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Preface

Spermatogenesis is a complex and highly efficient process producing millions of terminally differentiated and specialized sperm every day in males of most mammalian species. Indeed, the average man generates roughly 1300 sperm with every heartbeat, and this level of production is required for fertility. At the foundation of spermatogenesis are the actions of an undifferentiated spermatogonial population that consists of spermatogonial stem cell (SSC) and transit amplifying progenitor pools. Self-renewing divisions of SSCs maintain a reservoir from which progenitor spermatogonia arise that transiently amplify in number before transitioning from the undifferentiated type A to differentiating type A1 state under the influence of a retinoic acid pulse. This transition kick-starts a round of spermatogenesis and the differentiating spermatogonia gain competence for meiotic initiation. Not only are these behaviors essential for spermatogenesis during homeostatic conditions but also during regeneration of the spermatogenic lineage following cytotoxic insult or transplantation.

The previous two decades has seen an explosion of new information about the general biology of spermatogonia in mammals and development of methods to isolate, culture, genetically modify, and transplant SSCs. Mammalian spermatogonia have garnered the interest of researchers in the fields of developmental and germ cell biology for decades because the end product of their activities is sperm that are the conduit for transmission of a male's genetic information across generations and SSCs possess the capacity to regenerate the spermatogenic lineage. These attributes hold great potential for exploitation to engineer the genetics of the germline directly which has applications in both improving human health and enhancing the efficiency of animal agriculture.

In this book, we aim to provide a resource of current understandings about various aspects of the biology of spermatogonia in mammals. Considering that covering the entire gamut of all things spermatogonia is a difficult task, specific topics were selected that we believe provide foundational information that will be useful for seasoned researchers in the field of germ cell biology as well as investigators entering the area.

Looking to the future, we predict that the foundational information provided in this book combined with the advent of new tools and budding interests in the use of non-rodent mammalian models will produce another major advance in knowledge regarding the biology of spermatogonia over the next decade. In particular, we

anticipate that the core molecular machinery driving different spermatogonial states in most, if not all, mammals will be described fully, the extrinsic signals emanating from somatic support cell populations to influence spermatogonial functions will become fully known, and the capacity to derive long-term cultures of SSCs and transplant the population to regenerate spermatogenesis and fertility will become a reality for higher order mammals.

We would like to thank Portia Wong and Dana Bigelow and their staff at Springer Nature publishing for the patience and assistance in bringing this book to completion. Also, we would like to express sincere gratitude to all contributors of chapters in this book for sharing their expertise and insights with the research community.

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Part I

Spermatogenesis in Mammals

1 Organization of the Seminiferous Epithelium and the Cycle, and Morphometric Description of Spermatogonial Subtypes (Rodents and Primates)

Dirk G. de Rooij

Abstract

Spermatogenesis encompasses three main cell types: spermatogonia that proliferate, spermatocytes that carry out the process of meiosis, and haploid spermatids that develop into sperm. Differentiating spermatogonia are formed at species-specific intervals, and in each tubule cross-section 4 or 5 generations of spermatogenic cells can be observed. As the timing of the development of the germ cells is always similar, specific associations of germ cell types are seen in tubule cross-sections. Each epithelial area looks similar with regular intervals and goes through the cell associations in a specific order. This is called the cycle of the seminiferous epithelium and the cell associations are called stages (usually 12).

In non-primate mammals, the spermatogonial stem cells (SSCs) are single cells (A_s spermatogonia) that divide 2–3 times per epithelial cycle and render either two new singles (self-renewal) or the daughter cells stay together and form a pair. The niche for these SSCs likely is that part of the tubule basal lamina that borders on interstitial venules and arterioles. The pairs divide further into chains of spermatogonia that differentiate during epithelial stage VIII. The organization of the epithelium heavily depends on a peak in retinoic acid levels during stages VIII-IX that both induces spermatogonia to differentiate and preleptotene spermatocytes to enter meiotic prophase. Surprisingly, germ cells can develop normally while in abnormal epithelial stages, the strict epithelial organization is not required for qualitatively normal spermatogenesis.

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The present knowledge on primate SSCs and spermatogonial multiplication is discussed but does not yet allow clear conclusions.

Keywords
Spermatogonia • Cycle of the seminiferous epithelium • Stages of the seminiferous epithelial cycle • Wave of the seminiferous epithelium • Spermatogonial kinetics • Spermatogonial stem cells • Stem cell niche • Retinoic acid

1.1 Introduction

Spermatogenesis takes place in the seminiferous epithelium that lines the seminiferous tubules in the testis. The seminiferous tubules begin and end in the rete testis. Spermatozoa when released into the lumen of the tubules are transported to the rete testis and subsequently proceed through the efferent ducts to the epididymis. Besides the various types of germ cells, the seminiferous epithelium consists of the somatic Sertoli cells that produce many factors that are needed at various developmental steps during the spermatogenic process (for reviews see Griswold 2015).

During spermatogenesis, the germ cells have to pass through many different differentiation steps to develop into the highly specialized spermatozoa (Russell et al. 1990). The whole process can be subdivided into three main parts. First, there is a phase of cell proliferation which in many species involves about ten subsequent divisions from stem cells up to the formation of the next major cell type, called spermatocytes. Second, the spermatocytes carry out meiosis, a process that encompasses a recombination of hereditary characteristics and a reduction in the number of chromosomes by way of the two consecutive meiotic divisions. Third, through the meiotic divisions, spermatocytes give rise to haploid spermatids that during a long, intricate process transform and develop into spermatozoa that leave the testis. This book will specifically deal with the first of the three main parts of spermatogenesis, the proliferation phase. This part is carried out by a cell type called spermatogonia, among which there are the spermatogonial stem cells (SSCs) that ensure that spermatogenesis will be maintained throughout the lifespan of mammals.

An important particularity of spermatogenesis is that it is very strictly organized, both with respect to the duration of the whole process as well as to the localization of the subsequent cell types within the seminiferous epithelium. This organization determines the length of the period of time available for spermatogonial proliferation and even the timing of some crucial differentiation steps. This chapter describes the organization of the seminiferous epithelium, the various spermatogonial cell types, and how their cell kinetic behavior fits in the organization of the epithelium. The important role of spermatogonial stem cells (SSCs) will be highlighted, as well as their supposed localization in the epithelium, called niche.

1.2 The Organization of the Seminiferous Epithelium

As described above, spermatogenesis encompasses a great many subsequent types of germ cells. Nevertheless, looking at a cross-section of a seminiferous tubule, one does not see a random mixture of these cell types. There are multiple levels of organization that make the appearance of the seminiferous epithelium less confusing (Fig. 1.1).

1.2.1 The Clonal Organization of the Seminiferous Epithelium

As discussed below, the prevalent opinion is that SSCs are single cells. However, when SSCs differentiate, their daughter cells will stay connected by an intercellular

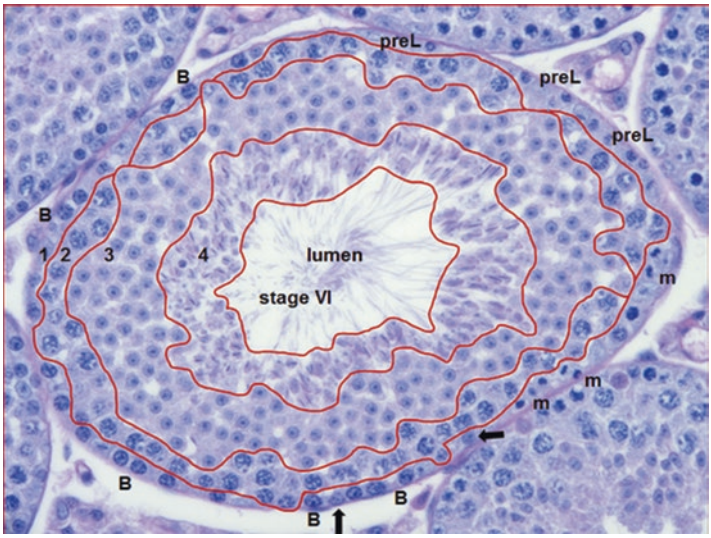

Fig. 1.1 Cross-section through a mouse seminiferous tubule showing the intricate organization of the epithelium. This cross-section is in stage VI of the cycle of the seminiferous epithelium. In the outermost layer (1) there are spermatogonia. In stage VI one finds late differentiating type B spermatogonia (some are indicated with B) which are numerous. In this stage, B spermatogonia go through a division to form preleptotene spermatocytes. Indeed, some of the B spermatogonia can be seen dividing (m) and some newly formed preleptotenes (preL) are already present. Intermingled with the B spermatogonia are $A_{s,pr,al}$ spermatogonia. These cells are few in number (*arrows*) and largely consist of A_{al} spermatogonia that will become differentiating type A1 spermatogonia in stage VIII. One layer up into the tubule (2) one can find pachytene spermatocytes which originate from B spermatogonia present in this area one epithelial cycle earlier (8.6 days). In layer 3 round spermatids can be found that (per definition) are in step 6 of their development. In layer 4 there are elongated spermatids that have already moved close to the lumen into which they will be shed during stage VIII. In total there are five generations of spermatogenic cells each differing 8.6 days of development from each other. Note that within each layer, each generation of germ cells is at the same moment of its development

bridge and form a pair. In subsequent divisions also, intercellular bridges remain between the daughter cells because of which larger and larger clones of interconnected spermatogonia are formed. The cells composing these syncytial clones will behave closely similar as regulating substances can pass freely through the bridges throughout the clone. Therefore one will always see large cohorts of germ cells being at the same step of development and with respect to spermatogonia even in the same phase of the cell cycle (Dym and Fawcett 1971). On top of the presence of clones of synchronized cells there is also synchronization between neighboring clones which makes the cohorts of germ cells at the same step of development even larger (Lok and de Rooij 1983a). So, in a tubule cross-section one does not see a mixture of many cell types in different phases of development but a restricted number of groups of similar looking cells (Fig. 1.1).

1.2.2 Timing of Germ Cell Development

The timing of the developmental steps germ cells go through is rather constant. There are species differences but for each species the total duration of the spermatogenic process, from stem cell to spermatozoa released from the epithelium, is always the same. Also, the duration of each step of development of the germ cells is fixed. In addition to the timing of the developmental steps the germ cells make, in any particular area of the seminiferous epithelium the time interval with which spermatogonia differentiate and go on their way to become spermatozoa is also fixed. For example, in the mouse a cohort of spermatogonia differentiates every 8.6 days. However, the development from newly formed differentiating spermatogonia to spermatozoa released into the tubule lumen takes about 34 days. Consequently, new cohorts of differentiating spermatogonia will be formed before the descendants of the previous cohorts have left the epithelium as spermatozoa. Therefore, in each area of the seminiferous epithelium 4–5 generations of germ cells can be observed and in the mouse each of the successive generations differ by 8.6 days of development. As a result of the fixed duration of all developmental steps and the interval with which differentiating spermatogonia are formed, one will always find the same associations of types of germ cells together Fig. 1.2). In the mouse, each area of the epithelium goes through a similar sequence of combinations of cell types every 8.6 days and will look similar with 8.6 day intervals. This is called the cycle of the seminiferous epithelium that can be observed in all mammals. While in the mouse the cycle takes 8.6 days (Oakberg 1956b), its duration differs for each species (Hess and Renato de Franca 2008). For example, in the rat the epithelial cycle takes 12.8–13.0 days (Hilscher et al. 1969; Huckins 1971a) and 16.0 days in the human (Heller and Clermont 1963).

1.2.3 Stages of the Cycle of the Seminiferous Epithelium

As in spermatogenesis one always sees the same associations of types of germ cells, it follows that when in a particular area of the epithelium one can identify one of the cell types, one can predict the presence of the other types of germ cells in the same

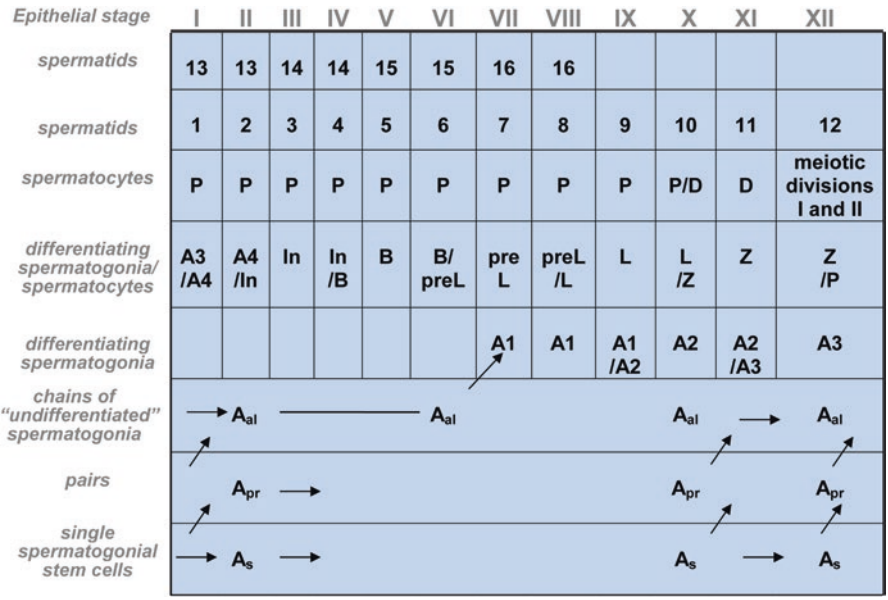

Fig. 1.2 Diagram of the cycle of the seminiferous epithelium in the mouse. The cycle is subdivided into 12 stages that are generally indicated by roman numerals. The stages are defined by the first 12 steps of spermatid development. The cell associations that can be seen in a stage are always similar. Therefore, when in a particular area the epithelial stage is defined looking at spermatid morphology, one will know which germ cell types will be present too. The A_s, A_{pr}, and A_{al} spermatogonia are always present and proliferate at random but are most active in stages X to II. The A_s spermatogonia can both self-renew and produce A_{pr} spermatogonia. Most of the A_{al} spermatogonia differentiate into A1 spermatogonia without division in stage VIII. Abbreviations: *A_s* A_{single} spermatogonia (spermatogonial stem cells), *A_{pr}* A_{paired}, *A_{al}* $A_{aligned}$, *preL* preleptotene spermatocytes, *L* leptotene spermatocytes, *Z* zygotene spermatocytes, *P* pachytene spermatocytes, *D* diplotene spermatocytes, *1 to 16* steps in the development of the spermatids

tubule area. Therefore, for many species the cell associations have been described and they have been called the stages of the cycle of the seminiferous epithelium. The most popular way to subdivide the epithelial cycle into stages is by using the clearly identifiable steps in the development of the acrosome in spermatids which can be easily made visible by staining using the periodic acid—Schiff reaction (Leblond and Clermont 1952b) (Fig. 1.2). This was first done for the rat (Clermont and Perey 1957; Leblond and Clermont 1952a) in which the cycle was subdivided in 14 stages, using the first 14 steps of the spermatid development to mark the various stages. For the mouse, Oakberg described 12 stages (Oakberg 1956a) and this number of stages has remained the most popular for other species for which seminiferous epithelial stages have been described. Unfortunately, the periodic acid Schiff (PAS) reaction does not work well on human spermatids and therefore, until recently the human epithelial cycle could only be subdivided into 6, very long, stages (Clermont 1963). This made it very difficult to compare findings on human spermatogenesis with those on experimental animals. Fortunately, the cycle of the human seminiferous epithelium has now also been subdivided in 12 stages, using

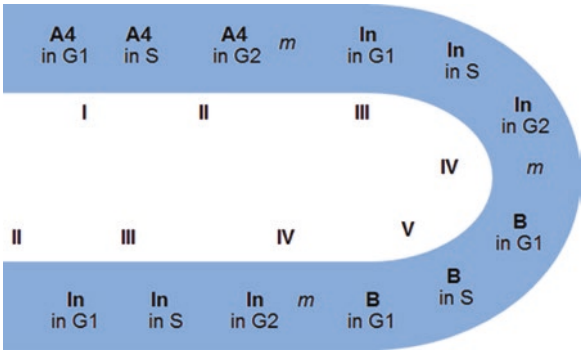

Fig. 1.3 Seminiferous tubule showing part of the wave of the seminiferous epithelium. The stages of the cycle of the seminiferous epithelium follow each other in order along the length of a seminiferous tubule but the order of stages can also reverse. In this tubule the wave goes from stage I to stage V and then the order reverses. Because of the wave one can also see the synchronous development of the differentiating type spermatogonia in order. In stage I, one will find A4 spermatogonia that divide into In spermatogonia in stage II which in turn will divide into B spermatogonia in stage IV. It is even known in which epithelial stage a particular type of differentiating spermatogonia goes through the various phases of the cell cycle, for example B spermatogonia are in S phase during stage V. The A_s, A_{pr}, and A_{al} spermatogonia cycle at random during the epithelial cycle but are most active during stages X to II (Lok and de Rooij 1983b)

immunohistochemical staining for acrosin in the acrosomes, which gives a rather comparable result as PAS staining in the mouse and other mammals (Muciaccia et al. 2013).

1.2.4 Wave of the Seminiferous Epithelium

In most mammals, but not in human and some other primates, there is a phenomenon called the wave of the seminiferous epithelium (Perey et al. 1961; Johnson 1994). Studying the spermatogenic process in whole mounts of seminiferous tubules one can see that each epithelial stage occupies a certain length of tubule and that the stages follow each other in order along the length of the tubules (Fig. 1.3). Although the order of the stages sometimes reverses, this is of great help when studying spermatogenesis in whole mounts. A further great advantage of studying whole mounts is that one can always observe the whole of the nuclei of the spermatogonia instead of parts of them, as in sections, enabling a much better distinction between the various spermatogonial cell types (Clermont and Bustos-Obregon 1968; Huckins 1971c; de Rooij 1973). In addition, one can study the topographical arrangement of the spermatogonia. The latter has been proven essential for a proper understanding of the spermatogonial compartment. Unfortunately, studying tubule whole mounts of the human does hardly provide these advantages as in the human, tubule areas in the same epithelial stage are very small. So small that even a cross-section of a human seminiferous tubule shows multiple epithelial stages (Clermont 1963). The same situation has been found in several other primate species (Wistuba et al. 2003).

1.3 Spermatogonial Cell Types

1.3.1 Non-primate Mammals

In the seminiferous epithelium, SSCs are at the start of the spermatogenic process and produce both new SSCs and spermatogonia that enter the differentiation pathway. Most of our knowledge on SSCs and spermatogonia comes from studies on rodents. In the prevailing scheme of spermatogonial multiplication and stem cell renewal, the SSCs are single spermatogonia (Huckins 1971c; Oakberg 1971; de Rooij 1973). These single spermatogonia are called A_s spermatogonia. Upon division, the A_s spermatogonia divide and either produce two new single cells that migrate away from each other or the daughter cells can stay together, connected by an intercellular bridge, forming a pair of cells called A_{pr} spermatogonia. At subsequent divisions the daughter cells will always remain connected by intercellular bridges because of which clones of increasing size will be formed at each division. Subsequently, chains of 4, 8 and 16 cells are formed that are called A_{al} spermatogonia. The scheme of spermatogonial multiplication and stem cell renewal is still debated (de Rooij and Griswold 2012). The opinions varying from stem cell renewal taking place by way of fragmentation of chains of A_{al} spermatogonia (Nakagawa et al. 2007, 2010) to only few of the A_s spermatogonia having stem cell properties (Chan et al. 2014; Oatley et al. 2011). The virtues of and problems with these alternative schemes are discussed in another chapter of this book.

The chains of A_{al} spermatogonia can differentiate, without an intervening division, into so-called A1 spermatogonia, the first generation of a cell type called differentiating spermatogonia. The differentiating spermatogonia comprise 6 generations of cells in mouse and rat which are called: A1, A2, A3, A4, In (Intermediate) and B spermatogonia. The B spermatogonia divide into spermatocytes called preleptotene spermatocytes at first and subsequently, when they enter meiotic prophase, leptotene spermatocytes. In all, in rodents there are about ten divisions in between the SSCs and the formation of spermatocytes (de Rooij and Russell 2000).

1.3.2 Primates

While one would not expect big differences between primate and non-primate mammals in a crucially important process as spermatogenesis, this seems nevertheless to be the case. In primates, a type of spermatogonia is distinguished that is totally missing in non-primates. These are the so-called A_{dark} spermatogonia (A_d). A_d spermatogonia are characterized by nuclei that stain darkly with hematoxylin. In addition, A_d spermatogonia show a rarefaction area in their nuclei. In human this rarefaction zone consists of a vacuole like space in the middle of the nuclei and in monkeys the rarefaction zone is at the circumference of the nuclei in between the nuclear membrane and the chromatin. Besides A_d, in primates A_{pale} (A_p) spermatogonia can be distinguished that stain more lightly with hematoxylin and do not show

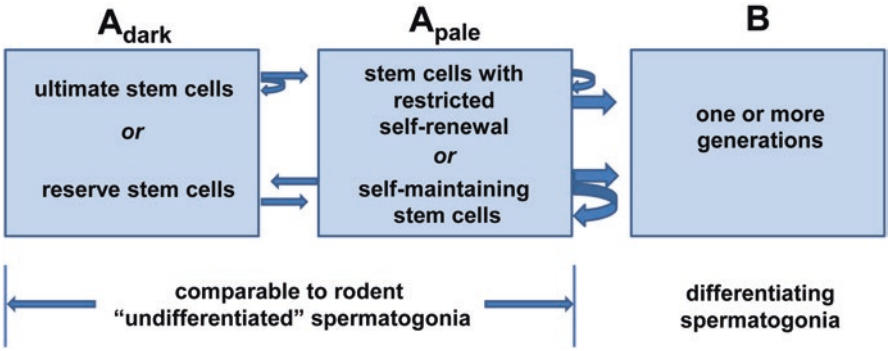

Fig. 1.4 Spermatogonial multiplication and stem cell renewal in primates. It is not yet clear how these events proceed. In primate spermatogenesis there are A_{dark} spermatogonia that possibly are the ultimate stem cells that very slowly proliferate and replenish A_{pale} spermatogonia that may not be able to fully maintain themselves. Alternatively, the A_{dark} spermatogonia are set aside by A_{pale} spermatogonia and function as reserve stem cells and are normally quiescent but become active A_{pale} spermatogonia again when locally the A_{pale} population becomes depleted, as after irradiation

a rarefaction zone in the nuclei. Unfortunately, the distinction between A_p and A_d spermatogonia is not straightforward. Spermatogonia with a different or intermediate morphology between A_p and A_d, have also been described (Rowley et al. 1971; Fouquet and Dadoune 1986; Simorangkir et al. 2005). Besides A_p and A_d spermatogonia, in monkeys and human, varying numbers of generations of B spermatogonia have been described.

In general, A_p and A_d spermatogonia together are supposed to be comparable to the category of A_s, A_{pr} and A_{al} ($A_{s,pr,al}$) spermatogonia in rodents, (review Hermann et al. 2010) (Fig. 1.4). A peculiar aspect of primate spermatogenesis is that the density of the spermatogonia is very high. This precludes a study of clonal sizes of A_p and A_d spermatogonia as the clones are often too close to each other to allow one to distinguish to which clone a particular cell belongs. Therefore, devising a scheme of spermatogonial multiplication and stem cell renewal for primate spermatogenesis has proven to be difficult. The A_d spermatogonia do not incorporate ^{3}H-thymidine and BrdU and also not become labeled one epithelial cycle after administration of either of the two S phase markers (Clermont 1969; Clermont and Antar 1973; Kluin et al. 1983; Fouquet and Dadoune 1986; Schlatt and Weinbauer 1994; Ehmcke et al. 2005a, b; Simorangkir et al. 2009). However, A_d spermatogonia can start proliferation again. During the first epithelial cycle after irradiation, the A_p spermatogonia become severely reduced in numbers because of the irradiation damage, killing these cells when they try to divide. Then the A_d spermatogonia become activated and become A_p spermatogonia (van Alphen et al. 1988). Therefore, the A_d spermatogonia are considered to be reserve stem cells that only become active when the A_p spermatogonia in a particular area fail. Alternatively, the A_d spermatogonia may be the real stem cells with a very long cell cycle, slowly giving rise to A_p with a restricted self-renewal capacity in order to maintain the numbers of these cells (Clermont 1966; Hermann et al. 2010; Clermont and Leblond 1959). The presence

of long-cycling SSCs has been suggested to occur in the rat (Huckins 1971b), but these findings could not be confirmed in the Chinese hamster (Lok et al. 1984). Clearly, more work will have to be carried out to understand how spermatogonial multiplication and stem cell renewal in primates is carried out. Too little is known yet about the nature and the behavior of both A_p and A_d spermatogonia.

1.4 Cell Kinetics

1.4.1 Non-primates

The A_s, A_{pr}, and A_{al} spermatogonia ($A_{s,pr,al}$) are often called undifferentiated spermatogonia but as discussed previously (de Rooij and Russell 2000), this is not a good term as most of these cells (A_{pr} and A_{al}) are on the differentiation pathway. The clones of $A_{s,pr,al}$ spermatogonia divide randomly throughout the epithelial stages. However, the overall proliferative activity of $A_{s,pr,al}$ spermatogonia varies with the stages of the epithelial cycle and these spermatogonia are most active in epithelial stages X to II. From stage II onwards, the proliferative activity decreases, especially that of A_{pr} and A_{al} spermatogonia (Lok and de Rooij 1983a, b). In the mouse and Chinese hamster, A_s, A_{pr} and A_{al} spermatogonia go on average through 2–3 divisions each epithelial cycle (Lok et al. 1983; Lok and de Rooij 1983b; Tegelenbosch and de Rooij 1993).

In stages VII and VIII, the A_{al} spermatogonia that were quiescent since stage II, differentiate into A1 spermatogonia, the first of the series of 6 generations of differentiating type spermatogonia. The differentiating spermatogonia have a different behavior compared to the $A_{s,pr,al}$ spermatogonia. Their cell cycle is shorter and importantly, in contrast to the $A_{s,pr,al}$ spermatogonia, the clones of these cells behave in a synchronized manner. Neighboring clones enter the cell cycle at about the same time (Huckins 1971a; Lok and de Rooij 1983a). The synchronization of neighboring clones is such that all clones of a generation of differentiating spermatogonia present in a particular area will traverse the cell cycle in unison. For example, in whole mounts of seminiferous tubules extended areas can be seen in which all differentiating spermatogonia are in S phase. Therefore, it is highly unlikely that clones of differentiating spermatogonia can skip a division.

1.4.2 Primates

Comparing spermatogonial multiplication in primates and non-primate mammals, one has to take into account the completely different ratio between the numbers of undifferentiated ($A_{s,pr,al}$ in non-primates and $A_p + A_d$ in primates) and differentiating type spermatogonia. In the mouse, there are 7.6 times more differentiating type spermatogonia per Sertoli cell than $A_{s,pr,al}$ spermatogonia (Tegelenbosch and de Rooij 1993). In contrast, in *Macaca fascicularis* there are 0.9 times less differentiating spermatogonia than $A_p + A_d$ spermatogonia (Zhengwei et al. 1997) and for the

human it can be calculated from data from Rowley (1971) that there are even 0.3 times less differentiating spermatogonia than $A_p + A_d$ spermatogonia. Nevertheless, the numbers of differentiating spermatogonia per Sertoli cell, produced in mice and primates do not differ all that much. In the mouse there are 0.83 differentiating spermatogonia per Sertoli cell, in *Macaca fascicularis* it is 0.43 and in the human 0.31.

What it comes down to is that in rodents the relatively few $A_{s,pr,al}$ spermatogonia go through many divisions to produce the required numbers of differentiating spermatogonia while in primates there are a great many A_p and A_d spermatogonia, the A_p of which divide once or twice every epithelial cycle, or even less, to produce an only somewhat smaller amount of differentiating spermatogonia.

1.5 Regulation of the Epithelial Cycle

As described above, the proliferative activity of the $A_{s,pr,al}$ spermatogonia strongly depends on the stages of the epithelial cycle. In the mouse, every 8.6 days a new cohort of A_{al} spermatogonia is induced to differentiate into A1 spermatogonia. This event takes place in stages VII and VIII of the epithelial cycle (Schrans-Stassen et al. 1999) and these newly formed A1 spermatogonia will divide into A2 spermatogonia in stage IX. The subsequent divisions will also take place in specific stages and ultimately B spermatogonia are formed. In stage VI, these B spermatogonia will divide to become preleptotene spermatocytes that will enter meiotic prophase at the end of stage VIII. How can all spermatogenic events be timed so accurately (de Rooij and Russell 2000)?

In recent years, it has become clear that retinoic acid plays an important role in the regulation of epithelial cycle events. It was already known that in case of vitamin A deficiency (VAD), in the mouse and rat, spermatogenesis becomes arrested (Mitranond et al. 1979; Unni et al. 1983). This arrest has been localized to the differentiation step of A_{al} into A1 spermatogonia and in the VAD rat preleptotene spermatocytes are unable to enter meiotic prophase (van Pelt and de Rooij 1990a, b; Ismail et al. 1990). It has been established that retinoic acid (RA) is necessary for preleptotene spermatocytes to enter meiotic prophase (Baltus et al. 2006; Anderson et al. 2008). Finally, RA has also been implicated in the release of spermatozoa during spermiation (Vernet et al. 2006, 2008). So, RA is required at three essential steps in spermatogenesis, the differentiation of A_{al} into A1 spermatogonia, the entrance of preleptotene spermatocytes into meiotic prophase and spermiation. All three steps take place in stage VIII of the epithelial cycle. Importantly, it has recently been established that RA levels in the seminiferous epithelium peak at stages VIII and IX (Hogarth et al. 2015). Apparently, it is this peak in RA levels that induces spermatogonial differentiation, entry of preleptotene spermatocytes into meiotic prophase and spermiation.

A recent study into the effects of RA administration on the spermatogenic process has revealed some aspects of how the strict organization of the seminiferous

epithelium is brought about (Endo et al. 2015). Injection of RA has a very drastic effect on the quiescent A_{al} spermatogonia present in stages II to VI at the time of administration. Normally these cells will differentiate into A1 spermatogonia during stage VIII but after injection of RA they do so within 24 h despite the fact that the surrounding epithelium is in stages II to VI. Apparently, the A_{al} in stages II to VI are already competent to differentiate into A1 spermatogonia but normally have to wait for the higher RA levels in stage VIII to do so. Furthermore, the administration of RA also induces preleptotenes to enter S phase, and subsequently meiotic prophase, at an earlier time than they normally do (Endo et al. 2015). Apparently, preleptotenes too are competent to take the next step in their development at an earlier time point than stage VIII. It can be concluded that the strict organization of the epithelium with its ever similar cell associations (stages) is at least for a large part due to the fact that both A_{al} spermatogonia and preleptotene spermatocytes are already competent to take their next developmental steps before stage VIII and the RA peak then causes these cells to simultaneously proceed their development. It is not known whether RA has a synchronizing effect on the development of pachytene spermatocytes and the onset of the elongation of step 8 spermatids in stage VIII. As mentioned earlier spermiation is dependent on RA levels (Vernet et al. 2006, 2008) but it is not known whether spermatids are already competent to be spermiated before stage VIII and are also waiting for the RA peak to occur.

The very strict organization of the seminiferous epithelium has always been interpreted as a requirement for a proper regulation of the spermatogenic process. For example, Sertoli cells will then be able to timely supply the various types of germ cells with necessary factors to take their next developmental step. The study by Endo et al. has thoroughly undermined such ideas (Endo et al. 2015). As mentioned above, RA administration drives A_{al} spermatogonia in stages II to VI to a prescheduled differentiation into A1 spermatogonia. Surprisingly, these A1 spermatogonia subsequently develop into A2, A3, A4, In and B spermatogonia at a normal pace and preleptotene spermatocytes can be seen in stages II to VI one epithelial cycle later. These preleptotenes then need RA to enter meiotic prophase and subsequently proceed through meiotic prophase normally. These observations strongly suggest that germ cells do not need a stage-specific guidance for their development, for example through the secretion of stage-specific factors by Sertoli cells. In support of that notion, rat germ cells transplanted into mouse testes develop at the same rate as in the rat testis despite the fact that they are surrounded by mouse somatic cells (Franca et al. 1998). Apparently, for their development the rat germ cells do not depend on guidance from the environment. In conclusion, although the seminiferous epithelium is highly organized, this organization does not seem required for a proper development of the germ cells. It may just be a consequence of the dependency of several crucial developmental steps on RA and the fact that there is only one peak in RA levels every epithelial cycle. Germ cells can develop perfectly well in inappropriate stages though it cannot be excluded that this may occur less efficiently.

1.6 The Spermatogonial Stem Cell Niche

In all tissues in which the functioning cells have a finite lifespan or, as in the testis, will leave the tissue, new cells will have to be constantly produced to replenish the lost cells. In such tissues, there will be stem cells to make sure that the tissue is maintained. Stem cells are able to both renew themselves and to produce cells that will become the functional cells of the tissue. In this way, the tissue will be maintained throughout life. It has been generally found that stem cells are not distributed at random in a tissue. Instead, for example in the intestine, hemopoiesis and liver they occupy specific parts in which surrounding cells provide an environment that stimulates stem cell self-renewal, (Schofield 1978; Stange and Clevers 2013; Snippert et al. 2010; Ugarte and Forsberg 2013; Smith and Calvi 2013; Kordes and Haussinger 2013). Such an area is called a stem cell niche. Outside of the stem cell niche the environment will induce differentiation of those daughter cells of the stem cells that venture out of the niche. In this way the tissue secures its necessary complement of stem cells and also takes care that those daughter cells of stem cells that spill out of the niche will differentiate. In the niche, stem cells will not get lost because differentiation of these cells is suppressed and on the other hand they will not be able to propagate in numbers and form a tumor outside of the niche because they will be forced to differentiate.

In the testis, all spermatogonia including the SSCs are on the basal lamina of the seminiferous tubules together with the somatic Sertoli cells. Sertoli cells play an important role in the functioning of the stem cell niche (de Rooij 2009, 2015). Importantly, it cannot be that all Sertoli cells serve as a SSC niche environment as in that case no differentiation would take place. For any tissue it is of equal importance that half of the cells produced by the stem cells differentiate because otherwise the tissue would keep growing or a tumor would even be formed.

For the testis, it has been shown that the clones of $A_{s,pr,al}$ spermatogonia are not distributed at random over the tubule basal lamina. Several reports show that those areas of the seminiferous tubules that border on the interstitial tissue and in particular on venules and arterioles in the interstitial tissue, contain most $A_{s,pr,al}$ spermatogonia (Chiarini-Garcia et al. 2001, 2003; Yoshida et al. 2007b; Shetty and Meistrich 2007; de Rooij 2009). The numbers of clones of $A_{s,pr,al}$ spermatogonia are highest in these areas suggesting that they originate in these areas and that the SSCs should reside there too. Unfortunately, it is very difficult to study the niche area directly. When preparing whole-mounts of seminiferous tubules the blood vessels are removed because it is difficult to see the spermatogonia under the blood vessels. Furthermore, in the live imaging system of the Yoshida group the niche area is in a plane perpendicular to the plane of observation making it impossible to follow spermatogonial behavior under the interstitial blood vessels. Therefore, a computer model of SSC behavior has been established in which the SSC niche is supposed to be directly opposite to the arterioles and venules in the interstitial tissue (de Rooij 2015; de Rooij and van Beek 2013). This model is based on parameters consistent with the many studies on spermatogonial and SSC proliferation. Within the niche the chance of self-renewal of the SSCs is very high, while out the niche the

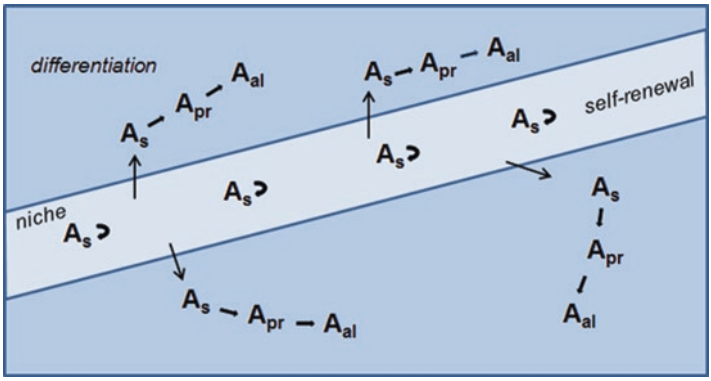

Fig. 1.5 The spermatogonial stem cell (SSC) niche is located in those areas of the tubule basal lamina that borders on arterioles and venules in the interstitial tissue (Yoshida et al. 2007a). Here many A_s spermatogonia are localized. In the niche there is primarily self-renewal and daughter cells will migrate away from each other. Those daughter cells that migrate out of the niche will at their next division form a pair of A_{pr} spermatogonia that eventually will become spermatozoa

chance of differentiation of SSCs is high. The SSCs are supposed to be single cells and when they go through a self-renewing division the daughter cells migrate away from each other. During this migration both daughter cells may stay in the niche and will not differentiate. However, in other cases one or both daughter cells may end up outside of the niche and at their next division differentiate and become A_{pr} spermatogonia (Fig. 1.5). Indeed, in the live-imaging system of the Yoshida group which enables one to follow the behavior of clones of $A_{s,pr,al}$ spermatogonia outside of the niche, virtually all A_s spermatogonia are seen to form A_{pr} spermatogonia after division (Hara et al. 2014). The computer model renders a stable situation in which the virtual spermatogenesis continues for more than one and a half year of a mouse life without the formation of a virtual tumor or SSC depletion (de Rooij and van Beek 2013).

Intriguingly, the situation may be more complicated than described above. In cell counts in whole mounts of mouse seminiferous tubules it was established that in the 3H1 mouse 10.6% of the undifferentiated spermatogonia are A_s (Tegelenbosch and de Rooij 1993). However, the only cells in the seminiferous epithelium capable to repopulate a recipient mouse testis after transplantation express *Id4* (*inhibitor of differentiation 4*) (Chan et al. 2014). These Id4+ spermatogonia are single cells and they comprise 1.9% of the Plzf+ spermatogonia, representing the total of undifferentiated spermatogonia. This means that there are many more A_s spermatogonia than there are SSCs capable of colonizing a recipient mouse testis. Moreover, the Id4+ A_s spermatogonia are not localized to the blood vessels in the interstitial tissue. Chan et al. (2014) suggest that the colonizing SSCs are a kind of stem cells that should be placed before the other A_s spermatogonia in the scheme of spermatogonial multiplication and stem cell renewal in the mouse. A hierarchy of stem cells is suggested in which the population of A_s spermatogonia does not fully maintain itself and needs A_s spermatogonia produced by ID4+ cells. Also, the Id4+ A_s

spermatogonia probably have another type of niche in a different location as they are not localized close to the interstitial blood vessels. Clearly, further work will be needed to get an understanding of the role of the Id4+ A_s spermatogonia in the normal seminiferous epithelium and of how the rodent spermatogonial stem cell compartment operates during steady state kinetics.

In conclusion, like in all other tissues, SSCs will not be spread over the seminiferous epithelium at random. There must be specific areas in which their chance of self-renewal is strongly enhanced while outside of the niche the differentiation of SSCs is strongly promoted. In this way, the sustained presence of SSCs in their niche is guaranteed while on the other hand any surplus SSCs that spill out of the niche will differentiate and eventually leave the epithelium as sperm. As to the localization of the niche, present data indicate that for most of the A_s spermatogonia it is located in areas opposing arterioles and venules in the interstitial tissue. In addition, there are relatively rare Id4+ A_s spermatogonia that are capable of colonizing a recipient mouse testis and likely have another type of niche and produce a kind of transient A_s spermatogonia to restock possible deficits in A_s numbers. As discussed elsewhere, Sertoli cells have a very important role in SSC maintenance and differentiation, producing mainly self-renewal factors within the niche and differentiation promoting factors outside of it (review de Rooij 2015). Recently, an important role in the regulation of SSC maintenance and differentiation has been detected with respect to peritubular macrophages (DeFalco et al. 2015). Further studies are needed to understand how macrophages and Sertoli cells orchestrate SSC behavior in- and outside of the niche. Unfortunately, in view of the lack of data on the nature of primate SSCs and also the very high density of A_p and A_d spermatogonia, the nature of the primate SSC niche remains unknown.

References

Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van Pelt AM, Page DC (2008) Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc Natl Acad Sci U S A 105(39):14976–14980. https://doi.org/10.1073/pnas.0807297105

Baltus AE, Menke DB, YC H, Goodheart ML, Carpenter AE, de Rooij DG, Page DC (2006) In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. Nat Genet 38(12):1430–1434. https://doi.org/10.1038/ng1919

Chan F, Oatley MJ, Kaucher AV, Yang QE, Bieberich CJ, Shashikant CS, Oatley JM (2014) Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev 28(12):1351–1362. https://doi.org/10.1101/gad.240465.114

Chiarini-Garcia H, Hornick JR, Griswold MD, Russell LD (2001) Distribution of type A spermatogonia in the mouse is not random. Biol Reprod 65(4):1179–1185. https://doi.org/10.1095/biolreprod65.4.1179

Chiarini-Garcia H, Raymer AM, Russell LD (2003) Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. Reproduction 126(5):669–680. https://doi.org/10.1530/rep.0.1260669

Clermont Y (1963) The cycle of the seminiferous epithelium in man. Am J Anat 112:35–51. https://doi.org/10.1002/aja.1001120103

Clermont Y (1966) Renewal of spermatogonia in man. Am J Anat 118(2):509–524. https://doi.org/10.1002/aja.1001180211

Clermont Y (1969) Two classes of spermatogonial stem cells in the monkey (Cercopithecus aethiops). Am J Anat 126(1):57–71. https://doi.org/10.1002/aja.1001260106

Clermont Y, Antar M (1973) Duration of the cycle of the seminiferous epithelium and the spermatogonial renewal in the monkey Macaca arctoides. Am J Anat 136(2):153–165. https://doi.org/10.1002/aja.1001360204

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(2):237–247. https://doi.org/10.1002/aja.1001220205

Clermont Y, Leblond CP (1959) Differentiation and renewal of spermatogonia in the monkey, Macacus-Rhesus. Am J Anat 104(2):237–273

Clermont Y, Perey B (1957) The stages of the cycle of the seminiferous epithelium of the rat: practical definitions in PA-Schiff-hematoxylin and hematoxylin-eosin stained sections. Rev Can Biol 16(4):451–462

de Rooij DG (1973) Spermatogonial stem cell renewal in the mouse. I. Normal situation. Cell Tissue Kinet 6(3):281–287. https://doi.org/10.1111/j.1365-2184.1973.tb01617

de Rooij DG (2009) The spermatogonial stem cell niche. Microsc Res Tech 72(8):580–585. https://doi.org/10.1002/jemt.20699

de Rooij DG (2015) The spermatogonial stem cell niche in mammals. In: Griswold MD (ed) Sertoli cell biology, 2nd edn. Elsevier, Amsterdam, pp 99–121. https://doi.org/10.1016/B978-0-12-417047-6.0000-1-1

de Rooij DG, Griswold MD (2012) Questions about spermatogonia posed and answered since 2000. J Androl 33:1085–1095. https://doi.org/10.2164/jandrol.112.016832

de Rooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21(6):776–798. https://doi.org/10.1002/j.1939-4640.2000.tb03408.x

de Rooij DG, van Beek ME (2013) Computer simulation of the rodent spermatogonial stem cell niche. Biol Reprod 88(5):131. https://doi.org/10.1095/biolreprod.113.108639

DeFalco T, Potter SJ, Williams AV, Waller B, Kan MJ, Capel B (2015) Macrophages contribute to the spermatogonial niche in the adult testis. Cell Rep 12(7):1107–1119. https://doi.org/10.1016/j.celrep.2015.07.015

Dym M, Fawcett DW (1971) Further observations on numbers of spermatogonia, spermatocytes, and spermatids connected by intercellular bridges in mammalian testis. Biol Reprod 4(2):195–215

Ehmcke J, Luetjens CM, Schlatt S (2005a) Clonal organization of proliferating spermatogonial stem cells in adult males of two species of non-human primates, Macaca mulatta and Callithrix jacchus. Biol Reprod 72(2):293–300. https://doi.org/10.1095/biolreprod.104.033092

Ehmcke J, Simorangkir DR, Schlatt S (2005b) Identification of the starting point for spermatogenesis and characterization of the testicular stem cell in adult male rhesus monkeys. Hum Reprod 20(5):1185–1193. https://doi.org/10.1093/humrep/deh766

Endo T, Romer KA, Anderson EL, Baltus AE, de Rooij DG, Page DC (2015) Periodic retinoic acid-STRA8 signaling intersects with periodic germ-cell competencies to regulate spermatogenesis. Proc Natl Acad Sci U S A 112(18):E2347–E2356. https://doi.org/10.1073/pnas.1505683112

Fouquet JP, Dadoune JP (1986) Renewal of Spermatogonia in the Monkey (Macaca-Fascilularis). Biol Reprod 35(1):199–207. https://doi.org/10.1095/biolreprod35.1.199

Franca LR, Ogawa T, Avarbock MR, Brinster RL, Russell LD (1998) Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol Reprod 59(6):1371–1377. https://doi.org/10.1095/biolreprod59.6.1371

Griswold MD (2015) Sertoli cell biology. Elsevier, Amsterdam

Hara K, Nakagawa T, Enomoto H, Suzuki M, Yamamoto M, Simons BD, Yoshida S (2014) Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. Cell Stem Cell 14(5):658–672. https://doi.org/10.1016/j.stem.2014.01.019

Heller CG, Clermont Y (1963) Spermatogenesis in man: an estimate of its duration. Science 140(3563):184–186. https://doi.org/10.1126/science.140.3563.184

Hermann BP, Sukhwani M, Hansel MC, Orwig KE (2010) Spermatogonial stem cells in higher primates: are there differences from those in rodents? Reproduction 139(3):479–493. https://doi.org/10.1530/Rep-09-0255

Hess RA, Renato de Franca L (2008) Spermatogenesis and cycle of the seminiferous epithelium. Adv Exp Med Biol 636:1–15. https://doi.org/10.1007/978-0-387-09597-4_1

Hilscher B, Hilscher W, Maurer W (1969) Autoradiographic studies on the modus of proliferation and regeneration of the seminiferous epithelium of Wistar rats. Z Zellforsch Mikrosk Anat 94(4):593–604

Hogarth CA, Arnold S, Kent T, Mitchell D, Isoherranen N, Griswold MD (2015) Processive pulses of retinoic acid propel asynchronous and continuous murine sperm production. Biol Reprod 92(2):37. https://doi.org/10.1095/biolreprod.114.126326

Huckins C (1971a) Cell cycle properties of differentiating spermatogonia in adult Sprague-Dawley rats. Cell Tissue Kinet 4(2):139–154. https://doi.org/10.1111/j.1365-2184.1971.tb01524.x

Huckins C (1971b) The spermatogonial stem cell population in adult rats. 3. Evidence for a long-cycling population. Cell Tissue Kinet 4(4):335–349

Huckins C (1971c) The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec 169(3):533–557. https://doi.org/10.1002/ar.1091690306

Ismail N, Morales C, Clermont Y (1990) Role of spermatogonia in the stage-synchronization of the seminiferous epithelium in vitamin-A-deficient rats. Am J Anat 188(1):57–63. https://doi.org/10.1002/aja.1001880107

Johnson L (1994) A new approach to study the architectural arrangement of spermatogenic stages revealed little evidence of a partial-wave along the length of human seminiferous tubules. J Androl 15(5):435–441. https://doi.org/10.1002/j.1939-4640.1994.tb00477.x

Kluin PM, Kramer MF, de Rooij DG (1983) Testicular development in Macaca irus after birth. Int J Androl 6(1):25–43. https://doi.org/10.1111/j.1365-2605.1983.tb00321.x

Kordes C, Haussinger D (2013) Hepatic stem cell niches. J Clin Invest 123(5):1874–1880. https://doi.org/10.1172/JCI66027

Leblond CP, Clermont Y (1952a) Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N Y Acad Sci 55(4):548–573

Leblond CP, Clermont Y (1952b) Spermiogenesis of rat, mouse, hamster and guinea pig as revealed by the periodic acid-fuchsin sulfurous acid technique. Am J Anat 90(2):167–215. https://doi.org/10.1002/aja.1000900202

Lok D, de Rooij DG (1983a) Spermatogonial multiplication in the Chinese hamster. I. Cell cycle properties and synchronization of differentiating spermatogonia. Cell Tissue Kinet 16(1):7–18

Lok D, de Rooij DG (1983b) Spermatogonial multiplication in the Chinese hamster. III. Labelling indices of undifferentiated spermatogonia throughout the cycle of the seminiferous epithelium. Cell Tissue Kinet 16(1):31–40

Lok D, Jansen MT, de Rooij DG (1983) Spermatogonial multiplication in the Chinese hamster. II. Cell cycle properties of undifferentiated spermatogonia. Cell Tissue Kinet 16(1):19–29

Lok D, Jansen MT, de Rooij DG (1984) Spermatogonial multiplication in the Chinese hamster. IV. Search for long cycling stem cells. Cell Tissue Kinet 17(2):135–143

