Chapter 10
Mechanisms Regulating Spermatogonial Differentiation

Jennifer M. Mecklenburg and Brian P. Hermann

Abstract Mammalian spermatogenesis is a complex and highly ordered process by which male germ cells proceed through a series of differentiation steps to produce haploid flagellated spermatozoa. Underlying this process is a pool of adult stem cells, the spermatogonial stem cells (SSCs), which commence the spermatogenic lineage by undertaking a differentiation fate decision to become progenitor spermatogonia. Subsequently, progenitors acquire a differentiating spermatogonia phenotype and undergo a series of amplifying mitoses while becoming competent to enter meiosis. After spermatocytes complete meiosis, post-meiotic spermatids must then undergo a remarkable transformation from small round spermatids to a flagellated spermatozoa with extremely compacted nuclei. This chapter reviews the current literature pertaining to spermatogonial differentiation with an emphasis on the mechanisms controlling stem cell fate decisions and early differentiation events in the life of a spermatogonium.

10.1 Introduction

Spermatogenesis is the process by which a diploid population of germline stem cells propagate and differentiate to give rise to millions of haploid flagellated spermatozoa every day throughout the life span of adult male mammals. Sustaining this tremendous productivity to maintain male fertility requires that germ cells undergo a series of programmed differentiation steps instructed by their microenvironment. Spermatogenic differentiation begins with commitment of spermatogonial stem cells (SSCs) to this differentiation pathway (versus the alternate cell fate, self-renewal) followed by a series of additional hallmark events, including spermatogonial differentiation concurrent with mitotic amplification, entry into meiosis, and post-meiotic spermiogenesis before ultimately culminating in sperm release. Intensive research efforts over the past several decades have revealed

J.M. Mecklenburg • B.P. Hermann, Ph.D. (✉)
Department of Biology, The University of Texas at San Antonio, One UTSA Circle, San Antonio, TX 78249, USA
e-mail: brian.hermann@utsa.edu

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many of the molecular and cellular cues, both germ cell intrinsic and arising from the somatic niche, which instruct these differentiation events and permit male fertility. Likewise, investigation of a variety of animal models and humans has revealed conservation of many, but not all, of the regulatory mechanisms controlling spermatogenic differentiation.

This chapter begins with a brief overview of the organization of spermatogenesis to frame an in-depth discussion of the key early events in which spermatogonia become committed to the differentiation pathway, initiate that differentiation, and known mechanisms of their control. To facilitate the translatability of this information, this chapter also highlights which processes are conserved or divergent among mammalian species, bringing to bear knowledge from rodent and nonhuman primate animal models, as well as data from humans.

10.2 Organization of Spermatogenesis

10.2.1 Testicular Anatomy and Cellular Components

Within the mammalian testis, there are two distinct partitions which physically separate the major biological functions of the testis. Seminiferous tubules comprise the gametogenic compartment in which spermatogenesis takes place, and the interstitial compartment between seminiferous tubules is the major site of testicular steroidogenesis (Russell et al. 1990). While the architecture of seminiferous tubules is highly organized, consisting of an epithelium of polar Sertoli cells and multiple layers of germ cells at different stages of spermatogenic development, the interstitial space is relatively disorganized and consists of numerous cell types and structures, including steroidogenic Leydig cells, blood and lymphatic vessels, macrophages, and connective tissue (Fawcett et al. 1973). Within the seminiferous epithelium, Sertoli cells envelop developing germ cells and provide substantial trophic support (Russell et al. 1990). Neighboring Sertoli cells are connected by tight junctions which further divide the seminiferous epithelium into adluminal and basal compartments and thereby separate meiotic and post-meiotic germ cells (leptotene spermatocytes and later) from the blood supply and prevent their immune recognition (Fawcett et al. 1973; Russell 1977). Beyond providing nutritional and structural support, Sertoli cells are considered to play a major role in regulating germ cell differentiation by providing microenvironmental cues for both stem cell maintenance and each of the major spermatogenic differentiation events. Beneath the Sertoli cell epithelium, the seminiferous tubule basement membrane is surrounded by a layer of peritubular myoid cells (PMCs) which may evoke peristaltic fluid movement in the tubule lumen to expel spermatozoa (Leeson and Forman 1981; Maekawa et al. 1996; Virtanen et al. 1986), but which have also recently been implicated in control of germ cell differentiation during spermatogenesis (Chen et al. 2014, 2016). Leydig cells in the interstitial space between

tubules are steroidogenic, producing testosterone which is required for spermatogonial proliferation, meiotic progression, and spermiation (reviewed by O'Shaughnessy 2014). Multiple distinct populations of macrophages are located in the interstitial compartment (DeFalco et al. 2015; Oatley et al. 2009). One macrophage population in particular is located on the outer surface of seminiferous tubules, immediately adjacent to the peritubular myoid cells, and are enriched at segments of seminiferous tubules that contain more undifferentiated spermatogonia (DeFalco et al. 2015). These macrophages have been implicated as regulators of spermatogonial differentiation, although the mechanisms of their involvement remain unclear.

10.2.2 Spermatogenic Lineage

Spermatogenesis occurs within the seminiferous epithelium and is divisible into three distinct developmental phases which occur sequentially in distinct cell types: (1) mitotic proliferation/clonal amplification, which takes place in spermatogonia, (2) meiosis in spermatocytes (primary and secondary), and (3) post-meiotic spermiogenesis in spermatids (round and elongating; Fig. 10.1). This organization is highly conserved among mammalian species (Fig. 10.1b), although the number of amplifying divisions of mitotic spermatogonia can vary quite substantially (Fig. 10.2).

At the foundation of the spermatogenic lineage is a population of spermatogonia that are adult stem cells, termed spermatogonial stem cells (SSCs). SSCs are responsible for maintaining spermatogenesis throughout the life span of adult mammals (Griswold and Oatley 2013; Oatley and Brinster 2008; Yang and Oatley 2014). As a population, SSCs must balance self-renewal to maintain the stem cell pool and differentiation to produce committed progenitor spermatogonia to meet the biological demand for sperm production and sustain the stem cell population (Jaenisch and Young 2008; Oatley and Brinster 2008, 2012; Yang and Oatley 2014) (Fig. 10.2). Indeed, excessive SSC differentiation or self-renewal impedes spermatogenesis and leads to male infertility by either depleting the stem cell pool or failing to produce differentiating germ cells to support spermatogenesis, respectively (de Rooij and Grootegoed 1998). At differentiating divisions of SSCs and at all subsequent divisions among differentiating male germ cells, division (mitotic or meiotic) is accompanied by incomplete cytokinesis which maintains daughter cells of the cell division as clones connected by intercellular cytoplasmic bridges (Fawcett 1959; Weber and Russell 1987; Greenbaum et al. 2006). Consequentially, spermatogenic cells develop in synchrony as syncytial clones which exchange transcripts, proteins, and organelles (Braun et al. 1989).

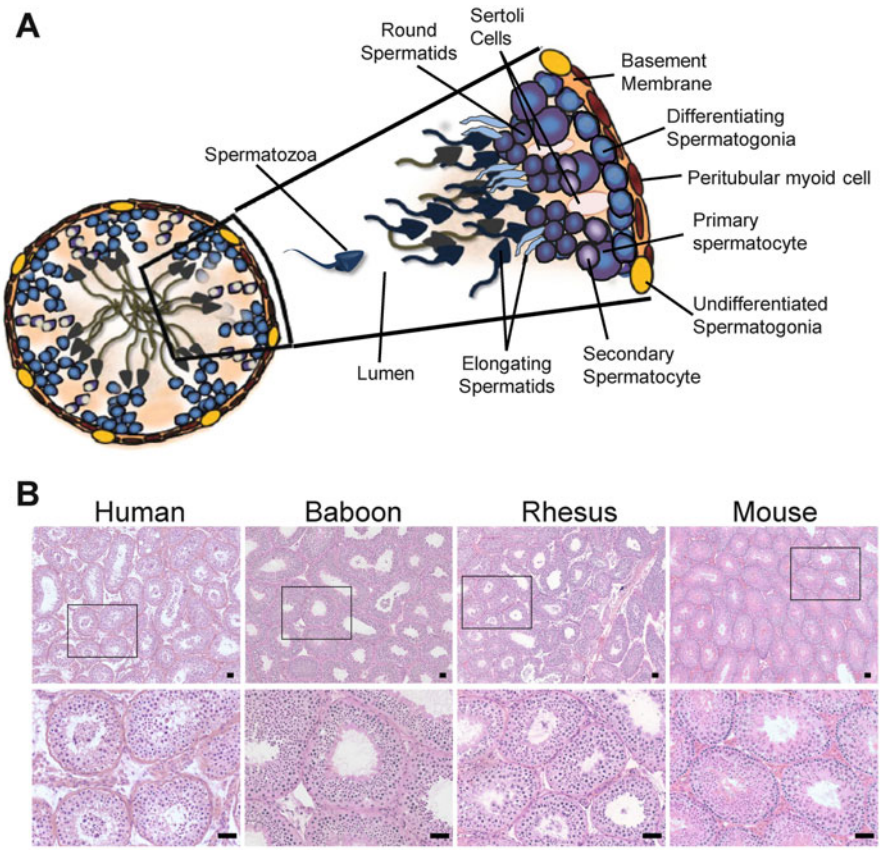

Fig. 10.1 Cellular organization of seminiferous tubules. (**a**) Seminiferous tubules are comprised of multiple layers of germ cells at distinct phases of spermatogenic development interdigitating between Sertoli cells. All spermatogonia, undifferentiated and differentiating, reside on the basement membrane. A layer of peritubular myoid cells is located on the outside of the seminiferous tubule basement membrane. (**b**) Histological sections through adult human, baboon, rhesus macaque, and mouse testes demonstrate extraordinary conservation in the organization of spermatogenesis among mammalian species. *Bar* = 50 μm

