *Id4*-null male animals revealed a reduction in testis weight and sperm concentration in the epididymis over an 8 month period and the progressive loss of fertility [23], all hallmarks of impaired SSC function. Also, SSC culture and transplantation experiments confirmed that the spermatogenic defect seen in *Id4*-null males was the result of a loss of SSC self-renewal rather than a defect in SSC proliferation. Spermatogenesis was never completely blocked in the *Id4*-null model, as some male knockout mice were still fertile at 8 months of age and their testes were found to contain a few tubules with a full complement of germ cells. Therefore, further investigation will be required in order to determine what protein(s) can partially compensate for the loss of ID4 in the SSC population.

Global expression array analyses have made significant contributions to our understanding of the molecular characteristics of SSCs. In addition, continued identification and functional analyses of proteins specific to undifferentiated spermatogonia are drawing us closer to being able to definitively pinpoint the true SSCs. ID4 is an extremely promising candidate and further colocalization studies will be important for integrating ID4 with the GFRα1/*Ngn3*/Nanos story. While the search for the elusive true SSC marker will and should continue, studies into how the undifferentiated spermatogonia interact with one another and the surrounding somatic compartment are equally important to the eventual goal of utilizing SSCs in therapeutics.

7.4 Regulating SSC Self-Renewal and Differentiation Through the Germ Cell Niche

The proper expression of SSC proteins that regulate their self-renewal and differentiation relies on signals derived from the surrounding somatic cells. These cells and signals build what is known as the germline stem cell niche and ongoing research efforts hope to dissect how this microenvironment balances the differentiation of SSCs with the maintenance of a healthy population of stem cells (for a recent review see [75]). The defining feature of each stem cell niche is the milieu of growth factors that not only home the stem cells to the niche but also keep them there and then direct them to either proliferate or differentiate. This section will summarize our current understanding of the growth factors important for testis niche function and how SSCs localize to their niche (summarized in Fig. 7.4).

7.4.1 Growth Factor Signals Promoting SSC Self-Renewal

7.4.1.1 Glial Cell Line-Derived Neurotrophic Factor (GDNF)

It has been known for over a decade that GDNF is essential for SSC function in mammals yet the

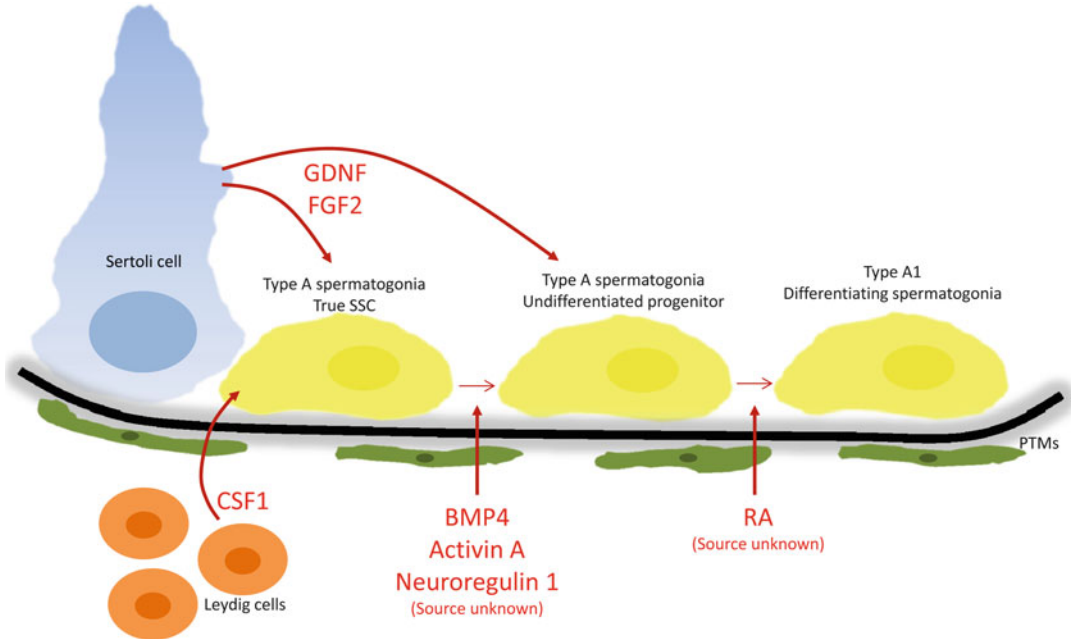

Fig. 7.4 Growth factor regulation of the germline stem cell niche in rodents. Schematic representing the current model for the growth factors and signaling molecules involved in regulating the rodent germline stem cell niche and the cell types responsible for their production. Sertoli cells are critical to the formation of the niche microenvironment and are known to secrete GDNF and FGF2. These two growth factors both act to support the self-renewal of the true SSCs and the maintenance of the progenitor Type A undifferentiated spermatogonia. CSF1 is produced by the Leydig cells in the testicular interstitium and there is evidence to suggest that its only function is to support the self-renewal of the true SSCs [43]. BMP4, Activin A and Neuroregulin 1, whose site of production within the testis is currently unknown, act upon the SSCs to promote their differentiation to the progenitor Type A spermatogonia, while RA is known to drive the transition from the progenitor undifferentiated Type A spermatogonia to the differentiating Type A1 spermatogonia. How RA is generated within the testis is still under investigation (Adapted from Oatley and Brinster [75])