Mitranond V, Sobhon P, Tosukhowong P, Chindaduangrat W (1979) Cytological changes in the testes of vitamin-A-deficient rats. I. Quantitation of germinal cells in the seminiferous tubules. Acta Anat (Basel) 103(2):159–168

Muciaccia B, Boitani C, Berloco BP, Nudo F, Spadetta G, Stefanini M, de Rooij DG, Vicini E (2013) Novel stage classification of human spermatogenesis based on acrosome development. Biol Reprod 89(3):60, 61-10. https://doi.org/10.1095/biolreprod.113.111682

Nakagawa T, Nabeshima YI, Yoshida S (2007) Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. Dev Cell 12(2):195–206. https://doi.org/10.1016/j.devcel.2007.01.002

Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S (2010) Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 328(5974):62–67. https://doi.org/10.1126/science.1182868

Oakberg EF (1956a) A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am J Anat 99(3):391–413. https://doi.org/10.1002/aja.1000990303

Oakberg EF (1956b) Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. Am J Anat 99(3):507–516. https://doi.org/10.1002/aja.1000990307

Oakberg EF (1971) Spermatogonial stem-cell renewal in the mouse. Anat Rec 169(3):515–531. https://doi.org/10.1002/ar.1091690305

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(2):347–356. https://doi.org/10.1095/biolreprod.111.091330

Perey B, Clermont Y, Leblond CP (1961) The wave of the seminiferous epithelium in the rat. Am J Anat 108:47–77. https://doi.org/10.1002/aja.1001080105

Rowley MJ (1971) Quantitation of cells of seminiferous epithelium of human testis employing Sertoli cell as a constant. Z Zellforsch Mikrosk Anat 115(4):461–472. https://doi.org/10.1007/BF00335713

Rowley MJ, Berlin JD, Heller CG (1971) Ultrastructure of 4 types of human Spermatogonia. Z Zellforsch Mikrosk Anat 112(2):139–157. https://doi.org/10.1007/BF00331837

Russell LD, Ettlin RA, Hikim APS, Clegg ED (1990) Histological and histopathological evaluation of the testis. Cache River Press, Clearwater, FL

Schlatt S, Weinbauer GF (1994) Immunohistochemical localization of proliferating cell nuclear antigen as a tool to study cell proliferation in rodent and primate testes. Int J Androl 17(4):214–222. https://doi.org/10.1111/j.1365-2605.1994.tb01245.x

Schofield R (1978) The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 4(1–2):7–25

Schrans-Stassen BH, van de Kant HJ, de Rooij DG, van Pelt AM (1999) Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology 140(12):5894–5900. https://doi.org/10.1210/en.140.12.5894

Shetty G, Meistrich ML (2007) The missing niche for spermatogonial stem cells: do blood vessels point the way? Cell Stem Cell 1(4):361–363. https://doi.org/10.1016/j.stem.2007.09.013

Simorangkir DR, Marshall GR, Ehmcke J, Schlatt S, Plant TM (2005) Prepubertal expansion of dark and pale type A spermatogonia in the rhesus monkey (Macaca mulatta) results from proliferation during infantile and juvenile development in a relatively gonadotropin independent manner. Biol Reprod 73(6):1109–1115. https://doi.org/10.1095/biolreprod.105.044404

Simorangkir DR, Ramaswamy S, Marshall GR, Pohl CR, Plant TM (2009) A selective monotropic elevation of FSH, but not that of LH, amplifies the proliferation and differentiation of spermatogonia in the adult rhesus monkey (Macaca mulatta). Hum Reprod 24(7):1584–1595. https://doi.org/10.1093/humrep/dep052

Smith JN, Calvi LM (2013) Concise review: current concepts in bone marrow microenvironmental regulation of hematopoietic stem and progenitor cells. Stem Cells 31(6):1044–1050. https://doi.org/10.1002/stem.1370

Snippert HJ, van der Flier LG, Sato T, van Es JH, van den Born M, Kroon-Veenboer C, Barker N, Klein AM, van Rheenen J, Simons BD, Clevers H (2010) Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell 143(1):134–144. https://doi.org/10.1016/j.cell.2010.09.016

Stange DE, Clevers H (2013) Concise review: the Yin and Yang of intestinal (cancer) stem cells and their progenitors. Stem Cells 31:2287–2295. https://doi.org/10.1002/stem.1475

Tegelenbosch RA, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 290(2):193–200. https://doi.org/10.1016/0027-5107(93)90159-D

Ugarte F, Forsberg EC (2013) Haematopoietic stem cell niches: new insights inspire new questions. EMBO J 32(19):2535–2547. https://doi.org/10.1038/emboj.2013.201

Unni E, Rao MRS, Ganguly J (1983) Histological and ultrastructural studies on the effect of vitamin-A depletion and subsequent repletion with vitamin-A on germ-cells and Sertoli cells in rat testis. Indian J Exp Biol 21(4):180–192

van Alphen MMA, van de Kant HJG, de Rooij DG (1988) Depletion of the spermatogonia from the seminiferous epithelium of the rhesus monkey after X irradiation. Radiat Res 113(3):473–486. https://doi.org/10.2307/3577244

van Pelt AMM, de Rooij DG (1990a) The origin of the synchronization of the seminiferous epithelium in vitamin A-deficient rats after vitamin A replacement. Biol Reprod 42(4):677–682. https://doi.org/10.1095/biolreprod42.4.677

van Pelt AMM, de Rooij DG (1990b) Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. Biol Reprod 43(3):363–367. https://doi.org/10.1095/biolreprod43.3.363

Vernet N, Dennefeld C, Guillou F, Chambon P, Ghyselinck NB, Mark M (2006) Prepubertal testis development relies on retinoic acid but not rexinoid receptors in Sertoli cells. EMBO J 25(24):5816–5825. https://doi.org/10.1038/sj.emboj.7601447

Vernet N, Dennefeld C, Klopfenstein M, Ruiz A, Bok D, Ghyselinck NB, Mark M (2008) Retinoid X receptor beta (RXRB) expression in Sertoli cells controls cholesterol homeostasis and spermiation. Reproduction 136(5):619–626. https://doi.org/10.1530/Rep-08-0235

Wistuba J, Schrod A, Greve B, Hodges JK, Aslam H, Weinbauer GF, Luetjens CM (2003) Organization of seminiferous epithelium in primates: relationship to spermatogenic efficiency, phylogeny, and mating system. Biol Reprod 69(2):582–591. https://doi.org/10.1095/biolreprod.103.015925

Yoshida S, Sukeno M, Nabeshima Y (2007a) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317(5845):1722–1726. https://doi.org/10.1126/science.1144885

Yoshida S, Sukeno M, Nabeshima YI (2007b) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317(5845):1722–1726. https://doi.org/10.1126/science.1144885

Zhengwei Y, McLachlan RI, Bremner WJ, Wreford NG (1997) Quantitative (stereological) study of normal spermatogenesis in the adult monkey (Macaca fascicularis). J Androl 18(6):681–687. https://doi.org/10.1002/j.1939-4640.1997.tb02445.x

Part II

Postnatal Development of the Spermatogonial Population

Transition of Prenatal Prospermatogonia to Postnatal Spermatogonia

2

John R. McCarrey

Abstract

In the mouse, the prospermatogonial phase of male germline development begins during the fetal stages and extends into the neonatal stages when the initial development of spermatogonia then occurs. In the immature testis, undifferentiated spermatogonia are heterogeneous with at least three distinct subpopulations—those spermatogonia that do not self-renew and give rise directly and only to the first spermatogenic wave, those spermatogonia that form spermatogonial stem cells (SSCs) that are capable of undergoing either self-renewal or differentiation such that they can sustain steady-state spermatogenesis throughout the reproductive lifespan of the male, and those spermatogonia that will undergo cell death. The mechanism that regulates which of these fates will be adopted by each developing spermatogonium remains unresolved. However, there is growing evidence that those prospermatogonia that ultimately give rise to SSCs may become predetermined to this fate during the early fetal stages of male germline development, such that these cells follow a unique developmental program that promotes accumulation of characteristics that are particularly advantageous to SSCs. This notion is supported by studies of the maintenance of genetic integrity in developing prospermatogonia and early undifferentiated spermatogonia that do or do not give rise to SSCs.

Keywords

Spermatogenesis • Specification of cell fate • Spermatogonial stem cells • Testis • Predetermination

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2.1 Developmental Dynamics of the Prospermatogonia to Spermatogonia Transition

Although germline stem cells are common to many Metazoan species (Reik and Surani 2015; Greenspan et al. 2015; Wu et al. 2013), the development of these cells in male mammals includes several unique features. As in many other Metazoan species, mammalian germ cells first emerge as primordial germ cells (PGCs)—the first distinct, unipotent cell lineage specified during embryonic development (Kurimoto and Saitou 2015). In the mouse, PGCs are specified in the epiblast between embryonic days 6.5–7.5 (E6.5–7.5). These PGCs then undergo mitotic expansion as they migrate from their site of origin to the developing genital ridges, which, in the case of males, then initiate testis development between E11.5–12.5. Although both the male and female germ cell lineages derive from indistinguishable PGCs, the subsequent development of the spermatogenic and oogenic cell lineages is marked by dramatic sexual dimorphisms. Thus, at about E12.5–14.5, germ cells in developing ovaries are induced by retinoic acid to enter meiosis, while germ cells in developing testes are inhibited from entering meiosis by CYP26B1, a protein expressed in the fetal testis that blocks the effects of retinoic acid thereby precluding initiation of meiosis in fetal testicular PGCs (Feng et al. 2014; Agrimson and Hogarth 2016). Indeed, this is one of, if not the first sex-specific phenotypic difference(s) that distinguish(es) male and female germline development (Reik and Surani 2015; Greenspan et al. 2015; Bowles and Koopman 2007).

The sexually dimorphic germline differentiation pathways are further distinguished by the differentiation of female PGCs to first form oogonia and then primary ooctyes, and the simultaneous differentiation of male PGCs to first form prospermatogonia and then spermatogonia, respectively (Zheng et al. 2009; Chan et al. 2014; Komai et al. 2014). During mouse development this distinction is initiated at E12.5–13.5. Specifically in the male, M-prospermatogonia, which form by E12.5, remain mitotically active until about E15.5 when they enter a state of mitotic quiescence, designated as T_1-prospermatogonia, which persists until 1–3 days postpartum (P1–3) when T_1-prospermatogonia then transition to T_2-prospermatogonia characterized by resumption of mitotic activity (McCarrey 2013). Between P4–P6, spermatogonia then arise. Interestingly, it has become clear that the population of spermatogonia at P6 is heterogeneous, and is made up of subpopulations of cells that can subsequently adopt alternate fates including either (1) giving rise uniquely to the initial wave of spermatogenesis (de Rooij 1998; Yoshida et al. 2006), (2) undergoing cell death (Rotgers et al. 2015), or (3) giving rise to foundational spermatogonial stem cells (SSCs) that will form the basis of ongoing spermatogenesis for the remainder of the reproductive lifespan in the male (Oatley et al. 2011), as depicted in Fig. 2.1.

The two spermatogonial subpopulations that remain viable in the P6 testis are committed to distinct fates—with one subpopulation giving rise to the first (or at most first and second) wave(s) of spermatogenesis in the rodent testis, and the second subpopulation giving rise to the foundational SSCs that then sustain all subsequent waves of spermatogenesis. A key distinction between these two subpopulations

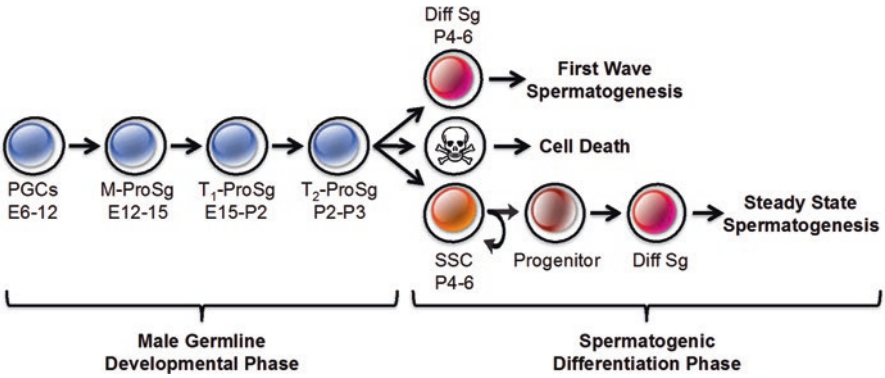

Fig. 2.1 The developmental transition from prospermatogonia to spermatogonia in the mouse. The male germ cell lineage emanates from primordial germ cells (PGCs) that first appear at embryonic day 6–7 (E6–7). In the male, PGCs give rise to prospermatogonia (ProSg) which are present during fetal and early postnatal development and include mitotically active M-prospermatogonia present from about E12–15, which then transition to mitotically quiescent T_1-prospermatogonia present from E15 until about postnatal day 2 (P2), and then to mitotically active T_2 prospermatogonia present at P2–3. Prospermatogonia then give rise to spermatogonia, including spermatogonial stem cells (SSCs), progenitor spermatogonia, and differentiating spermatogonia (Diff Sg). In rodents, the first wave of spermatogenesis emanates from spermatogonia that initiate differentiation without undergoing self-renewal (First Wave Spermatogenesis). Other spermatogonia undergo Cell Death. A small subset of developing prospermatogonia gives rise to the foundational pool of SSCs that then sustains all subsequent waves of spermatogenesis (Steady State Spermatogenesis) by undergoing an asymmetric division process yielding daughter cells that either (1) maintain the SSC phenotype thus achieving self-renewal of the SSC population or (2) initiate differentiation to give rise to successive waves of spermatogenesis that ultimately yield the male gametes. The SSC is the only cell type within the male germ cell lineage capable of self-renewal, and marks a transition between two overall phases of male germ cell development—the Developmental Phase that involves successive transitions from PGCs to prospermatogonia with no retention of the preceding cell type, and the Spermatogenic Differentiation Phase that involves reiterative waves of differentiating spermatogenic cells that arise from self-renewing SSCs (Modified version of original figure from Dr. Brian Hermann.)

is that the former progresses *directly* from an undifferentiated, prospermatogonial-like state into the spermatogenic differentiation pathway with no self-renewal, and therefore no persistence of the undifferentiated cell type, whereas the second subpopulation undergoes asymmetric replication (directly or indirectly) yielding progeny cells that either retain the spermatogonial stem cell fate (= self-renewal of SSCs) or initiate spermatogenic differentiation (= differentiating spermatogonia) to ultimately yield the male gametes.

Postnatal day 6 represents a key point in the postnatal development of the spermatogenic cell lineage in the mouse. By P6, prospermatogonia have all been replaced by spermatogonia. Indeed, prior to this stage, each type of developing male germ cell gives way to the subsequent cell type, with no persistence or renewal of the preceding cell type. Thus in the male the following transitions occur as the germ line develops—PGCs all transition to M-prospermatogonia, M-prospermatogonia all transition to T_1-prospermatogonia, T_1-prospermatogonia all transition to T_2-prospermatogonia, and

T_2-prospermatogonia all transition to spermatogonia (McCarrey 2013) (Fig. 2.1). The only alternative to progression through this developmental pathway is cell death, and many developing male germ cells do indeed succumb to this fate—typically during the spermatogonial stage (Komai et al. 2014).

The undifferentiated spermatogonial cell type at P6 differs from its predecessors in multiple ways. A subpopulation of these cells—that which will form the foundational SSCs—develops the ability to give rise to progeny cells that can adopt alternative fates—self-renewal or entry into the spermatogenic differentiation pathway as differentiating spermatogonia. Like the predecessors of the foundational SSCs, the progeny of SSCs that enter the differentiation pathway then proceed through another series of sequential, progressive transitions among spermatogenic cell types with no persistence or renewal of any of these cell types as they proceed to form spermatocytes that then undergo meiosis to produce spermatids that then undergo differentiation (spermiogenesis) to yield spermatozoa that are then released into the lumen of the seminiferous tubule. The progeny of SSCs which retain the SSC phenotype complete self-renewal to sustain the presence of stem cells at the base of the seminiferous epithelium. Therefore, although uniquely committed to the male germ cell lineage, foundational SSCs are multipotent in that they can give rise to either differentiating spermatogonia that lose the SSC phenotype or undifferentiated spermatogonia that retain the SSC phenotype. The SSCs are the *only* cell type in the male germline lineage capable of self-renewal—a capacity that is critical to the ongoing function of the seminiferous epithelium which, in human males, produces as many as 45 million sperm per day (Johnson et al. 1980).

Those T_2-prospermatogonia that progress directly into the spermatogenic differentiation pathway with no self-renewal contribute to the unique first wave of spermatogenesis in male rodents. This may represent a rodent-specific phenomenon. It appears that this process allows young male rodents to become fertile at least a month earlier during their lifetime than they otherwise would if they relied solely on spermatogenesis emanating from foundational SSCs. The foundational SSCs give rise to the self-renewing seminiferous epithelium in the testes of all mammalian males, and therefore appear to represent the primary functional product of the transition from prospermatogonia to spermatogonia common to all mammals.

In summary, the foundational SSCs are a unique subpopulation of undifferentiated spermatogonia that occur midway through the male germline developmental pathway that extends from PGCs to sperm. The SSC stage marks a transition from a single, progressive, nonrenewing developmental phase to a reiterative, self-renewing, spermatogenic differentiation phase (Fig. 2.1). Together, these two phases make up the complete male germ cell lineage. Importantly, it has become clear that not all prospermatogonia become foundational SSCs. Those prospermatogonia that do form foundational SSCs ultimately give rise to all male gametes after the initial wave of spermatogenesis in rodents, and likely give rise to all male gametes in most non-rodent mammals as well. Thus the allocation of a subset of prospermatogonia to form foundational SSCs represents a critical step toward fertility in an individual male as well as toward perpetuation of the species in general. Despite the

significance of this process, however, the mechanism by which a specific subset of prospermatogonia is allocated to form the foundational SSCs remains poorly understood.

2.2 Heterogeneity Among Spermatogonia in the Neonatal Testis

There is abundant evidence for heterogeneity among spermatogonia in the neonatal rodent testis (Yoshida et al. 2007; Morimoto et al. 2009; Grisanti et al. 2009; Suzuki et al. 2009; Zheng et al. 2009; Nakagawa et al. 2010; Shinohara et al. 2011; Oatley et al. 2011; Chan et al. 2014; Aloisio et al. 2014; Komai et al. 2014). On the basis of morphological analyses, Kluin and de Rooij (1981) suggested that following mitotic division of T_2-prospermatogonia (termed "gonocytes" by these authors) beginning between P1–P2 in the mouse testis, two subpopulations of daughter cells can be discerned which they termed "Type I cells" and "Type II cells." Type I cells resemble T_1-prospermatogonia, while Type II cells differ from Type I cells by the presence of larger nuclei, smaller nucleoli, and the absence of a distinct nuclear vacuole. By P2, the relative proportions of Type I and II cells are 30% and 70%, respectively. Type I cells exhibit a nuclear morphology similar to adult Type-A1 differentiating spermatogonia and are said to directly give rise to the differentiating spermatogonia in the neonatal testis to produce the first wave of spermatogenesis. On the other hand, the subpopulation of Type II cells is said to either give rise to the foundational SSC pool or to undergo cell death (Kluin and de Rooij 1981). Similarly, Orwig et al. (2002) described two subpopulations of prospermatogonia (also termed "gonocytes" by these authors) in the neonatal rat testis which they designated as "pseudopod" and "round" on the basis of morphological distinctions. These authors showed that the capacity to produce and maintain colonies of spermatogenesis upon transplantation into a recipient testis was limited almost completely to the pseudopod subpopulation.

Multiple reports have described heterogeneity of expression of a variety of genes and/or encoded protein markers among T_2 prospermatogonia and/or undifferentiated spermatogonia. Shinohara et al. (2000) reported that SSCs could be enriched by selecting for KIT-negative, alpha6-integrin-positive, alphav-integrin-low or -negative cells. Ohmura et al. (2004) found subpopulations of prospermatogonia that differed in levels of expression of OCT4 and suggested that expression of higher levels of OCT4 might be correlated with the capacity for self-renewal. In addition, visualization of spermatogonial clones in whole-mount preparations of seminiferous tubules revealed phenotypic heterogeneity among undifferentiated As, Apr, and Aal spermatogonia. Specifically, several proteins (e.g., BMI1, GFRA1, ID4, LIN28, NANOS2, NEUROG3, PAX7, ZBTB16/PLZF) show expression patterns that vary among undifferentiated spermatogonia with different clone lengths and between different spermatogonial clones of the same length (Grisanti et al. 2009; Suzuki et al. 2009; Zheng et al. 2009; Chan et al. 2014; Aloisio et al. 2014; Komai et al. 2014). In some cases, heterogeneous expression patterns have been reported within individual

spermatogonial clones for markers such as GFRA1 or NANOS2 (Grisanti et al. 2009; Suzuki et al. 2009) suggesting undifferentiated spermatogonia exist as multiple dynamic subpopulations.

Transplantation of putative spermatogonia as originally described by Brinster and colleagues (Brinster and Avarbock 1994) provides a direct functional assay for the capacity to seed spermatogensis—a central characteristic of SSCs. Shinohara et al. (2000) showed that spermatogonia derived from cryptorchid testes and capable of generating spermatogenic colonies following transplantation could be enriched 166-fold on the basis of sorting for Kit-negative/alpha6-integrin-positive/alphav-integrin-low or –negative cells. Ebata et al. (2005) confirmed that Kit-negative/alpha6-integrin-positive/alphav-integrin-low or –negative cells are enriched for SSCs and showed that expression of the GFRalpha1 marker correlated with SSC activity better in cells isolated from immature mouse testes than in those from adult mouse testes. Kubota et al. (2003, 2004) reported that stem cell activity was found in the MHC class I (MHC-I)-Thy-1+c-kit- cell fraction of the mouse cryptorchid testis, with little or no stem cell activity in any other fraction. The fact that SSCs can be enriched by sorting for different markers supports the contention that the population of undifferentiated spermatogonia in the P6 testis is heterogeneous with respect to potential to form foundational SSCs.

More recently, Yoshida and colleagues reported that in the rodent, the first wave of spermatogenesis initiates from a subset of differentiating prospermatogonia that lack self-renewal capability, fail to express the NGN3 marker (characteristic of self-renewing SSCs), but that do express galectin 1 mRNA. They reported that this subpopulation of prospermatogonia gives rise directly to KIT-positive differentiating spermatogonia during the first wave of spermatogenesis in the mouse, whereas all subsequent rounds of spermatogenesis emanate from self-renewing, NGN3+/KIT- SSCs (Yoshida et al. 2006). These self-renewing SSCs also sustain expression of multiple prospermatogonial markers including PLZF, RET, and OCT4, whereas these markers are not found in the KIT-positive spermatogonia produced directly from prospermatogonia during the first wave (Yoshida et al. 2006).

Another functional characteristic of spermatogonia in the neonatal testis is their ability to respond to retinoic acid (RA) signaling (Busada and Geyer 2016). Late prospermatogonia and early spermatogonia show variation in their response to RA signaling, which appears to regulate the prospermatogonia to spermatogonia and undifferentiated to differentiating spermatogonia transitions (Busada et al. 2014). In the neonatal testis, small patches of prospermatogonia become STRA8+/KIT+, which is indicative of initiation of spermatogonial differentiation, while large regions remain STRA8-/KIT- which is indicative of retention of the undifferentiated state (Busada et al. 2014, 2015). Those spermatogonia that remain unresponsive to RA avoid NOTCH and PDGF signaling which also contribute to entry of spermatogonia into the spermatogenic differentiation phase of male gametogenesis (Busada et al. 2014, 2015; Garcia et al. 2013; Garcia and Hofmann 2013; Manku and Culty 2015; Manku et al. 2015).

Niedenberger et al. (2015) found that some markers for the undifferentiated spermatogonial state, including PLZF and CDH1, are expressed in nearly all

spermatogonia from P1 through P7, whereas the differentiation markers, STRA8 and KIT, appear only in a subset of spermatogonia at P4, coincident with the onset of RA signaling. GFRA1, which is present in nearly all prospermatogonia at P1, persists only in STRA8/KIT- spermatogonia. From P10 and thereafter, distinct populations of undifferentiated and differentiating spermatogonia are marked by unique patterns of expression of spermatogonial fate markers indicative of either entry into the spermatogenic differentiation phase or retention of the undifferentiated SSC phenotype (Niedenberger et al. 2015).

Taken together, the many reports of differential characteristics among prospermatogonia and/or spermatogonia are strongly indicative of heterogeneity among male germ cells in the neonatal testis. Indeed, it has been reported that the number of As spermatogonia in a mouse testis (~35,000) is more than ten times larger than the number of SSCs with regenerative capacity (~3000) on the basis of transplantation experiments (Nagano 2003), demonstrating that there is also functional heterogeneity among undifferentiated spermatogonia which otherwise display similar morphological characteristics.

Oatley and colleagues identified the transcriptional repressor inhibitor of DNA binding 4 (ID4) as a putative SSC-specific marker in the mouse (Oatley et al. 2011), and then created a transgenic mouse line in which ID4 expressing cells become marked by expression of GFP (Chan et al. 2014). In the intact mouse testis, ID4-GFP+ cells exist primarily as a subset of the type As pool, and their frequency is greatest in the neonatal testis and then decreases proportionately during establishment of the spermatogenic lineage, eventually comprising ~2% of the undifferentiated spermatogonial population in the adult testis. Transplantation studies definitively demonstrated that SSCs were exclusively found within the ID4-GFP+ fraction of either undifferentiated spermatogonia from the intact mouse testis or cultured spermatogonia from mice bearing this marker transgene (Chan et al. 2014).

Most recently, Hermann et al. (2015) performed a single-cell gene expression study to determine the extent of gene expression heterogeneity among neonatal mouse spermatogonia. This analysis of expression of 172 different genes was conducted on 584 individual P6 testis cells enriched for spermatogonia by multiple methods, and revealed three spermatogonial subpopulations distinguishable on the basis of differential gene expression signatures resolvable by a principle component analysis. These authors suggested that the distinct gene expression signatures they described for each spermatogonial subpopulation may correlate with distinct functional characteristics, including SSCs, differentiating spermatogonia, and progenitor spermatogonia.

2.3 Specification of Foundational Spermatogonial Stem Cells

The quantity and quality of the evidence summarized above leaves no doubt that the spermatogonial pool in the neonatal rodent testis is heterogeneous with respect to the potential to form foundational SSCs. In turn, this begs mechanistic questions

including: (1) What characteristics distinguish those spermatogonia or prospermatogonia that will or will not go on to form foundational SSCs? (2) What is the fate of spermatogonia that do not contribute to the foundational SSC pool? and (3) How are the foundational SSCs specified from among the total prospermatogonial/spermatogonial pool in the neonatal testis?

At least three different functional schemes can be envisioned to potentially account for the manner in which the foundational SSCs are specified from among the total pool of developing spermatogonia, including: (1) the process could be largely stochastic in nature such that those developing spermatogonia that simply happen to be in closest proximity to the developing niches at the base of the seminiferous epithelium take up residence in those niches and initiate function as SSCs; (2) an active selection mechanism could influence which developing spermatogonia become the foundational SSCs such that those spermatogonia that display the most advantageous phenotypes (the specifics of which are yet to be defined) outcompete all other spermatogonia to become the foundational SSCs; or (3) the foundational SSCs may emanate from a specialized subpopulation of spermatogonia and earlier prospermatogonia that becomes "predetermined" during the fetal stages to subsequently give rise to the foundational SSCs.

Each of these schemes makes specific, testable predictions. The stochastic scheme predicts that specification of the foundational SSCs occurs contemporaneously with the initial appearance of the foundational SSCs and that no detectable molecular or cellular differences, other than position within the seminiferous cords, should distinguish individual cells among the spermatogonial pool at P4–6 in the neonatal mouse testis that do or do not become SSCs. The selection scheme predicts that distinctions in the form of epigenetic programming, gene expression patterns, and/or general cellular function or fitness will have developed randomly within the developing prospermatogonial/spermatogonial pool prior to the initial appearance of foundational SSCs and that an active selection process will then shunt the less robust cells into either a cell death pathway or directly into a differentiation pathway to give rise to the first wave of spermatogenesis, while retaining the more robust cells to form the foundational SSCs as the basis of the self-renewing seminiferous epithelium.

The predetermination scheme predicts that a subpopulation of fetal prospermatogonia becomes allocated as future foundational SSCs much earlier during the development of the male germ line via a directed process similar to those operating in other developing lineages (e.g. hematopoietic (Birbrair and Frenette 2016), neural (Mehler et al. 2000; Mehler 2002), or tumorigenic (Morrison and Kimble 2006) lineages) that progressively develop sub-lineages with different developmental potentials. As a part of this directed process, the fetal prospermatogonia that become predetermined to give rise to the foundational SSCs will then be nonrandomly induced to develop unique epigenetic programming, gene expression patterns and cellular characteristics that are particularly advantageous to the function of foundational SSCs via an active, directed process co-regulated as part of the SSC specification mechanism. Thus, the predetermination scheme suggests that specification of future foundational SSCs is a relatively early event—occurring during fetal stages

at the T_1-prospermatogonia stage or earlier, whereas the selection scheme suggests that specification of foundational SSCs is a relatively late event—occurring at, or just prior to the stage at which these cells first appear (P4–6 in the mouse).

The stochastic scheme can be easily excluded. The reports summarized above describing heterogeneity among undifferentiated spermatogonia in the neonatal testis, and especially the fact that spermatogonial subpopulations can be sorted on the basis of certain cellular characteristics that correlate directly with enrichment for, or depletion of foundational SSCs clearly demonstrate that detectable molecular and cellular differences do exist among undifferentiated spermatogonia and that these cells do not share equal potential to form foundational SSCs. Rather, it is clear that a sortable subpopulation of undifferentiated spermatogonia is indeed more likely to form the foundational SSCs and that the specification process is therefore not stochastic.

It is more challenging to distinguish between the two remaining schemes—selection or predetermination—as the primary mechanism by which foundational SSCs are specified. The key difference between these two scenarios is that the former (selection) suggests that a retrospective mechanism, functioning during a narrow developmental window spanning the transition from prospermatogonia to spermatogonia somehow distinguishes and selects among these cells to preferentially allow those with advantageous characteristics to form foundational SSCs, whereas the latter (predetermination) suggests that a prospective mechanism, initiated during the early fetal stages and persisting through the neonatal stages ensures that a specific subset of prospermatogonia becomes fated to ultimately form foundational SSCs and that the programming responsible for this predetermination also ensures development of cellular characteristics advantageous to foundational SSCs. Thus, the selection scheme suggests that it is *because* certain developing spermatogonia possess unique advantageous characteristics that they then preferentially form SSCs, whereas the predetermination scheme suggests that the developing spermatogonia that will form SSCs develop advantageous characteristics *because* they are fated to form SSCs.

Ongoing studies of gene expression patterns, epigenetic programming and cellular functions will contribute to a better understanding of the process of specification of foundational SSCs. In addition, however, analysis of the levels at which genetic integrity is maintained in developing male germ cells provides a unique opportunity to distinguish between the selection and predetermination schemes as the primary means by which foundational SSCs become specified (Walter et al. 1998; Murphey et al. 2013). Thus, a study that traced the accumulation of spontaneous point mutations in developing spermatogenic cells from PGCs to sperm revealed an overall progressive accumulation of spontaneous point mutations throughout the development and differentiation of the male germ line (Murphey et al. 2013) (Fig. 2.2). However this study also revealed an unexpected decrease in the frequency of point mutations during the spermatogonial phase of spermatogenesis—particularly that associated with the initial wave of spermatogenesis in the mouse. This decrease cannot be explained by the direct reversal of preexisting mutations because such reversals are exceedingly rare events (Mortelmans and Riccio 2000).

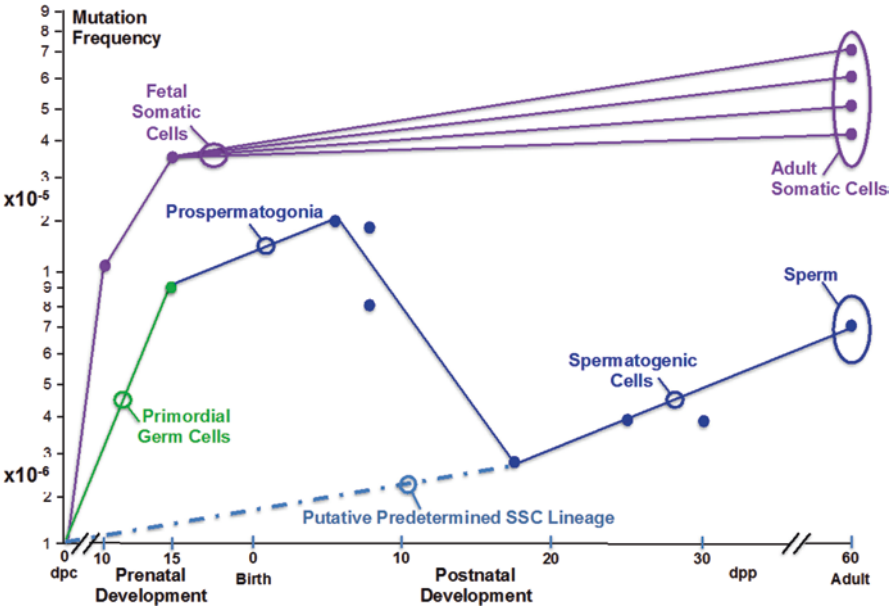

Fig. 2.2 Accumulation of spontaneous mutations in germ and somatic cells during murine development. The frequency of mutations detected in a *lacI* mutation-reporter transgene is shown as a function of pre- and postnatal development. At the time of fertilization, the *lacI* mutation-reporter transgene carries no mutations, but spontaneous point mutations begin to accumulate in this transgene following fertilization. The frequency of mutations rises quickly in somatic cells between fertilization and birth and then continues to rise at a lower rate after birth depending on the specific somatic cell lineage. By E15 (= 15.5 days postcoitum), male germ cells have accumulated significantly lower frequencies of mutations than developmentally matched somatic cells. Prospermatogonia then show an increase in mutation frequency between E15 and P6 (= 6 days postpartum), but then a dramatic decrease in the frequency of mutations occurs in spermatogonia, coincident with a known wave of apoptosis. This is followed by the accumulation of additional mutations in the spermatogenic cell lineage at a gradual rate. The possible dynamics of accumulation of spontaneous mutations in a subpopulation of developing prospermatogonia/spermatogonia predetermined to form spermagonial stem cells (SSCs) that would be consistent with mutation frequencies observed in more advanced spermatogenic cells is represented by the *dashed/dotted line* (putative predetermined SSC lineage) (Modified version of original figure from Murphey et al. 2013.)

Therefore this decrease in mutation frequency as a function of development of the spermatogenic cell lineage must be indicative of a nonrandom loss of a portion of developing spermatogonia that bear higher mutational loads.

Indeed, this drop in mutation frequency coincides with a known wave of cell death in developing spermatogonia (Mori et al. 1997), suggesting that spermatogonia with higher mutational loads preferentially undergo cell death or contribute uniquely to the first wave of spermatogenesis in a nonrandom manner, while spermatogonia with lower mutational loads preferentially give rise to the foundational SSC pool in an nonrandom manner. These nonrandom fates of spermatogonial subpopulations in the neonatal testis provide measurable outcomes that allow us to further discriminate between selection or predetermination as the mechanism(s) responsible for specification of the foundational SSCs.

The Disposable Soma Theory (Kirkwood 1977) predicts that the germ line will expend greater energy than the soma to maintain genetic integrity in a pristine state to ensure transmission of optimal genetic information from one generation to the next. To this end, it is critical that enhanced genetic integrity be maintained *throughout* the life history of the germ line, rather than only at certain stages of gametogenesis. This concept is supported by multiple observations that germline cells maintain genetic integrity more stringently than somatic cell types at all stages of the germ cell lineage (Walter et al. 1998; Murphey et al. 2013). Spermatogenic cells employ checkpoint mechanisms to selectively shunt cells bearing large-scale genetic defects such as aneuploidy or other gross chromosomal defects into a cell death pathway—especially during meiosis (Handel 1998; Hunt and Hassold 2002; Burgoyne et al. 2007). However, this cannot account for the observed nonrandom decrease in spermatogonia carrying higher frequencies of spontaneous point mutations, as there is no known checkpoint mechanism that can sense these small mutations.

It is possible that certain point mutations could negatively impact the phenotype of spermatogonia such that those cells could then be subject to selection that would lower the likelihood that they would subsequently form foundational SSCs. However, most spontaneous point mutations are silent and do not impact fitness or function of the cell, and therefore do not provide a basis for selection. In addition, the kinetics of the decline in mutation frequency observed during the prospermatogonia to spermatogonia transition (Fig. 2.2) suggest that the selection process would be confined to a very narrow window during postnatal development of the spermatogenic lineage. Overall, it is difficult to imagine that a sufficient preponderance of selectable point mutations regularly occurs during the spermatogonial phase of male germline development to an extent that could account for the consistent, nonrandom, extensive loss of spermatogonia that regularly occurs via cell death during neonatal development of the testis in all males. As a result, it is therefore difficult to envision any sort of active selection mechanism that functions during the brief developmental period coincident with specification of foundational SSCs Therefore, with no known surveillance mechanism to actively detect and eliminate spermatogonia bearing higher frequencies of point mutations, no selectable phenotype resulting from the large majority of such mutations, and no known selection mechanism capable of preferentially precluding a large majority of developing spermatogonia from forming SSCs, the concept that selection is directly responsible for determining which spermatogonia do or do not contribute to the foundational SSC pool is not well supported.

In the absence of a mechanism leading to specification of foundational SSCs on the basis of selection, predetermination of a subset of prospermatogonia to form the foundational SSC pool appears to be the scheme that is most consistent with the observation that a specific, small subset of developing spermatogonia selectively form foundational SSCs in a nonrandom manner. Further, a predetermination scheme has the potential to account for the nonrandom loss of developing spermatogonia bearing higher mutation frequencies based on co-regulation of cell fate and enhanced maintenance genetic integrity. Precedent for this concept comes from studies showing that the programming responsible for establishing and maintaining

a pluripotent cellular state interfaces with the gene network required to maintain enhanced of genetic integrity in embryonic stem cells or induced pluripotent stem cells (Murphey et al. 2009; Cooper et al. 2014). Thus, a similar mechanism could mechanistically link maintenance of enhanced genetic integrity with the programming responsible for predetermination of future SSCs.

As shown in Fig. 2.2, the predetermination scheme actually predicts that developing prospermatogonia will be heterogeneous—not only with respect to potential to form foundational SSCs but also with respect to maintenance of genetic integrity. The relative frequency of point mutations serves to permanently mark distinct subpopulations of spermatogonia because once present, point mutations are very rarely lost. Thus, the only feasible source of a low mutation frequency in a specific subpopulation of cells is that the predecessors of those cells maintained a low mutational load throughout development. In the case of developing prospermatogonia, a subpopulation fated to form SSCs could maintain a low mutation frequency throughout the fetal and neonatal stages, even while other prospermatogonia not fated to form SSCs are accumulating spontaneous point mutations at a relatively higher rate.

The dashed line in Fig. 2.2 represents the predicted mutation frequency in such a putative subset of developing prospermatogonia predetermined to form SSCs in a way that is consistent with the lower mutation frequencies observed during the subsequent spermatogenic differentiation phase. If it is the case that predetermined SSCs persist nonrandomly in the maturing postnatal testis while those not fated to form SSCs are preferentially lost due to either their contribution to the unique first wave of spermatogenesis or their nonrandom entry into a cell death pathway, then the SSCs remaining after this nonrandom loss of cells would derive predominantly, if not completely, from the protected subpopulation carrying a low mutational load in a manner consistent with the observed developmental dynamics of the accumulation of mutations in the spermatogenic cell lineage as shown in Fig. 2.2.

Taken together, the suggestion that specification of foundational SSCs involves predetermination of prospermatogonia that will subsequently form SSCs is consistent with the following documented observations: (1) that the population of undifferentiated prospermatogonia/spermatogonia at or prior to the emergence of foundational SSCs is heterogeneous with respect to epigenetic programming and gene expression (Hammoud et al. 2014; Hermann et al. 2015), (2) that a distinct subpopulation of neonatal undifferentiated spermatogonia preferentially includes cells displaying SSC function detectable by the transplantation assay (Shinohara et al. 2000; Kubota et al. 2003, 2004; Ebata et al. 2005; Chan et al. 2014), and (3) that the resulting population of cells that participate in the spermatogenic differentiation phase is highly enriched for cells that display characteristics consistent with those associated with the predetermined progenitors of the foundational SSCs—such as enhanced maintenance of genetic integrity (Murphey et al. 2013). These predictions differ from those of the selection scheme, which holds that foundational SSCs will derive from an otherwise equipotent population of undifferentiated prospermatogonia/spermatogonia based on selection of certain (currently undefined)

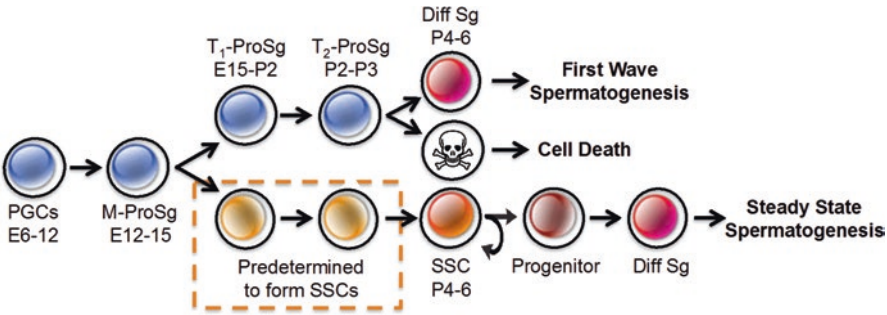

Fig. 2.3 Predetermination of foundational SSCs in the mouse. A developmental scheme consistent with the hypothesis that foundational SSCs are predetermined during fetal development is shown. This hypothesis suggests that a distinct subpopulation of male germline cells allocated at the T_1-prospermatogonia stage (or earlier) becomes selectively fated to subsequently form the foundational SSCs in the postnatal testis that then support reiterative Steady State Spermatogenesis, while all remaining prospermatogonia either give rise directly to differentiating spermatogonia that contribute solely to the unique first wave of spermatogenesis (First Wave Spermatogenesis) or undergo Cell Death. Following this segregational process in the neonatal testis, only the foundational SSCs and their descendants continue to contribute to Steady State Spermatogenesis throughout the remainder of the reproductive lifespan of the male

cellular phenotypes that vary among the undifferentiated spermatogonial population based on stochastic developmental events.

In summary, multiple observations of the prospermatogonia to spermatogonia transition are most consistent with the early allocation/predetermination of a subset of fetal prospermatogonia fated to form postnatal foundational SSCs. As part of this process, this predetermined subset of male germ cells can then become programmed to develop cellular characteristics that are paricularly advantageous to the function of foundational SSCs, and these foundational SSCs can then form the basis of steady state spermatogenesis ongoing in the seminiferous epithelium by undergoing asymmetric divisions that both maintain the SSC pool and contribute cells to the spermatogenic differentiation process. In this scheme, those prospermatogonia that are not predetermined to form foundational SSCs will preferentially enter the unique first wave of spermatogenesis or a cell death pathway. Overall, the result of this process will be persistence of the subset of the male germ cell lineage predetermined to form foundational SSCs and the coincident, nonrandom loss of the subset of the lineage not predetermined to form SSCs. The dynamics of this suggested scheme are depicted in Fig. 2.3.

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References

Agrimson KS, Hogarth CA (2016) Germ cell commitment to oogenic versus spermatogenic pathway: the role of retinoic acid. Results Probl Cell Differ 58:135–166

Aloisio GM, Nakada Y, Saatcioglu HD, Pena CG, Baker MD, Tarnawa ED, Mukherjee J, Manjunath H, Bugde A, Sengupta AL, Amatruda JF, Cuevas I et al (2014) PAX7 expression defines germline stem cells in the adult testis. J Clin Invest 124:3929–3944

Birbrair A, Frenette PS (2016) Niche heterogeneity in the bone marrow. Ann N Y Acad Sci 1370(1):82–96. https://doi.org/10.1111/nyas.13016; Epub 2016 Mar 25

Bowles J, Koopman P (2007) Retinoic acid, meiosis and germ cell fate in mammals. Development 134(19):3401–3411; Epub 2007 Aug 22. Review

Brinster RL, Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 91(24):11303–11307

Burgoyne PS, Mahadevaiah SK, Turner JM (2007) The management of DNA double-strand breaks in mitotic G2, and in mammalian meiosis viewed from a mitotic G2 perspective. BioEssays 29(10):974–986

Busada JT, Chappell VA, Niedenberger BA, Kaye EP, Keiper BD, Hogarth CA, Geyer CB (2015) Retinoic acid regulates Kit translation during spermatogonial differentiation in the mouse. Dev Biol 397:140–149

Busada JT, Geyer CB (2016) The role of retinoic acid (RA) in spermatogonial differentiation. Biol Reprod 94(1):10. https://doi.org/10.1095/biolreprod.115.135145; Epub 2015 Nov 11

Busada JT, Kaye EP, Renegar RH, Geyer CB (2014) Retinoic acid induces multiple hallmarks of the prospermatogonia-to-spermatogonia transition in the neonatal mouse. Biol Reprod 90:64

Chan F, Oatley MJ, Kaucher AV, Yang QE, Bieberich CJ, Shashikant CS, Oatley JM (2014) Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev 28:1351–1362

Cooper DJ, Walter CA, McCarrey JR (2014) Co-regulation of pluripotency and genetic integrity at the genomic level. Stem Cell Res 13:508–519

de Rooij DG (1998) Stem cells in the testis. Int J Exp Pathol 79(2):67–80. Review

Ebata KT, Zhang X, Nagano MC (2005) Expression patterns of cell-surface molecules on male germ line stem cells during postnatal mouse development. Mol Reprod Dev 72(2):171–181

Feng CW, Bowles J, Koopman P (2014) Control of mammalian germ cell entry into meiosis. Mol Cell Endocrinol 382:488–497

Garcia TX, DeFalco T, Capel B, Hofmann MC (2013) Constitutive activation of NOTCH1 signaling in Sertoli cells causes gonocyte exit from quiescence. Dev Biol 377:188–201

Garcia TX, Hofmann MC (2013) NOTCH signaling in Sertoli cells regulates gonocyte fate. Cell Cycle 12:2538–2545

Greenspan LJ, de Cuevas M, Matunis E (2015) Genetics of gonadal stem cell renewal. Annu Rev Cell Dev Biol 31:291–315

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

Hammoud SS, Low DH, Yi C, Carrell DT, Guccione E, Cairns BR (2014) Chromatin and transcription transitions of mammalian adult germline stem cells and spermatogenesis. Cell Stem Cell 15(2):239–253. https://doi.org/10.1016/j.stem.2014.04.006; Epub May 15

Handel MA (1998) Monitoring meiosis in gametogenesis. Theriogenology 49(2):423–430. Review

Hermann BP, Mutoji KN, Velte EK, Ko D, Oatley JM, Geyer CB, McCarrey JR (2015) Transcriptional and translational heterogeneity among neonatal mouse spermatogonia. Biol Reprod 92(2):54. https://doi.org/10.1095/biolreprod.114.125757; Epub 2015 Jan 7

Hunt PA, Hassold TJ (2002) Sex matters in meiosis. Science 296(5576):2181–2183. Review

Johnson L, Petty CS, Neaves WB (1980) A comparative study of daily sperm production and testicular composition in humans and rats. Biol Reprod 22(5):1233–1243

Kirkwood TB (1977) Evolution of ageing. Nature 270:301–304

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
Komai Y, Tanaka T, Tokuyama Y, Yanai H, Ohe S, Omachi T, Atsumi N, Yoshida N, Kumano K, Hisha H, Matsuda T, Ueno H (2014) Bmi1 expression in long-term germ stem cells. Sci Rep 4:6175
Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A 100(11):6487–6492; Epub 2003 May 8
Kubota H, Avarbock MR, Brinster RL (2004) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71(3):722–731; Epub Apr 28
Kurimoto K, Saitou M (2015) Mechanism and reconstitution in vitro of germ cell development in mammals. Cold Spring Harb Symp Quant Biol 80:147–154
Manku G, Culty M (2015) Mammalian gonocyte and spermatogonia differentiation: recent advances and remaining challenges. Reproduction 149:R139–R157
Manku G, Wang Y, Merkbaoui V, Boisvert A, Ye X, Blonder J, Culty M (2015) Role of retinoic acid and platelet-derived growth factor receptor cross talk in the regulation of neonatal gonocyte and embryonal carcinoma cell differentiation. Endocrinology 156:346–359
McCarrey JR (2013) Toward a more precise and informative nomenclature describing fetal and neonatal male germ cells in rodents. Biol Reprod 89:47
Mehler MF (2002) Mechanisms regulating lineage diversity during mammalian cerebral cortical neurogenesis and gliogenesis. Results Probl Cell Differ 39:27–52
Mehler MF, Mabie PC, Zhu G, Gokhan S, Kessler JA (2000) Developmental changes in progenitor cell responsiveness to bone morphogenetic proteins differentially modulate progressive CNS lineage fate. Dev Neurosci 22(1–2):74–85
Mori C, Nakamura N, Dix DJ, Fujioka M, Nakagawa S, Shiota K, Eddy EM (1997) Morphological analysis of germ cell apoptosis during postnatal testis development in normal and Hsp 70-2 knockout mice. Dev Dyn 208:125–136
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
Morrison SJ, Kimble J (2006) Asymmetric and symmetric stem-cell divisions in development and cancer. Nature 441(7097):1068–1074
Mortelmans K, Riccio ES (2000) The bacterial tryptophan reverse mutation assay with Escherichia coli WP2. Mutat Res 455(1–2):61–69
Murphey P, McLean DJ, McMahan CA, Walter CA, McCarrey JR (2013) Enhanced genetic integrity in mouse germ cells. Biol Reprod 88(1):6. https://doi.org/10.1095/biolreprod.112.103481
Murphey P, Yamazaki Y, McMahan CA, Walter CA, Yanagimachi R, McCarrey JR (2009) Epigenetic regulation of genetic integrity is reprogrammed during cloning. Proc Natl Acad Sci U S A 106:4731–4735
Nagano MC (2003) Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. Biol Reprod 69:701–707
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
Niedenberger BA, Busada JT, Geyer CB (2015) Marker expression reveals heterogeneity of spermatogonia in the neonatal mouse testis. Reproduction 149:329–338
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
Ohmura M, Yoshida S, Ide Y, Nagamatsu G, Suda T, Ohbo K (2004) Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. Arch Histol Cytol 67(4):285–296
Orwig KE, Ryu BY, Avarbock MR, Brinster RL (2002) Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testes. Proc Natl Acad Sci U S A 99(18): 11706–11711; Epub 2002 Aug 15

Reik W, Surani MA (2015) Germline and pluripotent stem cells. Cold Spring Harb Perspect Biol 7(11). https://doi.org/10.1101/cshperspect.a019422

Rotgers E, Nurmio M, Pietilä E, Cisneros-Montalvo S, Toppari J (2015) E2F1 controls germ cell apoptosis during the first wave of spermatogenesis. Andrology 3(5):1000–1014. https://doi.org/10.1111/andr.12090

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

Shinohara T, Ishii K, Kanatsu-Shinohara M (2011) Unstable side population phenotype of mouse spermatogonial stem cells in vitro. J Reprod Dev 57:288–295

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

Walter CA, Intano GW, McCarrey JR, McMahan CA, Walter RB (1998) Mutation frequency declines during spermatogenesis in young mice but increases in old mice. Proc Natl Acad Sci U S A 95:10015–10019

Wu J, Luo H, Wang H (2013) Germline stem cells. Curr Top Dev Biol 102:97–126

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

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

Zheng K, Wu X, Kaestner KH, Wang PJ (2009) The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol 9:38

Setting the Stage: The First Round of Spermatogenesis

3

Christopher B. Geyer

Abstract

The first round, or wave, of spermatogenesis is the postnatal process of male gametogenesis that begins with the differentiation of the first subset of spermatogonia and culminates with the formation of the first spermatozoa. This must occur in all mammals, although it occurs at widely variable times during development. It is during this time that fundamental cell populations are formed, the seminiferous epithelium matures, and macromolecular structures such as the blood–testis barrier are built that are required for lifelong fertility. Since spermatogenesis has been studied most extensively in mice and rats, this chapter will rely heavily on data from rodents in order to define the significant molecular and cellular changes that occur during this important period of spermatogonial development.