10.2.3 Undifferentiated and Differentiating Spermatogonia

Spermatogonia have historically been broadly classified as undifferentiated and differentiating, initially based upon histological appearance of nuclear morphology (Chiarini-Garcia et al. 2001; Russell et al. 1990; de Rooij and Grootegoed 1998). Undifferentiated spermatogonia generally lack heterochromatic nuclear architecture and, instead, have nuclei which appear more euchromatic (de Rooij and Russell 2000). In rodents, the multiple subtypes of spermatogonia are nearly all classified as differentiating: Type A_1, A_2, A_3, A_4, Intermediate (In), and B (Clermont et al. 1959; Roosen-Runge and Giesel 1950). Among Type A spermatogonia, the first five

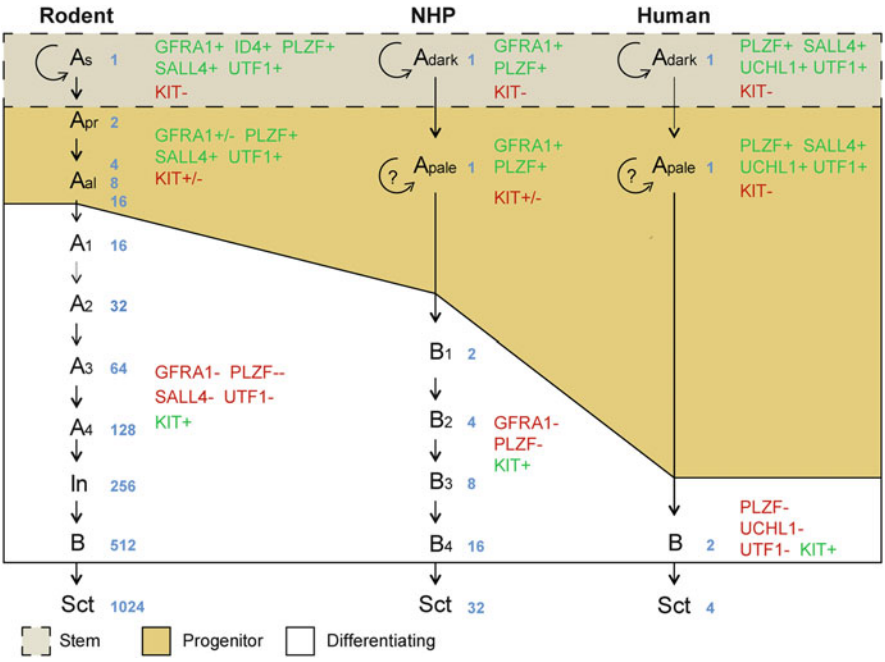

Fig. 10.2 The spermatogenic lineage. This schematic representation of the spermatogenic lineage depicts spermatogonial clonal amplification up to primary spermatocytes in rodents, nonhuman primates (NHP), and humans. Highlighting behind spermatogonial subtypes indicates whether cells are considered to be stem cells (*tan*), progenitor spermatogonia (*beige*), or differentiating spermatogonia (*white*). These subgroups also represent the major differentiation transitions among spermatogonia, initiation of differentiation among SSCs, and the conversion of progenitors to a differentiating spermatogonia phenotype (consistent with A_{al}–A_1 transition in rodents). Expression of select molecular markers is noted in *green* (expressed/detected) or red (not expressed/undetectable) and noted next to the respective lineages. *Blue* numbers indicate the degree of clonal amplification (cell numbers). For primate lineages, the precise number of transient amplifying divisions among A_{pale} progenitors is unknown (noted with *curved arrow* with *question mark*), but the absolute number of amplifying divisions from stem cells to sperm is considered to be fewer among primate species compared with rodents

generations of spermatogonia which precede differentiating Type A_1 are classified as undifferentiated, including A_{single}, A_{paired}, and $A_{aligned}$ clones of 4–16 spermatogonia based on the number of spermatogonia with similar nuclear morphology in a given clone (Fig. 10.2) (Huckins 1971b; Oakberg 1971; Oatley and Brinster 2012). Collectively, A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia constitute the undifferentiated spermatogonial pool in rodent testes, which can all be distinguished from differentiated spermatogonia histologically in tissue section, but can only be further subdivided based on clone size using intact (whole-mounted) seminiferous tubules (Phillips et al. 2010). Spermatogonia with similar nuclear morphology that have an inter-nuclear distance $\leq$25 μm are considered to be within a single clone (de Rooij and Russell 2000; Huckins 1971b), although exceptions to

this 25 μm rule have been noted (Tokuda et al. 2007). Thus, an A_{single} is an undifferentiated spermatogonium not found within 25 μm of other undifferentiated spermatogonia, A_{paired} are two undifferentiated spermatogonia $\leq$25 μm apart, but more than 25 μm from their nearest neighbors, and so forth.

At the midpoint in the cycle of the seminiferous epithelium, chains of $A_{aligned}$ spermatogonia will acquire a classical differentiated phenotype and become A_1 spermatogonia, a non-divisional process termed the $A_{aligned}$-to-A_1 transition (Fig. 10.2) (Schrans-Stassen et al. 1999). In rodents, resulting differentiating spermatogonia will subsequently go through five more clonal amplification mitoses giving rise to A_2, A_3, A_4, and type B spermatogonia, respectively. Type B spermatogonia will produce primary spermatocytes representing the beginning of the first meiotic prophase and the end of mitotic amplification (Oakberg 1956).

10.2.4 Stages of the Cycle of the Seminiferous Epithelium (Spermatogenic Stages)

During spermatogenesis, the cellular associations between different spermatogenic cell types are maintained in a repeated, cyclic fashion termed the cycle of the seminiferous epithelium (Clermont 1972; de Rooij and Russell 2000). For the sake of description, the cycle of the seminiferous epithelium cycle can be divided into a series of species-specific "stages," from as few as 6 in humans (Clermont 1963) to as many as 14 in rats (Leblond and Clermont 1952a, b), which correspond to segments of seminiferous tubules (or cross sections, when determined histologically) that always have the same complement of mitotic, meiotic, and post-meiotic germ cells. These spermatogenic stages occur sequentially along the length of the tubule, a characteristic referred to as the spermatogenic wave (Perey et al. 1961). This allows one to predict the subsequent or previous stages of the spermatogenic cycle based on a stage within one tubule cross section (de Rooij and Russell 2000). As a result, it is possible to follow the spermatogenic differentiation process over time along the length of a given seminiferous tubule. In both rodents and macaques, spermatogenic stages are arranged longitudinally, where each particular segment of a seminiferous tubule would have no more than one spermatogenic stage (Ehmcke and Schlatt 2006). However, in baboons and humans, multiple stages of spermatogenesis can be observed in each tubule cross section (Amann 2008; Chowdhury and Steinberger 1976; Chowdhury and Marshall 1980; Ehmcke et al. 2005a), likely the result of fewer amplifying mitoses (Hermann et al. 2010). The duration of one cycle of the seminiferous epithelium (the amount of time for a segment of seminiferous tubule to proceed from stage I through the final stage), which is also variable between species (Hermann et al. 2010), is a useful benchmark for the various hallmark differentiation events in the spermatogenic lineage because nearly all of these events happen in a repeated fashion in concert with the cycle. The duration of one cycle of the seminiferous epithelium is distinct from the complete duration of

spermatogenesis, from SSC to spermatozoa, which occurs over the span of several cycles of the seminiferous epithelium.

10.3 Hallmark Differentiation Events in the Life Span of Spermatogonia

After formation of the spermatogonial stem cell pool, spermatogonial differentiation can be simplified into two major transitions/events in rodents: (1) SSC fate determination (self-renewal or initiation of differentiation) and (2) the A_{al}-to-A_1 transition. Subsequently, spermatogonia undergo a programmed clonal amplification (A_1–A_4, In., B), enter and proceed through meiosis, and undertake spermiogenesis, the programmed post-meiotic differentiation and physiological maturation that produces flagellated spermatozoa. In the human testis, the terminology employed to describe pre-meiotic spermatogenic cell types is not nearly as refined as rodents, which makes direct interspecies comparison of the differentiation events challenging. Adding to the confusion, dogma holds that the stem cell system functions differently in primates than rodents. This section of the chapter will expound on the relevant mechanisms regulating the two hallmark spermatogonial differentiation events noted above and highlight key differences between rodents and primates.