downstream effects of this growth factor on SSCs are only beginning to be understood. GDNF was first identified as being secreted by glial cells but is now known to be produced by several organs during development (reviewed in [76]). Within the testis, it is produced and secreted by the Sertoli cells [77] with a recent report suggesting that the human peritubular myoid cells may also make GDNF [78]. The overexpression of GDNF results in excessive proliferation of undifferentiated spermatogonia, the disappearance of differentiating germ cells, and the formation of germ cell tumors, whereas mice deficient in GDNF lose germ cells with aging [79]. While these mouse models are only suggestive of a role for GDNF in SSC self-renewal, in vitro studies have provided definitive proof. Addition of recombinant GDNF to medium enhanced the ability of cultured undifferentiated spermatogonia to re-establish spermatogenesis after transplantation and GDNF is the only supplement required in serum-free chemically-defined medium to support the long term expansion of SSCs in populations of undifferentiated spermatogonia from DBA/2J mice [40, 80, 81]. In vitro studies with SSCs from different genetic backgrounds, such as C57BL/6, have shown that GDNF and either FGF2 or EGF are required for SSC expansion [40]. While it is clear that GDNF is essential for SSC self-renewal, its function is not specific to SSCs alone. The receptor for GDNF, GFRα1, is expressed by most undifferentiated spermatogonia, suggesting a general role for GDNF in the maintenance of these cells. Array studies of isolated undifferentiated spermatogonia cultured with and without GDNF have been a useful tool

for identifying novel targets of GDNF signaling in germ cells but dissecting out whether increased target expression is a direct or indirect result of the GDNF/GFRα1/RET signaling requires functional studies. Some of the most interesting and novel targets induced after GDNF signaling in culture were discussed above, so this section of the chapter will focus on two signaling pathways in undifferentiated spermatogonia that are known to be activated by GDNF.

Through GFRα1 and RET tyrosine kinase, GDNF can activate the SRC kinases and AKT intracellular cascades to promote SSC self-renewal and survival [82, 83]. Several kinases from the SRC family co-precipitate with RET after GDNF stimulation of SSCs in culture [82] and the SRC kinases are thought to play a predominant role in the immediate response of SSCs to GDNF signaling. As a result of SRC activation, the PI3K/AKT pathway is triggered in SSCs, leading to the expression of N-MYC and SSC proliferation [82]. In fact, studies with undifferentiated spermatogonia that constitutively overexpress AKT demonstrated that although the concentration of SSCs declined over time, SSC potential was observed long term in cultures without GDNF as long as either FGF2 or EGF were present [84]. These data suggest that GDNF promotes both expansion of SSC numbers and production of non-stem progenitor spermatogonia in vitro.

The second signaling pathway activated by GDNF is RAS. The RET tyrosine kinase has been shown to autophosphorylate its intracellular domain in response to GDNF stimulation [85], thereby generating numerous docking sites for many different signaling proteins. One particular site, Tyr 1062, serves as the docking site for RAS and as a result, the RAS/ERK1/2 signaling cascade is triggered in SSCs [86]. This pathway ultimately results in the activation of the transcription factors CREB-1, ATF-1 and CREM-1 as well as the increased expression of *Cyclina* and *Cdk2*, whose gene products promote the G1/2 phase mitotic transition, thereby driving the proliferation of SSCs. Taken together, these signaling studies indicate that GDNF plays a general role in the proliferation of undifferentiated spermatogonia, including the germline stem cells.

7.4.1.2 Colony Stimulating Factor 1 (CSF1)

The identification of CSF1 as being an important growth factor for SSC self-renewal came from array analyses of genes upregulated in THY1-enriched undifferentiated spermatogonia [43, 44]. Two different studies identified CSFR1, the receptor for CSF1, as being highly expressed in the THY1-enriched versus the THY1-depleted population and addition of recombinant CSF-1 to cultures of undifferentiated spermatogonia increased SSC numbers, determined by germ cell transplantation, but did not affect the proliferation of these cells [43]. This suggests that CSF1 is a specific regulator of the SSC pool rather than regulating the general population of progenitor spermatogonia. However, conflicting reports regarding the cellular localization of CSFR1 cast doubt over whether CSF1 can only regulate the stem cell pool. One study reported CSFR1 to be present on only a small percentage of single spermatogonia [43] whereas a second study reported CSFR1 protein on chained spermatogonia at a much higher incidence [44] (discussed above). If the receptor is only present on a small number of single spermatogonia, then this would provide additional in vivo evidence to support a specific role for CSF1 in SSC self-renewal whereas the detection of CSFR1 within chains of undifferentiated spermatogonia would suggest a more generalized role in the maintenance of these cells. Interestingly, unlike GDNF, CSF1 is not produced by Sertoli cells but instead, this growth factor appears to be exclusively expressed by Leydig cells and some peritubular myoid cells, and therefore, represents the first direct link between the interstitium and SSC self-renewal.

7.4.2 Growth Factor Signals Promoting Differentiation

It is vital, especially during the juvenile period of testis development, that the SSC pool divides such that the numbers of cells retaining stem cell potential is not only maintained, but perhaps favored. Equally important, however, is that these cells be regularly triggered to enter their differentiation pathway so that a continuous supply of

mature sperm are produced. Deciphering the growth factors and mechanisms responsible for driving SSC differentiation has been extremely difficult due to a lack of markers that distinguish between the true SSCs and the more differentiated A_{pr}/A_{al} cells. However, mouse models and culture systems set up to detect markers of differentiating spermatogonia have been useful for beginning to define the growth factors responsible for this process.