Keywords

Spermatogonia • Prospermatogonia • Gonocyte • Spermatogenesis • First wave • Testis

3.1 Initiation of Spermatogenesis

The initiation of spermatogenesis has classically been defined as the point in postnatal mammalian testis development when adult-like type A spermatogonia begin to proliferate and differentiate. They then undergo a precisely defined series of cell

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Table 3.1 Timing of spermatogenesis in nonseasonal breeding mammals

Species	First spermatogonia	First meiotic spermatocytes	Completed spermatogenesis
Mouse (Bellve et al. 1977; Janca et al. 1986; Nebel et al. 1961)	P3–4	P8	~P30–35
Rat (Clermont and Perey 1957; Hilscher et al. 1974; Malkov et al. 1998)	P4–5	P9–10	~P42–44
Rabbit (Gondos and Byskov 1981; Gondos et al. 1973)	7 weeks	9 weeks	14 weeks
Cat (Sanchez et al. 1993; Tiptanavattana et al. 2015)	4 months	6 months	8–9 months
Goat (Sarma and Devi 2012)	2–4 months	4–5 months	6 months
Donkey (Moustafa et al. 2015; Neves et al. 2002)	6–9 months	1–1.5 years	2 years
Bull (Abdel-Raouf 1960; Wrobel 2000; Wrobel et al. 1995; Curtis and Amann 1981)	3 months	4–6 months	8 months
Human (Paniagua and Nistal 1984)	2 months	10–11 years	11–13 years

divisions and morphogenetic changes that ultimately result in formation of the first testicular sperm within a few weeks' time. Organization of the testis and design of the spermatogenic program is highly similar in all mammalian species, with minimal variation in the actual length of time required for completion of one round of spermatogenesis. In addition, there is considerable conservation in the expression of spermatogonial protein markers such as ZBTB16, UCHL1, and mRNAs such as *Kit*, *Taf4b*, *Lin28*, and *Gfra1* between mice and humans (Wu et al. 2009). This suggests that the basic molecular mechanisms directing spermatogonial development are conserved among mammals, and supports the utility of rodents as a model organism to study this process. Multiple studies over the past 50–60 years have revealed that what does differ considerably between mammalian species is the timing of initiation of spermatogenesis and the interval following initiation until formation of the first testicular sperm (Table 3.1), which represent the finished product of the first and all subsequent rounds of spermatogenesis.

3.1.1 Initiation in Seasonal Breeders

Most mammals exhibit seasonal breeding behavior, which coincides with cyclical growth and regression of the seminiferous epithelium that results in specific temporal windows of fertility. The breeding season varies by species and by latitude, but is coordinated with the length of the gestation period such that offsprings are born in the most favorable time of the year for their survival (Bronson 2009; Jimenez et al. 2015). Outside of this breeding season, breeding behavior is reduced or absent, testes are small, and the seminiferous epithelia typically contain only Sertoli cells,

spermatogonia, and some spermatocytes. Changes in photoperiod, temperature, and/or food availability are correlated with activation of the hypothalamic-pituitary-gonadal axis, resulting in increased serum testosterone (T) levels and subsequent initiation of spermatogenesis and growth of the seminiferous epithelium. Within a few weeks' time following serum T elevation, spermatogenesis has completed and the cauda epididymides contain abundant spermatozoa. Changes in spermatogenesis have been documented for a variety of species including certain species of mice (e.g. *Peromyscus californicus* (Nelson et al. 1995)), grizzly bear (White et al. 2005), deer (Brown et al. 1979; Clarke et al. 1995), moles (Dadhich et al. 2013), armadillos (Luaces et al. 2013), most marsupials (reviewed in (Tyndale-Biscoe and Renfree 1987)), and even in marine mammals (Robeck and Monfort 2006; Urian et al. 1996). Certain strains of laboratory mice, which are not typically characterized as seasonal breeders, also show seasonal variation in litter size and sex ratios (Drickamer 1990).

As mentioned above, spermatogenesis in seasonal breeding mammals ceases at the end of each breeding season, and this is marked by regression of the seminiferous epithelium so that the only remaining germ cells are spermatogonia and some spermatocytes. It seems likely that low T levels help maintain this block to spermatogenesis, as the seminiferous epithelium appears similar to that of Sertoli cell androgen receptor knockout (SCARKO) mice (Denolet et al. 2006; Abel et al. 2008). At the beginning of the next breeding season, spermatogenesis reinitiates as T levels increase. It is unclear whether spermatogenesis is initiated from spermatogonial stem cells (SSCs), as in adult steady-state spermatogenesis, or whether subsets of spermatogonia are already committed to the program of spermatogenesis as progenitor or differentiating spermatogonia. Either way, a first round, or wave of spermatogenesis must initiate at the beginning of each breeding season. It is currently unclear what molecular cues in addition to increased T levels are involved. Their identification would significantly advance our understanding of spermatogenesis in both seasonal breeders as well as in higher-order mammals such as humans, in which the first round of spermatogenesis initiates along with (but perhaps not as a consequence of) elevated T levels at puberty (Ramaswamy and Weinbauer 2014; Walker 2011).

3.1.2 Initiation in Nonseasonal Breeders

As shown in Table 3.1, spermatogenesis proceeds in most nonseasonal breeding mammals largely in an uninterrupted fashion following the formation of type A spermatogonia from precursor prospermatogonia (also termed gonocytes (Culty 2009; McCarrey 2013)). In general, shorter-lived mammals (e.g. rodents) initiate and then complete the first round of spermatogenesis sooner than longer-lived mammals. The progression of spermatogenesis is controlled so that production of the first sperm is temporally coordinated with the onset of sexual maturity, which occurs at approximately 7 weeks of age in mice, but 12–13 years in humans.

In the human testis, type A spermatogonia (termed A_{pale} and A_{dark}, respectively) form in the infant testis, but then become developmentally arrested (except for a

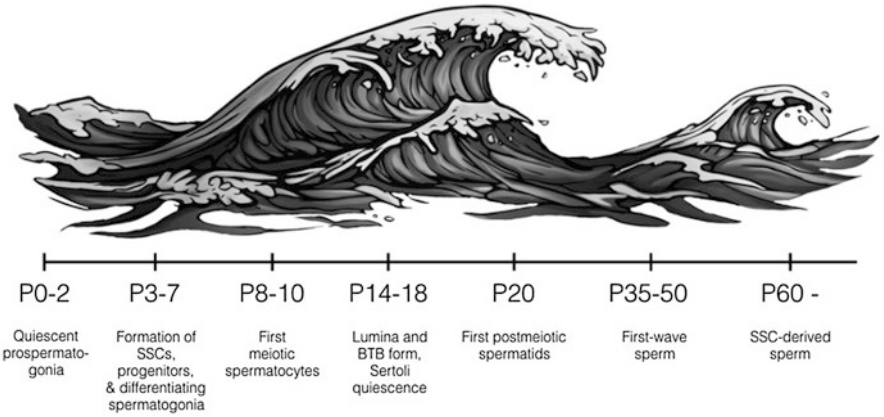

Fig. 3.1 Landmark events during the first round, or wave of spermatogenesis in mice. *SSC* spermatogonial stem cell, *BTB* blood–testis barrier

small population of type B spermatogonia that often form around 5–6 years of age) until sexual maturity occurs with the onset of puberty at ~12 years of age (Paniagua and Nistal 1984). It is unknown what mechanisms are responsible for this long delay in the progression of spermatogenesis in humans (and other nonhuman primates), but they likely involve regulatory endocrine signals such as testosterone (T) and follicle stimulating hormone (FSH), which both increase at puberty when spermatogenesis initiates (Ramaswamy and Weinbauer 2014; Walker 2011; Plant and Marshall 2001).

3.2 Germ Cell Kinetics During the First Wave of Spermatogenesis in Mice

Much of our knowledge of the development of mammalian spermatogenic cells comes from studies using mice. Therefore, we will focus our discussion here on the events that occur during the first few weeks of postnatal development in mice (see Fig. 3.1). The newborn mouse testis at postnatal day (P)0–1 contains a population of largely quiescent germ cells that have been termed type T1 prospermatogonia (Fig. 3.2, (Hilscher and Hilscher 1976)). They reside in a central position within the testis (or seminiferous) cords, which have not yet formed lumina. Beginning at P1–2, prospermatogonia move to the periphery of the cords and reenter the cell cycle in response to signals that have not been defined (see Fig. 3.2, P4 testis (Roosen-Runge and Leik 1968; Vergouwen et al. 1993; Vergouwen et al. 1991; Western et al. 2008)). It is also unclear whether these events are related or dependent upon each other. At this point, these mitotic germ cells are termed type T2 prospermatogonia (Hilscher and Hilscher 1976) to indicate that they are distinct from the quiescent type T1, but this nomenclature is not widely used. Instead, many researchers refer to male germ cells that have reentered the cell cycle as type A

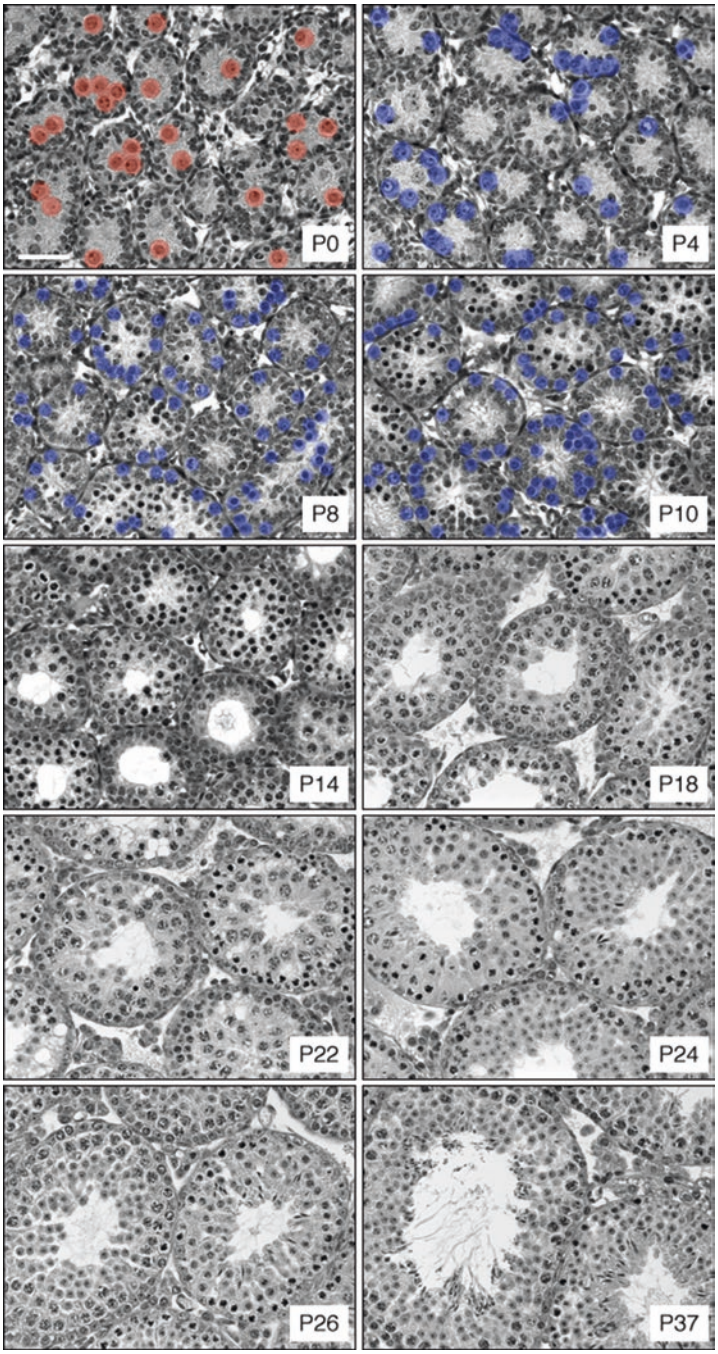

Fig. 3.2 Changes in the seminiferous epithelium during the first wave of spermatogenesis. Bouin's-fixed and paraffin-embedded testis sections are shown, with the age indicated on each image. Prospermatogonia are pseudocolored in *red*, while spermatogonia (A_{undiff} and differentiating) are pseudocolored in *blue*. Scale bar = 50 μm

spermatogonia. This initial population of type A spermatogonia is heterogeneous, containing both undifferentiated (A_{undiff}) and differentiating (A_{diff}) spermatogonia. Although it is challenging to reliably discern morphological differences in nuclear diameter, organelle organization, and chromatin structure (Kluin et al. 1982), by P3–4 spermatogonia differentially express established protein fate markers for the undifferentiated state such as "inhibitor of DNA binding 4" (ID4) (Chan et al. 2014; Oatley et al. 2011a) and "glial cell line-derived neurotrophic factor family receptor alpha 1" (GFRA1), or the differentiating state such as "stimulated by retinoic acid gene 8" (STRA8), and "kit oncogene" (KIT) (Niedenberger et al. 2015). It is currently unknown what mechanisms or signals direct spermatogonia to activate the expression of these markers, but their identification would significantly enhance our understanding of spermatogonial fate determination, both during the first wave of spermatogenesis and during adult steady-state spermatogenesis. It is clear that the A_{undiff} spermatogonial population contains both the foundational spermatogonial stem cell (SSC) pool as well as transit-amplifying progenitor spermatogonia that are poised to proliferate and differentiate on subsequent days of development.

In mice, the first round, or wave of spermatogenesis begins at ~P3, coincident with expression of differentiation markers by type A spermatogonia and culminates with the appearance of significant numbers of testicular spermatozoa by ~P35. The first wave proceeds in a highly organized manner, such that specific subtypes of mitotic spermatogonia, meiotic spermatocytes, and postmeiotic haploid spermatids appear at defined times in the postnatal testis, with minor strain-specific variation (Table 3.2).

Table 3.2 Approximate timing of appearance of cells during the first wave

Cell type	Approximate postnatal day of appearance in mice (Bellve et al. 1977; Janca et al. 1986; Nebel et al. 1961)	Approximate postnatal day of appearance in rats (Clermont and Perey 1957; Hilscher et al. 1974; Malkov et al. 1998)
Prospermatogonia	Through ~P2–3	Through ~P5–6
Type A_{undiff} spermatogonia	P2–3	P4
Type A_{diff} spermatogonia	P2–3	P4
Intermediate spermatogonia	P4	P6
Type B spermatogonia	P4–5	P6
Preleptotene spermatocytes	P8	P9–10
Leptotene spermatocytes	P10	P13–14
Zygotene spermatocytes	P12	P16–18
Pachytene spermatocytes	P14	P19–20
Round spermatids	P20	P24–26
Elongating spermatids	P24	P30–31
Condensed spermatids	P28	P36–37
Testicular sperm	~P30–35	~P42–44

3.3 Development of the Neonatal Spermatogonial Population

3.3.1 Timing and Regulation of Neonatal Spermatogonial Development

Spermatogenesis initiates in the neonatal testis with the transition of an apparently homogeneous population of prospermatogonia from P0–2 into a clearly heterogeneous population of spermatogonia by P3–4 (Kluin et al. 1982; Niedenberger et al. 2015; Hermann et al. 2015; Yoshida et al. 2006). These spermatogonia include the foundational pool of SSCs, the first progenitor spermatogonia, and the first differentiating spermatogonia. It is currently unclear how this diverse population of germ cells develops from the prospermatogonial pool, but there are two possibilities. First, the prospermatogonial pool may not be homogeneous as suspected, and subsets might be intrinsically preprogrammed to adopt these three spermatogonial fates. Second, all prospermatogonia may be equally capable of becoming SSCs or progenitor or differentiating spermatogonia, and this choice is made in response to cues present within their specialized microenvironment (niche). Although this latter option is a logical possibility, there is currently no direct evidence that one "niche" differs from another. What is clear, however, is that spermatogonial fate is maintained by the combined action of intrinsic and extrinsic signals. For example, the subset of undifferentiated spermatogonia containing the SSC population expresses GFRA1 and "ret proto-oncogene" (RET), which together form a co-receptor complex to bind "glial cell line derived neurotrophic factor" (GDNF) and activate pathways required for SSC self-renewal (reviewed in (Yang and Oatley 2014)). In contrast, another subset of spermatogonia responds to RA to activate pathways required for differentiation (Busada et al. 2014, 2015a; de Rooij 2001; Endo et al. 2015; Gely-Pernot et al. 2012; Ikami et al. 2015; Mark et al. 2015; Pellegrini et al. 2008; Snyder et al. 2010; van Pelt and de Rooij 1991).

3.3.2 Establishment of Spermatogonial Cell Fate Begins in the Neonatal Testis

Distinct spermatogonial fates are established by P6 in the mouse, with A_{undiff} (SSC and progenitor) and A_{diff} spermatogonia demarcated based on differential expression of mRNA and protein fate markers (Niedenberger et al. 2015; Hermann et al. 2015). Many of these markers have been directly correlated to spermatogonial fate based on analysis of the reproductive phenotypes of mice in which these genes have been deleted as well as by heterologous transplantation analyses, which measure the stem cell capacity of spermatogonia, or ability to repopulate a recipient testis that is devoid of germ cells (Niedenberger et al. 2015; Hermann et al. 2015; Yoshida et al. 2006; Oakberg 1956). Many protein markers have been identified that mark A_{undiff} spermatogonia, including CDH1 (Tokuda et al. 2007) ZBTB16/PLZF (Buaas et al. 2004; Costoya et al. 2004), GFRA1 (Buageaw et al. 2005; Grasso et al. 2012), RET

(Naughton et al. 2006), ITGA6 and ITGB1 (Shinohara et al. 1999), SALL4 (Gassei and Orwig 2013), PAX7 (Aloisio et al. 2014, 2017), and ID4 (see Fig. 3.3, (Chan et al. 2014; Oatley et al. 2011a)). A few have increased levels in A_{diff} spermatogonia, including SOHLH1 and SOHLH2 (Ballow et al. 2006a, b; Hao et al. 2008; Suzuki et al. 2012) and RHOX13 (Geyer and Eddy 2008; Geyer et al. 2012), or change their localization from nuclear to cytoplasmic such as FOXO1 (Goertz et al. 2011). Thus far, only two protein markers have been identified that are solely detectable in A_{diff} spermatogonia, STRA8 (Anderson et al. 2008; Hogarth et al. 2011; Oulad-Abdelghani et al. 1996; Zhou et al. 2008) and KIT (Busada et al. 2015a; Kissel et al. 2000; Packer et al. 1995; Prabhu et al. 2006; Yoshinaga et al. 1991).

The process of spermatogonial differentiation during the first week of life occurs in a manner analogous to singing a song in a round, with subsets of the spermatogonial population differentiating in succession. The commitment to differentiate is driven by the apparent exposure of discrete regions of the seminiferous cords to retinoic acid (RA) (Niedenberger et al. 2015; Busada et al. 2014, 2015a; Zhou et al. 2008). This differential response of spermatogonia to RA is essential for establishment of the asynchronous nature of spermatogenesis along the length of the seminiferous tubules that is seen in the adult testis. This asynchrony ensures the consistent production of sperm throughout the reproductive lifespan; at any given point in

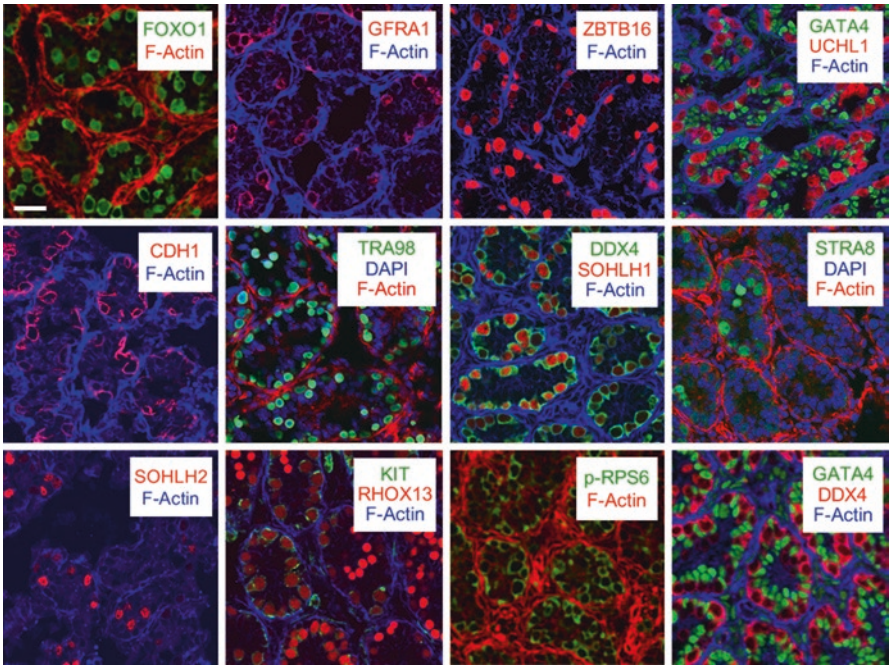

Fig. 3.3 Protein cell fate markers are differentially expressed in neonatal spermatogonia. Indirect immunofluorescence (IIF) was used to label proteins specified on each panel, and the text colors represent the color of the labeled protein. In some panels, DAPI labels nuclei, while in others fluorescently conjugated phalloidin labels F-Actin. Scale bar = 20 μm

time, numerous tubule segments are in stage VIII, which is when spermiation occurs. Several studies from the Griswold laboratory revealed that manipulating RA levels in neonates in vivo by addition of a bolus of exogenous RA or by blocking its synthesis using WIN 18,446 (also termed BDAD) significantly increased the synchronization of the stages of the seminiferous epithelium (Snyder et al. 2010; Snyder et al. 2011; Hogarth et al. 2011, 2013). The addition of exogenous RA to neonatal mice increases the number of STRA8+/KIT+ differentiating spermatogonia dramatically (Busada et al. 2014, 2015a), which reveals that many spermatogonia are poised to differentiate but by some unknown mechanism have not yet responded to endogenous RA. In contrast, WIN 18,446 treatment blocks RA synthesis, and therefore prevents spermatogonial differentiation. Upon treatment with exogenous RA, the majority of these A_{undiff} spermatogonia will simultaneously initiate differentiation, which leads to synchronization of the stages in the adult testis (Hogarth et al. 2011, 2013).

Once STRA8+/KIT+ A_{diff} spermatogonia initiate differentiation, they must proceed through successive stages (A_1, A_2, A_3, A_4, In, B) before entering meiosis as preleptotene spermatocytes at P8 (Oakberg 1956; Huckins 1971). It has been suggested that, following the appearance of preleptotene spermatocytes by P8, stages of the seminiferous epithelium can be assigned in juvenile mice, akin to those in the adult mouse testis (de Rooij, personal communication).

3.3.3 The First Wave of Spermatogenic Cells *May Not Be* Derived from SSCs

A unique feature of the first wave of spermatogenesis that has been demonstrated in mice is that, unlike subsequent waves, the first maturing germ cells may not originate from a self-renewing SSC pool. Instead, the spermatozoa produced from the first round of spermatogenesis that appear from ~P35 to P50 are thought to be derived directly from precursor prospermatogonia in the neonatal testis. There are three types of studies that support this notion.

The first type of studies used the characteristic morphology of prospermatogonia and both undifferentiated and differentiating type A spermatogonia to catalog their appearance during the first few days after birth. There are characteristic differences in nuclear shape and diameter, heterochromatin amount and localization, and nucleolar appearance that typify prospermatogonia and change during spermatogonial development (Chiarini-Garcia and Russell 2001; Drumond et al. 2011; Huckins 1971; Huckins and Oakberg 1978; Kluin and de Rooij 1981; Kluin et al. 1982; Oakberg 1956). The P0–1 testis contains an apparently homogeneous population of quiescent prospermatogonia. By P2, prospermatogonia begin to convert into type A spermatogonia, with some resembling undifferentiated (A_{undiff} and others resembling differentiating (A_{diff}, the first type being A_1). If A_1 spermatogonia do indeed form as early as P2, then one would expect to see later differentiating stages in subsequent days, and that is indeed the case. By P4, type A_3 and A_4 spermatogonia have appeared, followed closely by intermediate (In) spermatogonia. Type B

spermatogonia appear as early as P5–6, and preleptotene spermatocytes are observed by P7–8 (Kluin et al. 1982; Drumond et al. 2011). The timing of these sequential steps support the original differentiation of A_1 spermatogonia from prospermatogonia beginning at P2, although it is possible that a transient SSC-like intermediate was formed before differentiation.

The second type of studies utilized knockout (KO) mice. Numerous examples exist in the literature in which male mice carrying a mutation in a gene essential for SSC function are initially fertile as young adults (~P35–50), but then experience aging-related loss of the spermatogonial population (after ~P60). One clear example is provided by analyses of retinoblastoma (*Rb1*) male germ cell KO mice. When *Rb1* was conditionally deleted in prospermatogonia by the action of *Ddx4*-Cre transgene (Gallardo et al. 2007), males were initially fertile through ~P45, but become infertile after P60. A closer analysis of this phenotype indicates that *Rb1*-null SSCs fail to self-renew, and instead differentiate to enter meiosis during the first wave of spermatogenesis, leading to exhaustion of the SSC population (Hu et al. 2013; Yang et al. 2013). Germ cell KO mice for *Zbtb16/Plzf, Id4*, *Utp14b/jsd, Taf4b,* and *Etv5* led to a similar phenotype (Bradley et al. 2004; Buaas et al. 2004; Costoya et al. 2004; Falender et al. 2005; Hu et al. 2013; Lovasco et al. 2015; Tyagi et al. 2009; Yang et al. 2013). These types of studies underscore reduced role for SSCs in the first wave of spermatogenesis.

The third type of study used a lineage tracing approach. The "neurogenin 3" (*Ngn3*) gene is transcribed in A_{undiff} spermatogonia but not prospermatogonia or A_{diff} spermatogonia (Yoshida et al. 2004). Yoshida and colleagues took advantage of this expression pattern to irreversibly mark germ cells in which *Ngn3* was transcribed using an *Ngn3*-Cre transgenic mouse model. They reasoned that *Ngn3*+ germ cells had once passed through an undifferentiated spermatogonial stage, while *Ngn3*− germ cells had arisen directly from prospermatogonia. Their results indicate that ~60% of fertilizing sperm produced from the first wave originate from the *Ngn3*− population (Yoshida et al. 2006). This reveals that many first wave sperm did not originate from *Ngn3*+ undifferentiated spermatogonia, and that these sperm are indeed functional and capable of fertilization.

3.4 Development of the Somatic Cell Niche in the Mouse

Significant changes occur in the postnatal testis that affect Sertoli and peritubular myoid (PTM) cells, the two somatic cell types thought to be most involved in formation of the putative niche microenvironment based on their intimate association with spermatogonia at all phases of their development. Experimental evidence has supported this notion, which is described below.

Sertoli cells are positioned adjacent to spermatogonia within the seminiferous epithelium, and serve as "nurse cells" to support every phase of germ cell development. The Sertoli cell population increases dramatically as a result of cell proliferation from birth through approximately P15, when they cease dividing and remain mitotically quiescent. This is regulated in large part by thyroid hormone signaling,

which inhibits Sertoli cell proliferation, leading to their terminal differentiation (Cooke et al. 1991; Cooke and Meisami 1991; Hess et al. 1993; Holsberger et al. 2005; Van Haaster et al. 1992, 1993). In contrast, it has been shown that hypothyroidism, which can be induced experimentally by treatment with polythiouracil (PTU), extends the proliferation period, resulting in an ~30% increase in the Sertoli cell population (Joyce et al. 1993). The integral role for Sertoli cells in SSC niche formation was shown by the Oatley laboratory in experiments where they treated mice with PTU and discovered that increasing the numbers of Sertoli cells led to a greater than threefold increase in SSCs (Oatley et al. 2011b). This data reveals that the number of niches available for SSCs in the developing testis is limited, in essence by the number of Sertoli cells.

Another essential function of Sertoli cells after they differentiate during the first wave of spermatogenesis is the formation of the blood–testis barrier (BTB). This testis-specific adherens junction forms between adjacent Sertoli cells near the basement membrane. This extraordinarily tight junction is formed from tightly packed bundles of filamentous (F)-actin present between cisternae of endoplasmic reticulum and the apposing plasma membranes, and is also known as the basal ectoplasmic specialization (reviewed in (Cheng and Mruk 2012)). The BTB divides the seminiferous epithelium into adluminal and basal compartments, and provides a functional barrier to prevent passage of biomolecules and toxins from the circulatory and lymphatic systems into the adluminal compartment (Dym and Fawcett 1970; Fawcett et al. 1970; Hosoi et al. 2002; Russell 1977, 1978; Vitale et al. 1973). By doing so, it creates an immune-privileged area for germ cells to undergo meiosis and spermatid development (spermiogenesis). The basal compartment contains mitotic spermatogonia and preleptotene spermatocytes, the latter of which transit the BTB to enter meiosis in the adluminal compartment. Formation of the BTB is a critical process that is required for continuous spermatogenesis in the adult. However, it is interesting to note that male meiotic spermatocytes are already present in the juvenile testis prior to establishment of a functional BTB, and somehow are not recognized by the immune system since expression of the spermatogonial antigens induce a strong response in the adult.

Peritubular myoid (PTM) cells are a type of squamous smooth muscle cells that reside in the interstitium of the fetal testis and migrate to surround the exterior of the testis cords in the neonatal testis (Maekawa et al. 1996; Nurmio et al. 2012). Along with Sertoli cells, they provide structural support to the testis cords and tubules and secrete basement membrane components (Tung and Fritz 1987; Tung et al. 1984). In addition, in the adult the contraction of their extensive F-actin cytoskeleton provides a peristalsis-like motion to squeeze fluid containing immotile testicular sperm out of the tubules and into the rete testis (Ailenberg et al. 1990). It has more recently become apparent that PTM cells are also involved in directly regulating spermatogonial behavior. For years, it was thought that Sertoli cells were the only significant source of secreted GDNF, the ligand that binds to the GFRA1/RET co-receptors to help maintain spermatogonia in an undifferentiated state (Buageaw et al. 2005; He et al. 2008; Kubota et al. 2004b; Meng et al. 2000; Viglietto et al. 2000; Yomogida et al. 2003). Recently published results from the

Eddy laboratory reveal that PTM cells are also a requisite source of GDNF to maintain the A_{undiff} spermatogonial population in adult steady-state spermatogenesis (Chen et al. 2014, 2016).

3.5 Changes in Spermatogonial Gene Expression During the First Wave

There are dramatic changes in cell composition within the seminiferous cords during the first wave of spermatogenesis. The germ cell: somatic cell ratio increases as Sertoli cells stop proliferating, and the germ cell population expands dramatically through the proliferation and differentiation of the spermatogonial population and the accumulation of meiotic spermatocytes. Since progressively advanced populations of germ cells appear at predictable time points (Table 3.2), whole testis lysates can be prepared at different time points during the first wave, and gene product levels queried to provide important information about what germ cell population(s) express those genes. Global analyses of gene expression changes during postnatal development have been done at many different time points from birth through adulthood and have used two basic approaches prior to isolation of total RNA or protein. In the first, whole testes are immediately snap-frozen or lysed after removal from the body. In the second, single cell suspensions are generated by enzymatic and mechanical means, and germ and somatic cell populations isolated based on differences in size-based sedimentation through 2–4% bovine serum albumin (BSA) gradients (Bellve et al. 1977; Bryant et al. 2013), through differential expression of sortable cell surface markers (e.g. KIT, GFRA1, or THY1) (Hermann et al. 2015; Gassei et al. 2009; Hofmann et al. 2005; Morimoto et al. 2009; van der Wee et al. 2001; von Schonfeldt et al. 1999), or by fluorescence-activated cell sorting (FACS) based on the expression of transgenic reporter genes such as EGFP (Chan et al. 2014; Hermann et al. 2015). There is currently a need for the development of additional transgenic reporter mice with germ cell types differentially marked by expression of fluorescent reporter genes, as this would allow more labs to isolate pure populations of distinct germ cell populations.

There are inherent advantages and disadvantages to each of these approaches. The use of whole testis lysates limits the ability to assign gene expression to specific cell types, which is a distinct disadvantage. However, the isolation of single cell populations requires various manipulations and incubations over a several hour period, during which cells are maintained in various buffer conditions in oxygen levels that are 5–10 times higher than those in normal tissue (reviewed in (Carreau et al. 2011)). It is possible that steady-state levels of mRNA and protein change during these lengthy isolation procedures either because of altered rates of transcription/translation or degradation. Fortunately, multiple gene expression studies have been performed using both complementary approaches during the first wave of spermatogenesis (Vergouwen et al. 1993; Hoei-Hansen et al. 2004; Margolin et al. 2014; Schlecht et al. 2004; Schultz et al. 2003; Shima et al. 2004; Yu et al. 2003). Measured differences in the steady-state level of a particular mRNA during the first

wave of spermatogenesis must be interpreted with caution. These changes can reflect changes in transcription (generation of additional transcripts) or stability/decay (loss of transcripts), or from changes in the relative population of a particular cell type containing those mRNAs within the tissue. Interestingly, a number of the differences in mRNA abundance in undifferentiated vs. differentiating spermatogonia appear to involve genes whose products are required for meiosis (e.g. *Rec8*, *Stra8*, *Sycp3*, etc.). Therefore, it may be that the transcriptome changes do not reflect differences in spermatogonial function or biology, but rather preparation for the next phase of spermatogenesis (e.g. meiosis).

An overarching theme that has emerged from genome-wide expression analyses is that few genes appear to be differentially expressed (when considering mRNA levels) during spermatogonial development (Chan et al. 2014; Shima et al. 2004). However, the analysis of mRNA abundance using genome-wide approaches such as microarray and RNA-seq have utilized bulk populations of spermatogonia. A recent study quantified the mRNA levels of a large panel of genes whose products are involved in spermatogonial function using isolated single spermatogonia and a sensitive qRT-PCR based approach (Hermann et al. 2015). The results from this study reveal considerable heterogeneity in terms of mRNA abundance between undifferentiated and differentiating spermatogonia that was not found in previous studies using bulk populations of spermatogonia. Another theme reinforced from that study is that there is an apparent disconnect between mRNA and protein abundance for a number of genes.

Other recent studies have also found that the transcriptome does not always accurately predict the proteome (Li et al. 2014; Schwanhausser et al. 2011; Vogel and Marcotte 2012). Indeed, mRNAs may adopt and move between various fates including inefficient or efficient translation, storage, and decay. The dynamic regulation of mRNAs in this manner provides the conceptual basis of the "ribonome" (Mansfield and Keene 2009). This posttranscriptional regulation system involves the concerted action of translation initiation and elongation factors, RNA binding proteins, and mRNA decay machinery that can be in flux depending on the ever-changing needs of the cell. In support of this, our group has shown significant differences exist in the ribosome occupancy of ~3000 mRNAs during neonatal testis development (Chappell et al. 2013), including those for the essential differentiation factors KIT and "spermatogenesis and oogenesis specific basic helix-loop-helix" genes 1 and 2 (SOHLH1 and SOHLH2) (Busada et al. 2015a, b).

Multiple knockout studies have revealed the requirement for mRNA binding proteins (e.g. NANOS2, NANOS3, DAZL, TIAR/TIAL1, PIWIL2/MILI, PIWIL4/MIWI2, DDX4/VASA) in either repression or activation of translation during germ cell development. This underscores the importance of posttranscriptional control over gene expression during spermatogenesis. The most comprehensively studied example is NANOS2, whose expression is restricted to the male germline. NANOS2 is essential for maintenance of the male germline in the fetal testis; in its absence, prospermatogonia precociously express meiotic markers before dying by apoptosis prior to birth (Suzuki and Saga 2008; Suzuki et al. 2007; Tsuda et al. 2003). In the postnatal testis, NANOS2 is detectable in a subset of A_{undiff} spermatogonia

(primarily A_s and A_{pr}), and is required for stem cell self-renewal (Sada et al. 2009). Recent studies from that Saga laboratory have begun to reveal the mechanistic basis underlying the critical role of NANOS2 in suppressing germ cell differentiation and meiotic initiation. It is apparent that NANOS2 binds a subset of mRNAs in prospermatogonia and undifferentiated spermatogonia, likely through their 3′ UTRs, and facilitates their degradation in P-bodies through an interaction with the CCR4-NOT deadenylation complex (Geyer et al. 2012; Suzuki et al. 2010; Zhou et al. 2015). Several of these mRNAs encode proteins normally expressed later during spermatogenesis (e.g. STRA8, TAF7L, RHOX13, SYCP3 (Geyer et al. 2012; Suzuki et al. 2010), and therefore it is likely that NANOS2 regulates the proteome of prospermatogonia and undifferentiated spermatogonia by posttranscriptionally repressing expression of factors involved in differentiation and meiotic initiation.

3.6 Maintaining the Genome in First Wave Spermatogonia

The functional role of sperm is to deliver an intact haploid male genome to the egg at fertilization that can successfully direct development of the resulting embryo. There are two opposing forces that must be balanced during gametogenesis: (1) maintaining a high level of genome integrity to prevent the passage of deleterious mutations to the offspring, while (2) allowing rare de novo mutations to arise in order for evolutionary change to occur. In general, however, the relatively low number of mutations in the germline provide the basis for the "disposable soma theory," which posits that the germline has reduced mutation frequencies as compared to somatic cells, which do not contribute to the next generation (Kirkwood 1977). In support of this concept, studies from the Walter and McCarrey laboratories have shown that multiple male germ cell types examined during the first wave of spermatogenesis have DNA mutation frequencies that are 5–10 times lower than same-aged somatic cells (Intano et al. 2001, 2002; Walter et al. 1998). Within the P6 spermatogonial population, DNA mutation frequency is 3.8-fold lower in Thy1+ spermatogonia (Murphey et al. 2013). This subset of spermatogonia is enriched for SSCs, whose progeny become gametes during subsequent waves of spermatogenesis (Kubota et al. 2003, 2004b). Therefore, it seems that the putative SSC population has some mechanism, even as early as P6, to maintain lower mutation levels than Thy1− spermatogonia, most of which represent progenitor and differentiating spermatogonia. In addition, as germ cell development proceeds during the first wave, type B spermatogonia and preleptotene, leptotene, and zygotene spermatocytes had progressively lower mutation frequencies relative to a mixed population of type A spermatogonia (Walter et al. 1998).

There are two mechanisms that would be predicted to contribute to a reduced DNA mutation load in the male germline, and these are both active during the first wave of spermatogenesis, enhanced DNA repair, and apoptosis. It was found that germ cells had significantly higher rates of base excision repair (BER) activity than a panel of somatic tissues (Intano et al. 2001, 2002). BER is central to a low mutation frequency in the germline. Disruptions in BER result in elevated mutagenesis (Allan et al. 1992). The BER protein, APE1 plays

a key role in regulation germline mutagenesis as decreases in APE1 result in elevated mutation frequencies while elevated APE1 protects against germline mutagenesis. Also, type A and B spermatogonia exhibited increased ability to repair UV-induced lesions by nucleotide excision repair (NER) as compared to spermatocytes and spermatids (Xu et al. 2005).

There is also a relatively high rate of male germ cell apoptosis, particularly during the first wave of spermatogenesis. The levels of germ cell apoptosis are low in the neonatal mouse testis, with <10% of germ cells affected at P3, P5, and P7, respectively (Mori et al. 1997). However, the incidence increases dramatically at P8–10 and remains high through P22–30, mostly affecting spermatogonia and spermatocytes (Allan et al. 1992; Brinkworth et al. 1995; Clermont and Perey 1957; Mori et al. 1997). It is unclear why the incidence is so high during the first wave. Changes in hormone levels can affect apoptosis, and both extrinsic and intrinsic apoptotic pathways are sensitive to hormonal modulation (Shaha et al. 2010). An example of this is the observation that the peak of apoptosis is delayed by neonatal administration of the goitrogen propylthiouracil PTU, which makes animals hypothyroid and also delays Sertoli differentiation (Silva et al. 2011). It has been shown that apoptosis during the second week of life is dependent upon the action of the cysteine protease caspases, which mediate the intrinsic pathway; inhibition of caspase-2 by Z-VDVAD-FMK, blocked apoptosis in P15–16 mice (Furuchi et al. 1996; Knudson et al. 1995; Moreno et al. 2006; Zheng et al. 2006). The increased susceptibility to apoptosis during the first wave is due to high levels of p53-dependent expression of mediators such as BAX and BAD (Yan et al. 2000). There is less evidence for the involvement of the extrinsic pathway (via death receptors) for regulating/initiating apoptosis in rodent germ cells (Shaha et al. 2010).

It has been suggested that apoptosis during the first wave of spermatogenesis may participate in maturation of the seminiferous epithelium, possibly aiding in luminal formation within the developing tubules, which occurs around P14 (see Fig. 3.2, Billig et al. 1995). Since, as mentioned above, the majority of apoptotic cells during this time are spermatogonia and spermatocytes, there may be something inherently different about the initial cohort of male germ cells that develop directly from the precursor prospermatogonia rather than from SSCs. This is purely speculative at this point, but it does provide a potential explanation for the increase in apoptosis from ~P8 to P22 that was observed by the Eddy laboratory (Mori et al. 1997).

3.7 Differences Between First and Subsequent Waves

KO studies have revealed that there are differences in the requirements of spermatogonia in first and subsequent waves of spermatogenesis. As described above, there are multiple examples of gene products whose function is dispensable for the first wave, but required during steady-state spermatogenesis in the adult. This has been interpreted to mean that the first wave does not depend on SSC function, but it could

also indicate that there are important regulatory differences in germ cell development between first and subsequent waves.

There are a few KO studies that support this notion. Mice with germ cell deletion of "retinoic acid receptor gamma" (*Rarg*) had an apparently normal first wave of spermatogenesis, but increasingly lost differentiating spermatogonia in subsequent waves (Gely-Pernot et al. 2012). In contrast, KO mice have also been generated that exhibit a defective first wave of spermatogenesis, but that phenotype improves during subsequent waves. Another example is provided by the conditional deletion of *Rdh10* (Tong et al. 2013). It was found to be required in Sertoli cells for a normal first wave (deletion by *Amh*-Cre); there were very few differentiating spermatogonia or spermatocytes at P14 and P21. The phenotype was worse in testes of juvenile mice with RDH10 absent from both Sertoli cells and spermatogonia (deletion by *Stra8*-Cre). Both KO models were infertile. Interestingly, the phenotype improved such that adult (>9 week old) *Rdh10* KO mice had nearly normal-appearing seminiferous tubules and were fertile. Another example is provided in testes of mice following *Cyp26b1* deletion in fetal Sertoli cells (Hogarth et al. 2015; Li et al. 2009). There were a number of defects in the first wave of spermatogenesis that improved in subsequent waves. In a recent study from our laboratory, we reported that *Rhox13* KO mice on a C57BL/6 had a number of germ cell defects during the first wave including increased apoptosis, delayed appearance of round spermatids, and a twofold reduction in cauda sperm counts in young adult males (Busada et al. 2016). However, these defects were not observed in older adult mice undergoing steady-state spermatogenesis. In another study, mice lacking *E2f1* on a C57BL/6 genetic background had increased spermatogonial apoptosis during the first wave, and then a defect in SSC self-renewal during adult steady-state spermatogenesis (Rotgers et al. 2015). Constitutive and germ cell deletion of *Sox3* (*Sox3*$^{-/y}$ and *Sox3*$^{fl/fl}$;*Ddx4*-Cre) resulted in dramatic reductions in male germ cells during the first wave of spermatogenesis, with P20 tubules containing only undifferentiated spermatogonia (Laronda and Jameson 2011; Raverot et al. 2005). However, spermatogenesis was surprisingly recovered in adulthood, with 75% and 80% of tubules containing the full complement of germ cells at P56 and P84, respectively (Laronda and Jameson 2011). These results indicate that the transcription factor SOX3 is important for spermatogonial differentiation during the first wave of spermatogenesis, but dispensable for differentiation from SSCs in subsequent waves.

One of the possible reasons underlying differences between the first and subsequent waves of spermatogenesis may be anatomical in nature. The testes are incompletely descended into the scrotum during the first wave in rodents, and as a consequence spermatogenesis proceeds at or near the body temperature of 37 °C. After descent into the scrotum, spermatogenesis occurs at approximately 33 °C. In cryptorchid testes, there is a high incidence of CIS, the precursor lesion to most forms of testicular cancer (reviewed in Ferguson and Agoulnik 2013). The CIS is thought to originate from undifferentiated germ cells that failed to differentiate properly at the onset of puberty (Gondos et al. 1983; Hoei-Hansen et al. 2004; Skakkebaek et al. 2001; Sonne et al. 2008).

3.8 Conclusions

This chapter highlights various aspects of spermatogonial development that occur during the first wave of spermatogenesis, with a particular emphasis on the mouse since it has been the most extensively studied model to date. All mammals must have at least one first wave of spermatogenesis, with seasonal breeders having one at the beginning of each resumption of spermatogenesis. It is evident from studies in mice that the first wave of spermatogenesis is highly similar to subsequent waves, although there are subtle differences that have been uncovered through gene expression and KO mouse studies.

What is the utility of the first wave of spermatogenesis? There are at least two possibilities. First, it is well-established in mice that the stepwise differentiation of subsets of spermatogonia in the neonatal testis is responsible for the highly organized seminiferous epithelium in the adult testis that can be subdivided into discrete stages. This may be of potential benefit, as it allows for distinct stages to be influenced by different morphogens and hormones to efficiently regulate gene expression [188]. This level of organization is less obvious in the human testis, although the seminiferous epithelium can be subdivided into patches that contain germ cells in specific stages of development [189]. Second, in the wild, most animals live a rather perilous and often short existence fraught with dangers of predation and disease. Therefore, only productive mating encounters that result in pregnancy will lead to passage of a male's genes on to the next generation. Therefore, the production of functional sperm from the first wave of spermatogenesis allows males to be fertile earlier, (e.g. ~P35–P42 in mice) *before* the first SSC-derived sperm are produced.