10.3.1 Formation of the Foundational SSC Pool

In mice, after arrival of primordial germ cells (PGCs) to the developing testis at mid-gestation [~12.5 *dpc* (days *post coitum*); Fig 10.3] (McLaren 2003), M-prospermatogonia (mitotic-prospermatogonia) are formed and proliferate for a short time before becoming mitotically quiescent (designated T1-prospermatogonia; transitional-prospermatogonia) until birth (Hilscher et al. 1974; McCarrey 2013). Between postnatal days 0–3 (0–3 *dpp*, days *postpartum*) in mice, these prospermatogonia reenter the cell cycle (and are renamed T2-prospermatogonia) and proliferate in the middle of the seminiferous cords (Hilscher et al. 1974; McCarrey 2013). The terminology describing prospermatogonial types has been the subject of recent debate with some preferring a simplified description whereby all descendants of male PGCs are termed "gonocytes" until relocating to the basement membrane beginning at about 3 *dpp* (Culty 2013; McCarrey 2013). Between 3 and 6 *dpp*, nascent spermatogonia migrate to the basement membrane of seminiferous cords asynchronously and some will directly differentiate to A_2 spermatogonia and produce the first wave of spermatogenesis (Kluin and de Rooij 1981; Yoshida et al. 2006), while the remainder will be specified as foundational SSCs that will maintain spermatogenesis. There are two

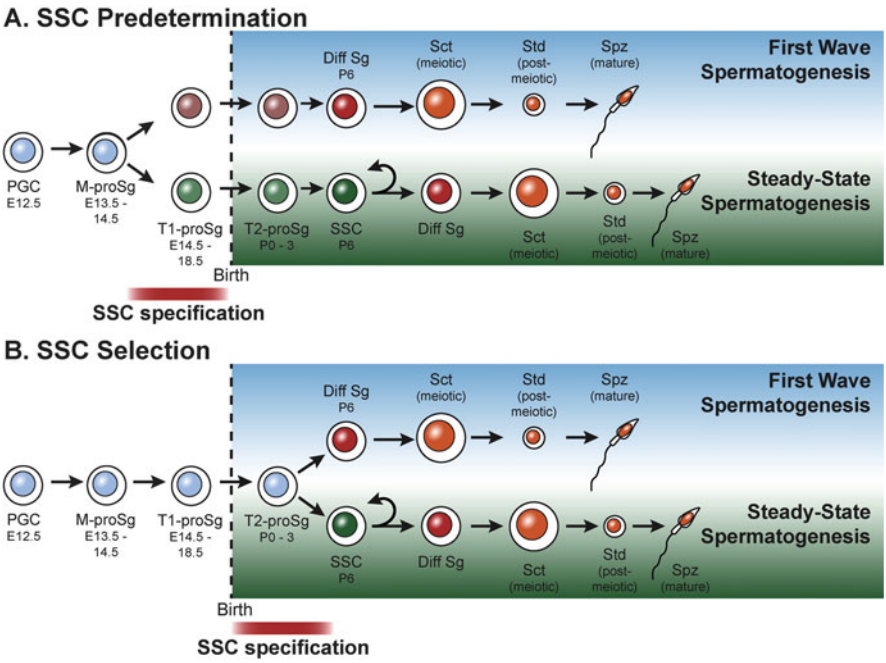

Fig. 10.3 Hypothetical modes of mouse SSC specification. Primordial germ cells (PGCs) arrive at the testis at mid-gestation (~embryonic day 12.5; E12.5), after which time they are considered to be prospermatogonia. These M-prospermatogonia proliferate for a short time before becoming mitotically quiescent (T1-prospermatogonia) until birth. Subsequently, in the first few days after birth (P0–P3), T2-prospermatogonia reenter the cell cycle and proliferate in the middle of the seminiferous cords. During the next 3 days (P3–P6), T2-prospermatogonia become spermatogonia and migrate to the basement membrane of seminiferous cords asynchronously. Some of the resulting spermatogonia will produce the first differentiated spermatogonia and produce the first wave of spermatogenesis, while the remainder will form the pool of foundational SSCs that will maintain spermatogenesis. There are two alternate theoretical models for how the SSCs are specified from prospermatogonia. (**a**) The predetermination model holds that there are subpopulations of prospermatogonia that are more or less likely to be specified as SSCs. (**b**) The selection model holds that prospermatogonia are equipotent and SSCs are selected stochastically. Modified from Hermann et al. (2015)

competing theories that describe potential mechanisms driving specification of the foundational SSCs from prospermatogonia—predetermination and selection—and we will discuss the evidence for each mechanism (Fig. 10.3).

The predetermination theory is based on the concept that there are actually yet-to-be-described subpopulations of prospermatogonia that are more or less likely to become SSCs (Fig. 10.3a). Alternately, a subpopulation of prospermatogonia could be selected stochastically to form the SSC pool (Fig. 10.3b). The predetermination theory was based on histological evaluation of fetal testes performed by Kluin and de Rooij, who suggested that a substantial proportion of mouse late fetal prospermatogonia exhibit a nuclear morphology similar to adult Type A_1

differentiating spermatogonia (Kluin and de Rooij 1981). Consequentially, those cells directly give rise to Type A_2 differentiating spermatogonia in the neonatal period, producing the first wave of spermatogenesis (Kluin and de Rooij 1981). Subsequently, Yoshida and colleagues suggested that the rodent first wave of spermatogenesis, which is not thought to have a human analog, initiates directly from differentiating prospermatogonia based on *Neurog3* and KIT marker expression (Yoshida et al. 2006). It follows that SSC specification may occur as a result of molecular divergence among prospermatogonia during an earlier developmental window that establishes subpopulations with higher or lower likelihoods of producing foundational SSCs (Fig. 10.3a). Results of analyses of spontaneous mutation frequency among prospermatogonia, spermatogonia, and later spermatogenic cells support this mechanism. Specifically, there appears to be a winnowing of mutation-bearing male germ cells between the prospermatogonia and SSC stage in which only those cells that bear a low mutation load produce the SSC pool, thereby suggesting therefore that these cells are predetermined to this fate (Walter et al. 1998; Murphey et al. 2013). We recently published the results of a single-cell gene expression study in which we defined the extent of molecular heterogeneity among neonatal mouse spermatogonia (Hermann et al. 2015). Single-cell qRT-PCR was done on a panel of 172 genes using enriched populations of spermatogonia from postnatal day 6 (6 *dpp*) testes, including cells from *Id4*-eGFP transgenic mice that express eGFP in a small fraction of undifferentiated spermatogonia (Chan et al. 2014). These analyses separated P6 testis cells into four major clusters based on distinct gene expression signatures, consisting of contaminating somatic cells and three groups of spermatogonia (Fig. 10.4). Thus, we found subpopulations of neonatal undifferentiated spermatogonia with discrete mRNA abundance signatures that may correlate with specific subtypes that differ in their functional capacities. In unpublished studies, we have since expanded our assessment of spermatogonial heterogeneity transcriptome wide and we have probed the functional implications of this heterogeneity with transplant analysis, confirming that these discrete transcriptomes separate cells with distinct function. Thus, the gene expression heterogeneity we observed at P6 spermatogonia supports the existence of multiple subtypes of undifferentiated spermatogonia at this stage (Fig. 10.4). Additional studies are needed to expand these results to earlier developmental time-points to determine if these discrete cell types emerge relatively earlier or later in development, which would tend to support the predetermination or selection theories, respectively. Direct evidence for the selection theory is largely lacking, although through examination of one or a few markers among fetal and neonatal germ cells has revealed much homogeneity in those markers (Busada et al. 2014), leading many to gravitate toward the selection hypothesis. Clearly, though, a more thorough examination of mRNA levels at the single-cell level indicates more substantial heterogeneity among spermatogonia and potentially their precursors.

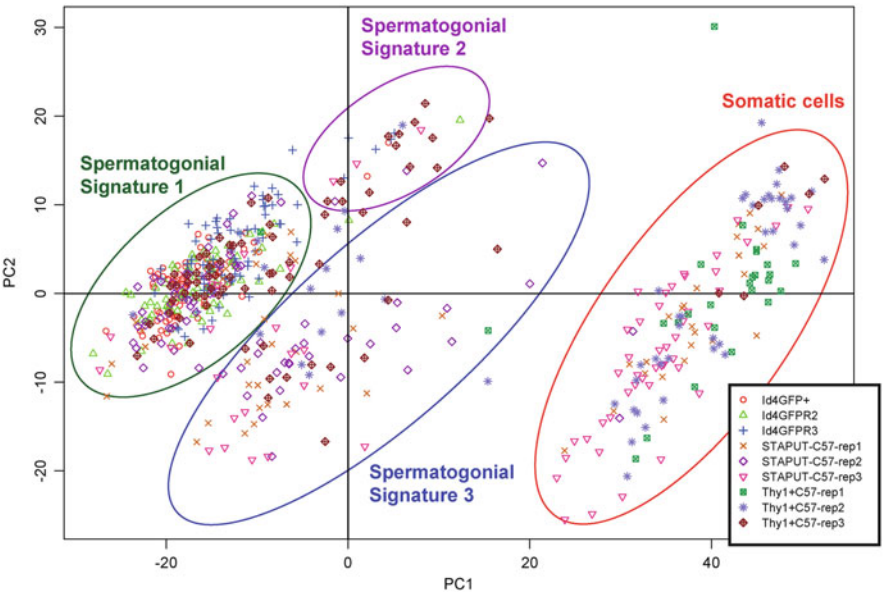

Fig. 10.4 Distinct clusters of P6 spermatogonia (signatures) revealed by mRNA abundance in single cells. Principal component analysis comparison of mRNA levels for 172 genes in 584 individual P6 testis cells (each point is an individual cell). Contaminating somatic cells (*red ellipse*) and three distinct spermatogonial signatures were noted—spermatogonial signatures 1, 2, and 3 (*green*, *violet*, and *blue ellipses*). Legend shows sample sources. Reprinted from Hermann et al. (2015)

10.3.2 Models of Spermatogonial Stem Cell Renewal and Differentiation

The first differentiation event in spermatogenesis results from SSC commitment to the differentiation pathway. Since this fate decision necessarily results from the mechanisms that balance stem cell self-renewal and initiation of differentiation, it is useful to review the kinetic models that have been proposed for this process. The undifferentiated spermatogonia discussed above are further divisible into subpopulations that differ in their biological capacities, including some cells which are considered to be SSCs that exhibit the ability to perpetually self-renew and initiate differentiation by producing committed progenitors (Fig. 10.2). Progenitors arising from SSCs have distinct developmental potential in that they have committed to the differentiation pathway and exhibit a finite transient-amplifying replicative capacity (Fig. 10.2; Hermann et al. 2015; Seaberg and van der Kooy 2003). The prevailing model for renewal and differentiation of rodent SSCs holds that the stem cells are A_{single} spermatogonia that symmetrically self-renew by completing cytokinesis to produce two new A_{single} spermatogonia or commit to differentiate and remain as an A_{paired} clone which will produce a chain of four progenitor cells

($A_{aligned\text{-}4}$) at the next mitosis (Fig. 10.2; Huckins 1971b; Oakberg 1971). This A_{single} model has gained wide (but not universal) acceptance in the field and is consistent with the basic tenets of stem cell biology (Potten 1992).