The TGFβ family of growth factors appears to be important for SSC differentiation, especially in the juvenile testis. BMP4 has been shown to increase the expression of *C-kit*, a marker of differentiating spermatogonia [87]. Exposure of cultured undifferentiated spermatogonia to either Activin A or BMP4 resulted in decreased SSC numbers [81]. BMP4 receptors are expressed by spermatogonia [87], however, a detailed analysis of which spermatogonial subtypes harbor this receptor has yet to be performed. In addition, increased levels of *C-kit* transcripts were also detected in a transgenic mouse model designed to produce lower levels of bioactive Activin A [88]. Collectively, these studies suggest that both Activin A and BMP4 are important for the maintenance of progenitor spermatogonia but stimulate a loss of stem cell maintenance. Characterization of an immortalized embryonic fibroblast cell line used as feeder cells for undifferentiated spermatogonia cultures identified Neuroregulin 1 as a secreted factor that can promote spermatogonial chain formation [89]. Neuroregulin 1 is a cell-signaling molecule that acts as a ligand for the ERBB family of receptor tyrosine kinases and has been shown to play essential roles in the nervous system, heart and breast [90]. Work in the newt has shown that Neuregulin 1 can promote spermatogonial proliferation [91] but there have yet to be any in vivo studies performed to verify its role in mammalian spermatogonia.

RA, one of the active metabolites of vitamin A, is also critical for spermatogonial differentiation. Mammals deficient in vitamin A develop testis tubules containing only Sertoli cells and undifferentiated spermatogonia, indicating that in the absence of RA, the undifferentiated A spermatogonia are unable to transition to A1 cells (reviewed in [17]). Treatment of THY1-enriched spermatogonia with RA in vitro leads to an increase in two markers of differentiating spermatogonia, *C-kit* and *Stra8* [92], and an inhibitor of the enzymes required for the production of RA from retinol, WIN 18,446, has recently been shown to prevent the expression of *Stra8* in cultures of neonatal testes and THY1-enriched spermatogonia [93]. What has yet to be determined, however, is how spermatogonia become more susceptible to RA signaling as they move away from the stem cell niche. Within the testis, RA signaling is believed to be active at very defined points along the tubule, and in the adult testis, these points of activity align with two particular stages of the seminiferous epithelium, Stages VII and VIII [17, 94, 95]. It's possible that the stem cell niche is located between these points of RA activity along the tubules so that the germline stem cells can be shielded from RA until they receive cues from within the niche to move to where RA signaling is taking place. The observation that undifferentiated spermatogonia, both in the neonatal testis and the adult testis, can respond to exogenous RA by expressing *C-kit* and *Stra8* prematurely [93–96] supports the theory that the undifferentiated spermatogonia are prepared to respond to RA but are shielded from it in some way. How the production of RA is controlled in such a precise manner within the testis tubule and which subtypes of spermatogonia can respond to RA signaling is still under investigation.

7.4.3 Homing SSCs to the Niche

While secreted signals are essential to the function of any stem cell niche, how a stem cell knows to stay within a particular area of an organ is key to maintaining the balance between self-renewal and differentiation. The homing of SSCs to the niche is likely due to a combination of secreted factors that draw migrating cells to the niche where adhesion molecules hold them there until they are triggered to differentiate. Two such adhesion molecules, α6- and β1-integrin, are transmembrane proteins known to bind laminin

and have been shown to be expressed by SSCs [97]. Disrupting the expression of β1-integrin inhibits the ability of SSCs to regenerate spermatogenesis after transplantation [98] even though migration of the SSCs to the basement of the recipient testis tubules was normal. This result suggests that while β1-intergin may be important for anchoring SSCs to the niche, it is not necessary for drawing them there. In addition, the expression of both α6- and β1-integrin is more widespread in undifferentiated spermatogonia and not localized to only SSCs, therefore these two proteins most likely play a general role in retaining spermatogonia at the basement membrane of the seminiferous tubule.

There is also evidence to suggest that germline niches reside in very specific areas of the seminiferous tubule. Histological analyses of mouse and rat testis cross sections revealed that undifferentiated spermatogonia are localized in higher concentrations where the basement membrane is in close association with the interstitium [99, 100]. This observation was recently supported by Yoshida et al. who performed live imaging tracking experiments to map how the undifferentiated spermatogonia move within the tubule [15]. Their imaging studies suggested that the male germline niches reside at the basement membrane areas of tubules closely associated with the vasculature. Over time, Yoshida et al. were able to visualize undifferentiated spermatogonia moving away from the vasculature upon differentiation. Whether this cue to move is a direct signal from the vasculature or whether the vasculature signals indirectly through the Sertoli cells has yet to be determined. Indeed, the number of Sertoli cells is thought to ultimately determine the number of niches present within a testis. This conclusion was drawn from the observation that the number of niches accessible for colonization by transplanted SSCs was increased in recipient animals which had been experimental altered to contain 50 % more Sertoli cells via the use of polythiouracil (PTU)-induced transient hypothyroidism [27]. Importantly, this study found no changes in the surface area of the PTU-induced tubules in contact with the interstitial tissue or in the percentage of tubules associated with the vasculature, indicating that neither the interstitial cells nor the vasculature could have contributed to the change in the numbers of available niches.

Clearly there is still much to be learned regarding how the somatic support cells of the testis interact to generate the male germline stem cell niche. Defining the components of this niche will be important for furthering our understanding of how spermatogenesis is founded and how the SSCs respond to the signals generated within the niche and its immediate surroundings to balance between self-renewal and differentiation as a population.

7.5 Regulating Translation in Undifferentiated Spermatogonia

An emerging area of research in testis development is the investigation of how small RNAs regulate transcription, RNA stability and translation. There are three major classes of small RNAs, classified based on their biogenesis, mechanism of action and function: (1) the small interfering RNAs (siRNAs); exogenous double stranded RNAs that are known to degrade mRNA or interfere with transcript translation; (2) the microRNAs (miRNAs); endogenous single-stranded RNAs that inhibit translation or result in mRNA instability; and (3) piwi-interacting RNAs (piRNAs); endogenous single-stranded RNAs that are expressed exclusively in spermatocytes and spermatids and are believed to cause gene silencing through interacting with the PIWI proteins (reviewed in [101]). Given that piRNAs have, to date, only been detected in meiotic and post-meiotic germ cells, and that the use of siRNAs in the investigation of SSC self-renewal and differentiation has been discussed above, this section will focus on our current understanding of the role that miRNAs play in spermatogonial differentiation.