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References

Abdel-Raouf M (1960) The postnatal development of the reproductive organs in bulls with special reference to puberty (incluing growth of the hypophysis and the adrenals). Acta Endocrinol Suppl (Copenh) 34(Suppl 49):1–109

Abel MH et al (2008) Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen. Endocrinology 149(7):3279–3285

Ailenberg M, Tung PS, Fritz IB (1990) Transforming growth factor-beta elicits shape changes and increases contractility of testicular peritubular cells. Biol Reprod 42(3):499–509

Allan DJ, Harmon BV, Roberts SA (1992) Spermatogonial apoptosis has three morphologically recognizable phases and shows no circadian rhythm during normal spermatogenesis in the rat. Cell Prolif 25(3):241–250

Aloisio GM et al (2014) PAX7 expression defines germline stem cells in the adult testis. J Clin Invest 124(9):3929–3944

Aloisio GM et al (2017) Visualization and lineage tracing of Pax7+ spermatogonial stem cells in the mouse. Methods Mol Biol 1463:139–154
Anderson EL et al (2008) Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc Natl Acad Sci U S A 105(39):14976–14980
Ballow D et al (2006a) Sohlh1 is essential for spermatogonial differentiation. Dev Biol 294(1):161–167
Ballow DJ et al (2006b) Sohlh2 is a germ cell-specific bHLH transcription factor. Gene Expr Patterns 6(8):1014–1018
Bellve AR et al (1977) Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol 74(1):68–85
Billig H et al (1995) Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages. Endocrinology 136(1):5–12
Bradley J et al (2004) An X-to-autosome retrogene is required for spermatogenesis in mice. Nat Genet 36(8):872–876
Brinkworth MH et al (1995) Identification of male germ cells undergoing apoptosis in adult rats. J Reprod Fertil 105(1):25–33
Bronson FH (2009) Climate change and seasonal reproduction in mammals. Philos Trans R Soc Lond Ser B Biol Sci 364(1534):3331–3340
Brown WB et al (1979) Effects of photoperiod on food intake, sexual condition and hormone concentrations in stags and rams [proceedings]. J Physiol 296:58p–59p
Bryant JM et al (2013) Separation of spermatogenic cell types using STA-PUT velocity sedimentation. J Vis Exp (80)
Buaas FW et al (2004) Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 36(6):647–652
Buageaw A et al (2005) GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol Reprod 73(5):1011–1016
Busada JT et al (2014) Retinoic acid induces multiple hallmarks of the prospermatogonia-to-spermatogonia transition in the neonatal mouse. Biol Reprod 90(3):64
Busada JT et al (2015a) Retinoic acid regulates Kit translation during spermatogonial differentiation in the mouse. Dev Biol 397(1):140–149
Busada JT et al (2015b) Mammalian target of rapamycin complex 1 (mTORC1) is required for mouse spermatogonial differentiation in vivo. Dev Biol 407(1):90–102
Busada JT et al (2016) Rhox13 is required for a quantitatively normal first wave of spermatogenesis in mice. Reproduction 152(5):379–388
Carreau A et al (2011) Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. J Cell Mol Med 15(6):1239–1253
Chan F et al (2014) Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev 28(12):1351–1362
Chappell VA et al (2013) Translational activation of developmental messenger RNAs during neonatal mouse testis development. Biol Reprod 89(3):61
Chen LY et al (2014) Peritubular myoid cells participate in male mouse spermatogonial stem cell maintenance. Endocrinology 155(12):4964–4974
Chen LY, Willis WD, Eddy EM (2016) Targeting the Gdnf Gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. Proc Natl Acad Sci U S A 113(7):1829–1834
Cheng CY, Mruk DD (2012) The blood-testis barrier and its implications for male contraception. Pharmacol Rev 64(1):16–64
Chiarini-Garcia H, Russell LD (2001) High-resolution light microscopic characterization of mouse spermatogonia. Biol Reprod 65(4):1170–1178
Clarke LA et al (1995) Expression of the prolactin receptor gene during the breeding and non-breeding seasons in red deer (Cervus elaphus): evidence for the expression of two forms in the testis. J Endocrinol 146(2):313–321
Clermont Y, Perey B (1957) Quantitative study of the cell population of the seminiferous tubules in immature rats. Am J Anat 100(2):241–267

Cooke PS, Meisami E (1991) Early hypothyroidism in rats causes increased adult testis and reproductive organ size but does not change testosterone levels. Endocrinology 129(1):237–243
Cooke PS et al (1991) Increased sperm production in adult rats after transient neonatal hypothyroidism. Endocrinology 129(1):244–248
Costoya JA et al (2004) Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 36(6):653–659
Culty M (2009) Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Res C Embryo Today 87(1):1–26
Curtis SK, Amann RP (1981) Testicular development and establishment of spermatogenesis in Holstein bulls. J Anim Sci 53(6):1645–1657
Dadhich RK et al (2013) Identification of live germ-cell desquamation as a major mechanism of seasonal testis regression in mammals: a study in the Iberian mole (Talpa occidentalis). Biol Reprod 88(4):101
de Rooij DG (2001) Proliferation and differentiation of spermatogonial stem cells. Reproduction 121(3):347–354
Denolet E et al (2006) The effect of a sertoli cell-selective knockout of the androgen receptor on testicular gene expression in prepubertal mice. Mol Endocrinol 20(2):321–334
Drickamer LC (1990) Seasonal variation in fertility, fecundity and litter sex ratio in laboratory and wild stocks of house mice (Mus domesticus). Lab Anim Sci 40(3):284–288
Drumond AL, Meistrich ML, Chiarini-Garcia H (2011) Spermatogonial morphology and kinetics during testis development in mice: a high-resolution light microscopy approach. Reproduction 142(1):145–155
Dym M, Fawcett DW (1970) The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. Biol Reprod 3(3):308–326
Endo T et al (2015) Periodic retinoic acid-STRA8 signaling intersects with periodic germ-cell competencies to regulate spermatogenesis. Proc Natl Acad Sci U S A 112(18):E2347–E2356
Falender AE et al (2005) Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. Genes Dev 19(7):794–803
Fawcett DW, Leak LV, Heidger PM Jr (1970) Electron microscopic observations on the structural components of the blood-testis barrier. J Reprod Fertil Suppl 10:105–122
Ferguson L, Agoulnik AI (2013) Testicular cancer and cryptorchidism. Front Endocrinol (Lausanne) 4:32
Furuchi T et al (1996) Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. Development 122(6):1703–1709
Gallardo T et al (2007) Generation of a germ cell-specific mouse transgenic Cre line, Vasa-Cre. Genesis 45(6):413–417
Gassei K, Orwig KE (2013) SALL4 expression in gonocytes and spermatogonial clones of postnatal mouse testes. PLoS One 8(1):e53976
Gassei K, Ehmcke J, Schlatt S (2009) Efficient enrichment of undifferentiated GFR alpha 1+ spermatogonia from immature rat testis by magnetic activated cell sorting. Cell Tissue Res 337(1):177–183
Gely-Pernot A et al (2012) Spermatogonia differentiation requires retinoic acid receptor gamma. Endocrinology 153(1):438–449
Geyer CB, Eddy EM (2008) Identification and characterization of Rhox13, a novel X-linked mouse homeobox gene. Gene 423(2):194–200
Geyer CB et al (2012) Rhox13 is translated in premeiotic germ cells in male and female mice and is regulated by NANOS2 in the male. Biol Reprod 86(4):127
Goertz MJ et al (2011) Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. J Clin Invest 121(9):3456–3466
Gondos B, Byskov AG (1981) Germ cell kinetics in the neonatal rabbit testis. Cell Tissue Res 215(1):143–151
Gondos B, Renston RH, Conner LA (1973) Ultrastructure of germ cells and Sertoli cells in the postnatal rabbit testis. Am J Anat 136(4):427–439

Gondos B, Berthelsen JG, Skakkebaek NE (1983) Intratubular germ cell neoplasia (carcinoma in situ): a preinvasive lesion of the testis. Ann Clin Lab Sci 13(3):185–192
Grasso M et al (2012) Distribution of GFRA1-expressing spermatogonia in adult mouse testis. Reproduction 143(3):325–332
Hao J et al (2008) Sohlh2 knockout mice are male-sterile because of degeneration of differentiating type A spermatogonia. Stem Cells 26(6):1587–1597
He Z et al (2008) Gdnf upregulates c-Fos transcription via the Ras/Erk1/2 pathway to promote mouse spermatogonial stem cell proliferation. Stem Cells 26(1):266–278
Hermann BP et al (2015) Transcriptional and translational heterogeneity among neonatal mouse spermatogonia. Biol Reprod 92(2):54
Hess RA et al (1993) Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli and germ cell numbers. Endocrinology 132(6):2607–2613
Hilscher W, Hilscher B (1976) Kinetics of the male gametogenesis. Andrologia 8(2):105–116
Hilscher B et al (1974) Kinetics of gametogenesis. I. Comparative histological and autoradiographic studies of oocytes and transitional prospermatogonia during oogenesis and prespermatogenesis. Cell Tissue Res 154(4):443–470
Hoei-Hansen CE et al (2004) Identification of genes differentially expressed in testes containing carcinoma in situ. Mol Hum Reprod 10(6):423–431
Hofmann MC, Braydich-Stolle L, Dym M (2005) Isolation of male germ-line stem cells; influence of GDNF. Dev Biol 279(1):114–124
Hogarth CA et al (2011) Suppression of Stra8 expression in the mouse gonad by WIN 18,446. Biol Reprod 84(5):957–965
Hogarth CA et al (2013) Turning a spermatogenic wave into a tsunami: synchronizing murine spermatogenesis using WIN 18,446. Biol Reprod 88(2):40
Hogarth CA et al (2015) CYP26 enzymes are necessary within the postnatal seminiferous epithelium for normal murine spermatogenesis. Biol Reprod 93(1):19
Holsberger DR, Kiesewetter SE, Cooke PS (2005) Regulation of neonatal Sertoli cell development by thyroid hormone receptor alpha1. Biol Reprod 73(3):396–403
Hosoi I et al (2002) Development of the blood-testis barrier in the mouse is delayed by neonatally administered diethylstilbestrol but not by beta-estradiol 3-benzoate. Andrologia 34(4):255–262
Hu YC, de Rooij DG, Page DC (2013) Tumor suppressor gene Rb is required for self-renewal of spermatogonial stem cells in mice. Proc Natl Acad Sci U S A 110(31):12685–12690
Huckins C (1971) The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec 169(3):533–557
Huckins C, Oakberg EF (1978) Morphological and quantitative analysis of spermatogonia in mouse testes using whole mounted seminiferous tubules, I. The normal testes. Anat Rec 192(4):519–528
Ikami K et al (2015) Hierarchical differentiation competence in response to retinoic acid ensures stem cell maintenance during mouse spermatogenesis. Development 142(9):1582–1592
Intano GW et al (2001) Mixed spermatogenic germ cell nuclear extracts exhibit high base excision repair activity. Nucleic Acids Res 29(6):1366–1372
Intano GW et al (2002) Base excision repair is limited by different proteins in male germ cell nuclear extracts prepared from young and old mice. Mol Cell Biol 22(7):2410–2418
Janca FC, Jost LK, Evenson DP (1986) Mouse testicular and sperm cell development characterized from birth to adulthood by dual parameter flow cytometry. Biol Reprod 34(4):613–623
Jimenez R, Burgos M, Barrionuevo FJ (2015) Circannual testis changes in seasonally breeding mammals. Sex Dev 9(4):205–215
Joyce KL, Porcelli J, Cooke PS (1993) Neonatal goitrogen treatment increases adult testis size and sperm production in the mouse. J Androl 14(6):448–455
Kirkwood TB (1977) Evolution of ageing. Nature 270(5635):301–304
Kissel H et al (2000) Point mutation in kit receptor tyrosine kinase reveals essential roles for kit signaling in spermatogenesis and oogenesis without affecting other kit responses. EMBO J 19(6):1312–1326

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(4):475–493
Kluin PM, Kramer MF, de Rooij DG (1982) Spermatogenesis in the immature mouse proceeds faster than in the adult. Int J Androl 5(3):282–294
Knudson CM et al (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270(5233):96–99
Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A 100(11):6487–6492
Kubota H, Avarbock MR, Brinster RL (2004a) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 101(47):16489–16494
Kubota H, Avarbock MR, Brinster RL (2004b) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71(3):722–731
Laronda MM, Jameson JL (2011) Sox3 functions in a cell-autonomous manner to regulate spermatogonial differentiation in mice. Endocrinology 152(4):1606–1615
Li H et al (2009) Cyp26b1 expression in murine Sertoli cells is required to maintain male germ cells in an undifferentiated state during embryogenesis. PLoS One 4(10):e7501
Li JJ, Bickel PJ, Biggin MD (2014) System wide analyses have underestimated protein abundances and the importance of transcription in mammals. PeerJ 2:e270
Linder CC et al (1991) Expression of receptors during the cycle of the seminiferous epithelium. Ann N Y Acad Sci 637:313–321
Lovasco LA et al (2015) TAF4b is required for mouse spermatogonial stem cell development. Stem Cells 33(4):1267–1276
Luaces JP et al (2013) Spermatogenesis is seasonal in the large hairy armadillo, Chaetophractus villosus (Dasypodidae, Xenarthra, Mammalia). Reprod Fertil Dev 25(3):547–557
Maekawa M, Kamimura K, Nagano T (1996) Peritubular myoid cells in the testis: their structure and function. Arch Histol Cytol 59(1):1–13
Malkov M, Fisher Y, Don J (1998) Developmental schedule of the postnatal rat testis determined by flow cytometry. Biol Reprod 59(1):84–92
Mansfield KD, Keene JD (2009) The ribonome: a dominant force in co-ordinating gene expression. Biol Cell 101(3):169–181
Margolin G et al (2014) Integrated transcriptome analysis of mouse spermatogenesis. BMC Genomics 15:39
Mark M et al (2015) Role of retinoic acid receptor (RAR) signaling in post-natal male germ cell differentiation. Biochim Biophys Acta 1849(2):84–93
McCarrey JR (2013) Toward a more precise and informative nomenclature describing fetal and neonatal male germ cells in rodents. Biol Reprod 89(2):47
McLean DJ et al (2003) Characterization of spermatogonial stem cell maturation and differentiation in neonatal mice. Biol Reprod 69(6):2085–2091
Meng X et al (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287(5457):1489–1493
Moreno RD et al (2006) Caspase activation throughout the first wave of spermatogenesis in the rat. Cell Tissue Res 325(3):533–540
Mori C et al (1997) Morphological analysis of germ cell apoptosis during postnatal testis development in normal and Hsp 70-2 knockout mice. Dev Dyn 208(1):125–136
Morimoto H et al (2009) Phenotypic plasticity of mouse spermatogonial stem cells. PLoS One 4(11):e7909
Moustafa MNK et al (2015) Morphological and morphometric study of the development of seminiferous epithelium of donkey (Equus asinus) from birth to maturity. J Cytol Histol 6(6):370
Muciaccia B et al (2013) Novel stage classification of human spermatogenesis based on acrosome development. Biol Reprod 89(3):60
Murphey P et al (2013) Enhanced genetic integrity in mouse germ cells. Biol Reprod 88(1):6
Naughton CK et al (2006) Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol Reprod 74(2):314–321

Nebel BR, Amarose AP, Hacket EM (1961) Calendar of gametogenic development in the prepuberal male mouse. Science 134(3482):832–833
Nelson RJ, Gubernick DJ, Blom JM (1995) Influence of photoperiod, green food, and water availability on reproduction in male California mice (Peromyscus californicus). Physiol Behav 57(6):1175–1180
Neves ES, Chiarini-Garcia H, Franca LR (2002) Comparative testis morphometry and seminiferous epithelium cycle length in donkeys and mules. Biol Reprod 67(1):247–255
Niedenberger BA, Busada JT, Geyer CB (2015) Marker expression reveals heterogeneity of spermatogonia in the neonatal mouse testis. Reproduction 149(4):329–338
Nurmio M et al (2012) Peritubular myoid cells have a role in postnatal testicular growth. Spermatogenesis 2(2):79–87
Oakberg EF (1956) Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. Am J Anat 99(3):507–516
Oatley MJ et al (2011a) 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(2):347–356
Oatley MJ, Racicot KE, Oatley JM (2011b) Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. Biol Reprod 84(4):639–645
Oulad-Abdelghani M et al (1996) Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. J Cell Biol 135(2):469–477
Packer AI, Besmer P, Bachvarova RF (1995) Kit ligand mediates survival of type A spermatogonia and dividing spermatocytes in postnatal mouse testes. Mol Reprod Dev 42(3):303–310
Paniagua R, Nistal M (1984) Morphological and histometric study of human spermatogonia from birth to the onset of puberty. J Anat 139(Pt 3):535–552
Pellegrini M et al (2008) ATRA and KL promote differentiation toward the meiotic program of male germ cells. Cell Cycle 7(24):3878–3888
Plant TM, Marshall GR (2001) The functional significance of FSH in spermatogenesis and the control of its secretion in male primates. Endocr Rev 22(6):764–786
Prabhu SM et al (2006) Expression of c-Kit receptor mRNA and protein in the developing, adult and irradiated rodent testis. Reproduction 131(3):489–499
Ramaswamy S, Weinbauer GF (2014) Endocrine control of spermatogenesis: role of FSH and LH/testosterone. Spermatogenesis 4(2):e996025
Raverot G et al (2005) Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. Dev Biol 283(1):215–225
Robeck TR, Monfort SL (2006) Characterization of male killer whale (Orcinus orca) sexual maturation and reproductive seasonality. Theriogenology 66(2):242–250
Roosen-Runge EC, Leik J (1968) Gonocyte degeneration in the postnatal male rat. Am J Anat 122(2):275–299
Rotgers E et al (2015) E2F1 controls germ cell apoptosis during the first wave of spermatogenesis. Andrology 3(5):1000–1014
Russell L (1977) Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. Am J Anat 148(3):313–328
Russell LD (1978) The blood-testis barrier and its formation relative to spermatocyte maturation in the adult rat: a lanthanum tracer study. Anat Rec 190(1):99–111
Sada A et al (2009) The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science 325(5946):1394–1398
Sanchez B et al (1993) Postnatal development of seminiferous tubules in the cat. J Reprod Fertil Suppl 47:343–348
Sarma K, Devi J (2012) Changes in the seminiferous epithelium of the testes during postnatal development in assam goat. Anat Res Int 2012:620924
Schlecht U et al (2004) Expression profiling of mammalian male meiosis and gametogenesis identifies novel candidate genes for roles in the regulation of fertility. Mol Biol Cell 15(3):1031–1043

Schultz N, Hamra FK, Garbers DL (2003) A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. Proc Natl Acad Sci U S A 100(21):12201–12206

Schwanhausser B et al (2011) Global quantification of mammalian gene expression control. Nature 473(7347):337–342

Shaha C, Tripathi R, Mishra DP (2010) Male germ cell apoptosis: regulation and biology. Philos Trans R Soc Lond Ser B Biol Sci 365(1546):1501–1515

Shima JE et al (2004) The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod 71(1):319–330

Shinohara T, Avarbock MR, Brinster RL (1999) beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 96(10):5504–5509

Silva D et al (2011) Propylthiouracil-induced hypothyroidism delays apoptosis during the first wave of spermatogenesis. Biol Res 44(2):181–188

Skakkebaek NE, Rajpert-De Meyts E, Main KM (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 16(5):972–978

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

Snyder EM et al (2011) Exposure to retinoic acid in the neonatal but not adult mouse results in synchronous spermatogenesis. Biol Reprod 84(5):886–893

Sonne SB et al (2008) Testicular dysgenesis syndrome and the origin of carcinoma in situ testis. Int J Androl 31(2):275–287

Suzuki A, Saga Y (2008) Nanos2 suppresses meiosis and promotes male germ cell differentiation. Genes Dev 22(4):430–435

Suzuki A, Tsuda M, Saga Y (2007) Functional redundancy among Nanos proteins and a distinct role of Nanos2 during male germ cell development. Development 134:77–83

Suzuki A et al (2010) NANOS2 interacts with the CCR4-NOT deadenylation complex and leads to suppression of specific RNAs. Proc Natl Acad Sci U S A 107(8):3594–3599

Suzuki H et al (2012) SOHLH1 and SOHLH2 coordinate spermatogonial differentiation. Dev Biol 361(2):301–312

Tiptanavattana N et al (2015) Determination phase at transition of gonocytes to spermatogonial stem cells improves establishment efficiency of spermatogonial stem cells in domestic cats. J Reprod Dev 61(6):581–588

Tokuda M et al (2007) CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol Reprod 76(1):130–141

Tong MH et al (2013) Retinol dehydrogenase 10 is indispensible for spermatogenesis in juvenile males. Proc Natl Acad Sci U S A 110(2):543–548

Tsuda M et al (2003) Conserved role of nanos proteins in germ cell development. Science 301(5637):1239–1241

Tung PS, Fritz IB (1987) Morphogenetic restructuring and formation of basement membranes by Sertoli cells and testis peritubular cells in co-culture: inhibition of the morphogenetic cascade by cyclic AMP derivatives and by blocking direct cell contact. Dev Biol 120(1):139–153

Tung PS, Skinner MK, Fritz IB (1984) Cooperativity between Sertoli cells and peritubular myoid cells in the formation of the basal lamina in the seminiferous tubule. Ann N Y Acad Sci 438:435–446

Tyagi G et al (2009) Loss of Etv5 decreases proliferation and RET levels in neonatal mouse testicular germ cells and causes an abnormal first wave of spermatogenesis. Biol Reprod 81(2):258–266

Tyndale-Biscoe H, Renfree M (1987) Reproductive physiology of marsupials. Monographs on marsupial biology. Cambridge University Press, New York

Urian KW et al (1996) Seasonality of reproduction in bottlenose dolphins, Tursiops truncatus. J Mammal 77(2):394–403

van der Wee KS et al (2001) Immunomagnetic isolation and long-term culture of mouse type A spermatogonia. J Androl 22(4):696–704

Van Haaster LH et al (1992) The effect of hypothyroidism on Sertoli cell proliferation and differentiation and hormone levels during testicular development in the rat. Endocrinology 131(3):1574–1576
van Haaster LH et al (1993) High neonatal triiodothyronine levels reduce the period of Sertoli cell proliferation and accelerate tubular lumen formation in the rat testis, and increase serum inhibin levels. Endocrinology 133(2):755–760
van Pelt AM, de Rooij DG (1991) Retinoic acid is able to reinitiate spermatogenesis in vitamin A-deficient rats and high replicate doses support the full development of spermatogenic cells. Endocrinology 128(2):697–704
Vergouwen RP et al (1991) Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J Reprod Fertil 93(1):233–243
Vergouwen RP et al (1993) Postnatal development of testicular cell populations in mice. J Reprod Fertil 99(2):479–485
Viglietto G et al (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(4):689–694
Vitale R, Fawcett DW, Dym M (1973) The normal development of the blood-testis barrier and the effects of clomiphene and estrogen treatment. Anat Rec 176(3):331–344
Vogel C, Marcotte EM (2012) Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. Nat Rev Genet 13(4):227–232
von Schonfeldt V et al (1999) Magnetic cell sorting is a fast and effective method of enriching viable spermatogonia from Djungarian hamster, mouse, and marmoset monkey testes. Biol Reprod 61(3):582–589
Walker WH (2011) Testosterone signaling and the regulation of spermatogenesis. Spermatogenesis 1(2):116–120
Walter CA et al (1998) Mutation frequency declines during spermatogenesis in young mice but increases in old mice. Proc Natl Acad Sci U S A 95(17):10015–10019
Western P et al (2008) Dynamic regualtion of mitotic arrest in fetal male germ cells. Stem Cells 26(2):339–347
White D, Berardinelli JG, Aune KE (2005) Seasonal differences in spermatogenesis, testicular mass, and serum testosterone concentrations in the grizzly bear. Ursus 16(2):198–207
Wrobel KH (2000) Prespermatogenesis and spermatogoniogenesis in the bovine testis. Anat Embryol (Berl) 202(3):209–222
Wrobel KH et al (1995) Evolution and ultrastructure of the bovine spermatogonia precursor cell line. Cell Tissue Res 281(2):249–259
Wu X et al (2009) Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. Proc Natl Acad Sci U S A 106(51):21672–21677
Xu G et al (2005) Nucleotide excision repair activity varies among murine spermatogenic cell types. Biol Reprod 73(1):123–130
Yan W, Suominen J, Toppari J (2000) Stem cell factor protects germ cells from apoptosis in vitro. J Cell Sci 113(Pt 1):161–168
Yang QE, Oatley JM (2014) Spermatogonial stem cell functions in physiological and pathological conditions. Curr Top Dev Biol 107:235–267
Yang QE et al (2013) Retinoblastoma protein (RB1) controls fate determination in stem cells and progenitors of the mouse male germline. Biol Reprod 89(5):113
Yomogida K et al (2003) Dramatic expansion of germinal stem cells by ectopically expressed human glial cell line-derived neurotrophic factor in mouse Sertoli cells. Biol Reprod 69(4):1303–1307
Yoshida S et al (2004) Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. Dev Biol 269(2):447–458
Yoshida S et al (2006) The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. Development 133(8):1495–1505

Yoshinaga K et al (1991) Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. Development 113(2):689–699
Yu Z et al (2003) Gene expression profiles in different stages of mouse spermatogenic cells during spermatogenesis. Biol Reprod 69(1):37–47
Zheng S, Turner TT, Lysiak JJ (2006) Caspase 2 activity contributes to the initial wave of germ cell apoptosis during the first round of spermatogenesis. Biol Reprod 74(6):1026–1033
Zhou Q et al (2008) Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine testes. Biol Reprod 79(1):35–42
Zhou Z et al (2015) RNA binding protein Nanos2 organizes post-transcriptional buffering system to retain primitive state of mouse spermatogonial stem cells. Dev Cell 34(1):96–107

Part III

Spermatogonial Stem Cells

Defining the Phenotype and Function of Mammalian Spermatogonial Stem Cells

4

Kazadi N. Mutoji and Brian P. Hermann

Abstract

Spermatogonial stem cells (SSCs) are a subpopulation of undifferentiated spermatogonia that maintain spermatogenesis throughout adult life and are essential for male fertility. At each cell division, an SSC produces daughter cells that will either self-renew to produce more SSCs or initiate differentiation to ultimately produce spermatozoa. Consequently, fertility throughout the mammalian male lifespan depends on formation of a foundational SSC pool and then balanced SSC self-renewal and differentiation once steady-state spermatogenesis is achieved. Fundamental studies of SSCs, however, are complicated by their extraordinary rarity in the adult testis (0.01%) and lack of definitive molecular markers that have allowed their prospective identification at any stage of testis development in any species. Despite these challenges, powerful experimental strategies such as transplantation and lineage tracing, which provide retrospective stem cell assessments, have revealed considerable phenotypic information about SSCs over the past two decades. This chapter provides an overview of the key phenotypic and functional characteristics of SSCs, the relative value of differing assessment methods, and the best-substantiated markers of SSCs. Particular emphasis will be placed on emerging technologies, such as single-cell molecular profiling and the use of ID4 reporters, which are facilitating the first prospective, comprehensive molecular characterizations of SSCs that will transform our understanding of the underlying regulatory framework controlling their function.

Keywords

Spermatogonial stem cells • Markers • Transplantation • Lineage-tracing • Single-cell

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4.1 Introduction

Spermatogenesis is maintained throughout adulthood by a pool of adult stem cells termed spermatogonial stem cells (SSCs). SSCs must balance self-renewal and differentiation to sustain the pool of stem cells over time and meet the biological demand for sperm production required for a normal male reproductive lifespan. Defects in formation or maintenance of SSCs are considered the primary causes of Sertoli cell-only (SCO) syndrome, which results in non-obstructive azoospermia (NOA) and male infertility. Rodent SSCs arise from prospermatogonia in the first week after birth, yet the mechanisms responsible for specification of these foundational SSCs have eluded the male reproduction field since the middle of the twentieth century. In mice, post-migratory primordial germ cells (PGCs) give rise to M-prospermatogonia by embryonic day (E) 13.5 (McLaren 2003), which proliferate until ~E15.5 when they become quiescent T1-prospermatogonia (Hilscher et al. 1974; McCarrey 2013). Between postnatal days (P) 0–3, T2-prospermatogonia reenter the cell cycle, proliferate in the middle of the seminiferous cords and migrate to the basement membrane by P6 (Hilscher et al. 1974; McCarrey 2013). Some prospermatogonia produce foundational SSCs, while the remainder die or directly differentiate to produce the first spermatogenic wave (Yoshida et al. 2006; Kluin and de Rooij 1981). There are at least two mechanistic theories for the origin of foundational SSCs, which will be reviewed in Chaps. 2–3, predetermination and selection. Subsequently, once steady-state spermatogenesis has been achieved, each division of an SSC requires a fate decision—self-renew to maintain the pool of SSCs *or* initiate differentiation to produce committed progenitors that will ultimately produce spermatozoa—and these fate decisions must be balanced to sustain spermatogenesis (Jaenisch and Young 2008; Oatley and Brinster 2008, 2012; Yang and Oatley 2014). Chapter 5 is devoted to describing the mechanisms controlling these alternate SSC fate decisions. Ultimately, though, understanding both SSC specification and fate requires an appreciation of the distinguishing features of SSCs. This has proven to be a difficult objective because SSCs are extremely rare [~3000 per adult testis (Nagano 2003)] and there is a paucity of strategies for their prospective identification (Valli et al. 2015). Despite these sizeable hurdles, experimental approaches *have* been devised which can be used to reveal key characterization of SSCs and their progeny that undertake either self-renewal or differentiation fates. This chapter reviews the methodologies enabling phenotypic and functional SSC characterization, limitations, and cautions of prevailing approaches, our current understanding of their key molecular features, and cutting-edge strategies that are catapulting our understanding of their complete molecular signature.

4.2 Functionally Defining SSCs

By definition, SSCs are a subpopulation of undifferentiated spermatogonia that are capable of (1) perpetual self-renewal to sustain the stem cell population AND (2) the capacity to initiate differentiation to produce committed progenitor

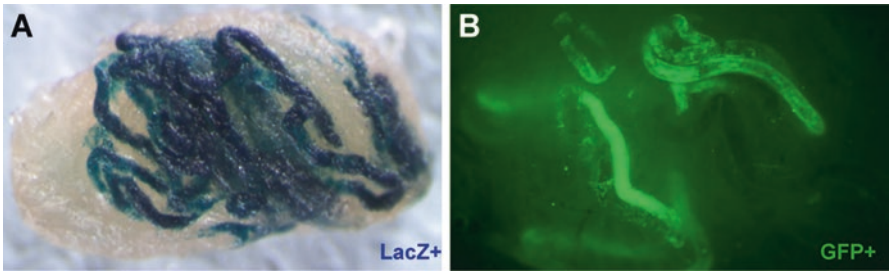

Fig. 4.1 Spermatogonial stem cell transplant recipient testes. Donor-derived spermatogenesis in recipient testes resulting from spermatogonial stem cell transplantation can be visualized using donor cells that bear ubiquitous transgenic reporters, including (**a**) *Rosa-lacZ* (Mutoji et al. 2016) and (**b**) *Actb-eGfp* (unpublished results from Hermann Lab). SSC colonization events constituting segments of seminiferous tubules containing donor-derived spermatogenesis can be subsequently counted microscopically to derive an index of colonization (colonies per 10^5 cells transplanted)

spermatogonia that will initiate spermatogonial differentiation in response to retinoic acid to produce the remainder of the spermatogenic lineage (see Chaps. 3 and 6). Since progenitor spermatogonia have a finite transient-amplifying replicative capacity, they are functionally distinct from SSCs and any experimental definitions of SSCs must therefore distinguish these cells on the basis of their differing biological activities. To that end, the gold-standard experimental approach, initially reported by Ralph Brinster's group in 1994 (Avarbock et al. 1996; Brinster and Zimmermann 1994), involves donor SSC transplantation to recipient testes to measure their regenerative capacity by production and maintenance of complete spermatogenesis in recipient seminiferous tubules. In rodents, definitive identification of donor-derived spermatogenesis in recipient testes can be facilitated by transplant of donor cells that bear ubiquitous transgenic reporters (e.g., *Rosa26-LacZ*, *Actb-eGfp*) into non-transgenic recipient testes. Recipient testes must be (mostly) devoid of endogenous spermatogenesis, such as W/W^v KIT mutants (Brinster et al. 2003) or busulfan-treated adults (Brinster and Avarbock 1994). In this way, segments of recipient testes bearing donor-derived spermatogenesis can be definitively recognized based upon expression of *βgalactosidase* (LacZ+, Fig. 4.1a) or fluorescent reporters (e.g., GFP+; Fig. 4.1b).

Presence of donor-derived spermatogenesis in recipient testes, however, does not alone point to origin from transplanted SSCs. Indeed, differentiating donor cells can engraft within recipient seminiferous tubules and produce complete spermatogenesis (Nagano et al. 1999; Yoshida et al. 2007a). Since differentiating spermatogonia lack self-renewal capacity, donor-derived spermatogenesis arising from non-SSCs is cleared from recipient testes in a time-frame consistent with the duration of spermatogenesis (Oakberg 1956). Thus, in order for SSC transplantation to *selectively* identify donor-derived spermatogenesis arising from *SSCs* (as opposed to non-stem spermatogonia), transplant recipients are allowed to "cook" for 2–3 months prior to analysis. Therefore, when SSC transplant studies are properly executed, only SSC-derived spermatogenic colonies are recognized in recipient testes, while

colonization by SSC progeny that have committed to or initiated differentiation fail to sustain donor-derived spermatogenesis and are not observed. Ultimately, since each colony of donor-derived spermatogenesis arises from a single SSC (Dobrinski et al. 1999b; Nagano et al. 1999; Zhang et al. 2003; Kanatsu-Shinohara et al. 2006), enumeration of donor colony numbers (per 10^5 cells transplanted) constitutes a quantification of donor SSC concentration that enables comparisons between different cell populations. Indeed, SSC transplantation combined with cell sorting, in vitro gene knockdown, and gene knockout approaches have enabled systematic characterization of the phenotype of mouse SSCs (Shinohara et al. 1999, 2000b; Kubota et al. 2003; Costoya et al. 2004; Kanatsu-Shinohara et al. 2004, 2014a; Buaas et al. 2004; Buageaw et al. 2005; Tokuda et al. 2007; Oatley et al. 2011; Yang et al. 2013a, b; Aloisio et al. 2014; Chan et al. 2014) (discussed below in more detail). Importantly, the SSC transplant assay can also be used to help distinguish cell-autonomous and non-autonomous contributors to spermatogenic defects since mutant germ cells can be transplanted into wild-type recipient testes and vice-versa. For instance, transplantation of testis cells from the sterile Steel/Steel(Dickie) (Sl/Sl^d) mutant mice, which contain a mutation in the KIT-ligand, revealed stem cell activity in donor cells upon transplantation (Shinohara et al. 2000a), confirming a non-germ cell autonomous defect in the niche.

Despite the experimental power of the SSC transplant assay, there are drawbacks of this approach that are worth noting. *First* and foremost, SSC transplantation serves as a retrospective assay for SSCs which thwarts prospective studies of SSC biology. *Second*, transplant studies are tedious, variable, and time-consuming. The full quantitative utility of SSC transplant is an art that is only realized with substantial care and practice unrealized in all but a few laboratories around the world. *Third*, SSC transplant results (colonies/10^5 cells transplanted) inform upon the purity of SSCs within the transplanted donor cell suspension, but require assumptions about colonization efficiency. Typically, investigators assume that only a proportion of actual SSCs are able to home to and engraft within available recipient SSC niches, and therefore multiply the colony counts by an assumed "colonization efficiency" value, subsequently dividing the resultant number by the number of cells transplanted (e.g., per 10^5). Estimated colonization/homing efficiency (CE) values of 5–12% have been reported (Ogawa et al. 2003; Shinohara et al. 2000b, 2001). Since these CE values are simply assumptions and could be amount to a "fudge factor" in assessment of SSC purity, alternative and more stringent approaches are needed to definitively prove SSC purity. For instance the gold-standard for demonstrating purity of hematopoietic stem cells for decades has been limiting dilution analysis [reviewed in (Sieburg et al. 2002)]. Applied to SSC transplantation, a sequential dilution of donor SSCs, to as few as one putative SSC are transplanted into each recipient testis. Regeneration of spermatogenesis from one cell would provide definitive evidence of SSC purity. *Fourth*, cells for SSC transplant are subject to considerable manipulation during preparation from donor testes and are subsequently placed into a non-normal testis (e.g., W/W^v, busulfan, etc.) devoid of steady-state spermatogenesis, thereby reducing competition for niche occupancy. Consequently, a concern was raised that the SSC transplant assay may over-report

SSC concentration (Nakagawa et al. 2007). That is, in addition to detecting SSCs, results of transplant experiments may detect colonization events that arise from progenitor spermatogonia that reacquire stem-ness upon introduction into the permissive environment afforded by the recipient seminiferous tubules, but which would not have ordinarily behaved as SSCs, in vivo. *Lastly*, while oft ignored or unrecognized, since the SSC transplant assay requires that the progeny of engrafted SSCs differentiate to produce complete spermatogenesis, it is impossible to use this assay to distinguish changes in SSC number from defects in SSC differentiation. That is, observing a reduction in transplant colony numbers might be consistent with both a reduction in SSC numbers and increased block to differentiation (which would prevent recognition of engraftment events). These limitations aside, transplantation still provides the unique opportunity for relative quantification of SSC content that has proven invaluable for establishing the molecular phenotype of SSCs.

An alternative (yet equally retrospective) approach to definitive identification of SSCs is lineage tracing. For example, if spermatogonia are genetically labeled in vivo based on expression of a particular gene of interest, presence of segments of seminiferous tubules that contain labeled spermatogenesis, at some distant time relative to the initiation of labeling, would be indicative of that gene's expression among SSCs. Thus, rather than isolating the cells of interest, SSCs can be defined and characterized in the context of steady-state spermatogenesis. Since the rodent spermatogenic lineage begins with undifferentiated spermatogonia, labeling should start with A_{single}, A_{paired}, and $A_{aligned}$ clones of 4–16 spermatogonia (Huckins 1971; Oakberg 1971; Oatley and Brinster 2012), followed by differentiating spermatogonia, spermatocytes, and spermatids (Mecklenburg and Hermann 2016). The expectation for this progression of lineage-tracing label is based on the prevailing model of stem cell contribution to spermatogenesis, termed the A_{single} model, in which SSCs are A_{single} spermatogonia which can undertake symmetrical self-renewal or differentiation fate decisions [(Huckins 1971; Oakberg 1971); discussed in more detail in Chap. 5]. Gene knockout studies to demonstrate loss of spermatogenesis (due to stem cell loss) following loss of specific gene products are often coupled such with lineage tracing to demonstrate expression of gene products in SSCs, in vivo (Meng et al. 2000; Buaas et al. 2004; Costoya et al. 2004; Yoshida et al. 2004, 2007b; Falender et al. 2005; Raverot et al. 2005; Ballow et al. 2006; Greenbaum et al. 2006; Nakagawa et al. 2007; Schlesser et al. 2008; Oatley et al. 2011; Goertz et al. 2011; Hobbs et al. 2012; Suzuki et al. 2012; Agbor et al. 2013; Hu et al. 2013a; Yang et al. 2013a; Aloisio et al. 2014; Kanatsu-Shinohara et al. 2014a; Lovasco et al. 2015a). Unlike SSC transplantation, though, it is impossible to quantify SSCs using lineage tracing, and thus, results are exclusively qualitative. Take, for example, a common approach in which lineage tracing is accomplished by conditional genetics (e.g., Cre/lox). Expression of a ubiquitous Cre reporter is activated by a *Gene X* Cre-driver to establish lineage tracing of cells expression *Gene X* (Fig. 4.2). If all SSCs are labeled in this lineage tracing scheme, all spermatogenesis would consequentially be labeled, making it impossible to distinguish label from individual SSCs. Conversely, if the lineage tracing does not label all SSCs, it is impossible to distinguish whether lack of labeling arises from labeling

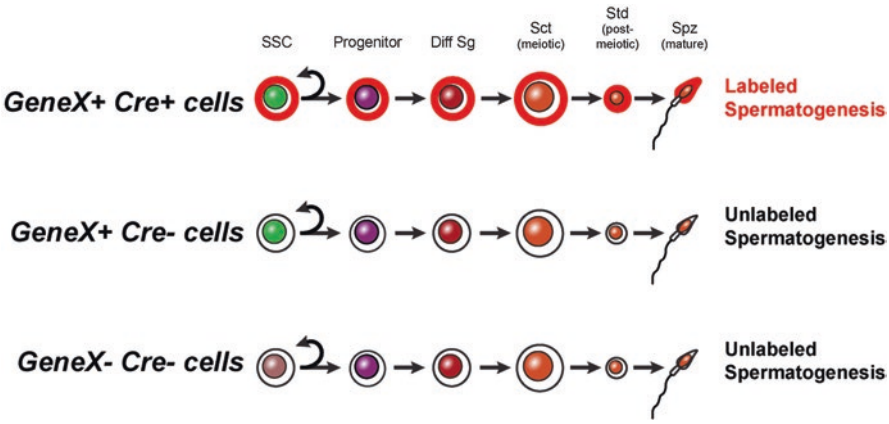

Fig. 4.2 Outcomes of spermatogonial stem cell lineage tracing. Lineage tracing experiments can provide definitive evidence that a gene is expressed by SSCs during steady-state spermatogenesis, in vivo. However, the results of such lineage tracing experiments, and in particular, those that employ Cre/lox genetics, can be difficult to interpret quantitatively in the testis. Take for example an experiment in which Cre recombinase is driven by the regulatory sequences that specify the normal expression of *GeneX* (promoter transgenic, BAC transgenic, knock-in, etc.). If this transgenic line is crossed with a Cre recombinase reporter induces membrane targeted red fluorescent reporter (mT) in response to Cre-dependent recombination, some or all of spermatogenesis would be labeled if Cre is sufficiently expressed (*top row*). If, however, only some *GeneX*-expressing cells sufficiently express Cre, then some *GeneX*+ cells will remain unlabeled (*middle row*), along with all spermatogenesis derived from those cells. This result may be hard to distinguish from unlabeled spermatogenesis that is derived from *GeneX*-negative cells (*bottom row*) in the absence of independent reporting of *GeneX* expression

inefficiencies by the *Gene X* Cre-driver (some Cre expressing SSCs are unlabeled) or true lack of *Gene X* expression (some SSCs do not express Cre; Fig. 4.2). Ultimately, however, lineage tracing methodologies play an important qualitative role for establishing that particular genes or gene products are or are not expressed by SSCs in steady-state spermatogenesis.

4.3 Can Xenotransplantation Quantify SSCs?

While transplantation has become a gold-standard functional readout for confirming presence of SSCs in a variety of animal models, including mice, rats (Orwig et al. 2002a, b), dogs (Kim et al. 2008), goats (Honaramooz et al. 2003a, b), bulls (Herrid et al. 2006; Izadyar et al. 2003), and most recently, nonhuman primates (Shetty et al. 2013; Hermann et al. 2012), for most outbred animal models (e.g., popular domestic species and primates), it is simply not feasible to perform autologous or allogeneic transplantation for routine SSC characterization. Moreover, it is not ethically palatable to perform experimental SSC transplantation for characterization purposes in humans. Therefore, shortly after the advent of mouse-to-mouse SSC transplantation, xenotransplantation into mouse recipient testes became a

popular alternative. Specifically, donor testicular cells from rats (Clouthier et al. 1996), hamsters (Ogawa et al. 1999a), bulls (Dobrinski et al. 2000; Oatley et al. 2002). rabbits (Dobrinski et al. 1999a). dogs (Dobrinski et al. 1999a), boars (Dobrinski et al. 2000). stallions (Dobrinski et al. 2000), baboons (Nagano et al. 2001), rhesus macaques (Hermann et al. 2007, 2009) and humans (Nagano et al. 2002; Dovey et al. 2013) have been successfully transplanted into the testes of immunocompromised mice (e.g., SCID, nu/nu) that were pre-conditioned with chemotherapy (e.g., busulfan) or irradiation to remove endogenous spermatogenesis and allow donor cell engraftment.

Much like mouse-to-mouse SSC transplants, xenotransplantation of germ cells between rodent species (rat or hamster to mouse, mouse to rat) produces complete donor-derived spermatogenesis in recipient testes within 2–3 months after transplant (Clouthier et al. 1996; Ogawa et al. 1999a, b), and therefore, rises to the level of a stringent assay for SSCs. Likewise, germ cells from more distant species (rabbits, boars, dogs, bulls, horses, primates) are able to colonize mouse seminiferous tubules and produce persistent chains of spermatogonia reminiscent of those produced shortly after rodent transplants [reviewed by (Hermann et al. 2010)]. However, complete spermatogenesis from such species is NOT produced in mouse testes, likely a consequence of evolutionary divergence that leaves mouse testicular somatic cells unable to support spermatogenic progression of more distant species. But, since patches of donor-derived xenogeneic spermatogonia can be recognized on the basement membrane of recipient mouse seminiferous tubules several months to a year after transplantation, we and others have applied xenotransplantation as a *colonization assay* that assesses at least some of the characteristics of SSCs in a variety of model species and humans in an experimentally tractable manner (Nagano et al. 2001, 2002; Hermann et al. 2007, 2009).

While xenotransplantation may yield potentially informative results when characterizing spermatogonia in species or experimental contexts for which autologous or allogeneic transplant are not feasible, results should be interpreted with caution given the following limitations. First, since regeneration of endogenous spermatogenesis in the testes of busulfan-treated immunocompromised recipients (e.g., SCID, nu/nu) is possible (and quite variable (Kotzur et al. 2016)), it cannot be assumed that germ cells observed in recipient testes after xenotransplantation are donor-derived. Thus, methods must be employed to label donor cells in xenotransplant experiments. Definitive recognition of donor origin has been accomplished previously using immunostaining with antibodies specific for the donor species of interest (Nagano et al. 2001, 2002; Hermann et al. 2007, 2009) or pre-labeling donor cells with fluorescent vital dyes [e.g., PKH26 or CFDA; (Honaramooz et al. 2002; Maki et al. 2009)], which are subject to dilution following cell divisions. Second, donor cells engraft and produce small colonization foci that should be rigorously evaluated for evidence of proliferation (cell number, connection by intercellular cytoplasmic bridges), presence of germ cell morphology (i.e., ovoid shape, high ratio nucleus:cytoplasm), position on the basement membrane, and, when possible, evidence of other germ cell phenotypes (e.g., germ cell marker expression such as DDX4; (Hermann et al. 2009; Dovey et al. 2013)) in order to provide

meaningful colonization data. Lastly, in the absence of production of complete donor-derived spermatogenesis in recipient seminiferous tubules, results from xenotransplantation *cannot* be used to substantiate claims about SSCs because the full regenerative capacity indicative of SSCs is not assessed. Indeed, transplantation of differentiating mouse spermatogonia engraft within recipient seminiferous tubules and give rise to transient waves of spermatogenesis before being cleared (Nakagawa et al. 2007). Thus, one cannot exclude the possibility that xenotransplanted germ cell colonization foci result in whole or in part from colonization by differentiated spermatogonia that fail to progress through the remainder of the spermatogenic lineage due to an evolutionary block. A thorough examination of the phenotype of xenotransplant colonization foci, including evaluation of marker expression (for undifferentiated and differentiating spermatogonia, and if possible, SSCs), evidence of ongoing proliferation (e.g., BrdU/EdU pulse-labeling), and potentially comparative transcriptomics with highly enriched populations of mouse SSCs should be undertaken.

4.4 What Information About SSCs Can Be Gleaned from Marker Analysis?

According to the A_{single} model, SSCs in the adult mouse testis exist only as isolated undifferentiated spermatogonia that reside on the basement membrane of seminiferous tubules. A_{single} spermatogonia, along with A_{paired} and $A_{aligned}$ spermatogonia, together comprise the population of spermatogonia considered to be morphologically and histologically undifferentiated. The morphological distinction between A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia is based on the number of interconnected spermatogonia within a clone bearing similar nuclear morphology (Huckins 1971; de Rooij and Russell 2000), which can only be recognized and distinguished within intact seminiferous tubules. In the absence of a stain or label that reveals intercellular cytoplasmic bridges between undifferentiated spermatogonia with similar nuclear morphology, cells with an internuclear distance of $\leq$25 μm have been considered to be within a single clone (Huckins 1971; de Rooij and Russell 2000). Surprisingly, labeling for CDH1 in mouse seminiferous tubules revealed connections between spermatogonia that were far in excess of 25 μm, suggesting that results using this arbitrary "25 μm rule" approach may erroneously assign clone sizes (Tokuda et al. 2007). Furthermore, while undifferentiated and differentiated spermatogonia can be distinguished histologically in tissue section based on the extent of heterochromatin (differentiated spermatogonial nuclei are recognizably heterochromatic, while undifferentiated spermatogonial nuclei are generally euchromatic), it is impossible to distinguish clone size in section because the three-dimensional arrangement of spermatogonial clones is lost in two-dimensional tissue sections (Phillips et al. 2010).