An alternative model, which we will term the "clone fragmentation" model has been advanced recently based on results from live imaging of transgenic mouse models bearing fluorescent reporters (Nakagawa et al. 2007; Yoshida et al. 2007a). In this model, renewal of spermatogonial stem cells is considered to arise from fragmentation of clones of $A_{aligned}$ spermatogonia. Moreover, while the vast majority of A_{paired} and $A_{aligned}$ typically have committed to differentiation in steady state, and thus, would normally not self-renew, this model holds that these progenitors still possess some latent stemness that can be mobilized under abnormal conditions, such as transplantation (Morimoto et al. 2009; Orwig et al. 2008; Yoshida et al. 2007a). This clone fragmentation model shares considerable similarities with an early alternative model of SSC self-renewal and differentiation put forth by Clermont and Bustos-Obregon (1968), termed the A_0/A_1 model and is also known as the "reserve stem cell" theory. Rather than a single pool of SSCs, this model describes an active stem cell pool that maintains steady-state spermatogenesis and a separate reserve stem cell pool that remains largely quiescent unless the testis is faced with a significant toxic insult. The A_0/A_1 model held that an "active" stem cell population (A_1) was renewed by fragmentation of differentiating Type A_1–A_4 spermatogonia, while a population of quiescent A_0 spermatogonia were "reserve" stem cells. Among the five generations of Type A spermatogonia (A_0, A_1, A_2, A_3, A_4) recognized by Clermont in this model, A_1 were arranged in clones of 4, 8, or 16 cells and most abundant in stages II–VII, much like $A_{aligned}$ spermatogonia from the A_{single} model, and the A_0 spermatogonia appear to reflect A_{single} and A_{paired} spermatogonia. The A_0/A_1 model was supplanted by the A_{single} model because the compendium of data were more consistent with the progressive, clonal amplification and renewal kinetics posited by the A_{single} model (Clermont and Bustos-Obregon 1968; de Rooij 1973; de Rooij and Russell 2000; Huckins 1971a, c; Huckins and Oakberg 1978; Oakberg 1971).

10.3.3 Models of Primate SSC Renewal and Differentiation

Historically, the identity of the SSC pool in primate testes and their mode of renewal has been distinct from the rodent A_{single} model. In the 1950s, Clermont's group initially described two morphologically distinct types of undifferentiated spermatogonia in the testes of rhesus macaques (Clermont and Leblond 1959) and designated these cells A_1 and A_2 (later renamed A_{dark} and A_{pale}). Initially, Clermont proposed that A_{dark} were bona fide SSCs, which undergo self-renewing divisions to maintain the stem cell pool and give rise to A_{pale} that subsequently generate differentiating type B spermatogonia (Clermont and Leblond 1959), much like the rodent model. Yet, a decade later, this model was revised based on observations in the vervet monkey (*Cercopithecus aethiops*) that A_{dark} failed to

label with ^{3}H-thymidine (i.e., they did not appear to proceed through S-phase and self-renew) (Clermont 1969). Rather, Clermont proposed based on these new results that A_{dark} and A_{pale} represented distinct "reserve" and "active" stem cells, respectively. Subsequently, three studies have reported results from S-phase pulse-labeling experiments in various nonhuman primate species and there is a lack of consensus about whether any labeling is observed in A_{dark} within a few hours or several days after the pulse (Clermont and Antar 1973; Ehmcke et al. 2005b; Fouquet and Dadoune 1986). According to this "reserve stem cell" model, ongoing spermatogenesis is maintained by the "active" pool of A_{pale} SSCs under normal circumstances, while the quiescent pool of A_{dark} is only mobilized to regenerate spermatogenesis when spermatogenesis (including A_{pale}) is destroyed by a gonadotoxic insult (e.g., radiation). This A_{dark}/A_{pale} "reserve stem cell" model of primate SSCs shares similarities with the A_0/A_1 model that Clermont advanced for rodents (Bartmanska and Clermont 1983; Clermont and Bustos-Obregon 1968; Clermont and Hermo 1975; Dym and Clermont 1970). As discussed above, the A_0/A_1 model fell out of favor when the alternative A_{single} model, in which there is a single population of stem cells (A_{single} spermatogonia) that divides regularly, but infrequently, was put forth for rodent (Huckins 1971b, c; Oakberg 1971). More recently, the numbers of A_{pale} divisions, which likely reflect progenitor amplification analogous to A_{pr}–A_{al} chains in rodents, have been subtly revised based on contemporary whole-mount immunofluorescent techniques (Ehmcke et al. 2006; Ehmcke and Schlatt 2006), yet the A_{dark}/A_{pale} "reserve stem cell" model has yet to be significantly revisited. Marker analyses (PLZF, GFRA1, NEUROG3, KIT) in adult rhesus monkey testes suggested that A_{dark} spermatogonia bear closer resemblance to rodent A_{single} spermatogonia, while A_{pale} spermatogonia, some of which were KIT+, mimicked A_{paired}–$A_{aligned}$ progenitors (Hermann et al. 2009, 2010). Additional studies will be required to more thoroughly characterize the A_{dark} and A_{pale} spermatogonia populations at the whole transcriptome levels in nonhuman primate and human testes to connect these populations with stem and progenitor phenotypes. Ultimately, though, a functional test (e.g., transplantation) may be needed to resolve uncertainty in the hierarchy among primate undifferentiated spermatogonia.

10.3.4 Identifying Spermatogonial Stem Cells

To further our understanding of the process by which SSCs undertake either self-renewal or differentiation, it is essential to identify the distinguishing features of renewing and differentiating progeny of SSCs. However, this has proven difficult because these cells are extremely rare (~3000 per adult testis based on transplantation; Nagano 2003), and there is currently no method to prospectively identify SSCs in any species (Valli et al. 2015). Conventional SSC identification has been accomplished retrospectively using a functional transplantation assay that measures the ability to produce and maintain spermatogenesis (Table 10.1; Aloisio

et al. 2014; Avarbock et al. 1996; Brinster and Zimmermann 1994; Buaas et al. 2004; Buageaw et al. 2005; Chan et al. 2014; Costoya et al. 2004; Kanatsu-Shinohara et al. 2004, 2014; Kubota et al. 2003; Oatley et al. 2011; Shinohara et al. 1999, 2000; Tokuda et al. 2007; Yang et al. 2013a, c). Knockout approaches have also been used quite extensively to confirm that loss of spermatogenesis follows loss of specific gene products, which, when coupled with lineage tracing, allows demonstration of gene expression that is required for SSC function, in vivo (Table 10.1; Agbor et al. 2013; Aloisio et al. 2014; Ballow et al. 2006; Buaas et al. 2004; Costoya et al. 2004; Falender et al. 2005; Goertz et al. 2011; Greenbaum et al. 2006; Hobbs et al. 2012; Hu et al. 2013; Kanatsu-Shinohara et al. 2014; Lovasco et al. 2015; Meng et al. 2000; Nakagawa et al. 2007; Oatley et al. 2011; Raverot et al. 2005; Schlesser et al. 2008; Suzuki et al. 2012; Yang et al. 2013a; Yoshida et al. 2004, 2007b). Furthermore, whole-mount immunostaining analyses have provided an avenue to define gene products which have testicular expression patterns that are limited, at least primarily, to undifferentiated spermatogonia. A number of such markers have been identified, including (but not limited to) glial cell line-derived neurotrophic factor receptor 1 alpha (GFRA1), inhibitor of DNA binding 4 (ID4), Neurogenin 3 (NEUROG3), Paired box 7 (PAX7), POU domain, class 5, transcription factor 1 (POU5F1; aka: OCT4), Sal-like 4 (SALL4, aka: Tex20), spermatogenesis- and oogenesis-specific basic helix-loop-helix transcription factor (SOHLH1), SRY-box containing gene 3 (SOX3), undifferentiated embryonic cell transcription factor 1 (UTF1), and zinc finger and BTB domain containing 16 (ZBTB16; aka: PLZF) (Table 10.1; Aloisio et al. 2014; Ballow et al. 2006; Buaas et al. 2004; Hobbs et al. 2012; Gassei and Orwig 2013; Greenbaum et al. 2006; Nakagawa et al. 2007; Oatley et al. 2011; Raverot et al. 2005; Schlesser et al. 2008; Tokuda et al. 2007; van Bragt et al. 2008; Wang et al. 2001; Yoshida et al. 2004, 2007b). Expression of many of these genes has been confirmed among spermatogonial types in nonhuman primate and human testes (Fig. 10.2; Altman et al. 2014; Dovey et al. 2013; Hermann et al. 2007, 2009, 2010, 2011; Izadyar et al. 2011; Valli et al. 2014). Yet, connecting these gene expression patterns to regulation of SSC fate decisions in any species has proven difficult because defects in either SSC renewal or differentiation produce an essentially indistinguishable block in spermatogenesis.