7.5.1 miRNA Regulation of SSC Self-Renewal and Differentiation

miRNAs are single-stranded RNAs between 19 and 25 nucleotides in length. They can be transcribed

from all regions of the genome, however, the majority reside in the introns of genes and their expression is usually governed by the same factors controlling the transcription of their host gene [102]. miRNAs are initially transcribed as single pri-miRNAs, or in a cluster of multiple pri-miRNAs, and form stem loop structures that can be cleaved by DROSHA within the nucleus. This cleavage event results in the production of pre-miRNAs that can be transported into the cytoplasm. The loop structure of the pre-miRNA is then cleaved by the endonuclease DICER, known to be essential for spermatogenesis [103–105], to form mature double-stranded miRNAs. Mature miRNAs are then able to associate with a group of proteins, known as the RISC complex, to mediate the post-transcriptional regulation of mRNA targets (recently reviewed in [102, 106]). miRNAs were first identified in *Caenorhabiditis elegans* in 1993 [107] but it has only been over recent years that the scientific community has begun to understand how they effect mammalian cell development. Most of our current insight into how they regulate SSC biology has been generated by array studies. However, advancements in SSC transfection and culture has allowed for more functional miRNA assays to be performed.

There are several publications outlining expression, microarray or sequence analysis of the miRNAs present in the mammalian testis at different stages of development [48, 49, 108–111]. Each of these studies was able to identify miRNAs that appear to be testis-enriched in comparison to other tissues but most expression studies have been focused on the miRNAs present in the meiotic and post-meiotic germ cells. There have only been a few studies published that have focused on SSCs or cultured germline stem cells. Jung et al. [109] performed a real time PCR analysis of two different miRNA families in testis-derived germ line stem cells and identified the members of the Let-7 miRNA family as being enriched in these cells [109]. This is somewhat contradictory to the observation that Let-7 family miRNAs are induced in response to RA treatment of spermatogonia, suggesting an increase in the expression of miRNAs after SSCs have differentiated [112], and so the role of the Let-7 mirRNA family in SSC function is the subject of continuing investigations.

Three different studies have performed microarray analyses on isolated gonocytes and/or spermatogonia, with two utilizing THY1-positive cell sorting [48, 49, 113]. Mclver et al. [113], found seven miRNAs that are differentially regulated between gonocytes and spermatogonia and in silico prediction software identified members of the PTEN and Wnt signaling pathways as targets of the miRNAs enriched in gonocytes versus spermatogonia [113]. These two pathways are both upstream regulators of Cyclin D, suggesting that miRNAs may co-ordinate the differentiation of gonocytes and participate in the maintenance of pluripotency in germ cells. Using microarray analyses, Tong et al. [49] identified the miR-17-92 cluster and miR-106b-25 as both being significantly downregulated in THY1-enriched cells incubated with RA [49]. These results were confirmed using real time PCR and the authors went on to characterize a germ-cell specific knockout of the miR-17-92 cluster. The observation that these mice had smaller testes but only a mild spermatogenic defect suggests that there is some level of compensation between different miRNAs and that the miR-17-92 cluster is not essential for spermatogenesis.

The second study to profile miRNA expression in THY1-enriched cell populations identified a potential player in SSC self-renewal. Niu et al. [48] compared the mature miRNA expression profile of freshly isolated 6 dpp THY1-enriched cells to freshly isolated 6 dpp THY1-depleted cells and cultured THY1-enriched cells [48]. This study identified 139 miRNAs that were differentially expressed between the freshly isolated THY1-enriched and THY1-depleted samples and interestingly, while the miRNAs present in the freshly isolated and cultured THY1-enriched samples were similar, there were several transcripts that were present at much higher levels in the cultured samples, probably due to the propagation and expansion of germ cells in vitro. Chromatin immunoprecipitation using mouse germ cell cultures revealed that the expression of one of these miRNAs, miR-21, may be directly regulated by ETV5, a Sertoli cell-expressed protein know to be essential for SSC self-renewal [114]. To further investigate the role of miR-21 in SSC self-renewal, THY1-enriched germ cell

cultures were transfected with an inhibitor of this miRNA and recipient testis transplants were performed with the transfected cells [48]. Analysis of the recipient testes revealed an increase in apoptosis within the transplanted germ cell population and a reduced number of germ cell colonies, suggesting that the knockdown of miR-21 inhibited the proliferation of SSCs. These data fit with the fact that miR-21 is a known antiapoptotic factor and has been shown to inhibit the production of the p21 tumor suppressor protein, thereby normally promoting proliferation [115]. In addition, miR-21 has been found to be overexpressed in human seminomas and germ cell tumors, testicular cancers thought to arise from the overproliferation of primordial germ cells [116]. Taken together, these data suggest a role for miR-21 in SSC proliferation, however, there is still a large amount to be learned with regards to the mRNAs that this and other miRNAs act upon to affect translation in SSCs and how manipulation of translation affects SSC self-renewal and differentiation.

7.6 Concluding Remarks

Clearly, the reproductive biology scientific community has made incredible progress over the last decade with regards to understanding the signals and factors involved in the balance between SSC self-renewal and differentiation and how SSCs respond at the molecular level. Making use of technical advances in sequencing techniques, e.g. next generation sequencing, will be extremely important for the identification of markers of the true SSC population, for mapping the molecular characteristics of SSCs in response to different signals and for investigating miRNA target mRNAs within SSCs. In addition to defining the molecular characteristics of SSCs, in vivo studies are required to further define how the secreted growth factors and signaling molecules regulate the niche microenvironment within the testis and the SSC pool. These data can then be integrated to provide us with a more detailed understanding of the niche environments and the resulting molecular response within SSCs that favors self-renewal versus differentiation.