Immunolocalization of proteins within clones of undifferentiated spermatogonia has been a fruitful avenue to delineate gene expression patterns among spermatogenic cell types and thereby focus attention on those most likely to play a role in

SSC function. Specifically, proteins which have been shown to exhibit spermatogenic expression patterns that are limited to (or predominantly expressed by) undifferentiated spermatogonia include (but are not limited to) GFRA1, ID4, NEUROG3, PAX7, POU5F1/OCT4, SALL4, SOHLH1, SOX3, UTF1, and ZBTB16 (PLZF) (Wang et al. 2001; Yoshida et al. 2004, 2007b; Ballow et al. 2006; Buaas et al. 2004; Raverot et al. 2005; Greenbaum et al. 2006; Nakagawa et al. 2007; Tokuda et al. 2007; Schlesser et al. 2008; Oatley et al. 2011; van Bragt et al. 2008; Hobbs et al. 2012; Gassei and Orwig 2013; Aloisio et al. 2014). Among these, only ID4 and PAX7 exhibit expression patterns that are restricted to A_{single} spermatogonia, and thus, are candidate SSC-specific markers (Chan et al. 2014; Oatley et al. 2011; Aloisio et al. 2014). While targeted mutation of *Id4* results in progressive loss of spermatogenesis, consistent with interpretation that it is required for SSC maintenance (Oatley et al. 2011), the *Pax7* gene appears to be dispensable for spermatogenesis (Aloisio et al. 2014). Jon Oatley's group was first to demonstrate using a GFP-recombineered BAC transgenic mouse that *Id4* expression was restricted to only A_{single} spermatogonia in the adult and cultured GFP-expression spermatogonia contained the entire stem cell pool (Chan et al. 2014). A recently reported knock-in *Id4*-2A-CreERT2-2A-tdTomato transgenic model exhibited an expression pattern consistent with the initial (expression by A_{single} spermatogonia, but also rare A_{paired} spermatogonia) (Sun et al. 2015). Importantly, recent transplant results demonstrated that not all *Id4*-eGFP+ spermatogonia are SSCs based on transplantation of subpopulations with different TSPAN8 cell surface phenotypes (Mutoji et al. 2016) or based on levels of EGFP epifluorescence (Helsel et al. 2017). Overall, though, ID4 (and transgenic fluorescent reporters) represents the most SSC-restricted marker identified to date in the spermatogenic lineage and will undoubtedly help transform our understanding of SSC biology by allowing the most robust enrichment (if not purification) of SSCs ever reported (see section on SSC purification, below).

In mouse testes, A_{single} spermatogonia comprise roughly 10.5% of undifferentiated spermatogonia and number approximately 35,000 per testis (Tegelenbosch and de Rooij 1993). However, based on transplantation studies, SSCs number only ~3000 per testis in the adult mouse (Nagano 2003), <10% of the number of A_{single} spermatogonia. Thus, while the A_{single} model may hold that all SSCs exhibit an A_{single} morphology, these data indicate that not all A_{single} spermatogonia are SSCs. Of course, the derivation of *estimated* absolute numbers of SSCs per testis (like the absolute number/concentration of SSCs in a cell population) is dependent upon assumptions [here Nagano assumed an engraftment efficiency of 12% based on his empirical data; (Nagano 2003)]. Therefore, until assumption-free quantification of SSC numbers is accomplished, the field should remain open to a wide range of SSC content/proportion among spermatogonia.

It is also important to note that while the A_{single} model is the prevailing model for SSC contribution to spermatogenesis, support for this model is far from universal. Indeed, an alternate "clone fragmentation" model has received considerable attention in recent years based on results of studies employing live imaging of undifferentiated spermatogonia in testes from transgenic reporter mouse models (Nakagawa

et al. 2007; Yoshida et al. 2007a; Hara et al. 2014). As the name suggests, the clone fragmentation model holds that SSC renewal arises from fragmentation of clones of A_{paired}-$A_{aligned}$ spermatogonia because a proportion of these retain latent stemness that can be mobilized to renew the SSC pool (Yoshida et al. 2007a; Morimoto et al. 2009). While the merits of these alternate models can certainly be debated, the disparity between these models illustrates the need to rely upon definitive and functional data to draw conclusions about SSCs. Therefore, while antibody-based localization of proteins (e.g., indirect immunofluorescence), RNA based on hybridization (e.g., in situ hybridization), or fluorescent transgenic reporter (e.g., GFP), either in section or in whole-mount, can certainly reveal gene expression profiles among spermatogenic cell types, it is *impossible* to extrapolate results of such studies to SSCs. Co-labeling for a marker of interest with other markers known to be expressed by undifferentiated spermatogonia or broadly among germ cells (e.g., PLZF/ZBTB16, DDX4/VASA, etc.) can provide additional general support for the extent of marker expression among spermatogenic cells, but again, cannot be extrapolated to support conclusions about SSCs.

4.5 Rigorously Defining the Phenotype of SSCs

Elucidating the mechanisms that specify SSCs in the testis and control their biological activities will undoubtedly reveal key players in the establishment and maintenance of spermatogenesis and underlying pathologies leading to male infertility. Consequently, studies that define expression of genes within the SSC compartment, and whether such genes are necessary and/or sufficient for some aspect of SSC function, will likewise be informative and important advances. Indeed, many features of SSC phenotype and the requirement of specific gene products for SSC function have been revealed using a combination of cell selection or genetic manipulations with SSC transplantation. For example, cell sorting (FACS or MACS) combined with SSC transplantation demonstrated that mouse SSCs are distinguished from other testicular cell types by the phenotype CD45−, CD9+, CDH1+, GPR125+, Hoechst side population+, ID4+, ITGAV−, ITGA6+, ITGB1+, KIT-, MHC-I−, $Rho123^{low}$, THY1+, $TSPAN8^{High}$ (Shinohara et al. 1999, 2000b; Kubota et al. 2003; Falciatori et al. 2004; Kanatsu-Shinohara et al. 2004; Lassalle et al. 2004; Fujita et al. 2005; Lo et al. 2005; Seandel et al. 2007; Chan et al. 2014; Mutoji et al. 2016; Tokuda et al. 2007). While not intended to present an exhaustive phenotype of mouse SSCs [see tables in recent reviews that provide this information (Phillips et al. 2010; Mecklenburg and Hermann 2016)], this list illustrates the considerable amount of phenotypic information gleaned over the past 20 years about SSCs. These markers exhibit considerable differences in their relative utility to exclusively mark SSCs, an issue that is discussed in detail later in this chapter. In steady state spermatogenesis, gene knockouts and lineage tracing have helped to confirm loss of spermatogenesis following loss of specific gene products and/or demonstration of gene expression among SSCs, in vivo (Meng et al. 2000; Buaas et al. 2004; Costoya et al. 2004; Falender et al. 2005; Raverot et al. 2005; Ballow et al. 2006; Greenbaum

et al. 2006; Nakagawa et al. 2007; Schlesser et al. 2008; Oatley et al. 2011; Goertz et al. 2011; Hobbs et al. 2012; Suzuki et al. 2012; Agbor et al. 2013; Hu et al. 2013b; Yang et al. 2013a; Kanatsu-Shinohara et al. 2014b; Lovasco et al. 2015b; Sun et al. 2015).

A related approach to investigating the phenotype and functional characteristics of SSCs takes advantage of the ability to propagate and manipulate SSCs in culture. Robust protocols for culturing rodent SSCs have been reported (Kanatsu-Shinohara et al. 2003; Kubota et al. 2004a, b; Ryu et al. 2005; Hamra et al. 2005) which allow them to be maintained indefinitely with significant expansion in numbers. These cultures contain a heterogeneous mixture of SSCs along with, presumably, spermatogonia that have lost stem cell capacity and become progenitor spermatogonia (akin to A_{paired} and $A_{aligned}$ spermatogonia), and thus, are more accurately termed undifferentiated spermatogonia cultures. Estimates of the stem cell concentration in undifferentiated spermatogonial cultures range from as high as 10% (Kubota et al. 2004b) to as low as 0.02% (Kanatsu-Shinohara et al. 2005). Using this culture system, measuring the response of SSCs to different environmental cues (e.g., growth factors) can be easily accomplished by varying/manipulating culture conditions. Spermatogonia can also be manipulated with RNA interference as a tractable method to determine the role of various intrinsic genetic characteristics in SSC biology. In both cases, such studies are best performed in conjunction with SSC transplant as a functional readout of any change in SSC number or quality. For example, transient siRNA-mediated (delivered by standard transfection approaches) or shRNA-mediated (lentiviral-delivered) knockdown have been used to demonstrate that *Bcl6b*, *Cdk4*, *Cdk6*, *Cldn3*, *Etv5*, *Lhx1*, *Mcam*, and *Pou3f1* enhance and are important for SSC self-renewal in vitro (Wu et al. 2010; Oatley et al. 2006, 2007; Tanaka et al. 2015; Kanatsu-Shinohara et al. 2012; Takashima et al. 2011), while *Stat3* and *Neurog3* inhibit SSC self-renewal in vitro (Kaucher et al. 2012; Oatley et al. 2010).

Rigorous application of definitive approaches is the only way to advance the understanding of SSC biology. Lax use of terminology referring to cells in the spermatogenic lineage and inappropriate use of experimental tools to define SSCs can muddy our understanding of SSC biology. It has become the "industry standard" to refer to the adult stem cells in the testis that produce and sustain the spermatogenic lineage as spermatogonial stem cells (SSCs). However, at least three other terms can be found in prominent, peer-reviewed, primary research articles to refer to SSCs, including adult germline stem cells (AGSCs), germline stem cells (GSCs), spermatogonial progenitor cells (SPCs), leading to confusion about exactly which cells are under investigation and how results can be compared between studies. Moreover, some studies employ "SSC markers" or use cell separation strategies to "isolate SSCs," when in fact, the approaches used did not allow for identification/selection of SSCs (or in some cases, even germ cells)—see section below on "The short-list of conserved, bona fide, mammalian SSC markers." This leads to results referring to the same cell population (SSCs) which were actually generated using different cell populations and mixtures of cells. A prototypical example has emerged recently following excellent work demonstrating THY1 can be used to *enrich* SSCs. THY1

(aka: CD90) is a GPI-tethered, homophilic cell-cell adhesion molecule that is expressed by cells located on the basement membrane of seminiferous tubules in mice, rats, bulls, rhesus monkeys and humans (Ryu et al. 2004; Kubota et al. 2003; Hermann et al. 2009; Reding et al. 2010; Izadyar et al. 2011; Maki et al. 2009). Transplantation studies in mice and rats demonstrated enrichment of regenerative activity (SSC activity) in THY1+ fractions from testis cell suspensions (Ryu et al. 2004; Kubota et al. 2003), and consequently, THY1 selection has become widely used as a means of enriching SSCs prior to culturing undifferentiated spermatogonia [see (Oatley and Brinster 2006)]. However, THY1 expression is neither restricted to SSCs nor even testicular germ cells (Hermann et al. 2015), and consequently, THY1 selection only enriches SSCs, it does not purify SSCs. Studies that DO employ THY1+ selection with the intent of isolating *pure* populations of mouse SSCs for bulk biochemical analyses (e.g., transcriptome, epigenome, proteome) consequently report erroneous results. In support of this argument, we have demonstrated that THY1+ testis cell suspensions are heterogeneous, containing both undifferentiated spermatogonia (some of which are SSCs) as well as somatic and other gem cell contaminants (Hermann et al. 2015). Therefore, valid reports of SSC or spermatogonial selection must provide definitive evidence (either from the literature or with de novo experimentation) to support claims made about the content of the cells/SSCs used in such reports. Use of lax terminology or failing to rigorously define the content of cell population used to study the biology of SSCs and/or undifferentiated spermatogonia carries with it the danger that the results obtained and conclusions drawn may not actually reflect SSC biology.

4.6 The Short-List of Conserved, Bona Fide, Mammalian SSC Markers

Given the considerable interest in extending the knowledge of SSC phenotype from rodent model species to more clinically relevant nonhuman primate models, and ultimately, to humans, there is still value in exploring the conservation of marker expression among spermatogonia from higher-order mammalian species even if there are no definitive SSC assessments available. With that said, here we will explore the most concise list of rodent SSC markers with respect to their expression conservation among likely primate SSCs.

To establish the short-list of bona fide rodent SSC markers, we first focused only on those markers for which functional validation had been performed (i.e., transplantation or lineage tracing) demonstrating enrichment of SSCs among expressing vs. non-expressing cells. In some cases, however, markers lacking conclusive transplant or lineage tracing evidence to support inclusion as a bona fide SSC marker were included if in situ expression data demonstrate restricted expression (e.g., A_{single} and A_{paired} spermatogonia) and genetic studies indicate the gene is required for spermatogenesis. These rodent SSC markers were subsequently separated into three categories, Tier (1) markers for which has demonstrated that gene product (mRNA or protein) expression *uniquely* marks SSCs (and perhaps some progenitor

Table 4.1 Classification of functionally validated rodent SSC by expression profile

Tier	Expression[a]	Markers	Relevant citations
1	A_{single}-A_{paired} spermatogonia (no somatic expression)	BMI1, GFRA1, ID4, NANOS2	Komai et al. (2014), Buageaw et al. (2005), Ebata et al. (2005), Sun et al. (2015), Chan et al. (2014), Helsel et al. (2017), and Sada et al. (2009)
2	A_{single}-A_{paired}-$A_{aligned}$ ± Adiff (A1–A4) spermatogonia (no somatic expression)	CDH1, EPCAM, FOXO1, GPR125, ITGA6, ITGB1, LIN28A, MCAM, NEUROG3, POU5F1, RET, RHOX10, SALL4, ZBTB16 (PLZF)	Tokuda et al. (2007), Kanatsu-Shinohara et al. (2011, 2012), Goertz et al. (2011), Seandel et al. (2007), Shinohara et al. (1999), Chakraborty et al. (2014), Shinoda et al. (2013), Zheng et al. (2009), Nakagawa et al. (2007), Ohbo et al. (2003), Naughton et al. (2006), Parker et al. (2014), Song et al. (2016), Gassei and Orwig (2013), Hobbs et al. (2012), Costoya et al. (2004), and Buaas et al. (2004)
3	Spermatogonia + testicular somatic cells	CD9, CD24A, CSF1R, THY1, TSPAN8	Kanatsu-Shinohara et al. (2004), Kubota et al. (2003, 2004b), Oatley et al. (2009), Mutoji et al. (2016)

[a]Gene product expression largely restricted to these cells

spermatogonia), Tier (2) markers exhibiting less restricted expression among undifferentiated and differentiating spermatogonia, and Tier (3) markers which are expressed by both germ cells and testicular somatic cells (Table 4.1). While markers that fall into Tier 1 are largely expressed only by A_{single} and A_{paired} spermatogonia and those in the second category are expressed throughout the undifferentiated spermatogonial pool and into differentiating spermatogonia, we did not consider many similar markers for which neither convincing functional readouts nor genetic studies were available. It is important to note, though, that a number of posited SSC markers failed to meet these strict criteria and were not evaluated further. For the purposes of establishing the short-list of conserved, bona fide, mammalian SSC markers, we have focused exclusively on SSC markers in Tier 1 because it is only these which could potentially be considered exclusive SSC markers. While genes in Tiers 2 and 3 are expressed by SSCs, they are not expressed by only SSCs in the testis.

BMI1. Among the four gene products considered to be the best markers of rodent SSCs to date, the case for BMI1 is the weakest. A single study examined BMI1 in mice, demonstrating using CreERT2 pulse-labeling that expression is chiefly among A_{single} spermatogonia (antibody staining was not possible using available reagents) and pulse-labeling ultimately marked ongoing spermatogenesis, consistent with expression among SSCs (Komai et al. 2014). To date, BMI1 expression has not been examined in putative SSCs in any primate species, so the degree of expression conservation is not known.

GFRA1. In mice, GFRA1 protein is well known to be primarily restricted to A_{single} and A_{paired} spermatogonia, SSCs fail to renew in *Gfra1* null animals (Naughton et al. 2006), and GFRA1 selection enriches SSCs from mouse pup testes

(Ebata et al. 2005; Buageaw et al. 2005). Indeed, the ligand for GFRA1, GDNF, is, without any doubt, the best understood niche factor required for normal SSC renewal (Meng et al. 2000; Oatley et al. 2006, 2007). Thus, the argument for GFRA1 as a Tier1 SSC marker is very strong. GFRA1 expression was first examined in primates in the rhesus testis using immunostaining methods (Hermann et al. 2009, 2010). In the adult monkey testis, all A_{dark} and A_{pale} spermatogonia in the adult testis were GFRA1+ with very limited overlap with KIT labeling in some A1 and A2 differentiating spermatogonia (Hermann et al. 2009). In human testes, GFRA1 staining is found in both A_{dark} and A_{pale} spermatogonia, although unlike the monkey, it did not appear that all human undifferentiated spermatogonia were GFRA1+ (Grisanti et al. 2009; von Kopylow et al. 2012). Further, GFRA1+ spermatogonia in intact seminiferous tubules were found to be very dense, but it was not possible to assign clone sizes among human GFRA1+ spermatogonia because their density was too high (Singh et al. 2017). Like in rodents, MACS has been used to isolate GFRA1+ cells from adult monkey and human testes (Gassei et al. 2010; He et al. 2012). MACS-isolated GFRA1+ cells from human testes also expressed several other SSC markers (He et al. 2012), but comprehensive genome-wide assessments have not been made to allow comparison to mouse spermatogonia in the absence of a definitive functional assay for human SSCs. Thus, it appears that primate GFRA1+ spermatogonia are similar to rodent counterparts, which may have implications for our understanding of the primate SSC pool (e.g., whether A_{dark} and/or A_{pale} spermatogonia are SSCs). Indeed, it appears likely that both A_{dark} and A_{pale} spermatogonia are part of the steady-state SSC pool in primate testes (Hermann et al. 2010).

ID4. The evidence supporting ID4 as a bona fide marker of rodent SSCs is extremely strong and is detailed above. In short, ID4 exhibits expression patterns that is primarily restricted to A_{single} spermatogonia (Chan et al. 2014; Oatley et al. 2011; Sun et al. 2015; Helsel et al. 2017), is required for SSC maintenance (Oatley et al. 2011; Chan et al. 2014), labels spermatogenesis arising from stem cells in steady state (Sun et al. 2015), and selecting for the brightest GFP-expressing spermatogonia using an *Id4*-eGFP transgenic mouse model facilitates near-purification of SSCs (Helsel et al. 2017). ID4 is further distinguished from the other three Tier1 SSC markers, though, by transplant studies which demonstrated that ALL SSCs express ID4 [at least the GFP transgene; (Chan et al. 2014)]. The vast majority of this evidence, however, emanates from experiments using transgenic models because existing ID4 antibodies are unreliable [see discussion in (Oatley et al. 2011)]. One study reported ID4 immunostaining in adult human testes which appeared to label cells on the basement membrane which exhibited partial staining overlap with PLZF (Sachs et al. 2014). Surprisingly, the reported ID4 immunostaining appeared to primarily localize to the cytoplasm in those cells (Sachs et al. 2014), which is unexpected for a transcription factor. Therefore, in the absence of high-quality reagents to immunolocalize ID4 protein in primate testes, connection of mouse results to clinically relevant species faces a substantial barrier. Further, unlike GFRA1, since ID4 protein is localized to the nucleus, cell sorting is considerably more difficult. Therefore, even if a high-quality commercial ID4 antibody were available, it is unlikely that substantial phenotypic or functional evaluation could be

performed by selecting ID4 (protein) expressing cells. Thus, until future studies examine ID4-expressing populations in novel ways, it will remain unclear to what extent ID4 may label primate SSCs.

NANOS2. In mice, CreERT2 pulse-labeling studies demonstrated that the *Nanos2* gene is expressed by at least some SSCs at any given time, knockout experiments showed that NANOS2 is required for normal SSC function and *Nanos2* overexpression led to accumulation of undifferentiated spermatogonia at the expense of spermatogenesis (Sada et al. 2009). *NANOS2* mRNA (RT-PCR) and NANOS2 protein (western blot) are detectable in human testes (Kusz et al. 2009), though, like ID4, there is a paucity of high-quality NANOS2 antibodies which preclude localization of expressing cells in primate testes. Therefore, is remains unclear whether putative primate SSCs express NANOS2.

Collectively, therefore, a very large knowledge gap exists between our understanding of the bona fide markers of SSCs in mice and primates. In order to advance the field of primate SSC biology, experiments to extend relevant rodent results (i.e., of Tier 1 markers) to primate species are needed. Indeed, a number of studies have examined the primate testis expression pattern of markers for which there is no convincing evidence in rodents indicating those are exclusive SSC markers (i.e., Tier 1). This is unfortunate because, in the absence of connection to functional evidence, such results have limited utility for advancing our understanding of human and nonhuman primate SSCs.

4.7 The Quest to Purify SSCs

Purification of SSCs from among a heterogeneous complex suspensions of testis cells or even heterogeneous populations of undifferentiated spermatogonia (Hermann et al. 2015) would provide the profound ability to *prospectively* study the cells that form the foundation of spermatogenesis. Achieving SSC selection purity has been an experimental objective since Ralph Brinster first reported SSC transplantation (Brinster and Avarbock 1994; Brinster and Zimmermann 1994) and the first enrichment studies were reported 5 years later (Shinohara et al. 1999, 2000b). Using results from SSC transplantation studies that are reported as colony #/10^5 cells transplanted and presuming an efficiency of colonization (typically 5%), SSC content within testis cell suspensions can be estimated and compared between studies. For example, GFRA1 selection from mouse pup testes produced 45 colonies/10^5 cells transplanted, roughly equating to an SSC purity of 1:111 SSC purity (Ebata et al. 2005). Purity of SSCs in GFRA1+ selection is substantially eclipsed by THY1 selection from mouse pup testes, which produced 124 colonies/10^5 cells transplanted [SSC purity of 1:40 (Kubota et al. 2004b)]. Among freshly isolated mouse pup testis cells, the greatest enrichment achieved was obtained recently in our unpublished studies using selection of ID4-EGFP+/TSPAN8^{High} spermatogonia, which produced 233 colonies/10^5 cells transplanted or 1:21 SSCs (Mutoji et al. 2016). Subsequently, selection of subsets of ID4-EGFP+ cells from neonatal testes on the basis of EGFP intensity (bright vs. dim) demonstrated a similar enrichment

of SSC activity by transplantation and limiting dilution analyses resulted in an estimated SSC purity of 1:0.92 cells (essentially pure) in the ID4-EGFP-bright population [(Helsel et al. 2017)]. Thus, these two studies may have succeeded in devising strategies to essentially purify SSCs from the neonatal mouse testis.

Two studies have reported substantial SSC enrichment from adult mouse testes. The first reported that the β2M- (MHC-I-) THY1+ cKIT- fraction of cryoptorchid adult testes produced 343 colonies/10^5 cells transplanted [1:15 SSCs (Kubota et al. 2003)], although the use of cryptorchidism to enrich SSCs may not represent normal spermatogonial biology. More recently, SSC enrichment to 1:6 purity was accomplished from normal adult mouse testes by selecting CD9+/EPCAM^{low}/MCAM+/KIT− cells (Kanatsu-Shinohara et al. 2012). Beyond these studies, though, there have been no legitimate claims of isolation methods that achieve SSC selection to purity. Interestingly, since there are roughly 3000 SSCs in the adult mouse testis (Nagano 2003) and roughly 6000 *Id4*-eGFP+ spermatogonia per adult mouse testis (Chan et al. 2014), nearly all of these A_{single} spermatogonia, it is tempting to speculate that the *Id4*-eGFP+ fraction of adult mouse testes may enrich for SSCs to as high 1:2.

4.8 Phenotyping SSCs on a Background of Male Germline Heterogeneity

Chan et al. (2014) reported a transgenic mouse line (*Id4*-eGFP) that is expressed by the most restricted proportion of undifferentiated spermatogonia to date (only some A_{single} spermatogonia) and can be used to substantially enrich for SSCs. Yet, only a subset of GFP+ spermatogonia appear to be SSCs. In the absence of a purification scheme for SSCs, this represents a key barrier to derivation of a refined and specific phenotype for SSCs. Indeed, conventional gene expression approaches that provide averaged results from thousands or millions of cells would therefore be ineffective for discerning signatures of individual cells or distinguishing among multiple signatures within heterogeneous cell populations. Exemplifying this challenge, the majority of studies performed to date using bulk preparations of undifferentiated spermatogonia have revealed surprisingly few gene expression differences that could point to the definitive transcriptome of SSCs (Shima et al. 2004; Oatley et al. 2006, 2009; Goertz et al. 2011; Aloisio et al. 2014; Chan et al. 2014; Hammoud et al. 2014, 2015; Margolin et al. 2014). Indeed, even comparison between ID4-EGFP+ and ID4-eGFP- subpopulations of cultured THY1+ spermatogonia revealed only 36 genes that were significantly differentially expressed between the two germ cell subpopulation (Chan et al. 2014). This result could emanate from molecular averaging among heterogeneous cell populations that are closely related.

To address this challenge, we reasoned that examining the differential expression of genes among cells within the population of ID4-EGFP+ spermatogonia might reveal distinguishing features that could be exploited to further enhance enrichment of SSCs. For this purpose, we performed single-cell gene expression analyses to defined the extent of molecular heterogeneity among neonatal mouse spermatogonia

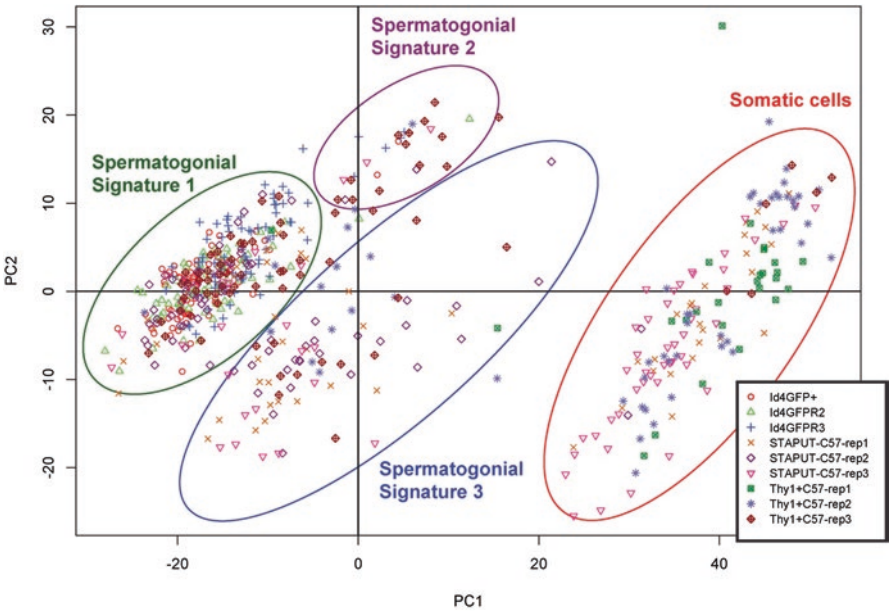

Fig. 4.3 Neonatal spermatogonial heterogeneity revealed by single-cell gene expression profiling. Single-cell qRT-PCR analyses measured mRNA levels for 172 genes among 584 individual neonatal mouse spermatogonia [P6; (Hermann et al. 2015)]. Testis cell suspensions were enriched for spermatogonia using StaPut gravity sedimentation, THY1+ magnetic cell sorting or FACS for GFP+ cells from the *Id4*-eGFP transgenic line. Principal component analysis of these data showed considerable heterogeneity in mRNA abundance for the panel of genes examined (comprised mainly of specific germ cell and stem cell mRNAs), which segregated into one somatic cell cluster (*red ellipse*) and three different clusters of spermatogonia (labeled Spermatogonial Signature 1, 2, and 3; green, violet, and blue ellipses, respectively). Spermatogonial suspensions prepared with either THY1 MACS or StaPut methods invariably contained contaminating somatic cell types, while *Id4*-eGFP+ cells were almost exclusively found in the green and violet spermatogonial populations. The legend shows sample replicate and source

(Hermann et al. 2015). Single-cell qRT-PCR was performed for a panel of 172 genes using enriched populations of spermatogonia from P6 testes, including cells from *Id4-eGfp* transgenic mice that express EGFP in a fraction of undifferentiated spermatogonia (Chan et al. 2014). From these analyses, we were able to separate P6 testis cells into four major clusters based on distinct gene expression signatures, including one population of contaminating somatic cells and three spermatogonial groups (Fig. 4.3). This demonstrated the existence of distinct subpopulations among neonatal undifferentiated spermatogonia with discrete gene expression signatures. We recently probed the functional implications of this heterogeneity with SSC transplantation (Mutoji et al. 2016). In these single-cell qRT-PCR studies (Hermann et al. 2015), several genes were expressed in a bimodal fashion among P6 ID4-EGFP+ spermatogonia (i.e., present/absent), suggesting they mark two or more discrete cell subpopulations of undifferentiated spermatogonia. Flow cytometry with antibodies against cell surface proteins encoded by three of these bimodal genes (TSPAN8,

EPHA2, and PVR) demonstrated that this heterogeneity extended to the protein level and defined subpopulations that could be tested for differences in function. Subsequent transplantation studies demonstrated substantial enrichment of SSCs in the TSPAN8^{High} vs. the TSPAN8^{Low} populations of P6 ID4-EGFP+ cells (Mutoji et al. 2016). These data indicate the presence of functionally distinct subpopulations of undifferentiated spermatogonia in the neonatal mouse testis and demonstrate that gene expression differences between these subpopulations are relevant to the distinct developmental state of SSCs vs. progenitor spermatogonia. Essentially, the single-cell gene expression profiling has already begun facilitating the first prospective, comprehensive molecular characterizations of SSCs. Arguably, though, the data we have generated to date using qRT-PCR for a defined gene set are biased by the choice of which genes were examined. These choices might ignore important contributors to spermatogonial heterogeneity and distinctions between subtypes. Therefore, the power of this approach will undoubtedly be advanced even further by use of single-cell RNA-seq methodologies that are beginning to garner widespread experimental use (Wu et al. 2014). Unbiased, whole-transcriptome, single-cell gene expression datasets will unquestionably transform our understanding of the underlying molecular distinctions between SSCs and progenitor spermatogonia, but will also likely reveal the regulatory framework controlling their function.

4.9 Conclusions

Spermatogonial stem cells are unique adult stem cells that must sustain sperm production in mammalian testes to ensure a normal reproductive lifespan in males. Elucidation of the basic biological characteristics of SSCs, the molecular underpinnings of their specification during development and mechanisms controlling of SSC self-renewal or differentiation has exploded over the past two decades as implementation of definitive assays for SSCs has become more regular. Yet, the field of SSC biology currently sits at the crossroad between rigorous science to uncover the true biology and borderline sloppy high-impact description that leads the field astray. Moreover, considerable technical barriers have prevented robust extension of knowledge of mouse SSCs to clinically relevant non-human primate species and humans. Still, novel approaches are emerging that may allow significant advances toward purification of SSCs and their progeny in bulk and interrogation of molecular variation among individuals in populations on the single-cell level. Together with scrupulous peer review, careful data interpretation, we will undoubtedly have extraordinary opportunities to advance the understanding of SSC biology.

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References

Agbor VA, Tao S, Lei N et al (2013) A Wt1-Dmrt1 transgene restores DMRT1 to sertoli cells of Dmrt1(−/−) testes: a novel model of DMRT1-deficient germ cells. Biol Reprod 88:51

Aloisio GM, Nakada Y, Saatcioglu HD et al (2014) PAX7 expression defines germline stem cells in the adult testis. J Clin Invest 124:3929–3944

Avarbock MR, Brinster CJ, Brinster RL (1996) Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nat Med 2:693–696

Ballow D, Meistrich ML, Matzuk M et al (2006) Sohlh1 is essential for spermatogonial differentiation. Dev Biol 294:161–167

Brinster RL, Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 91:11303–11307

Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 91:11298–11302

Brinster CJ, Ryu BY, Avarbock MR et al (2003) Restoration of fertility by germ cell transplantation requires effective recipient preparation. Biol Reprod 69:412–420

Buaas FW, Kirsh AL, Sharma M et al (2004) Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 36:647–652

Buageaw A, Sukhwani M, Ben-Yehudah A et al (2005) GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. Biol Reprod 73:1011–1016

Chakraborty P, Buaas FW, Sharma M et al (2014) LIN28A marks the spermatogonial progenitor population and regulates its cyclic expansion. Stem Cells 32:860–873

Chan F, Oatley MJ, Kaucher AV et al (2014) Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev 28:1351–1362

Clouthier DE, Avarbock MR, Maika SD et al (1996) Rat spermatogenesis in mouse testis. Nature 381:418–421

Costoya JA, Hobbs RM, Barna M et al (2004) Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 36:653–659

de Rooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21:776–798

Dobrinski I, Avarbock MR, Brinster RL (1999a) Transplantation of germ cells from rabbits and dogs into mouse testes. Biol Reprod 61:1331–1339

Dobrinski I, Ogawa T, Avarbock MR et al (1999b) Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice. Mol Reprod Dev 53:142–148

Dobrinski I, Avarbock MR, Brinster RL (2000) Germ cell transplantation from large domestic animals into mouse testes. Mol Reprod Dev 57:270–279

Dovey SL, Valli H, Hermann BP et al (2013) Eliminating malignant potential from therapeutic human spermatogonial stem cells. J Clin Invest 123:1833–1843

Ebata KT, Zhang X, Nagano MC (2005) Expression patterns of cell-surface molecules on male germ line stem cells during postnatal mouse development. Mol Reprod Dev 72:171–181

Falciatori I, Borsellino G, Haliassos N et al (2004) Identification and enrichment of spermatogonial stem cells displaying side-population phenotype in immature mouse testis. FASEB J 18:376–378

Falender AE, Freiman RN, Geles KG et al (2005) Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. Genes Dev 19:794–803

Fujita K, Ohta H, Tsujimura A et al (2005) Transplantation of spermatogonial stem cells isolated from leukemic mice restores fertility without inducing leukemia. J Clin Investig 115:1855–1861

Gassei K, Orwig KE (2013) SALL4 expression in gonocytes and spermatogonial clones of postnatal mouse testes. PLoS One 8:e53976

Gassei K, Ehmcke J, Dhir R et al (2010) Magnetic activated cell sorting allows isolation of spermatogonia from adult primate testes and reveals distinct GFRa1-positive subpopulations in men. J Med Primatol 39:83–91

Goertz MJ, Wu Z, Gallardo TD et al (2011) Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. J Clin Invest 121:3456–3466
Greenbaum MP, Yan W, MH W et al (2006) TEX14 is essential for intercellular bridges and fertility in male mice. Proc Natl Acad Sci U S A 103:4982–4987
Grisanti L, Falciatori I, Grasso M et al (2009) Identification of spermatogonial stem cell subsets by morphological analysis and prospective isolation. Stem Cells 27:3043–3052
Hammoud SS, Low DH, Yi C et al (2014) Chromatin and transcription transitions of mammalian adult germline stem cells and spermatogenesis. Cell Stem Cell 15:239–253
Hammoud SS, Low DH, Yi C et al (2015) Transcription and imprinting dynamics in developing postnatal male germline stem cells. Genes Dev 29:2312–2324
Hamra FK, Chapman KM, Nguyen DM et al (2005) Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. PNAS 102:17430–17435
Hara K, Nakagawa T, Enomoto H et al (2014) Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. Cell Stem Cell 14:658–672
He Z, Kokkinaki M, Jiang J et al (2012) Isolation of human male germ-line stem cells using enzymatic digestion and magnetic-activated cell sorting. Methods Mol Biol 825:45–57
Helsel AR, Yang QE, Oatley MJ et al (2017) ID4 levels dictate the stem cell state in mouse spermatogonia. Development 144:624–634
Hermann BP, Sukhwani M, Lin CC et al (2007) Characterization, cryopreservation and ablation of spermatogonial stem cells in adult rhesus macaques. Stem Cells 25:2330–2338
Hermann BP, Sukhwani M, Simorangkir DR et al (2009) Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. Hum Reprod 24:1704–1716
Hermann BP, Sukhwani M, Hansel MC, Orwig KE (2010) Spermatogonial stem cells in higher primates: are there differences to those in rodents? Reproduction 139:479–493
Hermann BP, Sukhwani M, Winkler F et al (2012) Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell 11:715–726
Hermann BP, Mutoji KN, Velte EK et al (2015) Transcriptional and translational heterogeneity among neonatal mouse spermatogonia. Biol Reprod 92:54
Herrid M, Vignarajan S, Davey R et al (2006) Successful transplantation of bovine testicular cells to heterologous recipients. Reproduction 132:617–624
Hilscher B, Hilscher W, Bulthoff-Ohnolz B et al (1974) Kinetics of gametogenesis. I. Comparative histological and autoradiographic studies of oocytes and transitional prospermatogonia during oogenesis and prespermatogenesis. Cell Tissue Res 154:443–470
Hobbs RM, Fagoonee S, Papa A et al (2012) Functional antagonism between Sall4 and Plzf defines germline progenitors. Cell Stem Cell 10:284–298
Honaramooz A, Megee SO, Dobrinski I (2002) Germ cell transplantation in pigs. Biol Reprod 66:21–28
Honaramooz A, Behboodi E, Blash S et al (2003a) Germ cell transplantation in goats. Mol Reprod Dev 64:422–428
Honaramooz A, Behboodi E, Megee SO et al (2003b) Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol Reprod 69:1260–1264
Hu YC, de Rooij DG, Page DC (2013a) Tumor suppressor gene Rb is required for self-renewal of spermatogonial stem cells in mice. Proc Natl Acad Sci U S A 110:12685–12690
Hu YC, de Rooij DG, Page DC (2013b) Tumor suppressor gene Rb is required for self-renewal of spermatogonial stem cells in mice. Proc Natl Acad Sci U S A 110:12685–12690
Huckins C (1971) The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec 169:533–557
Izadyar F, Den OK, Stout TA et al (2003) Autologous and homologous transplantation of bovine spermatogonial stem cells. Reproduction 126:765–774
Izadyar F, Wong J, Maki C et al (2011) Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. Hum Reprod 26:1296–1306

Jaenisch R, Young R (2008) Stem cells, the molecular circuitry of pluripotency and nuclear reprogramming. Cell 132:567–582
Kanatsu-Shinohara M, Ogonuki N, Inoue K et al (2003) Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 69:612–616
Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2004) CD9 is a surface marker on mouse and rat male germline stem cells. Biol Reprod 70:70–75
Kanatsu-Shinohara M, Ogonuki N, Iwano T et al (2005) Genetic and epigenetic properties of mouse male germline stem cells during long-term culture. Development 132:4155–4163
Kanatsu-Shinohara M, Inoue K, Miki H et al (2006) Clonal origin of germ cell colonies after spermatogonial transplantation in mice. Biol Reprod 75:68–74
Kanatsu-Shinohara M, Takashima S, Ishii K et al (2011) Dynamic changes in EPCAM expression during spermatogonial stem cell differentiation in the mouse testis. PLoS One 6:e23663
Kanatsu-Shinohara M, Morimoto H, Shinohara T (2012) Enrichment of mouse spermatogonial stem cells by melanoma cell adhesion molecule expression. Biol Reprod 87:139
Kanatsu-Shinohara M, Onoyama I, Nakayama KI et al (2014a) Skp1-Cullin-F-box (SCF)-type ubiquitin ligase FBXW7 negatively regulates spermatogonial stem cell self-renewal. Proc Natl Acad Sci U S A 111:8826–8831
Kanatsu-Shinohara M, Onoyama I, Nakayama KI et al (2014b) Skp1-Cullin-F-box (SCF)-type ubiquitin ligase FBXW7 negatively regulates spermatogonial stem cell self-renewal. Proc Natl Acad Sci U S A 111:8826–8831
Kaucher AV, Oatley MJ, Oatley JM (2012) NEUROG3 is a critical downstream effector for STAT3-regulated differentiation of mammalian stem and progenitor spermatogonia. Biol Reprod 86:164-1–164-11
Kim Y, Turner D, Nelson J et al (2008) Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. Reproduction 136:823–831
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
Komai Y, Tanaka T, Tokuyama Y et al (2014) Bmi1 expression in long-term germ stem cells. Sci Rep 4:6175
Kotzur T, Benavides-Garcia R, Mecklenburg JM et al (2016) Granulocyte colony-stimulating factor (G-CSF) promotes spermatogenic regeneration from surviving spermatogonia after high-dose alkylating chemotherapy. Reprod Biol Endocrinol 15:7. https://doi.org/10.1186/s12958-016-0226-1
Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A 100:6487–6492
Kubota H, Avarbock MR, Brinster RL (2004a) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 101:16489–16494
Kubota H, Avarbock MR, Brinster RL (2004b) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71:722–731
Kusz KM, Tomczyk L, Sajek M et al (2009) The highly conserved NANOS2 protein: testis-specific expression and significance for the human male reproduction. Mol Hum Reprod 15:165–171
Lassalle B, Bastos H, Louis JP et al (2004) 'Side Population' cells in adult mouse testis express Bcrp1 gene and are enriched in spermatogonia and germinal stem cells. Development 131:479–487
Lo KC, Brugh VM III, Parker M et al (2005) Isolation and enrichment of murine spermatogonial stem cells using rhodamine 123 mitochondrial dye. Biol Reprod 72:767–771
Lovasco LA, Gustafson EA, Seymour KA et al (2015a) TAF4b is required for mouse spermatogonial stem cell development. Stem Cells 33:1267–1276
Lovasco LA, Gustafson EA, Seymour KA et al (2015b) TAF4b is required for mouse spermatogonial stem cell development. Stem Cells 33:1267–1276
Maki CB, Pacchiarotti J, Ramos T et al (2009) Phenotypic and molecular characterization of spermatogonial stem cells in adult primate testes. Hum Reprod 24:1480–1491

Margolin G, Khil PP, Kim J et al (2014) Integrated transcriptome analysis of mouse spermatogenesis. BMC Genomics 15:39
McCarrey JR (2013) Toward a more precise and informative nomenclature describing fetal and neonatal male germ cells in rodents. Biol Reprod 89:47
McLaren A (2003) Primordial germ cells in the mouse. Dev Biol 262:1–15
Mecklenburg JM, Hermann BP (2016) Mechanisms regulating spermatogonial differentiation. Results Probl Cell Differ 58:253–287
Meng X, Lindahl M, Hyvonen ME et al (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287:1489–1493
Morimoto H, Kanatsu-Shinohara M, Takashima S et al (2009) Phenotypic plasticity of mouse spermatogonial stem cells. PLoS One 4:e7909
Mutoji K, Singh A, Nguyen T et al (2016) TSPAN8 expression distinguishes spermatogonial stem cells in the prepubertal mouse testis. Biol Reprod 95:117
Nagano MC (2003) Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. Biol Reprod 69:701–707
Nagano M, Avarbock MR, Brinster RL (1999) Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. Biol Reprod 60:1429–1436
Nagano M, McCarrey JR, Brinster RL (2001) Primate spermatogonial stem cells colonize mouse testes. Biol Reprod 64:1409–1416
Nagano M, Patrizio P, Brinster RL (2002) Long-term survival of human spermatogonial stem cells in mouse testes. Fertil Steril 78:1225–1233
Nakagawa T, Nabeshima Y, Yoshida S (2007) Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. Dev Cell 12:195–206
Naughton CK, Jain S, Strickland AM et al (2006) Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate. Biol Reprod 74:314–321
Oakberg EF (1956) Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium. Am J Anat 99:507–516
Oakberg EF (1971) Spermatogonial stem-cell renewal in the mouse. Anat Rec 169:515–531
Oatley JM, Brinster RL (2006) Spermatogonial stem cells. Methods Enzymol 419:259–282
Oatley JM, Brinster RL (2008) Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 24:263–286
Oatley JM, Brinster RL (2012) The germline stem cell niche unit in mammalian testes. Physiol Rev 92:577–595
Oatley JM, de Avila DM, McLean DJ et al (2002) Transplantation of bovine germinal cells into mouse testes. J Anim Sci 80:1925–1931
Oatley JM, Avarbock MR, Telaranta AI et al (2006) Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc Natl Acad Sci U S A 103:9524–9529
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
Oatley JM, Oatley MJ, Avarbock MR et al (2009) Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development 136:1191–1199
Oatley JM, Kaucher AV, Avarbock MR et al (2010) Regulation of mouse spermatogonial stem cell differentiation by STAT3 signaling. Biol Reprod 83:427–433
Oatley MJ, Kaucher AV, Racicot KE et al (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
Ogawa T, Dobrinski I, Avarbock MR et al (1999a) Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. Biol Reprod 60:515–521
Ogawa T, Dobrinski I, Brinster RL (1999b) Recipient preparation is critical for spermatogonial transplantation in the rat. Tissue Cell 31:461–472
Ogawa T, Ohmura M, Yumura Y et al (2003) Expansion of murine spermatogonial stem cells through serial transplantation. Biol Reprod 68:316–322

Ohbo K, Yoshida S, Ohmura M et al (2003) Identification and characterization of stem cells in prepubertal spermatogenesis in mice small star, filled. Dev Biol 258:209–225
Orwig KE, Ryu BY, Avarbock MR et al (2002a) Male germ-line stem cell potential is predicted by morphology of cells in neonatal rat testes. Proc Natl Acad Sci U S A 99:11706–11711
Orwig KE, Shinohara T, Avarbock MR et al (2002b) Functional analysis of stem cells in the adult rat testis. Biol Reprod 66:944–949
Parker N, Falk H, Singh D et al (2014) Responses to glial cell line-derived neurotrophic factor change in mice as spermatogonial stem cells form progenitor spermatogonia which replicate and give rise to more differentiated progeny. Biol Reprod 91:92
Phillips BT, Gassei K, Orwig KE (2010) Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond Ser B Biol Sci 365:1663–1678
Raverot G, Weiss J, Park SY et al (2005) Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. Dev Biol 283:215–225
Reding SC, Stepnoski AL, Cloninger EW et al (2010) THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. Reproduction 139:893–903
Ryu BY, Orwig KE, Kubota H et al (2004) Phenotypic and functional characteristics of spermatogonial stem cells in rats. Dev Biol 274:158–170
Ryu BY, Kubota H, Avarbock MR et al (2005) Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc Natl Acad Sci U S A 102:14302–14307
Sachs C, Robinson BD, Andres ML et al (2014) Evaluation of candidate spermatogonial markers ID4 and GPR125 in testes of adult human cadaveric organ donors. Andrology 2:607–614
Sada A, Suzuki A, Suzuki H et al (2009) The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science 325:1394–1398
Schlesser HN, Simon L, Hofmann MC et al (2008) Effects of ETV5 (ets variant gene 5) on testis and body growth, time course of spermatogonial stem cell loss, and fertility in mice. Biol Reprod 78:483–489
Seandel M, James D, Shmelkov SV et al (2007) Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature 449:346–350
Shetty G, Uthamanthil RK, Zhou W et al (2013) Hormone suppression with GnRH antagonist promotes spermatogenic recovery from transplanted spermatogonial stem cells in irradiated cynomolgus monkeys. Andrology 1:886–898
Shima JE, McLean DJ, McCarrey JR et al (2004) The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod 71:319–330
Shinoda G, de Soysa TY, Seligson MT et al (2013) Lin28a regulates germ cell pool size and fertility. Stem Cells 31:1001–1009
Shinohara T, Avarbock MR, Brinster RL (1999) β1- and α6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 96:5504–5509
Shinohara T, Avarbock MR, Brinster RL (2000a) Functional analysis of spermatogonial stem cells in Steel and cryptorchid infertile mouse models. Dev Biol 220:401–411
Shinohara T, Orwig KE, Avarbock MR et al (2000b) Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci U S A 97:8346–8351
Shinohara T, Orwig KE, Avarbock MR et al (2001) Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc Natl Acad Sci U S A 98:6186–6191
Sieburg HB, Cho RH, Muller-Sieburg CE (2002) Limiting dilution analysis for estimating the frequency of hematopoietic stem cells: uncertainty and significance. Exp Hematol 30:1436–1443
Singh D, Paduch DA, Schlegel PN et al (2017) The production of glial cell line-derived neurotrophic factor by human sertoli cells is substantially reduced in sertoli cell-only testes. Hum Reprod 32:1108–1117
Song HW, Bettegowda A, Lake BB et al (2016) The homeobox transcription factor RHOX10 drives mouse spermatogonial stem cell establishment. Cell Rep 17:149–164
Sun F, Xu Q, Zhao D et al (2015) Id4 marks spermatogonial stem cells in the mouse testis. Sci Rep 5:17594

Suzuki H, Ahn HW, Chu T et al (2012) SOHLH1 and SOHLH2 coordinate spermatogonial differentiation. Dev Biol 361:301–312

Takashima S, Kanatsu-Shinohara M, Tanaka T et al (2011) Rac mediates mouse spermatogonial stem cell homing to germline niches by regulating transmigration through the blood-testis barrier. Cell Stem Cell 9:463–475

Tanaka T, Kanatsu-Shinohara M, Shinohara T (2015) The CDKN1B-RB1-E2F1 pathway protects mouse spermatogonial stem cells from genomic damage. J Reprod Dev 61:305–316

Tegelenbosch RAJ, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 290:193–200

Tokuda M, Kadokawa Y, Kurahashi H et al (2007) CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol Reprod 76:130–141

Valli H, Phillips BT, Orwig KE, Gassei K, Nagano MC (2015) Spermatogonial stem cells and spermatogenesis. In: Plant TM, Zeleznik AJ (eds) Knobil and Neill's physiology of reproduction, 4th edn. Academic Press, Waltham, MA, pp 595–635

van Bragt MP, Roepers-Gajadien HL, Korver CM et al (2008) Expression of the pluripotency marker UTF1 is restricted to a subpopulation of early A spermatogonia in rat testis. Reproduction 136:33–40

von Kopylow K, Staege H, Spiess AN et al (2012) Differential marker protein expression specifies rarefaction zone-containing human Adark spermatogonia. Reproduction 143:45–57

Wang PJ, McCarrey JR, Yang F et al (2001) An abundance of X-linked genes expressed in spermatogonia. Nat Genet 27:422–426

Wu X, Oatley JM, Oatley MJ et al (2010) The POU domain transcription factor POU3F1 is an important intrinsic regulator of GDNF-induced survival and self-renewal of mouse spermatogonial stem cells. Biol Reprod 82:1103–1111

Wu AR, Neff NF, Kalisky T et al (2014) Quantitative assessment of single-cell RNA-sequencing methods. Nat Methods 11:41–46

Yang QE, Oatley JM (2014) Spermatogonial stem cell functions in physiological and pathological conditions. Curr Top Dev Biol 107:235–267

Yang QE, Gwost I, Oatley MJ et al (2013a) Retinoblastoma protein (RB1) controls fate determination in stem cells and progenitors of the mouse male germline. Biol Reprod 89:113

Yang QE, Racicot KE, Kaucher AV et al (2013b) MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells. Development 140:280–290

Yoshida S, Takakura A, Ohbo K et al (2004) Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis. Dev Biol 269:447–458

Yoshida S, Sukeno M, Nakagawa T et al (2006) The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. Development 133:1495–1505

Yoshida S, Nabeshima Y, Nakagawa T (2007a) Stem cell heterogeneity: actual and potential stem cell compartments in mouse spermatogenesis. Ann N Y Acad Sci 1120:47–58

Yoshida S, Sukeno M, Nabeshima Y (2007b) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317:1722–1726

Zhang X, Ebata KT, Nagano MC (2003) Genetic analysis of the clonal origin of regenerating mouse spermatogenesis following transplantation. Biol Reprod 69:1872–1878

Zheng K, Wu X, Kaestner KH et al (2009) The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol 9:38

Regulation of Spermatogonial Stem Cell Maintenance and Self-Renewal

5

Tessa Lord and Jon M. Oatley

Abstract

Spermatogonial stem cells (SSCs) reside within the stem cell niche along the basement membrane of the seminiferous tubules in the testis, and their actions provide the basis for continuity and regeneration of the spermatogenic lineage. SSCs must balance self-renewal with the production of progenitor spermatogonia in order to sustain optimal sperm production while preventing exhaustion of the stem cell reservoir. Regulation of SSC fate decision is in part influenced by signaling from growth factors, such as Gdnf and Fgf2, which are synthesized by somatic niche support cells. Such growth factors have been shown to directly influence expression of transcription factors such as Id4, Etv5, and Bcl6b within SSCs to stimulate self-renewal. Additionally, the undifferentiated state of both SSCs and progenitors is maintained by virtue of intracellular regulation at transcriptional, translational, and posttranslational levels; both independently and dependently of characterized growth factors released from the niche. This intrinsic regulation not only acts to enrich the expression of genes important for maintaining the undifferentiated state, but also supresses expression of differentiation-driving factors. Although progress in SSC research has previously been dampened by a lack of SSC-specific markers that can be used to isolate pure populations for analysis, recent advances have seen the development of mouse lines in which the SSC population alone is marked by expression of a fluorescent reporter transgene; for example the *Id4-eGfp* mouse line. Consequently, in-depth analysis of the SSC population in comparison to undifferentiated progenitors and differentiating spermatogonia is now possible. Further progress in characterizing factors involved in SSC maintenance and self-renewal is important for

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understanding potential underlying causes of idiopathic infertility, and further, is the basis for developing therapeutic strategies aimed at reinstating fertility in patients who have been rendered infertile as a consequence of chemotherapeutic treatments in pre-pubertal life.