Emerging out of these studies are examples of just a couple of genes which exhibit expression profiles that are restricted to only A_{single} spermatogonia, which contain the entire presumptive SSC pool. One such gene product, Inhibitor of DNA binding 4 (ID4), is a helix-loop-helix factor lacking a basic region which has an expression pattern restricted to a subpopulation of A_{single} spermatogonia among mitotic germ cells in the testis and is required for SSC maintenance and spermatogenesis (Oatley et al. 2011). The LT-11 *Id4*-eGFP BAC transgenic mouse exhibits eGFP+ expression only in a fraction of $A_{singles}$, and in cultures of THY1+ spermatogonia eGFP is observed in about 10 % of spermatogonia (Chan et al. 2014). Importantly, all stem cell activity from cultured spermatogonia was observed in the eGFP+ fraction (Chan et al. 2014). Thus, by transitive logic, if *Id4*-eGFP is restricted to A_{single} spermatogonia and only eGFP+ spermatogonia exhibit

Table 10.1 Key genes expressed by spermatogenic cell types involved in differentiation

Marker/ gene (alias)	Expression profile (method)	Mutant phenotype	Citation(s)
Atm	Undifferentiated spermatogonia (FACS, IHC, BrdU, TP, RT-PCR)	$Atm^{-/-}$ depletion of undifferentiated spermatogonia followed by cell cycle arrest as well as activation of pathways associated with DNA damage	Shiloh (2003); Takubo et al. (2008)
Bcl6b	Undifferentiated spermatogonia (siRNA, TP)	Sertoli-only tubules and complete loss of SSCs in BcI6b-deficient mice	Oatley et al. (2006)
Cd24a	SSCs (FACS, TG, THY1, Tr)	N/A	Kubota et al. (2003)
Cd81	Germline stem cells (FACS)	CD81 is a cell surface marker for SSCs highly expressed on GS cells	Kanatsu-Shinohara et al. (2012a)
Cd9	SSCs (Tr, FACS)	Selecting cells positive for CD9 show enrichment of SSCs	Kanatsu-Shinohara et al. (2004)
Cdh1	Undifferentiated spermatogonia (WM, IHC, WB, BrdU, Tr)	CDH1-positive cells formed clusters of one, two, four, and eight cells and survived after busulfan treatment colonizing recipient testes	Tokuda et al. (2007)
Cldn3	SSCs, Sertoli cells (RT-PCR, WB, IHC, siRNA)	Rac mutant cells show reduction in expression of *Cldn3*—role in the testis–blood barrier	Takashima et al. (2011)
Csf1	Leydig cells, peritubular myoid cells (IHC)	Addition of CSF1 to media increased the self-renewal capacity of SSCs in THY1 cultures without affecting germ cell expansion	Oatley et al. (2009)
Csf1r	Undifferentiated spermatogonia (IHC, ICC THY1, FACS)	N/A	Oatley et al. (2009)
Cxcr4	Undifferentiated spermatogonia (IHC, THY1, RT-PCR, Tr, FACS, shRNA, WB)	CXCL12/CXCR4 signaling necessary for transplant colonization, inhibition—SSC loss and buildup of progenitor spermatogonia	Yang et al. (2013b)
Dazl	Type A spermatogonia, intermediate, and type B spermatogonia (WM, IHC)	$Dazl^{-/-}$; blocked transition from A_{al} to A_1 spermatogonia	Schrans-Stassen et al. (2001)

(continued)

Table 10.1 (continued)

Marker/ gene (alias)	Expression profile (method)	Mutant phenotype	Citation(s)
Dmrt1	Spermatogonia and Sertoli cells (cKO, TG, BrdU, ChIP, IHC, WM, RT-PCR)	*Dmrt1*$^{-/-}$; premature meiotic entry, increased RA responsiveness	Matson et al. (2010); Agbor et al. (2013)
Dnmt3l	Prospermatogonia, Spermatocytes (Mu, KO, THY1)	*Dnmt3l*$^{-/-}$; meiotic-entry defect preceded by a progenitor differentiation defect (PLZF)	Webster et al. (2005); Liao et al. (2014)
Egr2	Undifferentiated spermatogonia (RT-PCR, Tr)	*Egr3* is GDNF responsive	Oatley et al. (2006)
Egr3	Undifferentiated spermatogonia (RT-PCR, Tr)	*Egr3* is GDNF responsive	Oatley et al. (2006)
Epcam	SSCs (FACS, siRNA, Tr, TG, Tu, RT-PCR, shRNA)	N/A	Anderson et al. (1999); Kanatsu-Shinohara et al. (2011)
Epha2	SSCs (FACS), subpopulation of *Id4*-eGFP+ (single-cell qRT-PCR)	N/A	Kanatsu-Shinohara et al. (2012a); Hermann et al. (2015)
Etv5	Sertoli cells, germ cells (Mu, TP)	*Etv5*$^{-/-}$; gradual germ cell depletion consistent with SSC renewal defect	Chen et al. (2005); Morrow et al. (2007)
Foxo1	Prospermatogonia, undifferentiated spermatogonia (KO, IHC, RT-PCR)	Deficiency of *Foxo1*, *Foxo3*, and *Foxo4* resulted in impairment of SSCs and block of differentiation	Goertz et al. (2011)
Gndf	Sertoli cells (TG, NB, WB, ISH, BrdU)	*Gdnf*$^{+/-}$; SSC depletion, overexpression leads to accumulation of undifferentiated spermatogonia	Meng et al. (2000); He et al. (2008); Johnston et al. (2011); Chen et al. (2014, 2016); Takashima et al. (2015)
Gfra1	Undifferentiated spermatogonia and some SSCs (WMIHC, Tr, siRNA)	*Gfra1* silencing leads to spermatogonial differentiation	Buageaw et al. (2005); He et al. (2007); Grasso et al. (2012); Hara et al. (2014)
Gpr125	Undifferentiated spermatogonia (IHC, WM, Tr, FACS)	N/A	Seandel et al. (2007)
Id4	Exclusive to A_{single} in adults (TG, IHC, FACS, TP, siRNA, WM, THY1, WB)	*Id4*$^{-/-}$ progressive loss of undifferentiated spermatogonia—essential for SSC self-renewal. *Id4*-eGFP BAC transgenic confirmed SSC activity in ID4-expressing cells	Oatley et al. (2011); Chan et al. (2014)

(continued)

Table 10.1 (continued)

Marker/ gene (alias)	Expression profile (method)	Mutant phenotype	Citation(s)
Itga6	SSCs (FACS, Tr), spermatogonia (IHC)	N/A	Shinohara et al. (1999, 2000)
Itgb1	SSCs (FACS, Tr), spermatogonia (IHC)	N/A	Shinohara et al. (1999, 2000)
Kdr (*Vegfr2, Flk1*)	Prospermatogonia, spermatogonia, Sertoli cells (IHC, RT-PCR, Tr)	Conditional *Vegfa*$^{-/-}$ with *Dmrt1*-Cre—defects in spermatogonial differentiation	Caires et al. (2012); Lu et al. (2013); Sargent et al. (2016)
Lin28a/b	PGCs, undifferentiated spermatogonia (WB, TG, IHC, WM, siRNA)	*Lin28a* needed for normal PGC expansion	Zheng et al. (2009); Shinoda et al. (2013); Chakraborty et al. (2014)
Lhx1	Undifferentiated spermatogonia (IHC, TP, siRNA)	*Lhx1* expression is upregulated by GDNF. *Lhx1* knockdown (in vitro) impaired SSC maintenance	Oatley et al. (2007)
Mcam	SSCs (siRNA, FACS/Tr)	*Mcam* knockdown (in vitro) reduced transplant colonization	Kanatsu-Shinohara et al. (2012b)
Mir221/ Mir222	Undifferentiated spermatogonia (THY1, IHC, RT-PCR, FACS, shRNA, ISH, WB, NB, Tr)	Overexpression prevents RA-dependent differentiation	Yang et al. (2013c)
Mycn	Undifferentiated spermatogonia (THY1, RT-PCR)	*N/A*	Braydich-Stolle et al. (2007)
Nanos2	A_{single} and A_{paired} (TG, WB, RT-PCR, IHC, cKO, KO, WM)	Conditional *Nanos2*$^{-/-}$; rapid progressive loss of SSCs. Overexpression—accumulation of undifferentiated spermatogonia	Suzuki et al. (2007, 2009); Sada et al. (2009); Zhou et al. (2015)
Nanos3	Undifferentiated spermatogonia (KO, NB, WB, IHC, FACS, IF)	*Nanos3* overexpression causes accumulation of cells in the G1 phase, blocks differentiation, and causes failure of meiotic entry and progression	Lolicato et al. (2008); Julaton and Reijo Pera (2011)
Neurog3 (*Ngn3*)	Undifferentiated spermatogonia (FACS, ISH, RT-PCR, TG, cKO, WM, THY1, TP, ChIP, shRNA, siRNA, WB)	Neurog3 marks early differentiating SSCs (perhaps coincident with entry into cell cycle)	Yoshida et al. (2004, 2006); Nakagawa et al. (2007); Ikami et al. (2015)