Acknowlegdements The author would like to acknowledge the help of Christopher Small and Michael Griswold for their critical reading and editing of the chapter.

References

1. Wade N (2004) Sperm stem cells are grown outside body. The New York Times Company, New York
2. Ying Y, Qi X, Zhao GQ (2001) Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways. Proc Natl Acad Sci USA 98:7858–7862
3. Ginsburg M, Snow MH, McLaren A (1990) Primordial germ cells in the mouse embryo during gastrulation. Development 110:521–528
4. McCarrey JR (1993) Development of the germ cell. In: Despardins C, Ewing L (eds) Cell and molecular biology of the testis. Oxford University Press, New York
5. Bendel-Stenzel M, Anderson R, Heasman J, Wylie C (1998) The origin and migration of primordial germ cells in the mouse. Semin Cell Dev Biol 9:393–400
6. Adams IR, McLaren A (2002) Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. Development 129:1155–1164
7. McLaren A (1981) The fate of germ cells in the testis of fetal Sex-reversed mice. J Reprod Fertil 61:461–467
8. McLaren A, Southee D (1997) Entry of mouse embryonic germ cells into meiosis. Dev Biol 187:107–113
9. Kluin PM, de Rooij DG (1981) A comparison between the morphology and cell kinetics of gonocytes and adult type undifferentiated spermatogonia in the mouse. Int J Androl 4:475–493
10. Western PS, Miles DC, van den Bergen JA, Burton M, Sinclair AH (2008) Dynamic regulation of mitotic arrest in fetal male germ cells. Stem Cells 26:339–347
11. Cooke HJ, Saunders PT (2002) Mouse models of male infertility. Nat Rev Genet 3:790–801
12. Yoshida S, Sukeno M, Nakagawa T, Ohbo K, Nagamatsu G, Suda T, Nabeshima Y (2006) The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. Development 133:1495–1505
13. Oatley JM, Brinster RL (2008) Regulation of spermatogonial stem cell self-renewal in mammals. Ann Rev Cell Dev Biol 24:263–286
14. Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S (2010) Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 328:62–67
15. Yoshida S, Nabeshima Y, Nakagawa T (2007) Stem cell heterogeneity: actual and potential stem cell compartments in mouse spermatogenesis. Ann N Y Acad Sci 1120:47–58

16. Morimoto H, Kanatsu-Shinohara M, Takashima S, Chuma S, Nakatsuji N, Takehashi M, Shinohara T (2009) Phenotypic plasticity of mouse spermatogonial stem cells. PLoS One 4:e7909
17. Hogarth CA, Griswold MD (2010) The key role of vitamin A in spermatogenesis. J Clin Invest 120:956–962
18. Ehmcke J, Wistuba J, Schlatt S (2006) Spermatogonial stem cells: questions, models and perspectives. Hum Reprod Update 12:275–282
19. Ehmcke J, Schlatt S (2006) A revised model for spermatogonial expansion in man: lessons from non-human primates. Reproduction 132:673–680
20. Clermont Y, Bustos-Obregon E (1968) Re-examination of spermatogonial renewal in the rat by means of seminiferous tubules mounted "in toto". Am J Anat 122:237–247
21. Dym M, Clermont Y (1970) Role of spermatogonia in the repair of the seminiferous epithelium following x-irradiation of the rat testis. Am J Anat 128:265–282
22. Clermont Y, Hermo L (1975) Spermatogonial stem cells in the albino rat. Am J Anat 142:159–175
23. Oatley MJ, Kaucher AV, Racicot KE, Oatley JM (2011) Inhibitor of DNA binding 4 is expressed selectively by single spermatogonia in the male germline and regulates the self-renewal of spermatogonial stem cells in mice. Biol Reprod 85:347–356
24. Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci USA 91:11298–11302
25. Buaas FW, Kirsh AL, Sharma M, McLean DJ, Morris JL, Griswold MD, de Rooij DG, Braun RE (2004) Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 36:647–652
26. Costoya JA, Hobbs RM, Barna M, Cattoretti G, Manova K, Sukhwani M, Orwig KE, Wolgemuth DJ, Pandolfi PP (2004) Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 36:653–659
27. Oatley MJ, Racicot KE, Oatley JM (2011) Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. Biol Reprod 84:639–645
28. Viglietto G, Dolci S, Bruni P, Baldassarre G, Chiariotti L, Melillo RM, Salvatore G, Chiappetta G, Sferratore F, Fusco A, Santoro M (2000) Glial cell line-derived neutrotrophic factor and neurturin can act as paracrine growth factors stimulating DNA synthesis of Ret-expressing spermatogonia. Int J Oncol 16:689–694
29. Dettin L, Ravindranath N, Hofmann MC, Dym M (2003) Morphological characterization of the spermatogonial subtypes in the neonatal mouse testis. Biol Reprod 69:1565–1571
30. Ebata KT, Zhang X, Nagano MC (2005) Expression patterns of cell-surface molecules on male germ line stem cell during postnatal mouse development. Mol Reprod Dev 72:171–178
31. He Z, Jiang J, Hofmann MC, Dym M (2007) Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase. Biol Reprod 77:723–733
32. Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functinoal characteristics with other stem cells. Proc Natl Acad Sci USA 100:6487–6492
33. Pesce M, Wang X, Wolgemuth DJ, Scholer H (1998) Differential expression of the Oct-4 transcription factor during mouse germ cell differentiation. Mech Dev 71:89–98
34. Yoshida S, Takakura A, Ohbo K, Abe K, Wakabayashi J, Yamamoto M, Suda T, Nabeshima Y (2004) Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. Dev Biol 269:447–458
35. Sada A, Suzuki A, Suzuki H, Saga Y (2009) The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science 325:1394–1398
36. Suzuki H, Sada A, Yoshida S, Saga Y (2009) The heterogeneity of spermatogonia is revealed by their topology and expression of marker proteins including the germ cell-specific proteins Nanos2 and Nanos3. Dev Biol 336:222–231
37. Ballow DJ, Xin Y, Choi Y, Pangas SA, Rajkovic A (2006a) Sohlh2 is a germ cell-specific bHLH transcription factor. Gene Expr Patterns 6:1014–1018
38. Ballow D, Meistrich ML, Matzuk M, Rajkovic A (2006b) Sohlh1 is essential for spermatogonial differentiation. Dev Biol 294:161–167
39. Tegelenbosch RA, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101F1 hybrid mouse. Mutat Res 290:193–200
40. Kubota H, Avarbock MR, Brinster RL (2004) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci USA 101:16489–16494
41. Oatley JM, Avarbock MR, Telaranta AI, Fearon DT, Brinster RL (2006) Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc Natl Acad Sci USA 103:9524–9529
42. Kokkinaki M, Lee TL, He Z, Jiang J, Golestaneh N, Hofmann MC, Chan WY, Dym M (2010) Age affects gene expression in mouse spermatogonial stem/progenitor cells. Reproduction 139:1011–1020
43. Oatley JM, Oatley MJ, Avarbock MR, Tobias JW, Brinster RL (2009) Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development 136: 1191–1199
44. Kokkinaki M, Lee TL, He Z, Jiang J, Golestaneh N, Hofmann MC, Chan WY, Dym M (2009) The molecular signature of spermatogonial stem/progenitor cells in the 6-day-old mouse testis. Biol Reprod 80:707–717
45. Orwig KE, Ryu BY, Master SR, Phillips BT, Mack M, Avarbock MR, Chodosh L, Brinster RL (2008) Genes involved in post-transcriptional regulation are overrepresented in stem/progenitor spermatogonia