Keywords
Spermatogonial stem cells • SSC niche • SSC self-renewal

5.1 Introduction

The foundation for continual and robust spermatogenesis in the mammalian testis is provided by actions of spermatogonial stem cells (SSCs). Mitotic division of SSCs is the basis for self-renewal that underlies maintenance of a foundational pool. From this pool, progenitor spermatogonia arise that amplify in number, also through mitotic divisions, prior to transitioning to a differentiating state under the influence of retinoic acid; an event that signifies commitment to terminal differentiation as spermatozoa (detailed in Chap. 6). In adulthood, SSC activities fuel the production of approximately 70 million sperm per day in rodents, and 100 million sperm per day in humans (Johnson et al. 1980). Historically, the rarity of SSCs in the testis [approximately 0.03% of cells in mouse and 1.25% of cells in human testes (Tagelenbosch and de Rooij 1993; Aponte et al. 2005)], in conjunction with a lack of established molecular markers available to distinguish SSCs from progenitor cells, has made the elucidation of pathways responsible for stem cell maintenance and self-renewal difficult. Fortunately, recent advances in the field, particularly the identification of factors expressed exclusively in SSCs, have paved the way for critical advances in our knowledge of these processes.

5.2 Developmental Origins of the SSC Population

SSCs originate from prospermatogonia that differentiate from primordial germ cells (PGCs) on the genital ridge during embryonic development. The majority of our understanding of this process comes from mouse studies which will be summarized here, while events in humans will be touched on briefly. Specification of PGCs is thought to begin from embryonic day 6–6.5 (Yoshimizu et al. 2001; Saitou et al. 2002), and complete specification can be identified by day 7–7.5; at which time expression of germ cell-specific markers, such as alkaline phosphatase, can be visualized (Chiquoine 1954; Ginsburg et al. 1990). In conjunction with the specification process, PGCs experience epigenetic reprogramming, including global gene demethylation and histone modification (Tseng et al. 2015). Following these events, PGCs undergo extensive mitotic proliferation up until embryonic day 13.5 (Nakatsuji and Chuma 2001). At this time, differentiation according to embryonic sex occurs, with PGCs in XY embryos transforming into prospermatogonia (De Felici et al. 2004).

The prospermatogonia continue to proliferate until day 15.5 at which point a period of quiescence is initiated and sustained until neonatal development (1–5 days postpartum depending on strain of mice) (Kluin and de Rooij 1981; Ginsburg et al. 1990). During the quiescent period, a new DNA methylation profile is initiated within prospermatogonia (Tseng et al. 2015), becoming fully established by the time of reentry into the cell cycle. Concomitant with cessation of de novo DNA methylation and resumption of proliferation, prospermatogonia transform into a primary population of undifferentiated spermatogonia comprised of SSCs and progenitors, and a differentiating population of spermatogonia that give rise to the first round of spermatogenesis at postnatal days 28–35. All other rounds of spermatogenesis arise from the primary undifferentiated population (Yang et al. 2013b).

As humans have a considerably longer pre-pubertal period than their murine counterparts, the transition of prospermatogonia into a population of postnatal spermatogonia does not occur until 2–3 months of age, while differentiation is not observed until approximately 12 years of age (Paniagua and Nistal 1984). The intricate transformation of prospermatogonia into spermatogonia during embryonic and neonatal life is clearly imperative for the formation of an SSC population that fuels sperm production in adulthood; however, impairment of this transition is also associated with pre-neoplastic changes that are thought to be the root of carcinoma in situ cells: the major source of testicular germ cell tumors (Skakkebæk 1972). Our understanding of the mechanisms and potential models for specification of the SSC pool from prospermatogonial precursors is detailed further in Chap. 2.

5.3 Models for Maintenance and Renewal of the SSC Pool

Following the onset of puberty, continuity of the spermatogenic lineage requires maintenance of the SSC pool. During steady-state conditions, progenitor spermatogonia periodically arise from SSCs; however, this is balanced by self-renewal to prevent depletion of the SSC population. Alternatively, certain situations require rapid regeneration of the spermatogonial population; such as following a cytotoxic insult that depletes a major portion of the population, in response to spermatogonial transplantation, or during neonatal development. In these circumstances self-renewal must predominate to rebuild the SSC pool. Again, in the absence of self-renewal, the SSC population will decline over time and eventually become exhausted, or regeneration will be stunted.

The most widely accepted model depicting the dynamics of the SSC and progenitor spermatogonial pools is referred to as the "A_{single} model". In rodent species, spermatogonia exist in three subclasses; type A, intermediate, and type B. It is well established that SSCs are a component of the type A spermatogonia, in particular the undifferentiated subpopulation. Mitotic division of the SSCs may either be symmetrical; producing two new SSCs (or two progenitors), or alternatively, asymmetrical; producing one SSC and one progenitor cell that will be committed to differentiation (Fig. 5.1a). Upon mitotic proliferation, progenitor cells remain connected by persistent cytoplasmic bridges, forming structures referred to as "A_{paired}"

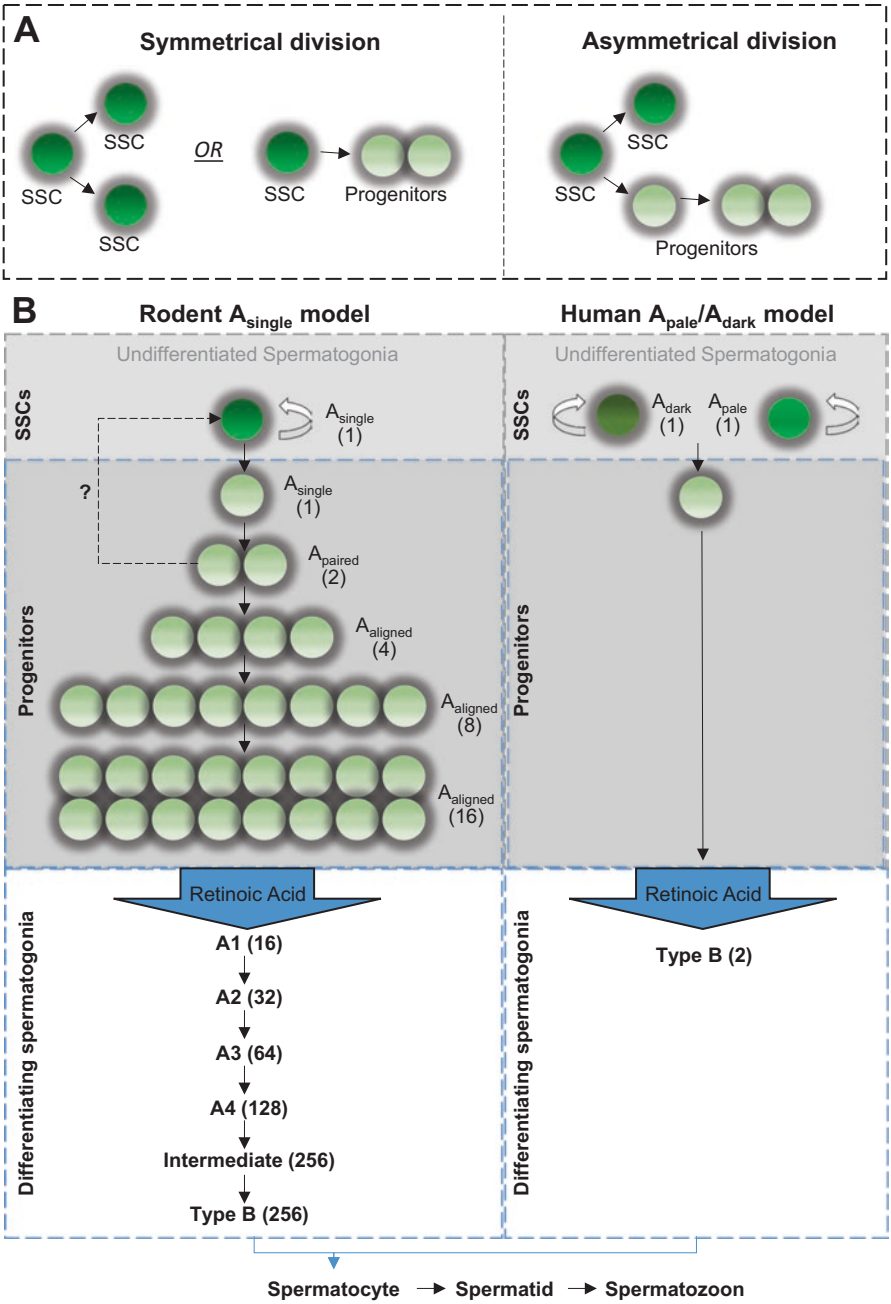

Fig. 5.1 Maintenance and renewal of the SSC pool. (**a**) Depending on systemic requirements, SSCs may either favor self-renewal to replenish the population, favor progenitor formation for the production of sperm, or maintain steady-state conditions. Thus, mitotic division of SSCs may be either symmetrical or asymmetrical. (**b**) The most widely accepted model for SSC maintenance and

spermatogonia. As a consequence of this syncytial connection, further mitotic divisions of these progenitors occur in synchrony, producing chains of spermatogonia referred to as "$A_{aligned}$," consisting of up to 16 cells (Fig. 5.1b). Cumulative evidence derived from rodent studies suggests that stem cell activity resides exclusively within the isolated A_{single} population, with the formation of an A_{paired} structure signifying commitment to a differentiation pathway. The first experimental evidence supporting this model came in the form of morphological and proliferative observations on spermatogonia in rodent testes in the 1970s (Huckins 1971; Oakberg 1971) and has been supported by more recent research efforts that utilize fluorescent reporters to identify SSCs specifically amongst the heterogeneous population of spermatogonia in the testes (Chan et al. 2014). Although the 'A_{single}' hypothesis depicts that all SSCs exist as A_{single} cells, conversely, not all A_{single} cells are believed to be SSCs. In fact, it has been estimated that under 10% of the A_{single} population retains the capacity for self-renewal and regeneration of the spermatogenic lineage (Nagano 2003; Chan et al. 2014). Thus, a 'revised' A_{single} model has been put forth to factor in this nuance (reviewed by Lord and Oatley 2017).

In contrast to the traditional A_{single} model, a recently emerged hypothesis, the 'fragmentation' hypothesis, proposes that contribution to the stem cell pool may be a more dynamic process than originally believed (reviewed by Lord and Oatley 2017). Time-lapse imaging studies have been used to demonstrate that spermatogonia can break off from $A_{aligned}$ chains to produce single cells (Hara et al. 2014); however, whether these cells, which can now be identified as "A_{single}," possess the functional attributes of an SSC remains to be unequivocally demonstrated. Despite this, results of pulse-chase experiments have suggested that a small subset of progenitors may be able to revert to a stem cell state and induce colonization in the testes. This occurrence is purportedly more common when regeneration of the germline is required (in this case, following transplantation or tamoxifen exposure), rather than during steady-state conditions (Nakagawa et al. 2010). Potentially, these data may depict that a subset of the rodent undifferentiated spermatogonia population act as "reserve" or "potential" stem cells, that contribute to the self-renewing population when replenishment of the germline is required; however, do not significantly contribute to the self-renewing population in steady-state conditions.

Fig. 5.1 (continued) progenitor production in rodents is the A_{single} model in which the A_{single} spermatogonia make up the SSC population. Upon transition into a progenitor state, cytokinesis following mitotic division is incomplete, forming pairs (A_{paired}), and chains ($A_{aligned}$) of spermatogonia. Undifferentiated progenitors commit to differentiation in response to retinoic acid signaling. Differentiating "A1" spermatogonia transition further to A2, A3, A4, Intermediate, and Type B spermatogonia, accompanied by additional mitotic divisions, before finally committing to the initiation of meiosis at the spermatocyte stage. Following two rounds of meiosis, haploid spermatids are formed that will mature into spermatozoa. In contrast to the rodent model, the human model for SSC maintenance is the A_{pale}/A_{dark} model. In this system, the A_{dark} cells are thought to be the "reserve" stem cells, while the A_{pale} cells actively contribute to fueling steady-state spermatogenesis. The primary difference between the rodent and human undifferentiated spermatogonial pool is the absence of rounds of successive mitotic division of progenitors in the human model. Instead, progenitor spermatogonia produced from division of A_{pale} or A_{dark} SSCs transition directly into Type B differentiating spermatogonia

In contrast to rodent species, the paradigm depicting SSC dynamics in primates is referred to as the A_{pale}/A_{dark} model (Fig. 5.1b). The primate type A spermatogonial population has been divided into these two subsets as a reflection of the different staining intensity of nuclei of spermatogonia by hematoxylin (Clermont and Antar 1973). Although not definitively classified with functional evidence, both A_{pale} and A_{dark} cells are thought to possess stem cell activity, with A_{dark} cells considered to be the reserve or "back-up" stem cells, while the A_{pale} cells actively self-renew and contribute to spermatogenesis in steady-state conditions (Clermont 1969). Progenitor cells produced from the A_{pale} division transition directly into type B spermatogonia (Clermont 1966), thus eliminating the clonal expansion step that is observed within mouse spermatogenesis. The smaller number of mitotic divisions during spermatogenesis in primates means that this process is highly inefficient when compared to that of rodents; particularly in the case of humans, whose spermatogonial progenitor cells undergo only one mitotic division prior to differentiation into a type B spermatogonium (Amann 2008).

5.4 The SSC Niche

Maintenance of the SSC pool is reliant on a niche microenvironment within the testis that is comprised of contributions from somatic cells that provide structural support as well as secrete growth factors that regulate the balance between self-renewal and differentiation. As mentioned previously, the requirement for intensive SSC renewal during neonatal development is a distinct contrast to the steady-state conditions required for maintenance during adulthood; representing the intricate and dynamic nature of the SSC niche. The SSC niche is comprised of the germ cells themselves, as well as the Sertoli cells; residing inside the epithelium of the seminiferous tubule (Fig. 5.2).

Observational studies have resulted in postulation that the formation of stem cell niche regions within the testis preferentially occurs adjacent to the blood vessels and interstitium, particularly associating with blood vessel branch points (Yoshida et al. 2007). Despite this, direct functional studies are yet to be conducted to confirm this observation. Also, another study observed that a majority of undifferentiated spermatogonia align in regions of seminiferous tubules associated with the interstitium at stages VII-VIII of the seminiferous cycle (Chiarini-Garcia et al. 2003). Considering that these stages are when most of the undifferentiated spermatogonia transition to a differentiating state, the association with vasculature and interstitial tissue may be driving the differentiating transition rather than influencing maintenance of the SSC pool. Furthermore, Inhibitor of DNA binding 4- (Id4)-eGfp+ spermatogonia which possess potent SSC capacity reside in areas of tubules that are not associated with the interstitial space (Chan et al. 2014). Moreover, experimental alteration of SSC niche number within seminiferous tubules does not alter the amount of area that associates with the vasculature or interstitium (Oatley et al. 2011a). Taking all of these observations into account, further investigation into whether the vasculature and interstitial tissue are key components of the SSC niche is clearly warranted.

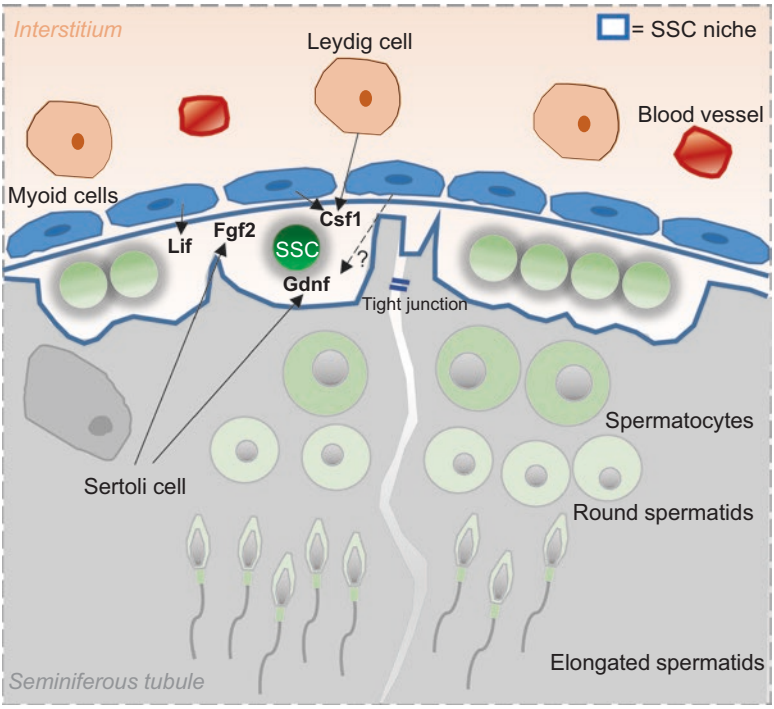

Fig. 5.2 The spermatogonial stem cell niche. The SSC niche-unit is comprised of the SSCs themselves, undifferentiated progenitor spermatogonia, and surrounding somatic support cells. The somatic cells of the SSC niche; the Sertoli, Leydig, and peritubular myoid cells; release growth factors that are thought to stimulate self-renewal of SSCs, and maintenance of an undifferentiated state in both SSCs and progenitors. The Sertoli cells, in addition to providing architectural support to the niche, produce the growth factors Gdnf and Fgf2, while both Leydig and myoid cells produce Csf1, and myoid cells produce Lif, and potentially Gdnf

Although the Sertoli cells are regarded to be the most influential regulators of the stem cell niche due to their direct interaction with germ cells, somatic cells within the interstitial space such as the Leydig and peritubular myoid cells also appear to be involved in niche regulation. Certainly, the positioning of the SSC population on the outer of the blood-testis barrier (formed via tight junctions between the Sertoli cells) would allow for exposure of these stem cells to regulatory factors that may be produced locally in the interstitium. The critical importance of the somatic support cells within the SSC niche is demonstrated by the direct association between their functionality/abundance and fluctuations in the SSC pool. Indeed, increasing the Sertoli cell population in the testes of mice using transplantation techniques has been shown to result in a threefold elevation in SSC number, as well as an improvement in stem cell niche formation following SSC transplantation (Oatley et al. 2011a). Additionally, the deterioration of niche quality, rather than the quality of the SSCs themselves, is thought to be causative of the decline in fertility that is associated with reproductive ageing. As such, SSCs transplanted from sub-fertile aged animals can support proficient spermatogenesis when transplanted into the testis of young animals (Ryu et al. 2006).

Support cell function in the SSC niche is likely to be a direct implication of the endocrine capacity of these cells. Both Leydig cells and Sertoli cells express gonadotropin receptors: these are luteinizing hormone (LH) and follicle stimulating hormone (FSH) receptors respectively. Certainly, repression of gonadotropin release from the anterior pituitary is directly linked with impaired spermatogonial proliferation in neonates (Kanatsu-Shinohara et al. 2004b). In response to gonadotropins, support cells (particularly the Sertoli cells) of both the murine and primate SSC niche have been demonstrated to release growth factors such as glial cell derived neurotrophic factor (Gdnf), that are directly implicated in maintenance of the spermatogonial population (Van Alphen et al. 1988; Crépieux et al. 2001; Kanatsu-Shinohara et al. 2004b; Mäkelä et al. 2014). In addition, circulating gonadotropins stimulate the production of testosterone by the Leydig cells, with testosterone also being hypothesized to promote growth factor production by both the peritubular myoid and Sertoli cells (Gonzalez-Herrera et al. 2006; Chen et al. 2014, 2016), as well as expression of miRNAs by Sertoli cells (Panneerdoss et al. 2012), that are potentially involved in SSC maintenance (Niu et al. 2011; He et al. 2013).

Perhaps the most extensively characterized paracrine regulator of SSC renewal is the Sertoli-secreted growth factor Gdnf. Interaction of Gdnf with its receptor complex (Ret receptor tyrosine kinase and Gdnf family α1 [Gfrα1]) that is known to be expressed on the surface of spermatogonia (He et al. 2007), not only supports maintenance of the SSC population, but concurrently inhibits differentiation of undifferentiated spermatogonia by retinoic acid; as demonstrated by Gdnf overexpression mouse models (Meng et al. 2000). Further, although *Gdnf*-, *Gfrα1*- and *Ret*-null mice do not survive beyond the first day of postnatal life (Schuchardt et al. 1994; Moore et al. 1996; Pichel et al. 1996; Enomoto et al. 1998), the deleterious effects of decreased Gdnf expression by Sertoli cells have been observed in $Gdnf^{+/-}$ mice. While these mice remain fertile, a high percentage of their seminiferous tubules contain impaired spermatogenesis due to depleted/reduced proliferation of spermatogonia (Meng et al. 2000). Like Gdnf, fibroblast growth factor 2 (Ffg2) is known to be secreted by the Sertoli cells in vivo (Chen and Liu 2015), and is thought to act along with Gdnf in a ratio-dependent manner to regulate SSC maintenance and self-renewal (Takashima et al. 2015). In conjunction with these growth factors secreted by the Sertoli cells; both Leydig and peritubular myoid cells in the mouse exhibit in vivo expression of colony stimulating factor-1 (Csf1), a ligand known to bind to the Csf1 receptor (Csf1r) expressed by a subset of undifferentiated spermatogonial population to enhance SSC renewal in vitro (Oatley et al. 2009). Additionally, peritubular myoid cells produce the growth factor leukemia inhibitory factor (Lif) (Piquet-Pellorce et al. 2000) which may work alongside Gdnf to stimulate SSC proliferation (Wang et al. 2014). Further, peritubular myoid cells may also potentially produce Gdnf themselves (Chen et al. 2016). Indeed, in mice with a Gdnf conditional knockout in peritubular myoid cells, an age-associated reduction in fertility is observed, purportedly as a consequence of the population of undifferentiated spermatogonia being reduced (Chen et al. 2016). A schematic representation of growth factor regulation within the stem cell niche by Sertoli, Leydig and peritubular myoid cells is provided in Fig. 5.2, and an extensive list of growth factors and their purported effects listed in Table 5.1.

Table 5.1 Extrinsic regulatory factors involved in directing fate decisions in SSCs and undifferentiated spermatogonia

Regulatory factor	Origin	Biological effect	Species	Reference
FSH and LH	Pituitary	Indirect effects on SSC self-renewal? Potential regulation of Gdnf secretion by Sertoli cells	Mouse Monkey	Mäkelä et al. (2014) and Crépieux et al. (2001) Kanatsu-Shinohara et al. (2004a) and Van Alphen et al. (1988)
Testosterone	Leydig cells	Indirect effects on SSC self-renewal. Stimulates Gdnf production by peritubular myoid cells and Fgf2 secretion by Sertoli cells	Mouse	Chen et al. (2014) and Gonzalez-Herrera et al. (2006)
Gdnf	Sertoli cells, peritubular myoid cells	Drives SSC self-renewal, impairs retinoic acid-driven differentiation	Mouse Rat Rabbit Hamster	Kubota et al. (2004b), Meng et al. (2000), Chen et al. (2014), and Chen and Liu (2015) Ryu et al. (2005) Kubota et al. (2011) Kanatsu-Shinohara et al. (2008a)
Fgf2	Sertoli cells	Supports/encourages SSC self-renewal	Mouse Rat Rabbit Hamster	Kubota et al. (2004b) Ryu et al. (2005) Kubota et al. (2011) Kanatsu-Shinohara et al. (2008a)
Lif	Peritubular myoid cells	Stimulates proliferation of undifferentiated spermatogonia in conjunction with Gdnf	Mouse	Wang et al. (2014)
Igf-1	Leydig cells	Enhances SCC renewal	Mouse	Kubota et al. (2004b)
Retinoic acid	Sertoli cells? Preleptotene spermatocytes?	Stimulates differentiation of progenitors	Mouse	Dann et al. (2008), Raverdeau et al. (2012), and Tong et al. (2013)
Csf1	Leydig cells, myoid cells	Stimulates self-renewal of SSCs	Mouse	Oatley et al. (2009)

5.5 Tools to Study the SSC Population

Historically, progress in characterizing the SSC population has been marred by several limitations. For instance, the rarity of SSCs within an undifferentiated spermatogonial population in which progenitors are much more abundantly represented, and also the lack of markers available to distinguish spermatogonial subtypes. As a consequence of these difficulties, the field has focused on advancing in vitro culture techniques to provide a sustainable population of cells to study, on further identifying SSC-specific factors expressed both intrinsically and on the surface of the cell, and on formulating novel methodologies to monitor and analyze SSC content within the heterogeneous spermatogonial population. The tools that are currently available for studying the SSC population are discussed below.

5.5.1 Primary Spermatogonial Cultures

Key limitations in studying SSCs are their rarity in the testis, along with the difficulty of directly manipulating these cells in vivo without interfering with the function of other germ cell populations or somatic support cells. As such, primary spermatogonial culture techniques have been continuously refined to provide a platform for producing larger numbers of cells for analysis, and a means for experimental manipulation that directly assesses the role of genes, molecular pathways, and extrinsic signals on self-renewal and maintenance of the SSC population. In refining cell culture techniques, replication of conditions within the stem cell niche is imperative, not only to maintain viability of these cells, but also to prevent entry into a differentiating pathway. As mentioned previously, the somatic support cells of the SSC niche release growth factors to stimulate self-renewal of the SSC population, and as such, a subset of these growth factors have been harnessed for the development of culture media that supports long-term maintenance of SSCs; including Gdnf, and Fgf2.

The assessment of culture conditions for supporting long-term maintenance of SSCs in vitro has been achieved using defined serum free media (Kubota et al. 2004b), to which desired growth factors are incrementally added to establish their effects on the SSC population. Importantly, SSCs are routinely cocultured with SIM mouse embryo-derived thioguanine and ouabain resistant (STO) feeder cells that are known to support culture of several stem cell types (Matsui et al. 1992; Nagano et al. 1998; Paratcha et al. 2003); likely by secreting several growth factors that are component of the respective niches in vivo. Medium that can promote SSC maintenance in vitro is identified by the formation and expansion of colonies of undifferentiated spermatogonia, as well as the ability for a portion of the cells to regenerate spermatogenesis in recipient testes posttransplantation. The addition of Gdnf alone to serum free culture medium has differing outcomes dependent on the strain of mouse from which the primary spermatogonial culture originated. For the spermatogonial population from mice with a DBA/2J genetic background, Gdnf supplementation alone supports colony expansion, and a subset of the population is able to

efficiently engraft in recipient testes following transplantation at various times of the culture period, thereby demonstrating self-renewal of SSCs (Kubota et al. 2004b). However, SSCs from other mouse genetic backgrounds (e.g. C57BL/6) do not thrive in these conditions. In these circumstances, SSC maintenance is achieved with the supplementation of Gdnf in combination with Fgf2 (Kubota et al. 2004b). Conflicting data exists as to whether Fgf2 alone can support in vitro proliferation of SSCs (Kubota et al. 2004b; Takashima et al. 2015). However, in combination with Gdnf, Fgf2 certainly improves long-term culture efficiency for spermatogonia from rabbits (Kubota et al. 2011), hamsters (Kanatsu-Shinohara et al. 2008b) and rats (Ryu et al. 2005).

Although several additional growth factors are known to stimulate proliferation and self-renewal of stem cells from other tissue types, the supplementation of these factors to SSC culture medium has been found to be ineffective or inconsistent. For instance, although Lif is critical for proliferation and maintenance of embryonic stem cell populations in vitro (Murray and Edgar 2001), and further, is known to be produced by the peritubular myoid cells in the testes (Piquet-Pellorce et al. 2000; Dorval-Coiffec et al. 2005); several studies have reported that supplementation of Lif into serum free culture medium has no demonstrable effect on the proliferation of rodent SSCs in vitro (Kubota et al. 2004b; Wang et al. 2014). Contrastingly, SSC proliferation was found to be improved when Lif was used in conjunction with Gdnf (Wang et al. 2014), but again, this is result is controversial (Nagano 2003). Numerous further growth factors have also been analyzed, including stem cell factor, epidermal growth factor, insulin-like growth factor 1 (Igf-1), and Noggin; however, of these factors only Igf-1 improved the maintenance of SSCs (Kubota et al. 2004b) (Table 5.1).

Although in vitro culture techniques for rodent spermatogonia have significantly advanced over the past 20 years, these cultures are still time-restricted; exhibiting a decline in stem cell number after prolonged periods (>6 months) (Kanatsu-Shinohara et al. 2005; Helsel et al. 2017a). Further, success with culture of human spermatogonia in these rodent-optimized conditions is severely limited, and remains a considerable challenge in the field (Zheng et al. 2014; Gassei and Orwig 2016). Together, these issues highlight the need for further refinement of culture conditions; potentially by reassessing the metabolomic requirements of undifferentiated spermatogonia, as well as the differences between rodent and primate models. Such advances are critical if SSC culture is to be considered a component of therapeutic strategies to reverse chemotherapy-induced infertility.

5.5.2 Cell Surface Markers

Clearly, characterization of the undifferentiated spermatogonial subtypes is more easily facilitated if pure populations of SSCs and progenitors can be isolated for analysis. The isolation of live SSC populations from the testes or from primary cultures of undifferentiated spermatogonia using antibody-driven strategies requires the elected “marker” to be expressed on the cell surface, following which magnetic

or fluorescence activated cell sorting techniques (MACS and FACS respectively) can be utilized. Unfortunately, to date, no surface markers have been identified that can unequivocally distinguish SSCs from undifferentiated progenitor spermatogonia. Despite this, a number of cell surface markers have been identified that can be utilized to enrich SSCs in a spermatogonial population when compared to an unselected control. A comprehensive list of cell surface markers that have been characterized for SSC enrichment is provided in Table 5.1, along with their efficiency. Cell adhesion molecules predominate this list, being an attractive area of investigation due to their purported role in "homing" of SSCs to the stem cell niche (Kanatsu-Shinohara et al. 2008a).

The cell adhesion molecules α6 and β1 integrin were the first candidates identified to have some capacity to facilitate enrichment of the SSC population. The expression of both molecules had been detected on the surface of spermatogonia within the undifferentiated population (Shinohara et al. 1999; Ebata et al. 2005); where their function was hypothesized to be the binding of laminin in the basement membrane of seminiferous tubules. Indeed, disruption of β1 integrin expression impaired the capacity of SSCs to regenerate spermatogenesis following transplantation into recipient testes (Kanatsu-Shinohara et al. 2008a); making these factors attractive candidates for SSC-specificity. Despite this, isolation of the $\alpha6^+$ and $\beta1^+$ populations from the testis resulted only in an eight and fourfold enrichment of the SSC population, respectively, when compared to an unselected control population (Shinohara et al. 1999); reflecting the expression of these markers not only in SSCs, but also cells within the progenitor population.

Several other cell adhesion molecules were characterized in the undifferentiated spermatogonial population in the years following the discovery of α6- and β1-integrin, including CD9, epithelial cell adhesion molecule (EpCAM), and Cadherin 1 (Cdh1). Both CD9 and EpCAM were originally classified as surface markers of embryonic stem cells. For the germline, selection of the CD9+ population leads to a sevenfold enrichment for SSCs in rodents (Kanatsu-Shinohara et al. 2004a); however, this is limited by the fact that CD9 is also expressed by differentiating germ cells and somatic cells. Similarly, EpCAM expression extends beyond SSCs to progenitors. As such, EpCAM enriched populations provide only a threefold increase in SSC content, as determined by spermatogonial transplantation analyses (Kanatsu-Shinohara et al. 2011). Isolation of the Cdh1+ population produces a similar result, again as a consequence of universal staining across the entirety of the undifferentiated spermatogonial population (Tokuda et al. 2007).

In order to increase enrichment efficiency, cell adhesion surface markers are regularly used in conjunction with other available surface antigens, or fluorescent assays. For example, isolation of $\alpha6^+$ testis cells that are also c-Kit- and MHC class I- significantly improves repopulation efficiency following transplantation (Kubota et al. 2003); with c-Kit being a marker of germ cell differentiation that is evident from the $A_{aligned}$ stage onwards (Schrans-Stassen et al. 1999), and MHC-I a marker thought to be present on the surface of almost all nucleated cells, however, absent from the spermatogonial population (Glynn 1988). Alternatively, the utilization Cdh1 labeling in conjunction with a fluorescent assay that monitors aldehyde

dehydrogenase (Aldh) activity allows for isolation of the Cdh1+, Aldh1− spermatogonial population that is enriched for SSC concentration when compared to the Cdh1+ population alone (Kanatsu-Shinohara et al. 2013). Although such multiparameter techniques facilitate the isolation of a population that is enriched for SSCs, such isolation strategies are associated with greater complexity and increased cell loss from testis preparations.

To date, Thy1; a phosphatidylinositol-anchored surface antigen that is known to be expressed in a hematopoietic (Spangrude et al. 1988), mesenchymal (Jiang et al. 2002) and embryonic (Ling and Neben 1997) stem cells; has arguably been the most valuable surface marker characterized as a single-parameter for SSC enrichment. The Thy1+ fraction isolated from mouse testes is enriched for SSC number by 30-fold compared to the nonselected total testis fraction of adult mice (Kubota et al. 2004a). As a consequence of the clearly superior SSC enrichment capacity of Thy1+ when compared to the other surface markers discussed, it is not surprising that this surface antigen is commonly employed in current SSC research, particularly for enriching SSC populations to generate primary spermatogonial cultures (Oatley et al. 2009; Helsel et al. 2017a). Additionally, the Thy1 enriched spermatogonial population has proved to be valuable for identifying intrinsic factors involved in regulating SSC maintenance and self-renewal (Oatley et al. 2007). Despite the value of Thy1 as an SSC surface marker, it still does not facilitate isolation of a pure SSC population; with the estimation of SSC content in the Thy1+ population being 1 in 15 cells (Kubota et al. 2003).

In the search for a SSC-specific marker, it is not surprising that receptors for growth factors produced by niche support cells to stimulate self-renewal of SSCs have been attractive candidates. As mentioned previously, Gfrα1 is part of the binding receptor complex for Gdnf (He et al. 2007); with Gdnf stimulating self-renewal of SSCs in vitro (Kubota et al. 2004b). Unfortunately, however, SSC content from Gfrα1+ populations of spermatogonia is only slightly enriched compared to that of unselected control cells in the pup testis, and in fact, SSC content is unchanged from the unselected control population in adult testes (Ebata et al. 2005); making this marker an undesirable choice for isolating pure SSC populations. Upon further investigation, it was found that Gfrα1 expression was seemingly uniform across the entire population of type-A spermatogonia in both mouse (Ebata et al. 2005; Grasso et al. 2012) and human testis (Grisanti et al. 2009; He et al. 2010), thus explaining the lack of SSC enrichment achieved by isolating cell fraction using this surface marker.

Although the capacity for the aforementioned surface markers to enrich the SSC population has been primarily focused on rodent species, the expression of these surface antigens appears to be relatively conserved between rodents and primates, with putative SSCs in rhesus macaque testis being $Thy1^{+}$, $Gfr\alpha1^{+}$ and c-Kit^{-} (Hermann et al. 2009). Additionally, some putative human SSC markers, such as stage-specific embryonic antigen 4 (Ssea4), have been identified (Kokkinaki et al. 2011). Despite this, a gap in knowledge clearly still exists in the elucidation of surface markers that are truly SSC-specific. Although this limitation has been somewhat circumvented in rodent species as a consequence of the identification of

intrinsic factors solely expressed in the SSC population, as well as the capacity to generate transgenic lines that express fluorescent reporters based on these intrinsic factors (discussed below); such strategies are clearly not translatable to the study of human SSC populations, and remain difficult in the majority of other mammalian species. As such, further investigation is required to establish surface markers that are SSC-specific, and exhibit expression that is conserved across a range of mammalian species.

5.5.3 Spermatogonial Transplantation

The development of a spermatogonial transplantation technique in rodents in 1994 (Brinster and Avarbock 1994; Brinster and Zimmermann 1994) was a turning point in the field of SSC research, as it provided a definitive means by which to determine SSC content within a heterogeneous spermatogonial population, and with which to make direct quantitative comparisons between spermatogonial populations; for example, between unselected spermatogonial populations and those enriched with one of the aforementioned cell surface markers. Indeed, while morphological observation of the spermatogenic lineage within a testis that has been subjected to fixation following in vivo genetic manipulation, or assessment of cell proliferation in response to in vitro manipulation, may provide insight into SSC dynamics, currently, the only unequivocal methodology to quantitatively assess SSC content is to analyze the ability of these cell to regenerate spermatogenic colonies in recipient testes.

Typically, the spermatogonial transplantation technique (Fig. 5.3) involves utilization of a donor mouse that possesses a LacZ transgene in the *Rosa26* locus; driving expression in every cell of the body; including germ cells. The recipient mouse utilized for this procedure must be devoid of endogenous spermatogenesis (so available niches are not occupied by endogenous SSCs); usually achieved via pretreatment with an alkylating chemotherapeutic agent (i.e. busulfan) that eliminates the germ cell population (Brinster 2002). Spermatogonia taken directly from the donor mouse, or from primary spermatogonial cultures established from the donor mouse, are injected into the rete testis of the recipient. Following this surgical procedure, the recipient testes are analyzed 2–5 months later to allow time for donor SSCs to regenerate colonies of persistent spermatogenesis. Clearly, only true SSCs in the donor population can incorporate into the stem cell niche and reestablish continual spermatogenesis. Thus, in knowing the number of cells injected into the recipient testis, determining the relative percentage of SSCs can be achieved by staining the recipient testis with X-gal and counting the number of LacZ expressing donor-derived colonies. The relative "SSC number" can be reported as the number of colonies generated per 10^5 cells injected in order to create standardization among experiments. To assess the "purity" of SSCs in the original suspension, colonization efficiency needs to be taken into consideration (i.e. not all SSCs injected will migrate to a niche and form a colony). Thus, it has been estimated that approximately 5–12% of transferred SSCs initiate colonization of a busulfan treated testis

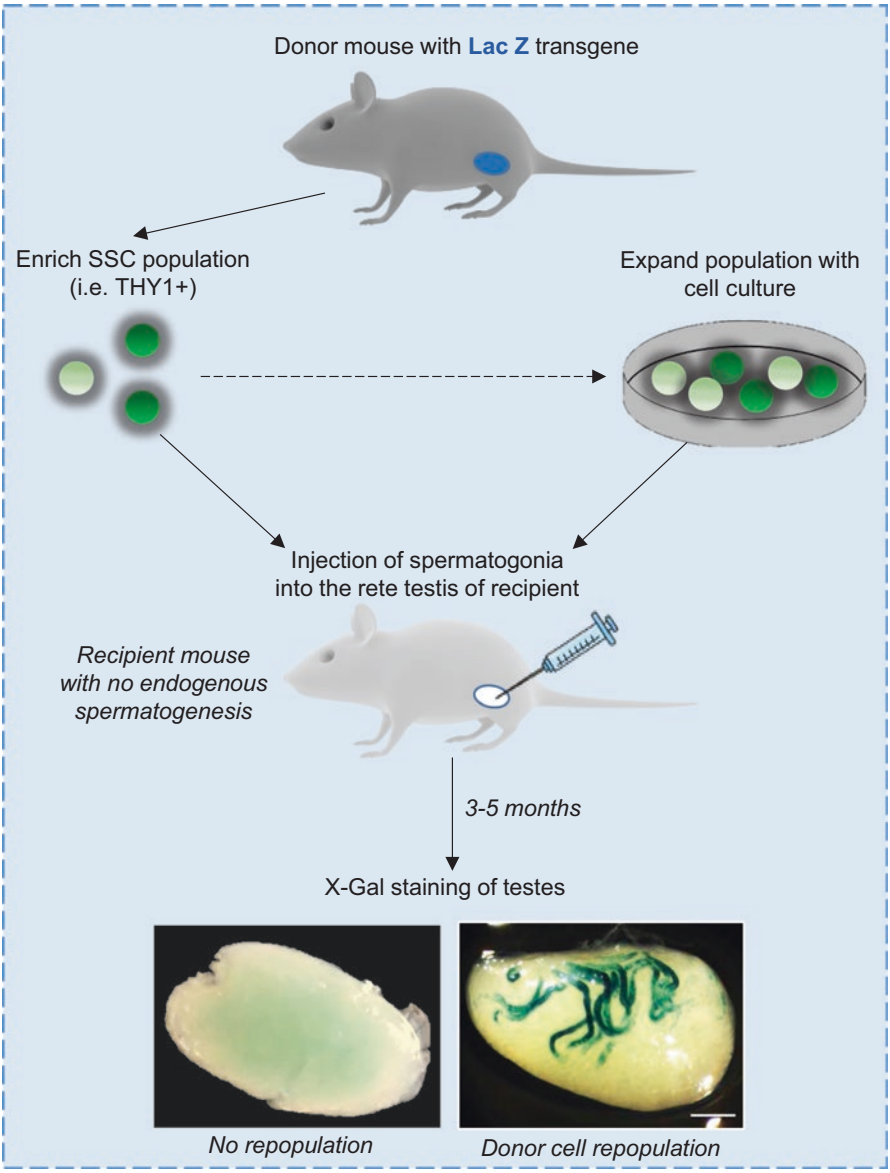

Fig. 5.3 Spermatogonial transplantation is performed using spermatogonia derived from a donor mouse with a Lac Z transgene in the *Rosa26* locus. Spermatogonia retrieved from this donor mouse may either be directly subjected to enrichment strategies (i.e. isolation of the Thy1+ population), or cultured for expansion and/or treatment of spermatogonia. Selected populations are injected into the rete testis of a recipient mouse whose endogenous spermatogenesis has been eliminated using treatment with alkylating chemotherapy. The recipient mouse is subjected to a 3–5 month recovery period to allow donor SSCs to migrate to vacant niches, and begin recolonization of the testes. Donor colonies can be visualized in recipient testis via X-Gal staining, and colonies can be counted to provide quantitative value that reflects SSC content

(Shinohara et al. 2001; Nagano 2003; Ogawa et al. 2003), with each of these colonies arising from a single SSC (Dobrinski et al. 1999).

Certainly, the spermatogonial transplantation technique is immensely useful for assessing the specificity of SSC markers used to enrich the spermatogonial population prior to transplantation, or alternatively, for highlighting changes to the SSC population in response to targeted depletion of factors hypothesized to be important for maintenance of the stem cell state. Further to this, although spermatogonial transplantation is not directly transferrable for the study of human SSC populations, successful autologous (donor and recipient are the same animal) and allogenic (donor and recipient are different animals) transplantation has been achieved in nonhuman primate species (Hermann et al. 2012). These studies potentially pave the way for the development of therapeutic treatments for human cancer survivors whom have been rendered infertile as a consequence of chemotherapy.

5.5.4 Intracellular Markers

With a lack of true SSC-specific surface markers available to isolate live stem cell populations, the field has turned to identifying and characterizing intracellular SSC markers to gain information on this population. This approach largely relies on antibody-driven experiments to make preliminary assessments on the population of interest; usually examining specificity of gene expression amongst the different germ cell populations, and within the undifferentiated spermatogonial population specifically, in fixed and sectioned rodent testes. As antibodies against these intracellular factors cannot be used to isolate the population of interest in the absence of fixation, historically, elucidation of the role of such intracellular spermatogonial markers in the maintenance of the undifferentiated population has been achieved using gene inactivation or knockdown strategies, both in vivo and in vitro. The limitation associated with this approach, in regards to the generation of null mice, is the possibility of causing an embryonic lethal phenotype. Indeed, this has been the case in a number of studies, particularly those assessing the role of pluripotency factors such as Pou5f1 (Nichols et al. 1998); that are expressed across the entire undifferentiated spermatogonial population (Ohbo et al. 2003) and play an important role in preimplantation embryo development. To circumvent this, RNAi-driven knockdown of these intrinsic factors is often conducted in primary cultures prior to spermatogonial transplantation (Dann et al. 2008), effectively demonstrating any involvement in maintenance of the undifferentiated spermatogonial population.

A number of intracellular spermatogonial markers have been identified and characterized in this manner; however, the vast majority of these markers are, again, not SSC-specific, but rather expressed throughout the undifferentiated population (i.e. in A_{single}, A_{paired} and $A_{aligned}$ cells). These factors include Zinc finger and BTB domain containing 16 (Zbtb16; i.e. Plzf) (Buaas et al. 2004; Costoya et al. 2004), Neurogenin3 (Neurog3) (Yoshida et al. 2004, 2006, 2007; Zheng et al. 2009), Nanos2 (Suzuki et al. 2009), Lin28 (Zheng et al. 2009) and Pou5f1 (Dann et al. 2008) (a comprehensive list of intracellular factors is provided in Table 5.3). While

helpful for distinguishing the undifferentiated spermatogonia from other germ cell types in the testes, as well as identifying heterogeneity in this population (Suzuki et al. 2009; Niedenberger et al. 2015), and allowing for study into how the undifferentiated population is maintained, such markers do not provide a direct means to study the SSC population specifically.