(continued)

Table 10.1 (continued)

Marker/ gene (alias)	Expression profile (method)	Mutant phenotype	Citation(s)
Notch1	Sertoli cells (Notch signaling reporter mice—ICC/IHC)	Conditional *Rbpj*$^{-/-}$ in Sertoli cells with *Amh*-Cre (mediator of NOTCH signaling) increases SSC number. Constitutive NOTCH activation leads to premature prospermatogonia differentiation	Dirami et al. (2001); Garcia and Hofmann (2013); Garcia et al. (2013); Garcia et al. (2014)
Nox1	Undifferentiated spermatogonia (IHC, KO, Tr)	Loss of *Nox1* results in suppression of spermatogonial proliferation (in vitro)	Morimoto et al. (2013)
Nox2 (*Cybb*)	Undifferentiated spermatogonia (FACS, RT-PCR, Tr)	*Nox2* initiates the AKT pathway inducing cellular proliferation and is important in NADPH oxidation	Morimoto et al. (2013)
Nox3	Undifferentiated spermatogonia (RT-PCR, shRNA, Tr)	shRNA-mediated knockdown prevents GDNF-stimulated ROS and SSC renewal in cultured and fresh spermatogonia	Morimoto et al. (2013, 2015)
Nox4	Undifferentiated spermatogonia (RT-PCR, shRNA)	Knockdown of *Nox4* (in vitro) suppresses spermatogonial proliferation	Morimoto et al. (2013)
Pax7	Subpopulation of A_{single} spermatogonia (IHC, LT, WM, Tr, cKO, WB)	Dispensable for spermatogenesis (*Pax7*$^{-/-}$)	Aloisio et al. (2014)
Pou3f1 (*Oct6*)	Undifferentiated spermatogonia (THY1, siRNA, IHC, RT-PCR, WB, TP)	Knockdown of *Pou3f1* in cultured spermatogonia induced apoptosis and impaired SSC maintenance	Wu et al. (2010)
Pvr	SSCs (FACS), subpopulation of *Id4*-eGFP (single-cell qRT-PCR)	N/A	Kanatsu-Shinohara et al. (2012a)
Rarg	$A_{aligned}$ spermatogonia and later spermatogonia	Spermatogonial differentiation incomplete in conditional *Rarγ*$^{-/-}$ (block A_{al}–A_1 transition)	Gely-Pernot et al. (2012)

(continued)

Table 10.1 (continued)

Marker/ gene (alias)	Expression profile (method)	Mutant phenotype	Citation(s)
Rb1	Prospermatogonia, undifferentiated spermatogonia, Sertoli cells (cKO, IHC, WB, siRNA, TP, RT-PCR)	Conditional *Rb1*$^{-/-}$ in prospermatogonia (*Ddx4*-Cre) have normal first wave, but are SSCs lost. Conditional *Rb1*$^{-/-}$ in progenitors (*Neurog3*-Cre) normal spermatogenesis	Yang et al. (2013a); Hu et al. (2013)
Sall4	Undifferentiated spermatogonia (IHC, WM, WB)	Conditional *Sall4*$^{-/-}$ (*Stra8*-Cre or *Ddx4*-Cre) loss of spermatogenesis consistent with defects in both SSC renewal and differentiation	Hobbs et al. (2012); Gassei and Orwig (2013)
Sohlh1	Type A1-B spermatogonia, spermatocytes (IHC, RT-PCR, Mu, BrdU)	*Sohlh1*$^{-/-}$; increased spermatogonial apoptosis, few spermatocytes in some tubules	Ballow et al. (2006)
Sohlh2	Undifferentiated spermatogonia (Mu, RT-PCR, WB, IHC, WM, ChIp)	*Sohlh2*$^{-/-}$; reduced numbers of A and B spermatogonia	Ballow et al. (2006); Toyoda et al. (2009)
Stat3	SSCs (siRNA, THY1, ChIP, IHC, shRNA, RT-PCR, WB, TP)	*Stat3*$^{-/-}$; block to *Neurog3* expression and differentiation in cultured A_{undiff}	Kaucher et al. (2012)
Sox3	Undifferentiated spermatogonia (Mu, IHC, RT-PCR)	SOX3 co-localizes with *Neurog3*; *Sox3*$^{-/-}$ mice have blocked spermatogonial differentiation beyond Type A spermatogonia (P10)	Raverot et al. (2005)
Stra8	A_{al}-B spermatogonia, spermatocytes (BrdU, IHC, RT-PCR, Mu)	*Stra8* mRNA and translation induced by RA and required for meiotic initiation and A_{al}–A_1 transition	Anderson et al. (2008); Snyder et al. (2011); Endo et al. (2015)
T (*Brachyury*)	Expressed by subpopulation of cultured spermatogonia (*Id4*-eGFP+)	*Etv5*$^{-/-}$ have decreased T activation	Wu et al. (2011); Chan et al. (2014)
Taf4b	Prospermatogonia, spermatocytes, Sertoli cells (Mu, IHC, TP, WB, RT-PCR, IF)	*Taf4b*$^{-/-}$; reduced prospermatogonia numbers at E18-P1, progressive loss of spermatogenesis consistent with SSC renewal defect	Falender et al. (2005); Lovasco et al. (2015)

(continued)

Table 10.1 (continued)

Marker/ gene (alias)	Expression profile (method)	Mutant phenotype	Citation(s)
Tspan8	Undifferentiated spermatogonia, SSCs (RT-PCR, Tr), subpopulation of *Id4*-eGFP (single-cell qRT-PCR)	*Tspan8* downregulated in cultured THY1+ spermatogonia following 1 h GDNF withdrawal	Oatley et al. (2006); Hermann et al. (2015)
Thy1 (*Cd90*)	SSCs (transplant), but also widely expressed by other somatic cells and progenitor spermatogonia	N/A	Kubota et al. (2003); Reding et al. (2010)
Uchl1 *(Pgp9.5)*	Undifferentiated spermatogonia	*Uchl1*-deficient gracile axonal dystrophy (GAD) mutant mice; seminiferous tubule atrophy, decreased germ cell numbers	Kwon et al. (2003, 2005); Luo et al. (2006)
Utf1	Prospermatogonia and early type A undifferentiated spermatogonia (RT-PCR, IHC)	UTF1 distribution suggests that it plays a role in maintaining the undifferentiated state	van Bragt et al. (2008)
Utp14b (*Jsd*)	Spermatogonia, higher in spermatocytes to round spermatids (RACE-PCR, TG, RT-PCR)	*Utp14b* mutants are sterile due to spermatogonial depletion	Rohozinski and Bishop (2004); Zhao et al. (2007)
Zbtb16 (*Plzf*)	Undifferentiated spermatogonia (Mu, ISH, RT-PCR, KO, TP, WM, IHC, FACS)	*Plzf* mutants (Luxoid or KO) exhibit progressive SSC loss due to compromised SSC renewal	Costoya et al. (2004); Buaas et al. (2004); Filipponi et al. (2007); Hobbs et al. (2010, 2012)

BrdU 5-bromo-2′-deoxyuridine DNA synthesis, *ChIP* chromatin-immunoprecipitation assay, *cKO* conditional knockout, *FACS* fluorescence-activated cell sorting, *IF* immunofluorescent staining, *IHC* immunohistochemistry, *ISH* in situ hybridization, *KO* knockout *LT* lineage tracing, *Mu* mutant animals, *RACE-PCR* rapid amplification of cDNA ends, *RT-PCR* real-time PCR, *TP* transplantation, *WB* Western Blot analysis, *WM* whole-mount, *THY1* THY1+ spermatogonia culture, *siRNA* in vitro knockdown experiment using siRNA, *shRNA* lentivirus vector transduction, *TG* transgenic animals, *Tu* TUNEL cell apoptosis assay

transplantable stem cell activity, then one could conclude that all SSCs are A_{single} spermatogonia. These results provide perhaps the most compelling argument in favor of the A_{single} model of SSC renewal and differentiation (Chan et al. 2014; Oatley et al. 2011). However, it does not appear that all *Id4*-eGFP+ spermatogonia exhibit stem cell activity (unpublished results and Chan et al. 2014; Oatley et al. 2011). Likewise, both endogenous *Id4* mRNA and *Id4*-eGFP are detectable in pachytene spermatocytes, and while the significance of *Id4* expression to spermatocyte function is not clear, this reinforces the concept that *Id4*-eGFP expression

alone is not sufficient to delineate stem cells in the testis. Indeed, there is currently no method, including using of molecular markers, that can distinguish SSCs from their differentiating progeny in the testis from any species.

Another candidate SSC marker, PAX7, was reported recently in the literature (Aloisio et al. 2014), which, like ID4, was observed exclusively among A_s spermatogonia in the neonatal and adult testis. While lineage-tracing experiments suggest that progeny of *Pax7*+ cells can produce complete spermatogenesis and have regenerative capacity after cytotoxic insult, this gene is also known to be dispensable for spermatogenesis. Examination of the transcriptomes of *Id4*-eGFP+ and *Id4*-eGFP– cultured spermatogonia and unpublished results from single-cell gene expression studies failed to identify any *Pax7* transcripts (Chan et al. 2014), raising the concern that PAX7 is not an effective marker of SSCs .