of cryptorchid mouse testes. Stem Cells 26:927–938
46. Schmidt JA, Avarbock MR, Tobias JW, Brinster RL (2009) Identification of glial cell line-derived neurotrophic factor-regulated genes important for spermatogonial stem cell self-renewal in the rat. Biol Reprod 81:56–66
47. von Kopylow K, Kirchhoff C, Jezek D, Schulze W, Feig C, Primig M, Steinkraus V, Spiess AN (2010) Screening for biomarkers of spermatogonia within the human testis: a whole genome approach. Hum Reprod 25:1104–1112
48. Niu Z, Goodyear SM, Rao S, Wu X, Tobias JW, Avarbock MR, Brinster RL (2011) MicroRNA-21 regulates the self-renewal of mouse spermatogonial stem cells. Proc Natl Acad Sci USA 108:12740–12745
49. Tong MH, Mitchell DA, McGowan SD, Evanoff R, Griswold MD (2012) Two miRNA Clusters, Mir-17-92 (Mirc1) and Mir-106b-25 (Mirc3), are involved in the regulation of spermatogonial differentiation in mice. Biol Reprod 86:72
50. Shinohara T, Avarbock MR, Brinster RL (2000) Functional analysis of spermatogonial stem cells in Steel and cryptorchid infertile mouse models. Dev Biol 220:401–411
51. Barna M, Merghoub T, Costoya JA, Ruggero D, Branford M, Bergia A, Samori B, Pandolfi PP (2002) Plzf mediates transcriptional repression of HoxD gene expression through chromatin remodeling. Dev Cell 3:499–510
52. Filipponi D, Hobbs RM, Ottolenghi S, Rossi P, Jannini EA, Pandolfi PP, Dolci S (2007) Repression of kit expression by Plzf in germ cells. Mol Cell Biol 27:6770–6781
53. Hobbs RM, Seandel M, Falciatori I, Rafii S, Pandolfi PP (2010) Plzf regulates germline progenitor self-renewal by opposing mTORC1. Cell 142:468–479
54. Payne C, Braun RE (2006) Histone lysine trimethylation exhibits a distinct perinuclear distribution in Plzf-expressing spermatogonia. Dev Biol 293:461–472
55. Mohapatra C, Barman HK, Panda RP, Kumar S, Das V, Mohanta R, Mohapatra SD, Jayasankar P (2010) Cloning of cDNA and prediction of peptide structure of Plzf expressed in the spermatogonial cells of Labeo rohita. Mar Genomics 3:157–163
56. Ozaki Y, Saito K, Shinya M, Kawasaki T, Sakai N (2011) Evaluation of Sycp3, Plzf and Cyclin B3 expression and suitability as spermatogonia and spermatocyte markers in zebrafish. Gene Expr Patterns 11:309–315
57. Buageaw A, Sukhwani M, Ben-Yehudah A, Ehmcke J, Rawe VY, Pholpramool C, Orwig KE, Schlatt S (2005) GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol Reprod 73:1011–1016
58. Naughton CK, Jain S, Strickland AM, Gupta A, Milbrandt J (2006) Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol Reprod 74:314–321
59. Jijiwa M, Kawai K, Fukihara J, Nakamura A, Hasegawa M, Suzuki C, Sato T, Enomoto A, Asai N, Murakumo Y, Takahashi M (2008) GDNF-mediated signaling via RET tyrosine 1062 is essential for maintenance of spermatogonial stem cells. Genes Cells 13:365–374
60. Grisanti L, Falciatori I, Grasso M, Dovere L, Fera S, Muciaccia B, Fuso A, Berno V, Boitani C, Stefanini M, Vicini E (2009) Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells 27:3043–3052
61. Grasso M, Fuso A, Dovere L, de Rooij DG, Stefanini M, Boitani C, Vicini E (2012) Distribution of GFRA1-expressing spermatogonia in adult mouse testis. Reproduction 143:325–332
62. Yoshida S, Sukeno M, Nabeshima Y (2007) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317:1722–1726
63. Wang C, Lehmann R (1991) Nanos is the localized posterior determinant in Drosophila. Cell 66:637–647
64. Tsuda M, Sasaoka Y, Kiso M, Abe K, Haraguchi S, Kobayashi S, Saga Y (2003) Conserved role of nanos proteins in germ cell development. Science 301:1239–1241
65. Sonoda J, Wharton RP (2001) Drosophila brain tumor is a translational repressor. Genes Dev 15:762–773
66. Kadyrova LY, Habara Y, Lee TH, Wharton RP (2007) Translational control of maternal Cyclin B mRNA by Nanos in the Drosophila germline. Development 134:1519–1527
67. Lolicato F, Marino R, Paronetto MP, Pellegrini M, Dolci S, Geremia R, Grimaldi P (2008) Potential role of Nanos3 in maintaining the undifferentiated spermatogonia population. Dev Biol 313:725–738
68. Saga Y (2010) Function of Nanos2 in the male germ cell lineage in mice. Cell Mol Life Sci 67:3815–3822
69. Julaton VT, Reijo Pera RA (2011) NANOS3 function in human germ cell development. Hum Mol Genet 20:2238–2250
70. Sada A, Hasegawa K, Pin PH, Saga Y (2012) NANOS2 acts downstream of glial cell line-derived neurotrophic factor signaling to suppress differentiation of spermatogonial stem cells. Stem Cells 30:280–291
71. Barrios F, Filipponi D, Pellegrini M, Paronetto MP, Di Siena S, Geremia R, Rossi P, De Felici M, Jannini EA, Dolci S (2010) Opposing effects of retinoic acid and FGF9 on Nanos2 expression and meiotic entry of mouse germ cells. J Cell Sci 123:871–880
72. Riechmann V, van Cruchten I, Sablitzky F (1994) The expression pattern of Id4, a novel dominant negative helix-loop-helix protein, is distinct from Id1, Id2 and Id3. Nucleic Acids Res 22:749–755
73. Sun XH, Copeland NG, Jenkins NA, Baltimore D (1991) Id proteins Id1 and Id2 selectively inhibit