Fortunately, recent research has identified two candidates whose expression profiles appear to exhibit a high degree of specificity to the SSC population; Id4 (Oatley et al. 2011b; Chan et al. 2014) and Paired box 7 (Pax7); whose theoretical roles in SSC maintenance and self-renewal will be explored later in this chapter. As mentioned previously, the identification of these SSC-specific factors, particularly Id4, arose from differential expression analyses on Thy1+ enriched spermatogonial populations as compared to Thy1− spermatogonia (Oatley et al. 2009); and further characterization was achieved using the aforementioned antibody-driven and gene knockdown techniques (Oatley et al. 2009). The true value of identifying these intracellular SSC-specific markers is realized when they are utilized for the generation of reporter transgene constructs and subsequent mouse lines, as will be discussed below.

5.5.5 Reporter Transgenes

The utilization of reporter transgenes has allowed for the development of mouse lines in which the spermatogonial population, and more recently the SSC population specifically, can be identified by expression of a fluorescent marker; usually Gfp, or alternatively a colorimetric marker such as LacZ. Reporter transgene constructs have been generated using the pan-undifferentiated markers Pou5f1 (Youn et al. 2013), Nanos3 (Yamaji et al. 2010) Neurog3 (Yoshida et al. 2004) and Sox2 (Arnold et al. 2011), as well as the putative SSC-specific markers Id4 (Chan et al. 2014) and Pax7 (Aloisio et al. 2014).

The advantage of such mouse lines are numerous. Firstly, reporter transgenes make it possible to perform live tissue "whole mount" analyses of testicular tubules, allowing for identification of spermatogonial cell subtypes that are expressing the factor of interest (i.e. single, paired and/or aligned structures) without the disruption that is caused by fixation and sectioning of tissues (Chan et al. 2014). Such analyses provide further clarification as to whether the factor of interest is likely to be a marker of pan-undifferentiated spermatogonia (i.e. expression in single, paired and aligned structures), or potentially a marker that is SSC-specific (i.e. only expressed in a small percentage of the A_{single} population). Such live imaging techniques have also been utilized to create three dimensional reconstruction of the testicular tubules, for instance with a specific focus on the placement of Neurog3-Gfp undifferentiated spermatogonia in relation to surrounding blood vessels (Yoshida et al. 2007).

The development of reporter mouse lines allows for FACS isolation of selected spermatogonial populations, again, circumventing both the requirement for selective factors to be expressed on the cell surface, and the need for antibodies and fixatives. Thus, the selected live spermatogonial population can be retrieved from the

testes, and purity of SSCs in the population (and thus specificity of the intrinsic marker) can easily be assessed via spermatogonial transplantation. For example, transplantation of isolated Gfp+ and Gfp− spermatogonia from in the *Id4-eGfp* mouse line (Chan et al. 2014) was integral in demonstrating the high degree of specificity of this marker for the SSC population; with >90% of regenerative spermatogonia being contained within the Gfp+ population. The capacity for SSC enrichment using this Id4-driven reporter transgene when compared to enrichment values achieved with other reported cell surface markers (Table 5.2), demonstrates the value of using such strategies for the study of the SSC population.

In addition to the aforementioned applications, spermatogonial populations derived from mouse lines containing reporter transgenes that label subsets of the undifferentiated spermatogonial population can be used to establish primary spermatogonial cultures. Such strategies provide the field with a means to rapidly monitor changes in transgene expression (as a function of fluorescence), and thus potential changes to the composition of the spermatogonial population. This application may be particularly useful for monitoring SSC dynamics in response to in vitro genetic manipulation (for instance RNAi knockdown), altered culture environments, and exogenous growth factors. Further, these technologies have provided

Table 5.2 Surface markers utilized for FACS or MACS isolation to enrich SSC populations in adult and pup testis suspensions

Surface marker	Expression/specificity	Colonization compared to unselected control (colonies per 10^5 cells)	Reference
Gfrα1	SSCs and progenitors	0.13 × *Adult* (7.12) 2.5 × *Pup* (45)	Ebata et al. (2005)
β1 integrin	SSCs and progenitors	4 × *Adult* (30)	Shinohara et al. (1999)
a6 integrin	SSCs and progenitors	8 × *Adult* (55)	Shinohara et al. (1999)
CD9	SSCs and some differentiated spermatogonia	7 × *Adult* (55)	Kanatsu-Shinohara et al. (2004b)
EpCAM	All spermatogonia; however, more strongly expressed in progenitors	3 × *Adult* (6)	Kanatsu-Shinohara et al. (2011)
Thy1	SSCs and progenitors	30 × *Adult* (48.1) 5 × *Pup* (69.6)	Kubota et al. (2004a)
Cdh1	SSCs and progenitors	N/A *Adult* (34.7 compared to 0 in CDH1-population)	Tokuda et al. (2007)

Enrichment efficiency is reported as a function of colonization of recipient testes following spermatogonial transplantation, as compared to an unselected control

a gateway for performing large-scale comparative analyses between putative SSC and progenitor populations; i.e. differential RNA-seq analysis (Chan et al. 2014); to further explore expression profiles that may be unique to SSCs, to identify how putative SSC-specific factors may be driving maintenance and self-renewal of this population, and which factors are integral for initiating the commitment to progenitor formation.

5.5.6 Lineage Tracing

Lineage tracing is a technique that provides an alternative to spermatogonial transplantation for assessing the specificity of a selected marker for the SSC population. Using this methodology, putative SSCs are permanently labeled (i.e. with LacZ or Gfp) by an inducible reporter transgene, and all daughter cells arising from this clone retain the label, allowing cell lineage to be traced back to the original cell. For example, using a tamoxifen-induced Cre, Sun et al. (2015) demonstrated that Id4+ cells in the adult testis gave rise to paired and aligned structures 5 days post-tamoxifen treatment, and continued to produce labeled clones at all stages of spermatogenesis at both 5 and 13 months post-tamoxifen injection. This strategy was also adopted by Aloisio et al. (2014) to demonstrate that Pax7+ spermatogonia undergo self-renewal, in addition to generating progenitors that experience clonal expansion, and eventually produce spermatozoa. The rationale behind this technique is that only true SSCs will continue to produce clones several months after tamoxifen-induced labeling, whereas progenitor spermatogonia, for instance, would only produce downstream labeled cells for the rounds of spermatogenesis that immediately follow tamoxifen treatment. Importantly, lineage tracing can also be utilized to track formation of the founder SSC population from prospermatogonia (Aloisio et al. 2014; Sun et al. 2015); providing a means to investigate this period in development that remains relatively elusive.

The advantage of lineage tracing above that of spermatogonial transplantation is the ability to monitor stem cell dynamics within physiological context (i.e. in steady-state conditions, without disruption to the population). Despite this, there are limitations to this technique in that, unlike transplantation, lineage tracing does not provide a quantitative assessment of SSC content, but simply depicts that a portion of the selected population does possess stem cell capacity. As such, lineage tracing is not particularly useful for direct quantitative comparisons between populations of spermatogonia. Additionally, the use of tamoxifen-induced Cre system to induce expression of these reporter transgenes creates concerns regarding the disruption of normal steady-state conditions in the testes, as tamoxifen has purported endocrine disrupting capacity (Yu et al. 2014). A diagrammatic comparison between lineage tracing and spermatogonial transplantation as techniques for assessing the SSC population is provided in Fig. 5.4, with a particular focus on the advantages and disadvantages of each technology.

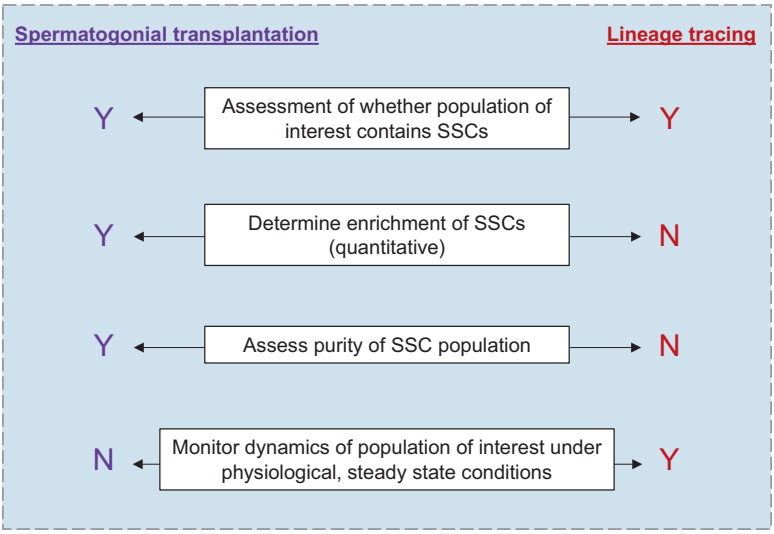

Fig. 5.4 Comparison of spermatogonial transplantation and lineage tracing strategies for assessing the SSC population

5.6 Molecular Regulation of SSC Maintenance; Transcription Factors, miRNAs, and Translational Regulators

Using the experimental techniques that have been discussed above, several intrinsic regulators, including transcription factors, miRNAs, and other posttranscriptional effectors have been identified that are involved in orchestrating SSC self-renewal, or alternatively, in maintenance of the undifferentiated state in both SSCs and progenitors. Below, we explore these factors in terms of their purported influence on SSC dynamics, and any established relationship between these factors and the extrinsic growth factors listed in Table 5.1. Key intrinsic regulatory molecules that are discussed below and their theoretical functions are summarized in Table 5.3, and a schematic of our current understanding of the processes controlling SSC renewal, and maintenance of the undifferentiated state in both SSCs and progenitors is provided in Fig. 5.5.

5.6.1 Transcription Factors

Not surprisingly, transcription factors appear to be at the spearhead of maintaining the stem cell state; with knockdown of a multitude of these factors resulting in significantly impaired SSC maintenance, stimulating loss of the undifferentiated population to differentiation, and in many cases resulting in infertility. Below we examine

Table 5.3 Intrinsic factors involved in regulation of undifferentiated spermatogonial populations

Regulatory factor	Classification	Effect on undifferentiated population	Reference
Bcl6b	Gdnf-regulated transcription factor	Involved in SSC self-renewal	Oatley et al. (2006, 2007)
Etv5	Gdnf-regulated transcription factor	Involved in SSC self-renewal	Oatley et al. (2006, 2007)
Lhx1	Gdnf-regulated transcription factor	Involved in SSC self-renewal	Oatley et al. (2006, 2007)
Pou3f1	Gdnf-regulated transcription factor	Involved in SSC self-renewal	Wu et al. (2011)
Foxo1	Gdnf-regulated transcription factor	May be involved in SSC maintenance and self-renewal	Goertz et al. (2011)
Plzf/Zbtb16	Zinc finger protein/transcription factor	Required for maintenance of the undifferentiated population	Buaas et al. (2004), Costoya et al. (2004), and Dann et al. (2008)
Taf4b	TATA binding protein	Required for maintenance of the undifferentiated population	Lovasco et al. (2015)
Oct4	Pluripotency transcription factor	Required for maintenance of the undifferentiated population	Dann et al. (2008)
Sox2	Gdnf-regulated, pluripotency transcription factor	Involved in maintaining the undifferentiated population?	Arnold et al. (2011) and Oatley et al. (2007)
Sall4	Pluripotency transcription factor	Required for maintenance of the undifferentiated population	Gassei and Orwig (2013)
Rb1	Transcription factor	Involved in formation of SSC pool and SSC maintenance	Hu et al. (2013) and Yang et al. (2013b)
Id4	Gdnf-regulated, SSC-specific HLH transcription factor	Required for SSC renewal/maintenance	Oatley et al. (2011b) and Chan et al. (2014)
Pax7	Putative SSC-specific transcription factor	Involved in stem cell renewal/maintenance?	Aloisio et al. (2014)
Sox3	"High mobility group" transcription factor	Required for progenitor differentiation	Raverot et al. (2005)
Stat3	Transcription factor	Required for progenitor differentiation	Kaucher et al. (2012) and Oatley et al. 2010)

(continued)

Table 5.3 (continued)

Regulatory factor	Classification	Effect on undifferentiated population	Reference
Ngn3	HLH transcription factor, Gdnf-regulated	Required for progenitor differentiation	Kaucher et al. (2012)
Sohlh1/2	HLH transcription factor	Required for progenitor differentiation	Ballow et al. (2006) and Suzuki et al. (2012)
miR21	microRNA	Involved in maintaining an undifferentiated state	Niu et al. (2011)
miR221, miR222	microRNA	Involved in maintaining an undifferentiated state	Yang et al. (2013b)
miR146	microRNA	Modulates differentiation in response to retinoic acid	Huszar and Payne (2013)
miR20, miR106a	microRNA	Involved in maintaining an undifferentiated state	He et al. (2013)
Nanos2	Translational repressor	Acts with mRNPS to repress translation of differentiation-related mRNAs	Zhou et al. (2015b)

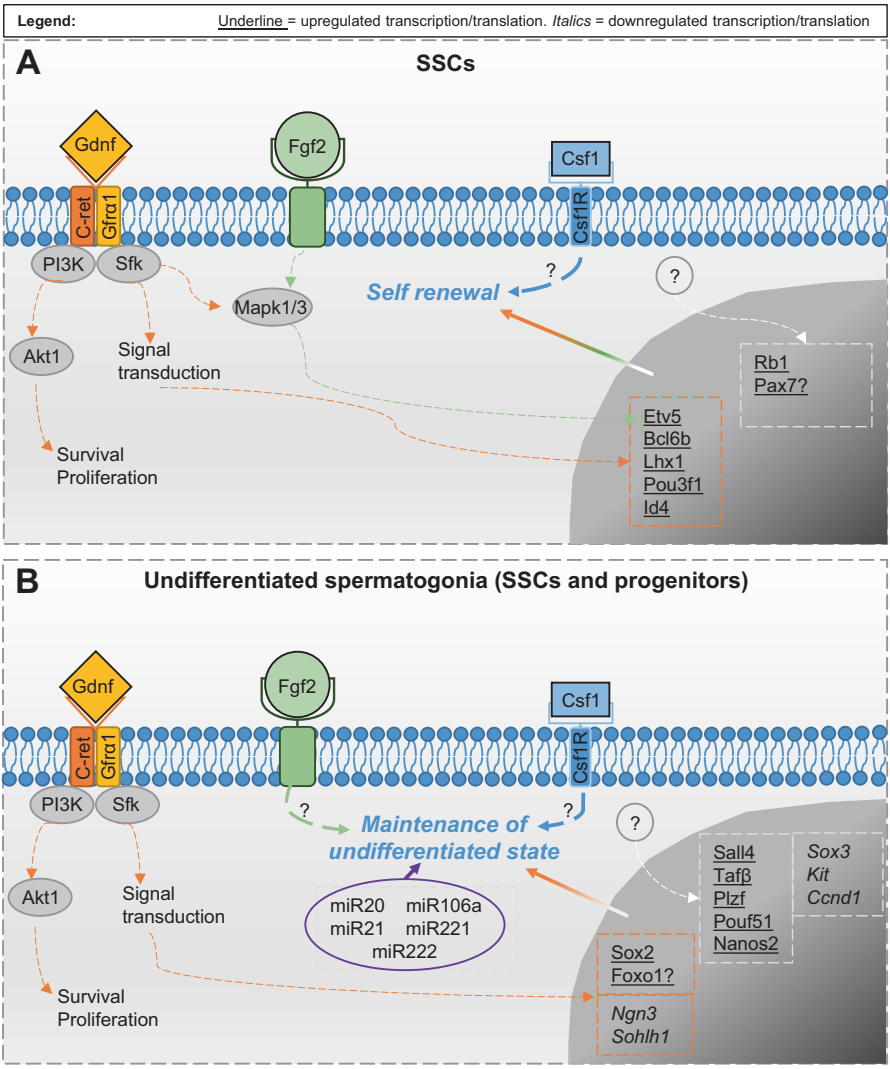

Fig. 5.5 Diagrammatic representation of factors involved in self-renewal of SSCs (**a**), and maintaining an undifferentiated state in SSCs and progenitors (**b**). (**a**) Gdnf signaling via the c-Ret/Gfrα1 receptor complex has been directly connected with increased self-renewal of SSCs in vitro. Etv5, Bcl6b, Lhx1, Pou3f1 and Id4 experience prominent upregulated expression in response to Gdnf signaling, purportedly via a PI3K/SFK-driven signal cascade. Further, Etv5 and Bcl6b are upregulated by an Fgf2-driven Mapk signaling cascade. (**b**) A number of intracellular factors have been identified that are expressed in both SSC and progenitor populations, whose knockdown results in loss of these populations to differentiation. While genes such as Sox2 and members of the Foxo gene family are upregulated by Gdnf, differentiation-driving factors such as Ngn3 and Sohlh1 are downregulated in response to Gdnf signaling pathways. A number of Gdnf-independent genes are also upregulated in response to unknown signaling pathways to maintain the undifferentiated state, while Sox3, Kit, and Ccnd1; again responsible for driving differentiation of spermatogonia, are downregulated in response to unknown external cues. Further, a number of miRNAs are known to be involved in maintaining the population of undifferentiated spermatogonia (*purple circle*), via the degradation of mRNAs that would normally drive differentiation in these cells

transcription factors known to be responsive to growth factor signals from the SSC niche (particularly Gdnf) that stimulate self-renewal of SSCs, transcription factors that appear to influence SSC function independently of Gdnf, factors that maintain pluripotency in embryonic stem cells that also play a role in SSC maintenance, and helix-loop-helix (HLH) factors that have recently emerged as key regulators of the SSC population. We also focus specifically on the putative SSC-specific transcription factors Id4 and Pax7, and their potential roles as "master regulators" of the stem cell state.

5.6.1.1 Gdnf-Dependent

When considering the propensity for growth factors such as Gdnf to stimulate self-renewal and proliferation of SSCs in vitro, it is not surprising that characterization of the transcription factors and signaling pathways that are activated within spermatogonia in response to these growth factors have been a primary focus of investigation. Pioneering studies in 2006 used microarray analyses to identify factors in Thy1+ spermatogonial cultures that were highly influenced by the addition and removal of Gdnf (Oatley et al. 2006). Transcription factors that were identified to be Gdnf responsive included B cell CLL/lymphoma 6 member B (Bcl6b), Ets variant 5 (Etv5), LIM homeobox 1 (Lhx1), Sox2, and Id4 (Oatley et al. 2006, 2007), and in later studies, Pou3f1 (Wu et al. 2011). The role of Bcl6b; the most highly upregulated Gdnf-responsive gene, was demonstrated using RNAi and spermatogonial transplantation. Specifically, following knockdown of Bcl6b, proliferation of spermatogonia in culture was curtailed; and SSC content following transplantation was found to be reduced by >8-fold (Oatley et al. 2006). Further, Bcl6b null mice experienced an age-related loss of fertility characterized by a Sertoli cell only phenotype (Oatley et al. 2006) in accordance with the first rounds of spermatogenesis occurring independently of the SSC population (Yoshida et al. 2004, 2006), while spermatogenesis in adulthood was stunted by the inability for SSCs to self-renew. Subsequent studies demonstrated that knockdown of Etv5, Lhx1, and Pou3f1 has an equivalent effect on proliferation of spermatogonial colonies in vitro, and SSC number following transplantation (Oatley et al. 2007; Wu et al. 2011). Further to these findings, overexpression of Etv5 and Bcl6b has been found to drive rapid self-renewal/proliferation in vitro, and in the case of Bcl6b, overexpression results in the formation of germ cell tumors following transplantation; presumably as a consequence of excessive self-renewal (Ishii et al. 2012). Cumulatively, these data suggest a key role for the Gdnf responsive genes Bcl6b, Etv5, Lhx1, and Pou3f1 in driving self-renewal of SSCs.

Transduction of the Gdnf signal upon binding with its receptor to influence transcription of these target factors has been found to be reliant on Src family kinase (Sfk) signaling downstream of the c-Ret component of the Gdnf receptor (Oatley et al. 2007). Etv5 expression appears to be directly influenced by this Gdnf signaling pathway, with Etv5 itself then stimulating downstream expression of Bcl6b and Lhx1 (Wu et al. 2011). In analyzing the genes targeted by Etv5, Bcl6b, and Pou3f1 using microarray analyses, surprisingly little overlap was found to exist; however,

genes commonly targeted were those involved in cellular proliferation and self-renewal, such as Brachyury (T) (Wu et al. 2011).

Although the Gdnf-Sfk pathway is thought to be directly involved in regulation of the aforementioned transcription factors, the PI3K/Akt pathway that is correspondingly effected by Gdnf-receptor binding has also been demonstrated to be important for SSC survival; with its inhibition resulting in apoptosis (Oatley et al. 2007). Further, more recent studies by Goertz et al. (2011) have identified this Gdnf-driven PI3K/Akt pathway as a regulator of Foxo1; an additional transcription factor that may be involved in SSC maintenance, with c-Ret itself being a potential gene target of this factor. Indeed, combined deficiency of the Foxo family of genes results in impaired fertility and loss of the undifferentiated population (Goertz et al. 2011).

Interestingly, not only has Gdnf signaling been shown to upregulate expression of genes involved in SSC maintenance and renewal, but also to downregulate genes that are required for spermatogonial differentiation. As such, the microarray study performed by Oatley et al. in 2006 identified Neurog3 expression to be significantly downregulated in response to Gdnf exposure; with more recent studies demonstrating that a loss of Neurog3 expression is associated with an inability for spermatogonia to differentiate (Kaucher et al. 2012). Further investigation revealed that Neurog3 is required for "Signal transducer and activator of transcription 3" (Stat3)-driven differentiation of spermatogonia as a consequence of direct binding of Stat3 to the Neurog3 promoter/enhancer (Kaucher et al. 2012).

Although literature on growth factor-regulated transcription factors in the undifferentiated spermatogonial population has been primarily focused on Gdnf, it should be noted that recent publications have also investigated Fgf2-responsive genes. Interestingly, two primary candidates that experienced upregulated expression in response to Fgf2 were also Gdnf responsive genes; namely Etv5 and Bcl6B (Ishii et al. 2012). The Fgf2-initiated response was reported to act via a Mapk1/3 signaling pathway, and dysregulation of this pathway prior to spermatogonial transplantation resulted in the formation of germ cell tumors; again supporting a role for these Gdnf-responsive transcription factors in driving self-renewal of the SSC population (Ishii et al. 2012).

5.6.1.2 Gdnf-Independent

Despite the integral nature of Gdnf signaling, a number of transcription factors that appear to be involved in SSC maintenance have been characterized that act independently of this growth factor; including Plzf, TATA-Box Binding Protein Associated Factor 4b (Taf4b) and retinoblastoma protein (Rb1) (Oatley et al. 2006); likely indicating a gap in knowledge surrounding extrinsic regulatory factors within the SSC niche. The transcriptional repressor Plzf was the first identified intrinsic regulator of undifferentiated spermatogonia and was described in 2004 (Buaas et al. 2004; Costoya et al. 2004). Plzf is known to be involved in regulation of cell cycle progression in other cell types, including hematopoietic stem cells where it appears to be involved in the G1 to S phase transition (Vincent-Fabert et al. 2016). In the testis, Plzf does not exhibit SSC-specific expression; rather, its expression can be identified throughout the undifferentiated spermatogonial population (Costoya et al.

2004). Although not SSC-specific, expression of Plzf is indispensable for maintenance of the SSC population. Plzf knockout mice exhibit sub-fertility and a diminished spermatogonial population, and their germline cells cannot colonize recipient testes upon transplantation (Buaas et al. 2004; Costoya et al. 2004). Regulation of Plzf expression is thought to occur via a phosphatase and tensin homolog (Pten) signaling cascade (Zhou et al. 2015b); and its potential modes-of-action to maintain the undifferentiated state include repression of genes that have been implicated in spermatogonial differentiation, including c-Kit (Filipponi et al. 2007) Cyclin D1 (Ccnd1) (Costoya et al. 2004), and mammalian target of rapamycin complex 1 (mTorc1) (Hobbs et al. 2010). Conversely, expression of Plzf is purportedly downregulated in response to retinoic acid; an event that is likely required to allow for differentiation to occur in progenitor spermatogonia (Dann et al. 2008). Interestingly, knockdown of a component of the TFIID general transcription factor complex, Taf4b, exhibits a similar phenotype to that seen with Plzf in that differentiation is favored over maintenance of the undifferentiated population (Lovasco et al. 2015). Again, however, the extrinsic signals controlling Taf4b expression are unknown, and its expression in undifferentiated spermatogonia is not altered by the presence/absence of Gdnf (Oatley et al. 2006, 2007).

Another factor involved in regulating transcription to maintain the SSC population, independently of Gdnf (Oatley et al. 2006), is Rb1. Like Plzf, Rb1 is known to be a key cell cycle regulator (Cobrinik 2005). In male mice with Rb1 germline inactivation, progressive germline loss is visible from 2 months of age (Hu et al. 2013). Rb1 appears to not only be involved in maintenance of the SSC population; as can be observed as a consequence of reduced testis-colonization following siRNA knockdown and spermatogonial transplantation of cultured spermatogonia; but also in formation of the SSC pool in neonatal development upon the prospermatogonial transition (Yang et al. 2013b). Thus, although the first (and possibly second) round(s) of spermatogenesis occur [a process known to be independent of the SSC population (Yoshida et al. 2004, 2006)], germline cells are progressively lost in mice with Rb1 inactivation, suggesting that the SSC pool was not formed (Yang et al. 2013b). Interestingly, spermatogonial cells with diminished Rb1 expression demonstrate increased tumorigenic properties; invading the basement membrane of the testis posttransplantation; potentially symbolizing a loss of cell cycle control resulting in dysregulated proliferation/self-renewal (Yang et al. 2013b).

5.6.1.3 Pluripotency Transcription Factors

Similar to Plzf and Taf4b, Pou5f1 is a Gdnf-independent transcription factor involved in maintaining the undifferentiated SSC population (Oatley et al. 2006). Pou5f1, Sox2 [Gdnf-dependent (Oatley et al. 2006)] and Spalt-like transcription factor 4 (Sall4), were originally characterized as a genes required for maintaining pluripotency in embryonic stem cells. Despite this, these factors are also known to be expressed in undifferentiated spermatogonia; a unipotent cell type (in physiological conditions). Pou5f1 is a homeobox transcription factor that is widely indispensable for stem cell activity. Pou5f1 knockout in mice causes an embryonic lethal phenotype, as the inner cell mass of the blastocyst does not retain pluripotency

(Nichols et al. 1998). Similarly, knockdown of Sox2 and Sall4 in ES cells results in a loss of pluripotency, purportedly due to the necessity of these factors for maintenance of the required levels of Pouf51 expression (Zhang et al. 2006; Masui et al. 2007).

In the germline, Pou5f1 is expressed throughout the undifferentiated spermatogonial population (Ohbo et al. 2003). Conflicting data exists as to the role of Pouf51 in SSC function, with one study reporting that knockdown of Pou5f1 in spermatogonial cultures resulted in significantly impaired colonization following transplantation (Dann et al. 2008), while another reported no detriment to SSC maintenance (Wu et al. 2010). Despite this, any activity exerted on the undifferentiated spermatogonia population by Pou5f1 appears to be via direct interaction with Sox2 (Takashima et al. 2013); as is the case within embryonic stem cells. In Sox2-reporter mouse lines, Sox2 expression can be identified within A_{single} spermatogonia (Arnold et al. 2011); however, expression cannot be identified using immunohistochemistry techniques, suggesting that this transcription factor is either not translated, or that protein levels are very low (Arnold et al. 2011). Despite this, like Pou5f1, Sox2 has been shown to be expressed by at least a subset of the SSC population; as demonstrated by lineage tracing analyses (Arnold et al. 2011). Finally, Sall4 expression is detectable across the entirety of the undifferentiated spermatogonial population (Gassei and Orwig 2013), with knockdown influencing maintenance of the SSC population. In addition to its potential role in interacting with co-expressed pluripotency factors Pou5f1 and Sox2, Sall4 has also been found to physically interact with Plzf (Hobbs et al. 2012), and target a large number of genes (>2500) in the undifferentiated spermatogonial population (Lovelace et al. 2016).

Interestingly, although SSCs express a suite of pluripotency genes, they themselves do not possess capacity to revert to pluripotency in vivo (Takashima et al. 2013). Theoretically, this may be due to absence of expression of an additional pluripotency factor, Nanog, in the SSC population (Oatley and Brinster 2008). Indeed, Nanog expression is directly related to self-renewal of embryonic stem cells, and is thought to act alongside Pou5f1 to control a myriad of pathways responsible for governing a pluripotent state (Loh et al. 2006).

5.6.1.4 HLH TFs

One family of transcription factors that have recently come to light as key players in SSC maintenance and renewal, as well as in spermatogonial differentiation, are those of the HLH family; which includes Id4, Neurog3, and spermatogenesis and oogenesis specific basic helix-loop-helix transcription factor (Sohlh1). As eluded to previously, Id4 is particularly interesting as is it one of only two identified intrinsic factors whose expression appears to exist within the SSC population specifically. While Id4 is involved in maintenance and self-renewal of SSCs, contrastingly, Neurog3 and Sohlh1 are required for differentiation.

As previously mentioned, Neurog3 expression is negatively regulated by Gdnf (Oatley et al. 2006), and is associated with Stat3-driven differentiation (Kaucher et al. 2012). The “high mobility group” transcription factor Sox3 has also been found to interact with Neurog3, with Sox3 expression being analogous to that of

Neurog3; i.e. expressed in A_{single}, A_{paired}, and $A_{aligned}$ spermatogonia. As such, Sox3 deletion results in infertility as a consequence of complete germline loss from postnatal day 10; with testes retaining only Sertoli cells and undifferentiated spermatogonia (Raverot et al. 2005). Interestingly, downregulation of Sox3 leads to elevated levels of Pou5f1 expression (Raverot et al. 2005), demonstrating the highly regulated balance between maintenance and differentiation of the undifferentiated spermatogonial population.

Similar to Neurog3; Sohlh1 and 2 are found to be uniformly expressed across the type A spermatogonial population. Sohlh1 expression is driven by Bmp4/Smad signaling (Li et al. 2014) and also appears to be influenced by Gdnf (Grasso et al. 2012). In accordance with their roles in driving spermatogonial differentiation, loss of Sohlh1/2 expression results in infertility accompanied by a reduction in expression of other key genes involved in differentiation (including Neurog3, c-Kit and Sox3), and increased expression of factors involved in stem cell maintenance (Ballow et al. 2006; Suzuki et al. 2012). Both Sohlh1 and 2 individual knockouts show similar phenotypes, namely, these adult mice possess only Sertoli and spermatogonial cells in their testes (Suzuki et al. 2012).

5.6.1.5 SSC-Specific Transcription Factors

As previously mentioned, a milestone has been achieved in recent years in the identification of two factors that are potentially SSC-specific; Id4 and Pax7. Although a number of factors, discussed above, have been reported to be important for maintenance of the SSC population, the expansive expression of these genes between the SSC and progenitor populations suggest that there must be a gap-in-knowledge in factors regulating SSC dynamics. Indeed, the expression of factors such as Id4 and Pax7 in SSC but not progenitor populations makes them strong candidates for orchestrating self-renewal; a characteristic that closely related undifferentiated progenitors do not share.

The inhibitor of DNA binding proteins are transcriptional repressors, traditionally expressed in undifferentiated populations of cells. While Id2 and 3 have been identified in Sertoli cells, and Id1 within spermatocytes, Id4 expression has been established to exist solely within populations of type A spermatogonia within the testis (Oatley et al. 2011b). In conjunction with the revised A_{single} model of spermatogonial maintenance (Helsel et al. 2017b; Lord and Oatley 2017), Id4 is expressed heterogeneously in the A_{single} pool of cells; within less than 10% of the population (Oatley et al. 2011b; Chan et al. 2014). The frequency of Id4 expressing cells peaks in the testis during neonatal development, then decreases significantly in adulthood (Chan et al. 2014), in-line with the developmental kinetics of the SSC population. Importantly, male Id4 null mice possess impaired spermatogenesis that is exacerbated with age; a hallmark characteristic of impaired maintenance of the SSC population. Specifically, seminiferous tubules within the testes of these mice attain a Sertoli-cell-only phenotype (Oatley et al. 2011b). Further, Id4 knockdown in spermatogonial cultures, followed by transplantation, depicts impaired maintenance of the SSC pool (Oatley et al. 2011b). As mentioned earlier

in this chapter, the generation of an *Id4-eGfp* reporter mouse line has allowed for further confirmation that expression of this factor is highly specific to the SSC population; with FACS sorted Id4-eGfp+ spermatogonia derived from primary cultures encompassing >90% of the regenerative spermatogonial population in mice (Chan et al. 2014). Further, it was determined using a limiting dilution transplantation approach that the spermatogonia in the testis expressing the highest levels of Id4 (denoted the Id4-eGfp "bright" cells) represent a population in which 1 in every 0.94 cells is an SSC; i.e. this is an essentially pure SSC pool (Helsel et al. 2017b).

Regarding functional roles in SSCs; expression of Id4 is upregulated by Gdnf signaling and may act to repress expression of genes that drive progenitor formation, such as Neurog3 (Oatley et al. 2009; Helsel et al. 2017b). Additionally, it is plausible that Id4 expression influences stem cell state via regulation of the cell cycle. Quiescence or "slow cycling" is a common property of stem cells; for instance, slow cycling HSCs have the highest long-term stem cell potential, while the more rapidly cycling cells have little to no stem cell capacity (Fuchs 2009). Indeed, it has recently been reported that Id4-eGfp "bright" spermatogonia rarely exhibit EdU incorporation in vivo (Zhang et al. 2016). Further, Id4 has been found to directly interact with key cell cycle regulator Rb1 in the undifferentiated spermatogonial population (Yang et al. 2013b); an interaction that has been implicated to influence cell cycle regulation in other cell types (Zebedee and Hara 2001). Certainly, it will be important to continue investigation into Id4 modes-of-action in rodent SSC populations, as well as establish any conserved expression of this factor amongst other mammalian species including humans.

More recently, Pax7 has been characterized as a transcription factor with an expression profile purportedly specific for SSCs (Aloisio et al. 2014). As described for Id4, $Pax7^{+}$ cells were found to be abundant in neonatal testis; however, they made up only a small portion of the A_{single} cells in the adult testis. Lineage-tracing studies demonstrated that the $Pax7^{+}$ spermatogonia in the adult testis could undergo both self-renewal and produce progenitors to fuel spermatogenesis, suggesting that this factor is expressed in at least a subset of the SSC population. Interestingly, the Pax7 expressing population of spermatogonia has been found to persist following chemotherapy/radiotherapy treatment, and subsequently to be capable of repopulating the testis to restore fertility (Aloisio et al. 2014). Although Pax7 is a high-priority contender for further analysis, the consequences of Pax7 deficiency on the SSC population are yet to be elucidated, and quantitative comparisons of SSC content between Pax7+ and Pax7− populations using transplantation analyses have not yet been conducted. Thus, it is too early to confirm whether this factor is truly SSC-specific. Importantly, however, expression of Pax7 in the undifferentiated spermatogonial population does appear to be relatively phylogenetically conserved, with expression detected in testis sections from domestic animals, nonhuman primates and humans (Aloisio et al. 2014). As such, continued research in to the extrinsic molecules and signaling pathways that regulate Pax7 expression certainly have clinical implications.

5.6.2 miRNAs

Although the role of transcription factors in maintaining the SSC population is clearly integral, regulation of stem cell dynamics and fate decision is also known to occur at the posttranscriptional level; for instance, via transcript degradation or inhibition of translation by miRNAs. The generation of miRNAs within germ cells has been demonstrated to be important for the maintenance of spermatogenesis, as the ablation of DICER and DROSHA; processing enzymes critical for the formation of mature miRNAs, results in infertility as a consequence of azoospermia (Wu et al. 2012). A myriad of different miRNAs are enriched in undifferentiated spermatogonia specifically (Wang and Xu 2015), including miR20 and miR106a (He et al. 2013). Following spermatogonial differentiation (induced by retinoic acid), expression of these miRNAs is greatly reduced. Both in vivo and in vitro analyses utilizing miRNA mimetics and inhibitors suggest a role for miR20 and miR106a in regulation of SSC proliferation/renewal, purportedly via direct interaction with Stat3 and Ccnd1 at the posttranscriptional level (He et al. 2013). Similarly, miR21 expression has been found to be enriched in the Thy1+ fraction of testis cells, with inhibition resulting in reduced colonization of recipient testes following transplantation, and elevated levels of apoptosis (Niu et al. 2011). Micro RNAs 221 and 222 also appear to be involved in maintaining spermatogonia in an undifferentiated state, as inhibition of these miRNAs results in transition of spermatogonia from a c-Kit- to c-Kit+ state (Yang et al. 2013a); a hallmark of the transition from undifferentiated to differentiating spermatogonia. Additionally, in contrast to the aforementioned miRNAs, miR146 appears to be involved in modulating differentiation rather than self-renewal of SSCs, specifically in response to retinoic acid signaling (Huszar and Payne 2013).

5.6.3 Translational Regulators

Finally, in addition to the aforementioned transcription factors and miRNAs involved in intrinsic control of SSC maintenance and self-renewal, current research has identified a further layer of regulation of these processes at the posttranscriptional level. Specifically, Nanos2, an evolutionarily conserved RNA-binding protein whose expression is enriched in the undifferentiated spermatogonial population (A_{single} and A_{paired}), has been found to directly interact with messenger ribonucleoprotein (mRNP) complexes to form a "buffering system" that controls fate decision. In the presence of Nanos2, mRNPs condense to trap differentiation-related mRNAs such as Solhlh2, and signal transducers such as mTOR; preventing their translation and keeping SSCs and progenitors in an undifferentiated state. Alternatively, when Nanos2 expression is reduced, mRNP granules breakdown, releasing the entrapped differentiation-driving mRNAs and allowing for their expression (Zhou et al. 2015a). The key role for Nanos2 in maintenance of SSCs was demonstrated by knockout of this gene in the spermatogonial population, which caused stem-cell depletion. Contrastingly, overexpression of Nanos2 in

SSCs resulted in an accumulation of undifferentiated spermatogonia, suggesting impaired entry into the differentiating pathway (Sada et al. 2009). Although the key role of this posttranscriptional regulatory process in SSCs has been relatively well defined, the extrinsic signals controlling Nanos2 expression have not yet been identified. It has, however, been proposed that the characterized Nanos2 system is a relatively stable, cell autonomous mechanisms that is not effected by extrinsic spermatogenic signals regulating cycling between self-renewal and differentiation (Zhou et al. 2015a).

5.7 Implications of SSC Research from a Clinical Perspective

In understanding the biology and regulation of SSCs, it may be possible to harness these cells for use in therapeutic treatments; specifically for pre-pubertal boys with a cancer diagnosis that are subsequently facing chemotherapeutic intervention that may render them permanently infertile. While adult males facing such treatments have the opportunity to cryopreserve their spermatozoa for future use in IVF or ICSI, pre-pubertal boys are not yet producing sperm, thus do not have this option. As such, cryopreservation of tissue biopsies taken from the testes is a procedure that has begun to be offered to these patients in selected clinics worldwide (Ginsberg et al. 2010; Picton et al. 2015); despite the fact that treatments associated with such procedures remain purely experimental (Gassei and Orwig 2016). Theoretically, SSCs contained within testis tissue biopsies could be retrieved post-cryopreservation (many years in the future, when the patient has reached adulthood), proliferated in vitro, and injected back into the testis of the patient in order to recolonize the stem cell niche and thus reestablish fertility. Concerns surrounding this technique include the unknown capacity for human SSCs to remain viable within frozen tissue samples for long periods of time, the very low number of SSCs that are likely to be contained within a single tissue biopsy, the lack of characterized SSC-specific markers for human testis which could be used to isolate these cells specifically, the currently poor proliferation of putative human SSCs in culture, and the potential for reintroduction of cancer-causing cells into the patient when injecting spermatogonia back into the testes [reviewed by Sadri-Ardekani and Atala 2014]. A theoretical alternative to the autologous transplantation of cryopreserved spermatogonia is the initiation of in vitro spermatogenesis using these cells, followed by IVF or ICSI. However, while some recent success has been achieved with this technique in rodent models; producing haploid sperm that are fertile (Sato et al. 2011); concerns remain regarding the genetic integrity of these spermatozoa, particularly epigenetic changes that could have transgenerational effects [Reviewed by Cheung and Rennert 2011]. Clearly, with cancer survival rates for prepubertal patients now estimated to be above 80% (Howlander et al. 2016) due to ever-improving intervention strategies, a high importance is placed on the continuation of SSC research to fill the gaps-in-knowledge hindering therapeutic infertility treatments.

5.8 Conclusions

Regulation of SSC maintenance and self-renewal is not only integral for formation and expansion of the SSC pool during neonatal development, but is an absolute requirement for maintaining steady-state spermatogenesis in adulthood. The SSC pool, thought to be comprised by a subset of the A_{single} spermatogonial population, resides within the stem cell niche in the testis and responds to extrinsic cues; such as the growth factors Gdnf and Fgf2 to undergo self-renewal and sustain the reservoir from which the entirety of the spermatogenic lineage arises. We now have a comprehensive tool kit to study this rare subset of spermatogonia; using a suite of extrinsic and intrinsic markers to identify and isolate undifferentiated spermatogonial subsets, cell culture techniques to expand and sustain these cells, and transplantation and lineage tracing techniques to assess SSC content within heterogeneous spermatogonial populations. Perhaps the most useful platform for studying SSCs that has recently been developed is the formation of mouse lines with SSC-specific reporter transgenes; such as the Id4-eGfp mouse. For the first time, live populations of putatively pure SSCs can be isolated, and critically analyzed in comparison to progenitor populations and differentiating spermatogonial cell types. In the wake of these technologies we are likely to see an exponential increase in the identification of intrinsic regulatory molecules involved in the SSC fate decision. Improvement in our wealth of knowledge surrounding these processes will aid to facilitate advances in our current understanding of azoospermic infertility, and support the development of therapeutic strategies to treat this pathology, as well as provide options to pre-pubertal male chemotherapy recipients whose fertility may be compromised.

References

Aloisio GM, Nakada Y, Saatcioglu HD, Pena CG, Baker MD, Tarnawa ED, Mukherjee J, Manjunath H, Bugde A, Sengupta AL, Amatruda JF, Cuevas I, Hamra FK, Castrillon DH (2014) PAX7 expression defines germline stem cells in the adult testis. J Clin Invest 124(9):3929–3944

Amann RP (2008) The cycle of the seminiferous epithelium in humans: a need to revisit? J Androl 29(5):469–487

Aponte PM, Van Bragt MPA, De Rooij DG, Van Pelt AMM (2005) Spermatogonial stem cells: characteristics and experimental possibilities. APMIS 113(11–12):727–742

Arnold K, Sarkar A, Yram MA, Polo JM, Bronson R, Sengupta S, Seandel M, Geijsen N, Hochedlinger K (2011) Sox2(+) adult stem/progenitor cells are important for tissue regeneration and survival of mice. Cell Stem Cell 9(4):317–329

Ballow D, Meistrich ML, Matzuk M, Rajkovic A (2006) Sohlh1 is essential for spermatogonial differentiation. Dev Biol 294(1):161–167

Brinster RL (2002) Germline stem cell transplantation and transgenesis. Science 296(5576): 2174–2176

Brinster RL, Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. PNAS 91(24):11303–11307

Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. PNAS 91(24):11298–11302

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(6):647–652

Chan F, Oatley MJ, Kaucher AV, Yang Q-E, Bieberich CJ, Shashikant CS, Oatley JM (2014) Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev 28(12):1351–1362

Chen L-Y, Willis WD, Eddy EM (2016) Targeting the Gdnf gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. Proc Natl Acad Sci 113(7):1829–1834

Chen L, Brown PR, Willis WB, Eddy EM (2014) Peritubular Myoid cells participate in male mouse spermatogonial stem cell maintenance. Endocrinology 155(12):4964–4974

Chen, S., Liu, Y. (2015). Regulation of spermatogonial stem cell self-renewal and spermatocyte meiosis by Sertoli cell signaling. Reproduction 149(4); R159–R167.