10.3.5 Regulation of SSC Self-Renewal: Instruction from the Niche

The fate decision of dividing SSCs to either self-renew or initiate differentiation represents the first differentiation step in the spermatogenic lineage. It is well recognized that cells comprising the SSC niche produce signals (e.g., GDNF, KITL, RA, FGF2, CSF1, NOTCH2) which help instruct this balance by promoting either self-renewal or differentiation (Busada et al. 2015a; Chen et al. 2014, 2016; Dann et al. 2008; Garcia et al. 2014; He et al. 2008; Ishii et al. 2012; Kubota et al. 2004; Meng et al. 2000; Oatley et al. 2006; Oatley et al. 2007, 2009; Ohta et al. 2000; Schrans-Stassen et al. 1999; Takashima et al. 2015). Molecules that promote SSC renewal (e.g., GDNF, FGF2, CSF1) appear to play a predominant role in this process since SSCs do not appear to be responsive to differentiation signals (e.g., RA, KITL), but rather, their capacity to respond to differentiation signals is acquired after commitment to differentiation by progenitors.

10.3.5.1 GDNF and GFRA1/RET

Among the best studied niche-derived factor is glial cell line-derived neurotrophic factor (GDNF), which was initially described in 2000 as a key regulator of this critical balance (Meng et al. 2000). GDNF is a paracrine factor produced by testicular somatic cells in the niche (e.g., Sertoli cells, peritubular myoid cells) which must be produced in the correct amount to sustain spermatogenesis (Chen et al. 2014, 2016; Meng et al. 2000). Animals bearing one null allele of *Gdnf* produce roughly half the normal GDNF leads and exhibit reduced SSC self-renewal and exhaustion of the stem cell pool (Meng et al. 2000). Reciprocally, transgenic overexpression of *Gdnf* leads to excessive SSC self-renewal and failure to produce differentiating spermatogenic cells (Meng et al. 2000). This concept that GDNF

levels must be exquisitely regulated to remain in a narrow range to promote balanced SSC self-renewal and differentiation has since been supported by the results of numerous in vitro and in vivo studies (Grasso et al. 2012; He et al. 2007, 2008; Jain et al. 2004; Jijiwa et al. 2008; Kubota et al. 2004, 2011; Lee et al. 2007; Naughton et al. 2006; Oatley et al. 2006, 2007; Parker et al. 2014; Ryu et al. 2005; Savitt et al. 2012; Tadokoro et al. 2002; Wu et al. 2010). GDNF acts by binding glial cell line-derived neurotrophic factor family receptor alpha 1 (GFRA1), which induces signaling through the RET tyrosine kinase receptor, both of which are expressed by undifferentiated type A spermatogonia (Jijiwa et al. 2008; Naughton et al. 2006). GDNF-dependent signaling through GFRA1/RET (Arighi et al. 2005) involves activation of at least three cascades in SSCs including PI3kinase/AKT (Lee et al. 2007; Oatley et al. 2007), RAS/ERK1/2 (He et al. 2008), and SRC family kinases (SFKs; Oatley et al. 2007). Manipulation of GDNF levels in the medium of THY1+ spermatogonia cultured leads to changes in the expression of at least 269 genes, including several known to be involved in SSC self-renewal and differentiation, such as B cell CLL/lymphoma 6 member b (*Bcl6b*), Ets variant gene 5 (*Etv5*), Forkhead box protein O1 (*Foxo1*), and Lim homeobox protein 1 (*Lhx1*) (Goertz et al. 2011; Morrow et al. 2007; Oatley et al. 2006, 2007; Schlesser et al. 2008; Wu et al. 2011). More recently, the cellular source of GDNF required for spermatogenesis has come into question. Initially, it was presumed that Sertoli cells were the primary source of GDNF required for spermatogenesis, based on expression studies (Fouchecourt et al. 2006; Johnston et al. 2011; Katoh-Semba et al. 2007). However, it now appears that peritubular myoid cells also produce GDNF in a manner dependent on testosterone (Chen et al. 2014) and that GDNF production by peritubular myoid cells is required for normal spermatogenesis (Chen et al. 2016). It remains to be seen whether Sertoli cell-derived GDNF is also necessary for sustaining SSC self-renewal and spermatogenesis.

10.3.5.2 Colony-Stimulating Factor 1

Colony-stimulating factor 1 (CSF1) is a cytokine that is produced by cells in the testicular interstitium (near clusters of Leydig cells) and some peritubular myoid cells which is known to promote SSC self-renewal (Oatley et al. 2009). Colony-stimulating factor 1 receptor (*Csf1r*) was found to be highly expressed in THY1+ germ cells, indicating that CSF1 acts directly on SSCs. Exposure of cultures of THY1+ spermatogonia to CSF1 in the presence of GDNF enhances mouse SSC self-renewal in vitro (Oatley et al. 2009). Interestingly, exposure of cultured mouse THY1+ spermatogonia to CSF1 alone (i.e., in the absence of GDNF) does not expand SSCs (Oatley et al. 2009), suggesting a requisite cooperation between both growth factors that likely involves signal transduction cross talk. Furthermore, testicular macrophages were recently shown to participate in regulation of SSC or spermatogonial fate, perhaps by secreting CSF1 or another related factor (DeFalco et al. 2015). A related cytokine, granulocyte colony-stimulating factor (G-CSF or CSF3), was recently identified as a potential somatic-derived self-renewal factor

based on the potential of exogenous CSF3 to protect spermatogenesis from alkylating chemotherapy (Benavides-Garcia et al. 2015). The CSF3 receptor (CSF3R) is present at the mRNA and protein level in undifferentiated spermatogonia, but the cellular source of CSF3 in normal testes is not clear (Benavides-Garcia et al. 2015). These data support the concept that other testicular somatic cell types, beyond Sertoli cells, may play critical roles in driving SSC fate during normal steady-state spermatogenesis.

10.3.5.3 FGF2 (Basic FGF)

Like CSF1, fibroblast growth factor 2 (FGF2) is known to enhance mouse, rat, and hamster SSC self-renewal in vitro, but also like CSF1, this factor is insufficient to support SSC renewal and expansion alone (Kanatsu-Shinohara et al. 2008; Kubota et al. 2004; Ryu et al. 2005). In these studies, addition of FGF2 to the medium for cultured THY1+ spermatogonia is able to promote SSC expansion only in the presence of GDNF. Like GDNF, though, it appears that the primary testicular source of FGF2 is Sertoli cells (Mullaney and Skinner 1991). FGF2 promotes self-renewal of SSCs by enhancing expression of GDNF-regulated genes and acting as a mitogen (Ishii et al. 2012; Takashima et al. 2015).

10.3.6 Intrinsic Control of SSC Fate

In order to regulate the alternative fates among progeny of SSCs, niche-derived signals (e.g., GDNF) must be mediated by alternative mechanisms within SSCs that translate these signals into distinct gene expression patterns. Intrinsic control of SSC fate decisions, however, has remained poorly understood due to the extraordinary technical challenge of studying a cell population that comprises roughly 0.003 % of the germ cells in the adult testis (Nagano 2003; Tegelenbosch and de Rooij 1993). Thus, the field has focused on understanding the roles of gene products which exhibit an expression pattern that is restricted (or primarily restricted) to undifferentiated A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia in early spermatogenesis. As a result, while numerous gene products with this restricted expression profile and which are necessary for spermatogenesis have been identified (Table 10.1), for the most part, it is not clear at which point in the spermatogenic lineage (i.e., in which male germ cells) such genes are essential, and thus, their precise role(s) in SSC fate is not clear. Despite this uncertainty, it is clear from such studies that spermatogenesis can be disrupted at the very earliest stages by deletion or perturbation of a variety of genes and gene products (Table 10.1).

Curiously, a majority of gene products examined which are required for undifferentiated spermatogonial function are involved in transcriptional regulation (e.g., ID4, PLZF, and SALL4). Thus, as is the case in other developmental programs, such genes might play roles as master regulators of cell fate decisions by

executing essential gene expression programs. Here we will focus on three examples of potential master transcriptional regulators of SSC fate, ID4, PLZF, and SALL4, for which functional evidence is available. Knockouts for all three factors have confirmed that they are essential for proper control of SSC fate (Buaas et al. 2004; Costoya et al. 2004; Hobbs et al. 2012; Oatley et al. 2011). Two different PLZF mutants exhibit the same phenotype, a progressive depletion of the SSC pool after the first wave of spermatogenesis leading to azoospermia (Buaas et al. 2004; Costoya et al. 2004). This defect appears to result from a shift in the balance in SSC fate away from self-renewal and toward differentiation, indicating that PLZF either inhibits genes involved in SSC differentiation or activates SSC self-renewal genes. In support of this, we recently reported the full regulatory repertoire for PLZF in undifferentiated spermatogonia using ChIP-seq and found that both renewal and differentiation genes were bound by and required PLZF for expression (Lovelace in press). While whole-animal knockouts for *Sall4* are embryonic lethal (Sakaki-Yumoto et al. 2006), conditional *Sall4* knockouts generated using the *Vasa*-Cre transgene exhibit a progressive spermatogenic deficiency reminiscent of the PLZF mutants, suggesting that both maintenance and differentiation of SSCs are perturbed (Hobbs et al. 2012). Similar ChIP-seq results for SALL4 in undifferentiated spermatogonia demonstrate it is targeted to the genome by PLZF and the differentiation factor DMRT1 and appears to be required for expression of both self-renewal and differentiation genes (Lovelace in press). Most recently, *Id4*-null animals also exhibited a progressive spermatogenic loss defect consistent with failure of SSC renewal (Oatley et al. 2011). ID4 is a helix-loop-helix protein that lacks a basic region, meaning that it can dimerize with other HLH factors and act as a dominant-negative protein to prevent their DNA binding. It is not clear which proteins complex with ID4 in A_{single} spermatogonia, but two possibilities include E2F and NEUROG3, which may have implications for spermatogonial proliferation and differentiation, respectively. Thus, ID4, PLZF, and SALL4, which have expression patterns restricted to subpopulations of undifferentiated spermatogonia, including SSCs and progenitor spermatogonia, and which are known to be functionally required for ongoing spermatogenesis, likely are involved in creating the favorable transcriptomes for SSC self-renewal and/or differentiation. Future studies further examining the cistromes for these and other transcription factors with similar expression profiles will undoubtedly reveal key regulatory networks that predispose the ability of SSCs to renew or differentiate.