DNA binding by one class of helix-loop-helix proteins. Mol Cell Biol 11:5603–5611
74. Sablitzky F, Moore A, Bromley M, Deed RW, Newton JS, Norton JD (1998) Stage- and subcellular-specific expression of Id proteins in male germ and Sertoli cells implicates distinctive regulatory roles for Id proteins during meiosis, spermatogenesis, and Sertoli cell function. Cell Growth Differ 9:1015–1024
75. Oatley JM, Brinster RL (2012) The germline stem cell niche unit in mammalian testes. Physiol Rev 92:577–595
76. Hofmann MC (2008) Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. Mol Cell Endocrinol 288:95–103
77. Mullaney BP, Skinner MK (1992) Basic fibroblast growth factor (bFGF) gene expression and protein production during pubertal development of the seminiferous tubule: follicle-stimulating hormone-induced Sertoli cell bFGF expression. Endocrinology 131:2928–2934
78. Spinnler K, Kohn FM, Schwarzer U, Mayerhofer A (2010) Glial cell line-derived neurotrophic factor is constitutively produced by human testicular peritubular cells and may contribute to the spermatogonial stem cell niche in man. Hum Reprod 25:2181–2187
79. Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H et al (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287:1489–1493
80. Kanatsu-Shinohara M, Ogonuki N, Inoue K, Miki H, Ogura A, Toyokuni S, Shinohara T (2003) Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 69:612–616
81. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brinster RL (2003) Maintenance of mouse male germ line stem cells in vitro. Biol Reprod 68:2207–2214
82. Braydich-Stolle L, Kostereva N, Dym M, Hofmann MC (2007) Role of Src family kinases and N-Myc in spermatogonial stem cell proliferation. Dev Biol 304:34–45
83. Oatley JM, Avarbock MR, Brinster RL (2007) Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J Biol Chem 282:25842–25851
84. Lee J, Kanatsu-Shinohara M, Inoue K, Ogonuki N, Miki H, Toyokuni S, Kimura T, Nakano T, Ogura A, Shinohara T (2007) Akt mediates self-renewal division of mouse spermatogonial stem cells. Development 134:1853–1859
85. Encinas M, Crowder RJ, Milbrandt J, Johnson EM Jr (2004) Tyrosine 981, a novel ret autophosphorylation site, binds c-Src to mediate neuronal survival. J Biol Chem 279:18262–18269
86. He Z, Jiang J, Kokkinaki M, Golestaneh N, Hofmann MC, Dym M (2008) Gdnf upregulates c-Fos transcription via the Ras/Erk1/2 pathway to promote mouse spermatogonial stem cell proliferation. Stem Cells 26:266–278
87. Pellegrini M, Grimaldi P, Rossi P, Geremia R, Dolci S (2003) Developmental expression of BMP4/ALK3/SMAD5 signaling pathway in the mouse testis: a potential role of BMP4 in spermatogonia differentiation. J Cell Sci 116:3363–3372
88. Mithraprabhu S, Mendis S, Meachem SJ, Tubino L, Matzuk MM, Brown CW, Loveland KL (2010) Activin bioactivity affects germ cell differentiation in the postnatal mouse testis in vivo. Biol Reprod 82:980–990
89. Hamra FK, Chapman KM, Nguyen D, Garbers DL (2007) Identification of neuregulin as a factor required for formation of aligned spermatogonia. J Biol Chem 282:721–730
90. Falls DL (2003) Neuregulins: functions, forms, and signaling strategies. Exp Cell Res 284:14–30
91. Oral O, Uchida I, Eto K, Nakayama Y, Nishimura O, Hirao Y, Ueda J, Tarui H, Agata K, Abe S (2008) Promotion of spermatogonial proliferation by neuregulin 1 in newt (Cynops pyrrhogaster) testis. Mech Dev 125:906–917
92. Zhou Q, Li Y, Nie R, Friel P, Mitchell D, Evanoff RM, Pouchnik D, Banasik B, McCarrey JR, Small C, Griswold MD (2008) Expression of stimulated by retinoic acid gene 8 (Stra8) and maturation of murine gonocytes and spermatogonia induced by retinoic acid in vitro. Biol Reprod 78:537–545
93. Hogarth CA, Evanoff R, Snyder E, Kent T, Mitchell D, Small C, Amory JK, Griswold MD (2011) Suppression of Stra8 expression in the mouse gonad by WIN 18,446. Biol Reprod 84:957–965
94. Snyder EM, Small C, Griswold MD (2010) Retinoic acid availability drives the asynchronous initiation of spermatogonial differentiation in the mouse. Biol Reprod 83(5):783–790
95. Snyder EM, Davis JC, Zhou Q, Evanoff R, Griswold MD (2011) Exposure to retinoic acid in the neonatal but not adult mouse results in synchronous spermatogenesis. Biol Reprod 84:886–893
96. Pellegrini M, Filipponi D, Gori M, Barrios F, Lolicato F, Grimaldi P, Rossi P, Jannini EA, Geremia R, Dolci S (2008) ATRA and KL promote differentiation toward the meiotic program of male germ cells. Cell Cycle 7:3878–3888
97. Shinohara T, Avarbock MR, Brinster RL (1999) beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci USA 96:5504–5509
98. Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S, Raducanu A, Nakatsuji N, Fassler R, Shinohara T (2008) Homing of mouse spermatogonial stem cells to germline niche depends on beta1-integrin. Cell Stem Cell 3:533–542

99. Chiarini-Garcia H, Hornick JR, Griswold MD, Russell LD (2001) Distribution of type A spermatogonia in the mouse is not random. Biol Reprod 65:1179–1185
100. Chiarini-Garcia H, Raymer AM, Russell LD (2003) Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. Reproduction 126:669–680
101. He Z, Kokkinaki M, Pant D, Gallicano GI, Dym M (2009) Small RNA molecules in the regulation of spermatogenesis. Reproduction 137:901–911
102. Shomron N, Levy C (2009) MicroRNA-biogenesis and Pre-mRNA splicing crosstalk. J Biomed Biotechnol 2009:594678
103. Hayashi K, Chuva de Sousa Lopes SM, Kaneda M, Tang F, Hajkova P, Lao K, O'Carroll D, Das PP, Tarakhovsky A, Miska EA, Surani MA (2008) MicroRNA biogenesis is required for mouse primordial germ cell development and spermatogenesis. PLoS One 3:e1738
104. Papaioannou MD, Pitetti JL, Ro S, Park C, Aubry F, Schaad O, Vejnar CE, Kuhne F, Descombes P, Zdobnov EM, McManus MT, Guillou F et al (2009) Sertoli cell Dicer is essential for spermatogenesis in mice. Dev Biol 326: 250–259
105. Maatouk DM, Loveland KL, McManus MT, Moore K, Harfe BD (2008) Dicer1 is required for differentiation of the mouse male germline. Biol Reprod 79:696–703
106. Kim VN, Han J, Siomi MC (2009) Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 10:126–139
107. Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75:843–854
108. Buchold GM, Coarfa C, Kim J, Milosavljevic A, Gunaratne PH, Matzuk MM (2010) Analysis of microRNA expression in the prepubertal testis. PLoS One 5:e15317
109. Jung YH, Gupta MK, Shin JY, Uhm SJ, Lee HT (2010) MicroRNA signature in testes-derived male germ-line stem cells. Mol Hum Reprod 16:804–810
110. Ro S, Park C, Sanders KM, McCarrey JR, Yan W (2007) Cloning and expression profiling of testis-expressed microRNAs. Dev Biol 311:592–602
111. Shin JY, Gupta MK, Jung YH, Uhm SJ, Lee HT (2011) Differential genomic imprinting and expression of imprinted microRNAs in testes-derived male germ-line stem cells in mouse. PLoS One 6:e22481
112. Tong MH, Mitchell D, Evanoff R, Griswold MD (2011) Expression of Mirlet7 family microRNAs in response to retinoic acid-induced spermatogonial differentiation in mice. Biol Reprod 85:189–197
113. Mclver SC, Stanger SJ, Santarelli DM, Roman SD, Nixon B, McLaughlin EA (2012) A unique combination of male germ cell miRNAs coordinates gonocyte differentiation. PLoS One 7:e35553
114. Chen C, Ouyang W, Grigura V, Zhou Q, Carnes K, Lim H, Zhao GQ, Arber S, Kurpios N, Murphy TL, Cheng AM, Hassell JA et al (2005) ERM is required for transcriptional control of the spermatogonial stem cell niche. Nature 436:1030–1034
115. Zheng J, Xue H, Wang T, Jiang Y, Liu B, Li J, Liu Y, Wang W, Zhang B, Sun M (2011) miR-21 downregulates the tumor suppressor P12 CDK2AP1 and stimulates cell proliferation and invasion. J Cell Biochem 112:872–880
116. Gillis AJ, Stoop HJ, Hersmus R, Oosterhuis JW, Sun Y, Chen C, Guenther S, Sherlock J, Veltman I, Baeten J, van der Spek PJ, de Alarcon P et al (2007) High-throughput microRNAome analysis in human germ cell tumours. J Pathol 213:319–328