10.3.7 Spermatogonial Progenitor Response to Differentiation Signals

The reciprocal SSC fate, differentiation, is only discernable once progenitor spermatogonia acquire the capacity to respond to niche-derived differentiation factors (e.g., RA, KITL). At postnatal day 6 (6 *dpp*), nearly all *Id4*-eGFP+ spermatogonia in the mouse testis express mRNAs for *Stra8* and *Kit*, including presumptive SSCs

and progenitor spermatogonia (Hermann et al. 2015), which would be indicative of an intact retinoid receptor response and be indicative of spermatogonial differentiation. Yet, it is also clear that neither STRA8 nor KIT protein was detectable in the vast majority of *Id4*-eGFP+ spermatogonia (Hermann et al. 2015). Indeed, in that study, KIT and STRA8 protein were only localized to a small percentage of spermatogonia with weak eGFP fluorescence intensity which is thought to be indicative of spermatogonia that have transitioned out of an SSC state to become progenitor spermatogonia (Chan et al. 2014; Hermann et al. 2015). These results raise an important concept of early spermatogonial differentiation in which regulation of cell fate appears to occur predominantly at the level of regulation of mRNA utilization (i.e., translational control), rather than transcriptional regulation (Busada et al. 2014; Chappell et al. 2013). Acquisition of RA responsiveness and KIT protein expression appears to be the rate-limiting step in poising progenitor spermatogonia for competency to undergo the A_{al}–A_1 transition (Ikami et al. 2015).

10.3.7.1 Retinoic Acid

Retinoic acid (RA) is a biologically active metabolite of vitamin A (retinol) and is essential for male fertility (Anderson et al. 2008; Bowles et al. 2006). The requirement for RA in spermatogenesis is easily recognized using the vitamin A-deficient (VAD) model in which dietary-derived RA is essentially absent. VAD mice or rats exhibit a block in spermatogenesis at the undifferentiated spermatogonia stage (Morales and Griswold 1987). VAD followed by release of the RA deficiency also synchronizes spermatogenesis and 24–48 h after injection with either RA or retinol (vitamin A); previously arrested A_{al} reenter the cell cycle and differentiate into A_1 spermatogonia (Morales and Griswold 1987). Likewise, inhibition of ALDH1A2, the rate-limiting enzyme responsible for RA biosynthesis from retinaldehyde, using the bisdichloroacetyldiamine (BDAD) WIN 18,446, mimics VAD and induces spermatogenic arrest by blocking A_{al}–A_1 transition (Heller et al. 1961). It is not clear which retinoid receptors (RARs or RXRs) are involved in cellular changes induced by RA in progenitor spermatogonia, although one publication suggests that RARγ is required for the A_{al}–A_1 transition (Gely-Pernot et al. 2012). Independent of the nuclear RAR/RXR response, it appears that a primary consequence of RA stimulation of progenitor spermatogonia is to induce translation of mRNAs encoding the KIT tyrosine kinase and STRA8, via a mechanism involving P13K/AKT/mTORC1 signaling (Busada et al. 2015a, b).

During the cycle of the seminiferous epithelium, a mid-cycle pulse of RA (centered on stages VI–VII) drives transition of $A_{aligned}$ spermatogonia to A_1 spermatogonia (Endo et al. 2015; Hogarth et al. 2015a), although not all $A_{aligned}$ appear to differentiate. It is not clear why only some undifferentiated spermatogonia respond to RA, although it is possible that expression of CYP26A1 and CYP26B1 enzymes, which catalyze the degradation of RA, may restrict RA action to subpopulations of progenitor spermatogonia. Recent conditional mutants of CYP26A1 and CYP26B1 within either Sertoli cells (*Amh*-Cre) or differentiating

spermatogonia (*Stra8*-iCre) suggest that neither enzyme is essential for spermatogenesis, since these animals were fertile (Hogarth et al. 2015b). Subtle spermatogenic defects were observed in these animals, most markedly, an increase in the number of STRA8+ differentiating spermatogonia, but overall suggesting that redundancy might preclude complete differentiation of the undifferentiated spermatogonial pool when RA levels cannot be reduced.

10.3.7.2 KIT and KIT-Ligand

Acquisition of KIT expression among A_{al} spermatogonia is another crucial step promoting differentiation to Type A_1 spermatogonia. KIT is type III receptor tyrosine kinase which is considered to be required for normal spermatogonial differentiation (Besmer et al. 1993; Dym et al. 1995; Koshimizu et al. 1992; Manova and Bachvarova 1991; Sorrentino et al. 1991; Yoshinaga et al. 1991; Zhang et al. 2011). KIT is the membrane receptor for stem cell factor (SCF, aka: KIT ligand) and is expressed in some A_{al} progenitor spermatogonia (chains of 8–16 cells), differentiating spermatogonia, and up through preleptotene spermatocytes (Schrans-Stassen et al. 1999; Yoshinaga et al. 1991). Male mice bearing mutant alleles of *Kit* (W/W^v, dominant-white spotting locus) or *Kitl* (Steele) are largely sterile (Besmer et al. 1993; Coulombre and Russell 1954; Geissler et al. 1981; Koshimizu et al. 1992). Absence of KIT or SCF expression does not affect proliferation of progenitor spermatogonia, demonstrated by both transplantation and in vitro THY1+ culture experiments (Kubota et al. 2009; Ohta et al. 2003). However, under certain transplant conditions (W/W^v pup recipients), KIT-mutant germ cells are still able to differentiate to produce complete spermatogenesis (Kubota et al. 2009).

The *Kit* gene is also perhaps the best characterized putative PLZF target (Filipponi et al. 2007; Hobbs et al. 2012; Puszyk et al. 2013). PLZF binding to the *Kit* promoter in spermatogonia occurred through a "consensus" PLZF binding motif (5′-ATACAGT-3′) which was identified by chromatography with a GST fusion to the seven most carboxy-terminal Zn fingers found in human PLZF (Li et al. 1997). Unpublished ChIP-seq results for PLZF from the Hermann lab demonstrate modest binding to a site in first intron of *Kit*, but no evidence of PLZF binding to the *Kit* promoter in THY1+ spermatogonia. Moreover, the in vitro-selected putative PLZF binding motif (5′-A-T/G-G/C-T-A/C-A/C-A-G-T-3′) was not among the top ten motifs from PLZF ChIP-seq in undifferentiated spermatogonia and was not significantly represented among PLZF binding sites. Regardless, it is hard to conceive how PLZF directly represses transcription of *Kit* in undifferentiated spermatogonia given that PLZF and *Kit* mRNA are co-expressed, as confirmed recently by single-cell gene expression studies in undifferentiated spermatogonia (Hermann et al. 2015). That is, expression of both PLZF and *Kit* in the vast majority of *Id4*-eGFP+ spermatogonia makes it unlikely that PLZF directly represses *Kit* transcription. Still, *Kit* mRNA and KIT translation are both induced by RA and represent among the first cellular consequences of RA-induced spermatogonial differentiation (Busada et al. 2014, 2015a; Dann et al. 2008).

10.4 Conclusions

Spermatogonial differentiation is essential for spermatogenesis and male fertility, but is also a highly complex process requiring exquistite extrinsic and intrinsic instruction to properly control cell fate. The mechanisms responsible for executing the earliest phase of this differentiation cascade, stem cell commitment to a progenitor phenotype, have been the most elusive. That is, the mechanisms which cause SSC progeny to differentially respond to niche-derived signals and undertake alternate self-renewal or differentiation fate decisions are not known because it has been impossible to precisely identify and selectively recover these cell types from among the heterogeneous pool of undifferentiated spermatogonia in the testis. Consequently, new studies that can separate cells undertaking these alternate fates will help resolve long-standing questions in the field—*how is the balance between SSC renewal and differentiation controlled?* Ultimately, knowledge of such processes will permit insight into the etiology of certain types of male infertility (e.g., non-obstructive azoospermia), as well as revealing new therapeutic avenues to address naturally occurring defects in sperm production, mitigate iatrogenic male infertility (e.g., arising from cancer therapy), or intervene for male contraception.

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