Cheung H-H, Rennert OM (2011) Generation of fertile sperm in a culture dish: clinical implications. Asian J Androl 13(4):618–619

Chiarini-Garcia H, Raymer AM, Russell LD (2003) Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. Reproduction 126(5):669–680

Chiquoine AD (1954) The identification, origin, and migration of the primordial germ cells in the mouse embryo. Anat Rec 118(2):135–146

Clermont Y (1966) Renewal of spermatogonia in man. Am J Anat 118(2):509–524

Clermont Y (1969) Two classes of spermatogonial stem cells in the monkey (Cercopithecus aethiops). Am J Anat 126(1):57–71

Clermont Y, Antar M (1973) Duration of the cycle of the seminiferous epithelium and the spermatogonial renewal in the monkey Macaca arctoides. Am J Anat 136(2):153–165

Cobrinik D (2005) Pocket proteins and cell cycle control. Oncogene 24(17):2796–2809

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(6):653–659

Crépieux P, Marion S, Martinat N, Fafeur V, Le Vern Y, Kerboeuf D, Guillou F, Reiter E (2001) The ERK-dependent signalling is stage-specifically modulated by FSH, during primary Sertoli cell maturation. Oncogene 20(34):4696–4709

Dann CT, Alvarado AL, Molyneux LA, Denard BS, Garbers DL, Porteus MH (2008) Spermatogonial stem cell self-renewal requires OCT4, a factor downregulated during retinoic acid-induced differentiation. Stem Cells 26(11):2928–2937

De Felici M, Scaldaferri ML, Lobascio M, Iona S, Nazzicone V, Klinger FG, Farini D (2004) Experimental approaches to the study of primordial germ cell lineage and proliferation. Hum Reprod Update 10(3):197–206

Dobrinski I, Ogawa T, Avarbock MR, Brinster RL (1999) Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice. Mol Reprod Dev 53(2):142–148

Dorval-Coiffec I, Delcros J, Hakovirta H, Toppari J, Jégou B, Piquet-Pellorce C (2005) Identification of the leukemia inhibitory factor cell targets within the rat testis. Biol Reprod 72(3):602–611

Ebata KT, Zhang X, Nagano MC (2005) Expression patterns of cell-surface molecules on male germ line stem cells during postnatal mouse development. Mol Reprod Dev 72(2):171–181

Enomoto H, Araki T, Jackman A, Heuckeroth RO, Snider WD, Johnson EM Jr, Milbrandt J (1998) GFRα1-deficient mice have deficits in the enteric nervous system and kidneys. Neuron 21(2):317–324

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(19):6770–6781

Fuchs E (2009) The tortoise and the hair: slow-cycling cells in the stem cell race. Cell 137(5):811–819

Gassei K, Orwig KE (2013) SALL4 expression in gonocytes and spermatogonial clones of postnatal mouse testes. PLoS One 8(1):e53976

Gassei K, Orwig KE (2016) Experimental methods to preserve male fertility and treat male factor infertility. Fertil Steril 105(2):256–266

Ginsberg JP, Carlson CA, Lin K, Hobbie WL, Wigo E, Wu X, Brinster RL, Kolon TF (2010) An experimental protocol for fertility preservation in prepubertal boys recently diagnosed with cancer: a report of acceptability and safety. Hum Reprod 25(1):37–41

Ginsburg M, Snow MH, McLaren A (1990) Primordial germ cells in the mouse embryo during gastrulation. Development 110(2):521–528
Glynn LE (1988) Natural history of the major histocompatibility complex. Jan Klein, John Wiley and Sons: New York. 775 pages, £90.75 (1986). Cell Biochem Funct 6(3):222–222
Goertz MJ, Wu Z, Gallardo TD, Hamra FK, Castrillon DH (2011) Foxo1 is required in mouse spermatogonial stem cells for their maintenance and the initiation of spermatogenesis. J Clin Invest 121(9):3456–3466
Gonzalez-Herrera IG, Prado-Lourenco L, Pileur F, Conte C, Morin A, Cabon F, Prats H, Vagner S, Bayard F, Audigier S, Prats A (2006) Testosterone regulates FGF-2 expression during testis maturation by an IRES-dependent translational mechanism. FASEB J 20(3):476–478
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(3):325–332
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(12):3043–3052
Hara K, Nakagawa T, Enomoto H, Suzuki M, Yamamoto M, Simons BD, Yoshida S (2014) Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. Cell Stem Cell 14(5):658–672
He Z, Jiang J, Hofmann M, Dym M (2007) Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase. Biol Reprod 77(4):723–733
He Z, Jiang J, Kokkinaki M, Tang L, Zeng W, Gallicano I, Dobrinski I, Dym M (2013) MiRNA-20 and MiRNA-106a regulate spermatogonial stem cell renewal at the post-transcriptional level via targeting STAT3 and Ccnd1. Stem Cells 31(10). https://doi.org/10.1002/stem.1474
He Z, Kokkinaki M, Jiang J, Dobrinski I, Dym M (2010) Isolation, characterization, and culture of human spermatogonia. Biol Reprod 82(2):363–372
Helsel AR, Oatley MJ, Oatley JM (2017a) Glycolysis-Optimized Conditions Enhance Maintenance of Regenerative Integrity in Mouse Spermatogonial Stem Cells during Long-Term Culture. Stem Cell Reports 8(5):1430–1441
Helsel AR, Yang Q, Oatley MJ, Lord T, Sablitzky F, Oatley JM (2017b) ID4 levels dictate the stem to progenitor transition in the mammalian male germline. Development 144(4):624–634
Hermann BP, Sukhwani M, Simorangkir DR, Chu T, Plant TM, Orwig KE (2009) Molecular dissection of the male germ cell lineage identifies putative spermatogonial stem cells in rhesus macaques. Hum Reprod 24(7):1704–1716
Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, Valli H, Rodriguez M, Ezzelarab M, Dargo G, Peterson K, Masterson K, Ramsey C, Ward T, Lienesch M, Volk A, Cooper DK, Thomson AW, Kiss JE, Penedo MCT, Schatten GP, Mitalipov S, Orwig KE (2012) Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell 11(5):715–726
Hobbs RM, Fagoonee S, Papa A, Webster K, Altruda F, Nishinakamura R, Chai L, Pandolfi PP (2012) Functional antagonism between Sall4 and Plzf defines germline progenitors. Cell Stem Cell 10(3):284–298
Hobbs RM, Seandel M, Falciatori I, Rafii S, Pandolfi PP (2010) Plzf regulates germline progenitor self-renewal by opposing mTORC1. Cell 142(3):468–479
Howlander N, Noone AM, Krapcho M, Miller D, Bishop K, Altekruse SF, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KA (2016) SEER cancer statistics review 1975–2013. N. C. Institute, Bethesda, MD
Hu Y-C, de Rooij DG, Page DC (2013) Tumor suppressor gene Rb is required for self-renewal of spermatogonial stem cells in mice. Proc Natl Acad Sci U S A 110(31):12685–12690
Huckins C (1971) The spermatogonial stem cell population in adult rats. Cell Prolif 4(4):335–349
Huszar JM, Payne CJ (2013) MicroRNA 146 (Mir146) modulates spermatogonial differentiation by retinoic acid in mice. Biol Reprod 88(1):15

Ishii K, Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2012) FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP 2K1 activation. Development 139(10):1734–1743

Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418(6893):41–49

Johnson L, Petty CS, Neaves WB (1980) A comparative study of daily sperm production and testicular composition in humans and rats. Biol Reprod 22(5):1233–1243

Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T (2005) Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod 72(4):985–991

Kanatsu-Shinohara M, Mori Y, Shinohara T (2013) Enrichment of mouse spermatogonial stem cells based on aldehyde dehydrogenase activity. Biol Reprod 89(6):140, 141–110

Kanatsu-Shinohara M, Morimoto T, Toyokuni S, Shinohara T (2004b) Regulation of mouse spermatogonial stem cell self-renewing division by the pituitary gland. Biol Reprod 70(6):1731–1737

Kanatsu-Shinohara M, Muneto T, Lee J, Takenaka M, Chuma S, Nakatsuji N, Horiuchi T, Shinohara T (2008b) Long-term culture of male germline stem cells from hamster testes. Biol Reprod 78(4):611–617

Kanatsu-Shinohara M, Takashima S, Ishii K, Shinohara T (2011) Dynamic changes in EPCAM expression during spermatogonial stem cell differentiation in the mouse testis. PLoS One 6(8):e23663

Kanatsu-Shinohara M, Takehashi M, Takashima S, Lee J, Morimoto H, Chuma S, Raducanu A, Nakatsuji N, Fässler R, Shinohara T (2008a) Homing of mouse spermatogonial stem cells to germline niche depends on β1-integrin. Cell Stem Cell 3(5):533–542

Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2004a) CD9 is a surface marker on mouse and rat male germline stem cells. Biol Reprod 70(1):70–75

Kaucher AV, Oatley MJ, Oatley JM (2012) NEUROG3 is a critical downstream effector for STAT3-regulated differentiation of mammalian stem and progenitor spermatogonia. Biol Reprod 86(5):164, 161–111

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(1–6):475–493

Kokkinaki M, Djourabtchi A, Golestaneh N (2011) Long-term culture of human SSEA-4 positive spermatogonial stem cells (SSCs). J Stem Cell Res Therapy 2(2):2488

Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. PNAS 100(11):6487–6492

Kubota H, Avarbock MR, Brinster RL (2004a) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71(3):722–731

Kubota H, Avarbock MR, Brinster RL (2004b) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. PNAS 101(47):16489–16494

Kubota H, Wu X, Goodyear SM, Avarbock MR, Brinster RL (2011) Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. FASEB J 25(8):2604–2614

Li Y, Zhang Y, Zhang X, Sun J, Hao J (2014) BMP4/Smad signaling pathway induces the differentiation of mouse spermatogonial stem cells via Upregulation of Sohlh2. Anat Rec 297(4):749–757

Ling V, Neben S (1997) In vitro differentiation of embryonic stem cells: Immunophenotypic analysis of cultured embryoid bodies. J Cell Physiol 171(1):104–115

Loh Y, Wu Q, Chew J, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong K, Sung KW, Lee CWH, Zhao X, Chiu K, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei C, Ruan Y, Lim B, Ng H (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nat Genet 38(4):431–440

Lord T, Oatley JM (2017) A revised Asingle model to explain stem cell dynamics in the mouse male germline. Reproduction 154:R55–R64
Lovasco LA, Gustafson EA, Seymour KA, de Rooij DG, Freiman RN (2015) TAF4b is required for mouse spermatogonial stem cell development. Stem Cells 33(4):1267–1276
Lovelace DL, Gao Z, Mutoji K, Song YC, Ruan J, Hermann BP (2016) The regulatory repertoire of PLZF and SALL4 in undifferentiated spermatogonia. Development 143(11):1893–1906
Mäkelä J, Toppari J, Rivero-Müller A, Ventelä S (2014) Reconstruction of mouse testicular cellular microenvironments in long-term seminiferous tubule culture. PLoS One 9(3):e90088
Masui S, Nakatake Y, Toyooka Y, Shimosato D, Yagi R, Takahashi K, Okochi H, Okuda A, Matoba R, Sharov AA, Ko MSH, Niwa H (2007) Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol 9(6):625–635
Matsui Y, Zsebo K, Hogan BLM (1992) Derivation of pluripotential embryonic stem-cells from murine primordial germ-cells in culture. Cell 70(5):841–847
Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287(5457):1489–1493
Moore MW, Klein RD, Farinas I, Sauer H, Armanini M, Phillips H, Reichardt LF, Ryan AM, Carver-Moore K, Rosenthal A (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature 382(6586):76–79
Murray P, Edgar D (2001) The regulation of embryonic stem cell differentiation by leukaemia inhibitory factor (LIF). Differentiation 68(4–5):227–234
Nagano M, Avarbock MR, Leonida EB, Brinster CJ, Brinster RL (1998) Culture of mouse spermatogonial stem cells. Tissue Cell 30(4):389–397
Nagano MC (2003) Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. Biol Reprod 69(2):701–707
Nakagawa T, Sharma M, Nabeshima Y, Braun RE, Yoshida S (2010) Functional hierarchy and reversibility within the murine spermatogenic stem cell compartment. Science 328(5974):62–67
Nakatsuji N, Chuma S (2001) Differentiation of mouse primordial germ cells into female or male germ cells. Int J Dev Biol 45:541–548
Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Schöler H, Smith A (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95(3):379–391
Niedenberger BA, Busada JT, Geyer CB (2015) Marker expression reveals heterogeneity of spermatogonia in the neonatal mouse testis. Reproduction (Cambridge, England) 149(4):329–338
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 108(31):12740–12745
Oakberg EF (1971) Spermatogonial stem-cell renewal in the mouse. Anat Rec 169:515–531
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(35):25842–25851
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 U S A 103(25):9524–9529
Oatley JM, Brinster RL (2008) Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 24:263–286
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(7):1191–1199
Oatley JM, Kaucher AV, Avarbock MR, Brinster RL (2010) Regulation of mouse spermatogonial stem cell differentiation by STAT3 signaling. Biology of Reproduction 83(3):427–433
Oatley MJ, Racicot KE, Oatley JM (2011a) Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. Biol Reprod 84(4):639–645

Oatley MJ, Kaucher AV, Racicot KE, Oatley JM (2011b) 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(2):347–356

Ogawa T, Ohmura M, Yumura Y, Sawada H, Kubota Y (2003) Expansion of murine spermatogonial stem cells through serial transplantation. Biol Reprod 68(1):316–322

Ohbo K, Yoshida S, Ohmura M, Ohneda O, Ogawa T, Tsuchiya H, Kuwana T, Kehler J, Abe K, Schöler HR, Suda T (2003) Identification and characterization of stem cells in prepubertal spermatogenesis in mice. Dev Biol 258(1):209–225

Paniagua R, Nistal M (1984) Morphological and histometric study of human spermatogonia from birth to the onset of puberty. J Anat 139(3):535–552

Panneerdoss S, Chang Y, Buddavarapu KC, Chen HH, Shetty G, Wang H, Chen Y, Kumar TR, Rao MK (2012) Androgen-responsive MicroRNAs in mouse sertoli cells. PLoS One 7(7):e41146

Paratcha G, Ledda F, Ibanez CF (2003) The neural cell adhesion molecule NCAM is an alternative signaling receptor for GDNF family ligands. Cell 113(7):867–879

Pichel JG, Shen L, Sheng HZ, Granholm A-C, Drago J, Grinberg A, Lee EJ, Huang SP, Saarma M, Hoffer BJ, Sariola H, Westphal H (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. Nature 382(6586):73–76

Picton HM, Wyns C, Anderson RA, Goossens E, Jahnukainen K, Kliesch S, Mitchell RT, Pennings G, Rives N, Tournaye H, van Pelt AMM, Eichenlaub-Ritter U, Schlatt S (2015) A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. Hum Reprod 30(11):2463–2475

Piquet-Pellorce C, Dorval-Coiffec I, Pham M, Jégou B (2000) Leukemia inhibitory factor expression and regulation within the testis. Endocrinology 141(3):1136–1141

Raverdeau M, Gely-Pernot A, Féret B, Dennefeld C, Benoit G, Davidson I, Chambon P, Mark M, Ghyselinck NB (2012) Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. PNAS 109(41):16582–16587

Raverot G, Weiss J, Park SY, Hurley L, Jameson JL (2005) Sox3 expression in undifferentiated spermatogonia is required for the progression of spermatogenesis. Dev Biol 283(1):215–225

Ryu BY, Kubota H, Avarbock MR, Brinster RL (2005) Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. PNAS 102(40):14302–14307

Ryu BY, Orwig KE, Oatley JM, Avarbock MR, Brinster RL (2006) Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. Stem Cells 24(6):1505–1511

Sada A, Suzuki A, Suzuki H, Saga Y (2009) The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. Science 325(5946):1394–1398

Sadri-Ardekani H, Atala A (2014) Testicular tissue cryopreservation and spermatogonial stem cell transplantation to restore fertility: from bench to bedside. Stem Cell Res Ther 5(3):68–68

Saitou M, Barton SC, Surani MA (2002) A molecular programme for the specification of germ cell fate in mice. Nature 418(6895):293–300

Sato T, Katagiri K, Yokonishi T, Kubota Y, Inoue K, Ogonuki N, Matoba S, Ogura A, Ogawa T (2011) In vitro production of fertile sperm from murine spermatogonial stem cell lines. Nat Commun 2:472

Schrans-Stassen BHGJ, van de Kant HJG, de Rooij DG, van Pelt AMM (1999) Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia. Endocrinology 140(12):5894–5900

Schuchardt A, D'Agati V, Larsson-Blomberg L, Costantini F, Pachnis V (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature 367(6461):380–383

Shinohara T, Avarbock MR, Brinster RL (1999) β1- and α6-integrin are surface markers on mouse spermatogonial stem cells. PNAS 96(10):5504–5509

Shinohara T, Orwig KE, Avarbock MR, Brinster RL (2001) Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc Natl Acad Sci 98(11):6186–6191

Skakkebæk N (1972) Possible carcinoma-in-situ of the testis. Lancet 300(7776):516–517

Spangrude GJ, Heimfeld S, Weissman IL (1988) Purification and characterization of mouse hematopoietic stem cells. Science 241:58–62

Sun F, Xu Q, Zhao D, Degui Chen C (2015) Id4 marks spermatogonial stem cells in the mouse testis. Nat Sci Rep 5(17594)

Suzuki H, Ahn HW, Chu T, Bowden W, Gassei K, Orwig KE, Rajkovic A (2012) SOHLH1 and SOHLH2 coordinate spermatogonial differentiation. Dev Biol 361(2):301–312

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(2):222–231

Tagelenbosch RAJ, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 290(2):193–200

Takashima S, Hirose M, Ogonuki N, Ebisuya M, Inoue K, Kanatsu-Shinohara M, Tanaka T, Nishida E, Ogura A, Shinohara T (2013) Regulation of pluripotency in male germline stem cells by Dmrt1. Genes Dev 27(18):1949–1958

Takashima S, Kanatsu-Shinohara M, Tanaka T, Morimoto H, Inoue K, Ogonuki N, Jijiwa M, Takahashi M, Ogura A, Shinohara T (2015) Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. Stem Cell Rep 4(3):489–502

Tokuda M, Kadokawa Y, Kurahashi H, Marunouchi T (2007) CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes. Biol Reprod 76(1):130–141

Tong M, Yang Q, Davis JC, Griswold MD (2013) Retinol dehydrogenase 10 is indispensible for spermatogenesis in juvenile males. PNAS 110(2):543–548

Tseng Y-T, Liao H-F, Yu C-Y, Mo C-F, Lin S-P (2015) Epigenetic factors in the regulation of prospermatogonia and spermatogonial stem cells. Reproduction 150(3):R77–R91

Van Alphen MMA, Van De Kant HJG, De Rooij DG (1988) Follicle-stimulating hormone stimulates spermatogenesis in the adult monkey. Endocrinology 123(3):1449–1455

Vincent-Fabert C, Platet N, Vandevelde A, Poplineau M, Koubi M, Finetti P, Tiberi G, Imbert A-M, Bertucci F, Duprez E (2016) PLZF mutation alters mouse hematopoietic stem cell function and cell cycle progression. Blood 127(15):1881–1885

Wang L, Xu C (2015) Role of microRNAs in mammalian spermatogenesis and testicular germ cell tumors. Reproduction 149(3):R127–R137

Wang P, Suo L, Wang Y, Shang H, Li G, Hu J, Li Q (2014) Effects of GDNF and LIF on mouse spermatogonial stem cells proliferation in vitro. Cytotechnology 66(2):309–316

Wu Q, Song R, Ortogero N, Zheng H, Evanoff R, Small CL, Griswold MD, Namekawa SH, Royo H, Turner JM, Yan W (2012) The RNase III enzyme DROSHA is essential for MicroRNA production and spermatogenesis. J Biol Chem 287(30):25173–25190

Wu X, Goodyear SM, Tobias JW, Avarbock MR, Brinster RL (2011) Spermatogonial stem cell self-renewal requires ETV5-mediated downstream activation of Brachyury in mice. Biol Reprod 85(6):1114–1123

Wu X, Oatley JM, Oatley MJ, Kaucher AV, Avarbock MR, Brinster RL (2010) The POU domain transcription factor POU3F1 is an important intrinsic regulator of GDNF-induced survival and self-renewal of mouse spermatogonial stem cells. Biol Reprod 82(6):1103–1111

Yamaji M, Tanaka T, Shigeta M, Chuma S, Saga Y, Saitou M (2010) Functional reconstruction of NANOS3 expression in the germ cell lineage by a novel transgenic reporter reveals distinct subcellular localizations of NANOS3. Reproduction 139(2):381–393

Yang Q-E, Gwost I, Oatley MJ, Oatley JM (2013b) Retinoblastoma protein (RB1) controls fate determination in stem cells and progenitors of the mouse male germline. Biol Reprod 89(5):113, 111–111

Yang Q, Racicot KE, Kaucher AV, Oatley MJ, Oatley JM (2013a) MicroRNAs 221 and 222 regulate the undifferentiated state in mammalian male germ cells. Development 140(2):280–290

Yoshida S, Sukeno M, Nabeshima Y (2007) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317:1722–1726

Yoshida S, Sukeno M, Nakagawa T, Ohbo K, Nagamatsu G, Suda T, Nabeshima Y-i (2006) The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage. Development 133(8):1495–1505

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(2):447–458

Yoshimizu T, Obinata M, Matsui Y (2001) Stage-specific tissue and cell interactions play key roles in mouse germ cell specification. Development 128(4):481–490

Youn H, Kim S-H, Choi KA, Kim S (2013) Characterization of Oct4-GFP spermatogonial stem cell line and its application in the reprogramming studies. J Cell Biochem 114(4):920–928

Yu M, Wang J, Liu W, Qin J, Zhou Q, Wang Y, Huang H, Chen W, Ma C (2014) Effects of tamoxifen on the sex determination gene and the activation of sex reversal in the developing gonad of mice. Toxicology 321:89–95

Zebedee Z, Hara E (2001) Id proteins in cell cycle control and cellular senescence. Oncogene 20(58):8317–8325

Zhang J, Tam W, Tong G, Wu Q, Chan H, Soh B, Lou Y, Yang J, Ma Y, Chai L, Ng H, Lufkin T, Robson P, Lim B (2006) Sall4 modulates embryonic stem cell pluripotency and early embryonic development by the transcriptional regulation of Pou5f1. Nat Cell Biol 8(10):1114–1123

Zhang T, Oatley J, Bardwell VJ, Zarkower D (2016) DMRT1 is required for mouse spermatogonial stem cell maintenance and replenishment. PLoS Genet 12(9):e1006293

Zheng K, Wu X, Kaestner KH, Wang P (2009) The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol 9(1):1–11

Zheng Y, Thomas A, Schmidt CM, Dann CT (2014) Quantitative detection of human spermatogonia for optimization of spermatogonial stem cell culture. Hum Reprod 29(11):2497–2511

Zhou W, Shao H, Zhang D, Dong J, Cheng W, Wang L, Teng Y, Yu Z (2015b) PTEN signaling is required for the maintenance of spermatogonial stem cells in mouse, by regulating the expressions of PLZF and UTF1. Cell Biosci 5:42

Zhou Z, Shirakawa T, Ohbo K, Sada A, Wu Q, Hasegawa K, Saba R, Saga Y (2015a) RNA binding protein Nanos2 organizes post-transcriptional buffering system to retain primitive state of mouse spermatogonial stem cells. Dev Cell 34(1):96–107

Part IV

Spermatogonial Differentiation

Role of Retinoic Acid Signaling in the Differentiation of Spermatogonia

6

My-Thanh Beedle, Cathryn A. Hogarth, and Michael D. Griswold

Abstract

The three major exogenous signaling systems that regulate spermatogenesis are follicle stimulating hormone, testosterone, and retinoic acid. In mice the absence of follicle stimulating hormone affects testis size but the animals are still fertile while the absence of testosterone signaling results in a complete block in spermatogenesis in the early spermatid stages. Similar to the case for testosterone, the absence of retinoic acid signaling results in a complete block of spermatogonial differentiation and the failure to enter meiosis; the stem cells produce progenitor cells (undifferentiated A spermatogonia) that proliferate to form syncytia but never progress to type A differentiating spermatogonia. In the presence of retinoic acid the undifferentiated A spermatogonia progress through timed steps of spermatogonial differentiation to ultimately form preleptotene spermatocytes and enter meiosis. The action of retinoic acid allows major changes in the germ cell nuclear architecture and gene expression that ultimately lead to meiotic prophase. One of the induced gene products is *Stra8*, which is required for normal meiosis in male and female germ cells and serves as a molecular marker for retinoic acid signaling in the testis. The process by which the retinoic acid signal is regulated ultimately leads to and maintains the cycle of the seminiferous epithelium with species-specific timing.

Keywords

Exogenous signaling systems • Spermatogenesis • Follicle stimulating hormone • Testosterone • Retinoic acid

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Abbreviations

ALDH	Aldehyde dehydrogenase
dpp	Days post partum
E	Embryonic day
RA	Retinoic acid
RAR	Retinoic acid receptor
ROL	Retinol
RXR	Retinoid X receptor
SSC	Spermatogonial stem cell
VAD	Vitamin A deficient
WIN	WIN 18,446

6.1 Introduction

Fertility in the males of nonseasonal mammals requires the production of large numbers of sperm over a lifetime. In humans, for example, sperm production begins at puberty and usually continues until death. The process by which sperm are produced is known as spermatogenesis and consists of three distinct biological processes: (1) the renewal of stem cells and the production and expansion of progenitor cells by mitosis, (2) the reduction, to the haploid number of chromosomes in each progenitor cell by meiosis, and (3) the unique differentiation of haploid cells into mobile gametes, often termed spermiogenesis. In mice and humans early progenitor cells are designated as A spermatogonia and are globally defined as being "undifferentiated." This term is somewhat misleading, as these cells have the limited potential to become gametes but have not yet committed to the process and very little is known about the spermatogonial differentiation process in primates. However, it is well accepted that in vertebrates undifferentiated spermatogonia divide mitotically to form a pool of progenitor cells that undergo spermatogenesis. In mice, once spermatogonia leave the pool of undifferentiated spermatogonia to enter their differentiation pathway they become known as A1 spermatogonia and begin a series of irreversible and temporally controlled differentiation steps leading to meiosis and spermiogenesis (de Rooij and Russell 2000; Griswold 2016). Differentiating spermatogonia in adult mice undergo five mitotic divisions before entering meiosis and becoming preleptotene spermatocytes. After the formation of preleptotene spermatocytes, the recognizable steps and cell types of spermatogenesis are relatively conserved between mice and humans.

It has been known since 1925 that vitamin A is required for normal spermatogenesis, and when male rodents are made vitamin A deficient (VAD), spermatogenesis ceases (Livera et al. 2002). Examination of VAD rodent testes revealed only

undifferentiated spermatogonia and Sertoli cells within the seminiferous epithelium (Mitranond et al. 1979; Unni et al. 1983; van Pelt and de Rooij 1990a; McLean et al. 2002a; Griswold et al. 1989). Treatment of VAD mice or rats with retinol (ROL) or retinoic acid (RA), the active metabolite of vitamin A, triggered the release of the block on spermatogonial differentiation and resulted in the simultaneous differentiation of A spermatogonia to A1 spermatogonia (A to A1 transition) and synchronous spermatogenesis. Since these studies, our understanding of spermatogonial biology has been significantly enhanced through investigations of how RA triggers spermatogonial differentiation and the downstream effects of RA on these cells. In addition, experiments that manipulate RA levels or signaling within the seminiferous epithelium have proven to be excellent tools for studying spermatogonia. Several principal approaches have been utilized, including RA receptor knockouts and excess and depleted RA models. This chapter focuses specifically on the effects of the RA signaling pathway on spermatogonia and the use of RA depletion models in studying this cell type.

6.2 RA Synthesis and Signaling in the Testis

RA activity in the testis is made possible by transport, metabolism, and degradation of retinoids, compounds related chemically to vitamin A (Livera et al. 2002). The storage and transport retinoid is ROL. Dietary ROL can be stored in the liver and transported via serum to target tissues where oxidation to RA generally takes place. The stellate cells of the liver and Sertoli cells of the testis appear to take up and maintain large pools of ROL (Livera et al. 2002; Bishop and Griswold 1987). Spermatids and testicular and epididymal sperm can also store retinoids (Ren and Bishop 1989). Early studies speculated that Sertoli cells were the main site of RA synthesis in the testis and it was proposed that Sertoli cells supplied RA to germ cells (Livera et al. 2002). It is becoming increasingly clear that while Sertoli cells are a source of RA (Raverdeau et al. 2012; Tong et al. 2013), there are additional cell types including germ cells that can synthesize RA.

All of the required intracellular binding proteins and retinoid metabolism enzymes are expressed in the testis so that the production and degradation of RA can be tightly regulated (Molotkov et al. 2004). In general, very low levels of the active ligand (RA) are produced in target tissues and the measured half-life of RA in the testis has been determined to be 1.3 h (Arnold et al. 2015a). Within cells, RA binds to two families of intracellular receptors termed retinoic acid receptors (RARα, RARβ, and RAR), and retinoid X receptors (RXRα, RXRβ, and RXRγ) (Livera et al. 2002; Mark et al. 1849). These receptors, in the form of homo- and heterodimers, regulate gene expression by binding to specific elements in the promoter regions of genes under the control of RA. The RAR and RXR isoforms are found in various testis cell types at different developmental stages. RARα is essential in Sertoli cells for normal fertility (Vernet et al. 2006a) and RARγ acts in early germ cells (Gely-Pernot et al. 2012).

6.3 STRA8 as a Marker of Spermatogonial Differentiation and the RA Response

Stra8 (Stimulated by retinoic acid gene 8) was first described as one of a group of RA-responsive genes in P19 cells (Oulad-Abdelghani et al. 1996). Later the *Stra8* transcript was shown to be gonad-specific and highly expressed only in the ovary at embryonic day (E) 14.5 and in the neonatal postnatal testis during the progression of spermatogonial differentiation and entry into meiosis (Koubova et al. 2006; Shima et al. 2004; Small et al. 2005; Anderson et al. 2008; Bowles et al. 2006). RA has been shown to induce *Stra8* expression in the adult mouse testis and in isolated germ cells both in vivo and in culture (Zhou et al. 2008a, b). In normal mouse testes, STRA8 protein was found by immunocytochemistry in spermatogonia as early as 3 days post partum (dpp) and in the adult mouse testes, the highest levels of *Stra8* mRNA and protein were associated with seminiferous tubules in Stages VI–VIII of the cycle of the seminiferous epithelium (Zhou et al. 2008b; Hogarth et al. 2015). The expression of *Stra8* is necessary for germ cells to complete meiosis and is an excellent marker for the action of RA in mice.

The deletion of the *Stra8* gene results in the prevention of meiosis in germ cells of both sexes (Anderson et al. 2008; Baltus et al. 2006). In both male and female embryonic urogenital ridges RA appears to be synthesized but its action, as determined by the induction of STRA8, is inhibited by the presence of the enzyme cytochrome P450, family 26, subfamily b, polypeptide 1 (CYP26B1) that degrades RA (Koubova et al. 2006; Bowles et al. 2006). Following either chemical inhibition of enzymatic activity or genetic deletion of *Cyp26b1*, *Stra8* mRNA is synthesized in male mouse primordial germ cells and meiosis is initiated (Koubova et al. 2006; Bowles et al. 2006; MacLean et al. 2007). Three independent studies investigated the effects of a complete loss of *Stra8* in the testis (Anderson et al. 2008; Mark et al. 2008; Endo et al. 2015). *Stra8* null male mice are infertile, due to a block during meiotic prophase and reduced numbers of differentiating spermatogonia, and their testes also display an accumulation of undifferentiated spermatogonia when compared to controls (Endo et al. 2015). While some cells do progress as far as preleptotene spermatocytes, a major defect in the *Stra8* knockout mouseline is the failure of the A to A1 transition. Interestingly, the appearance of STRA8-positive preleptotene spermatocytes and the A to A1 transition both occur during Stage VIII of the cycle of the seminiferous epithelium, coincident with when RA levels are highest within the testis. However, while STRA8 is an excellent marker for the A to A1 spermatogonial transition, the absence of STRA8 is not equivalent to RA deficiency. In the *Stra8* knockouts the A to A1 transition is impaired but not totally blocked, yet in the absence of RA the A to A1 transition is completely blocked. To date, STRA8 has only been found in vertebrates and the expression is confined to the germ cells but its function remains elusive. Nuclear and cytoplasmic localization of STRA8 have both been reported, suggesting that the protein can shuttle between different cellular compartments (Hogarth et al. 2015; Tedesco et al. 2009), and a putative DNA binding domain has been identified within its amino acid sequence (Tedesco et al. 2009), indicative of perhaps altering gene expression or chromatin structure.

6.4 Investigating Spermatogonial Biology via RA Depletion

Dietary depletion of RA has been a vital tool for the study of multiple aspects of spermatogonial biology, as the process essentially creates testes enriched with undifferentiated spermatogonia. The spermatogonial cell cycle in VAD testes has been investigated by several laboratories, with different conclusions being reached as to what point spermatogonia exit the cell cycle in response to vitamin A depletion. While there is agreement that spermatogonia enter mitotic arrest (Ismail et al. 1990; van Pelt and de Rooij 1990b) and that the proliferative index of the undifferentiated spermatogonia decreases over time (van Pelt et al. 1995) in VAD testes, data has been collected to suggest that this arrest may occur during either G1 (van Pelt and de Rooij 1990b; van Pelt et al. 1995), S (Wang et al. 1993) or G2 phase (Ismail et al. 1990) of the cell cycle. The effects of the different derivatives of RA on spermatogonia have also been tested using VAD testes (Gaemers et al. 1996; Gaemers et al. 1998a) and there is now an extensive collection of gene expression data assessing the response of the VAD testis to RA at multiple time points following RA administration (Doyle et al. 2009; Lee et al. 2004; Gaemers et al. 1998b). The spermatogonial stem cell (SSC) population has also been assessed following dietary vitamin A depletion. Van Pelt et al. used VAD rats to isolate a more enriched population of undifferentiated A spermatogonia when compared to isolations using control testes (van Pelt et al. 1996) and McLean et al. observed increased colonization of recipient testes following transplantation of germ cells isolated from a VAD testis compared to controls (McLean et al. 2002b). However, based on the small numbers of cells recovered from the VAD testes, the total number of SSCs within a VAD animal was calculated to be approximately 12.5% of that in adult controls (McLean et al. 2002b). This observation implies that the SSC population may undergo apoptosis or lose the expression of factors essential for maintenance of their "stemness" in a low vitamin A environment in the adult animal.

Data on the effects of dietary vitamin A depletion on spermatogenesis has only been collected for adult rodents as complete vitamin A depletion requires weeks to be achieved. Breeder female mice must be fed a VAD diet 4 weeks prior to mating, maintained on the diet while breeding and nursing offspring, and the male offspring must be placed on the diet at weaning and left for 10–12 weeks before complete deficiency in the testis is reached. Taken together, it can take upwards of 28 weeks to generate VAD male mice and therefore experiments with this model are expensive and timely to perform and must be carefully planned. To counteract this and as a means of investigating RA action in the neonatal and juvenile testis, investigators have utilized animals carrying null mutations of the retinoid storage (LRAT) or transport (RBP4) enzymes to assess the effects of retinoid deficiency during these early developmental windows. Although spermatogenesis in both the *Lrat*- and *Rpb4*-null mice is perfectly normal on a vitamin A sufficient diet, both models are much more vulnerable to dietary vitamin A depletion compared to wild-type littermates and rapidly develop testicular defects when feed a VAD diet (Ghyselinck et al. 1999; Liu and Gudas 2005). The *Lrat*-null model was used to demonstrate that vitamin A is required for meiotic initiation during the first round

of spermatogenesis, as histological analysis revealed the absence of condensed chromosomes and SYCP3, which marks the synaptonemal complex, within meiotic cells (Li et al. 2011). Further analysis demonstrated that this inability to enter meiosis was not due to impaired germ cell numbers (Li et al. 2011). In fact, spermatogonia which failed to enter meiosis in the *Lrat*-null model were arrested in an undifferentiated state (Li et al. 2011). This arrest in spermatogonial differentiation was also observed in the *Rbp4*-null mouse model (Ghyselinck et al. 2006). Using this model Ghyselinck et al. reported the absence of germ cell layers in *Rbp4*-null animals fed a VAD diet (Ghyselinck et al. 2006). As terminal deoxynuxleotidyl transferase-mediated deoxyuridinetriphosphate nick end-labeling (TUNEL) assays did not reveal an increase in apoptotic spermatocytes and round spermatids, these authors reasoned that the missing germ cell layers arose through delayed spermatogonial differentiation (Ghyselinck et al. 2006). Collectively, these findings suggest that vitamin A and its metabolites regulate spermatogonial differentiation in both juvenile and adult animals.

In addition to the available dietary and genetic approaches, chemical approaches have been utilized to study the effect of vitamin A deficiency on spermatogenesis. In particular, a group of compounds known as the BDADs (Bis-(dichloroacetyl)-diamines) were found to have selective effects on spermatogenesis (Berberian et al. 1961). The oral administration of one specific BDAD, WIN 18,446 (WIN), was shown to safely and reversibly block spermatogenesis in several species, including dogs (Drobeck and Coulston 1962; Coulston et al. 1960), monkeys (Drobeck and Coulston 1962; Coulston et al. 1960), wolves (Asa et al. 1996), cats (Munson et al. 2004), shrews (Singh and Dominic 1980), rodents (Coulston et al. 1960; Singh and Dominic 1995; Beyer et al. 1961), and man (Heller et al. 1961). Although this compound was initially pursued as a promising candidate for an oral contraceptive, further research was rapidly abandoned when it was identified that WIN also potently inhibits the enzymes in the liver responsible for the breakdown of alcohol (Amory et al. 2011). Men treated with WIN who also consumed alcohol experienced an unpleasant disulfiram reaction, characterized by flushing, nausea, and vomiting. Interest in the WIN compound has been recently renewed in favor of understanding the mechanisms by which it exerts its spermatogenic effects. Recent studies demonstrated that WIN acts by inhibiting the RA metabolizing enzymes, the aldehyde dehydrogenases (ALDHs) (Amory et al. 2011). Ex vivo testis explant studies illustrated that WIN is capable of blocking RA production and expression of *Stra8*, while in vivo studies in rabbits and mice revealed that WIN results in reduced RA levels, reduced expression of RA responsive genes, and an accumulation of undifferentiated spermatogonia (Amory et al. 2011; Hogarth et al. 2013a; Hogarth et al. 2011). Histological analysis demonstrated that the testes of 2 dpp animals treated with seven daily consecutive doses of WIN were similar to VAD testes, in that they contained only Sertoli cells and undifferentiated spermatogonia (Brooks and van der Horst 2003). An injection RA after WIN treatment in these mice triggered the simultaneous transition of the undifferentiated spermatogonia into differentiating spermatogonia (Hogarth et al. 2013a). Similar to the VAD situation, the first synchronized spermatogenic cycle after RA replacement in

WIN-treated rodents contained only a few successive germ cell populations. As a result, the treatment of neonatal mice with WIN followed by an RA injection induced synchronized spermatogenesis and this method allows for the study of enriched populations of advanced germ cells and individual stages of the cycle of the seminiferous epithelium.

This synchronization technique has been instrumental in allowing researchers to study the first round of spermatogenesis after the RA injection. Specifically, Evans et al. utilized the WIN/RA synchronization protocol combined with a novel mouse line, RiboTag (Sanz et al. 2009, 2013), to investigate gene expression differences in germ and Sertoli cells across a synchronized first round of spermatogenesis (Evans et al. 2014). Agrimson et al. also utilized the WIN/RA synchronization protocol to further investigate the kinetics of the differentiating populations of spermatogonia following the onset of spermatogenesis (Agrimson et al. 2016). Busada et al. utilized the WIN compound to investigate the relationship between RA signaling and KIT protein expression in spermatogonia. Analysis of adult testes from animals treated with the WIN/RA treatment scheme as neonates demonstrated that spermatogenic synchrony was maintained through at least 50 days post RA injection, as the tubules contained only two to three closely related stages (Hogarth et al. 2013a). These data demonstrate that the WIN/RA treatment regime results in testes that proceed through the first and subsequent waves synchronously. As a result, this induced synchrony protocol provides a simplified and powerful model through which to study spermatogonial biology.

Germ cell development is heterogeneous in nature, with a variety of developmental steps occurring in tandem. Therefore, investigating specific developmental steps or specific cell populations is a highly difficult task. Synchronization of spermatogenesis via the VAD model has simplified the task for researchers and has allowed for initial investigations into stage-specific events that occur during the spermatogenic cycle. However, synchrony via dietary deficiency is difficult to achieve, requires an extended amount of time and the health of the animal is greatly compromised. The use of animals with null mutations for the retinoid storage enzyme and transport enzyme as a postnatal model for RA depletion is also hampered by similar difficulties. The WIN/RA method is much faster, safer and a more reproducible alternative to dietary VAD (Hogarth et al. 2013a). The WIN/RA synchronization protocol produces testes that are nearly homogenous in germ cell content, thus greatly simplifying the difficulties encountered in the study of spermatogonia in a stage-specific manner.

6.5 RA and the Cycle of the Seminiferous Epithelium

The initial evidence that supported a role for RA in the establishment of the mammalian cycle of the seminiferous epithelium was derived from analysis of VAD rats. Weanling rats were kept on a VAD diet until loss of testicular germ cells occurred after 12–14 weeks. The only germ cells remaining in the testes of the VAD rats were a few preleptotene spermatocytes and undifferentiated spermatogonia. When ROL

was given back to these animals, spermatogenesis was reinitiated by stimulating spermatogonial differentiation in a synchronized manner throughout the entire testis. The spermatogenic wave disappeared and only closely very related stages could be found in tubules across a histological cross-section of these testes (Griswold et al. 1989). This synchronization of the testes of these animals did not affect the length of the cycle of the seminiferous epithelium and was maintained for many months. Similar studies were done in mice where synchronization resulted in the release of spermatozoa only every 8.6 days and the vitamin A-driven synchronization resulted in pulsatile rather than continuous sperm production (van Pelt and de Rooij 1990a; Griswold et al. 1989). While these early studies provided important new insights into the organization of the testis, the difficulty of creating the VAD condition in rodents limited the approaches to questions regarding the establishment of the cycle and the wave and the sources of RA within the testis.

The availability of new research tools, including WIN described above, cell-specific gene deletions, and a mouse line that expressed beta galactosidase in the presence of an active RA signaling mechanism (receptors and ligand), have led to a much more complete understanding of the organization of the testicular epithelium. The beta galactosidase reporter mice were used to show that the A to A1 transition occurred in discrete blue patches of seminiferous tubules of 2–3 dpp mice (Snyder et al. 2011; Snyder et al. 2010). These patches were interpreted to represent the first functional RA activity in the postnatal testes, the first A to A1 transition, and the first evidence of the initiation of the spermatogenic wave. However, if these 2–3 dpp mice are given exogenous RA the tubules became completely positive for beta galactosidase with no patchy appearance (Snyder et al. 2010; Davis et al. 2013). When these RA-treated animals were maintained until adulthood, synchronous spermatogenesis was observed (Snyder et al. 2010). These studies concluded that the first A to A1 transition occurs in the testes of 2–3 dpp mice because of patchy availability of RA along the length of the tubule and the patches of RA activity gave rise to the spermatogenic wave.

The source of the RA required to stimulate the first A to A1 transition and to initiate the wave could not be determined from the reporter mice. Patchy RA during the "first wave" or initiation of spermatogenesis appears to result from synthesis in the Sertoli cells. It had previously been shown that Sertoli cells contained RDH10 that could carry out the oxidation of ROL to retinal (Tong et al. 2013) and the ALDH enzymes that could carry out the final oxidation step (Raverdeau et al. 2012). Deletion of *Rdh10* in both germ and Sertoli cells delayed the initiation of the A to A1 transition (Tong et al. 2013) and deletion of the aldehyde dehydrogenases (*Aldh1a1*, *Aldh1a2*, and *Aldh1a3*) in Sertoli cells blocked spermatogonial differentiation (Raverdeau et al. 2012) similar to the block induced by WIN. This block at the A to A1 transition could be relieved by a single injection of RA and spermatogenesis surprisingly continued unimpeded for months in the absence of these key enzymes in Sertoli cells (Raverdeau et al. 2012). These results have been interpreted to mean that the Sertoli cells are the source of RA for the first round of spermatogenesis but the source for subsequent rounds has yet to be

clearly defined, although it is hypothesized to be the more advanced germ cells (Raverdeau et al. 2012; Davis et al. 2013; Sugimoto et al. 2012). Based on all of the data the most likely germ cells to be a source of RA after the first wave are the preleptotene spermatocytes that appear in Stage VIII of the cycle, coincident with the pulse of RA (Davis et al. 2013; Sugimoto et al. 2012).

The design, initiation, and maintenance of the cycle of the seminiferous epithelium have been reviewed in detail (Griswold 2016). It was proposed that relatively higher levels of RA were present in these stages of the cycle but quantification of RA levels in each stage of the cycle could not be performed. Gradients of RA have been proposed to regulate many developmental processes in many tissues and in many organisms but direct measurement of this gradient has been impossible because levels of RA in target tissues are very low and short-lived. However, using the WIN protocol described above to synchronize spermatogenesis, testes were collected 42–50 days after RA injection and were found to be comprised of only one or a few closely related stages of the cycle (Hogarth et al. 2013b). When synchronized testes covering the entire spectrum of stages were obtained, mass spectrometry could then be used to measure levels of RA across the cycle (Hogarth et al. 2015). From this data it was determined that the highest levels of RA are associated with Stages VIII-IX of the cycle of the seminiferous epithelium. This observation, combined with gene and protein expression data published by other laboratories (Sugimoto et al. 2012; Vernet et al. 2006b), has led to the hypothesis that pulses of RA are generated by coordinated enzymatic synthesis and degradation and must move along the tubule correlated with the appearance of Stages VIII-IX.

For the first time we have some major mechanistic insights into the classic cyclic system described in detail by Leblond and Clermont in 1952 (Leblond and Clermont 1952). In mice, it appears that the cycle of the seminiferous epithelium is initiated by RA synthesized in patches of adjacent Sertoli cells at 2–3 dpp, and is maintained by the cyclic synthesis of RA by advanced germ cells coincident with Stage VIII of the cycle. This hypothesis is supported by the following observations: (1) the development of RA signaling in patches in the neonatal testis that drives asynchronous spermatogenesis can be prevented by injection of exogenous RA, leading to spermatogenic synchrony (Snyder et al. 2010; Davis et al. 2013; Busada et al. 2014), (2) RA levels vary along the length of a testis tubule, with the highest levels coincident with Stage VIII (Hogarth et al. 2015), (3) RA synthesized by Sertoli cells is not required for the second and subsequent rounds of spermatogenesis (Raverdeau et al. 2012), (4) if a rodent has testes containing undifferentiated A spermatogonia as the only germ cell type, e.g. neonatal, VAD, or WIN-treated mice, and receives exogenous RA, synchronous spermatogenesis ensues (van Pelt and de Rooij 1990a; Snyder et al. 2010; Davis et al. 2013; Hogarth et al. 2013b), and (5) advanced germ cells contain the machinery to be able to synthesize RA (Sugimoto et al. 2012; Vernet et al. 2006b). The questions to now be addressed are the mechanism/s controlling the synthesis of RA only in patches in the neonatal testis, how the RA pulses are coordinated along testis tubules and the direct demonstration of the ability of the advanced germ cells to synthesize RA.

6.6 RA Regulation of Human Spermatogenesis

There is very little data concerning RA and its action in human spermatogenesis. Vitamin A deficiency occurs in humans and is one of the leading causes of blindness, especially in third world countries (Sommer 2009); however, there is no information regarding reproductive capabilities in these people. WIN treatment has been shown to reduce sperm count in men to below 0.1 million/mL (Coulston et al. 1960) but there is only minimal information available regarding the effect of RA on human male germ cells and the expression of RA metabolism enzymes in men. Similar to what was reported for germ cells cultured from mouse fetal testes, the germ cells in fetal human testes cultured with RA were found to undergo apoptosis after they were stimulated to proliferate (Lambrot et al. 2006; Trautmann et al. 2008). Human Sertoli cells reportedly express different enzymes of the vitamin A metabolism pathway (Lindqvist et al. 2005; Paik et al. 2004), similarly to the mouse, and *STRA8* is expressed in the human embryonic ovary and testis (Houmard et al. 2009) and the postnatal testis (Miyamoto et al. 2002). Levels of ALDH1A2 but not ALDH1A1 or ALDH1A3 were shown to correlate well with germ cell numbers in infertile men (Arnold et al. 2015b). In a pilot study involving 24 men there was a correlation between abnormal semen analyses and levels of 13-cis RA but not all trans RA (Nya-Ngatchou et al. 2013). Clearly there are large gaps in our knowledge with respect to RA regulation of human spermatogenesis; however, the rodent studies and the high level of cell type conservation in the testis between humans and rodents imply that vitamin A could very well be critical in humans for spermatogonial differentiation, meiosis, and the cycle of the seminiferous epithelium.

6.7 Conclusions

Follicle stimulating hormone, testosterone, and RA are all essential for normal mammalian spermatogenesis. Follicle stimulating hormone and testosterone act directly on Sertoli cells and indirectly on the germ cells while RA acts on both Sertoli and germ cells and triggers undifferentiated spermatogonia to enter their differentiation pathway and eventually, meiotic prophase. Given the complexities associated with retinoid storage, transport, and uptake; the number of elements in the retinoid signaling pathway; the multiple intracellular receptors; and the control of the overall organization of spermatogenesis, the potential for male fertility pathologies associated with vitamin A activity is large. However, given the importance of vitamin A in nearly every other organ system it is likely that there is a great deal of genetic redundancy designed to protect the organism. Looking forward, the focus of future research will be to determine the precise mechanism of retinoid metabolism within the testis, not any easy task given the multitude of different enzymes that could regulate this process. There is also much to be learned about whether RA is as critical to human spermatogenesis as it is in mice, and elaboration of the testicular RA production pathway in mice could provide screening targets for natural cases of human infertility.

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References

Agrimson KS, Onken J, Mitchell D, Topping TB, Chiarini-Garcia H, Hogarth C, Griswold M (2016) Characterizing the spermatogonial response to retinoic acid during the onset of spermatogenesis and following synchronization in the neonatal mouse testis. Biol Reprod 95(4):81

Amory JK, Muller CH, Shimshoni JA, Isoherranen N, Paik J, Moreb JS, Amory DW Sr, Evanoff R, Goldstein AS, Griswold MD (2011) Suppression of spermatogenesis by bisdichloroacetyl-diamines is mediated by inhibition of testicular retinoic acid biosynthesis. J Androl 32:111–119

Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van Pelt AM, Page DC (2008) Stra8 and its inducer, retinoic acid, regulate meiotic initiation in both spermatogenesis and oogenesis in mice. Proc Natl Acad Sci U S A 105:14976–14980

Arnold SL, Kent T, Hogarth CA, Griswold MD, Amory JK, Isoherranen N (2015a) Pharmacological inhibition of ALDH1A in mice decreases all-trans retinoic acid concentrations in a tissue specific manner. Biochem Pharmacol 95:177–192

Arnold SL, Kent T, Hogarth CA, Schlatt S, Prasad B, Haenisch M, Walsh T, Muller CH, Griswold MD, Amory JK, Isoherranen N (2015b) Importance of ALDH1A enzymes in determining human testicular retinoic acid concentrations. J Lipid Res 56:342–357

Asa C, Zaneveld L, Munson L, Callahan M, Byers A (1996) Efficacy, safety and reversibility of a bisdiamine male-directed oral contraceptive in grey wolves (*Caris lupus*). J Zoo Wildl Med 27:501–506

Baltus AE, Menke DB, YC H, Goodheart ML, Carpenter AE, de Rooij DG, Page DC (2006) In germ cells of mouse embryonic ovaries, the decision to enter meiosis precedes premeiotic DNA replication. Nat Genet 38:1430–1434

Berberian D, Slighter R, Surrey A (1961) In vitro and in vivo amebicidal activity of N, N′Bis(dichloroacetyl)diamines. Antibiot Chemother 11(4):245–255

Beyer A, Potts G, Coulston F, Surrey A (1961) The selective testicular effects of certain bis-(dichloroacetyl) diamines. Endocrinology 69(4):819–833

Bishop PD, Griswold MD (1987) Uptake and metabolism of retinol in cultured Sertoli cells: evidence for a kinetic model. Biochemistry 26:7511–7518

Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, Hamada H, Koopman P (2006) Retinoid signaling determines germ cell fate in mice. Science 312:596–600

Brooks NL, van der Horst G (2003) Short-term effects of N′N-bis(dichloroacetyl)-1,8-octamethylenediamine (WIN 18446) on the testes, selected sperm parameters and fertility of male CBA mice. Lab Anim 37:363–373

Busada JT, Kaye EP, Renegar RH, Geyer CB (2014) Retinoic acid induces multiple hallmarks of the prospermatogonia-to-spermatogonia transition in the neonatal mouse. Biol Reprod 90:64

Coulston F, Beyler AL, Drobeck HP (1960) The biologic actions of a new series of bis(dichloroacetyl) diamines. Toxicol Appl Pharmacol 2:715–731

Davis JC, Snyder EM, Hogarth CA, Small C, Griswold MD (2013) Induction of spermatogenic synchrony by retinoic acid in neonatal mice. Spermatogenesis 3:e23180

de Rooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21:776–798

Doyle TJ, Oudes AJ, Kim KH (2009) Temporal profiling of rat transcriptomes in retinol-replenished vitamin A-deficient testis. Syst Biol Reprod Med 55:145–163

Drobeck HP, Coulston F (1962) Inhibition and recovery of spermatogenesis in rats, monkeys, and dogs medicated with bis(dichloroacetyl)diamines. Exp Mol Pathol 1:251–274

Endo T, Romer KA, Anderson EL, Baltus AE, de Rooij DG, Page DC (2015) Periodic retinoic acid-STRA8 signaling intersects with periodic germ-cell competencies to regulate spermatogenesis. Proc Natl Acad Sci U S A 112:E2347–E2356

Evans E, Hogarth C, Mitchell D, Griswold M (2014) Riding the spermatogenic wave: profiling gene expression within neonatal germ and sertoli cells during a synchronized initial wave of spermatogenesis in mice. Biol Reprod 90:108

Gaemers IC, van Pelt AM, van der Saag PT, de Rooij DG (1996) All-trans-4-oxo-retinoic acid: a potent inducer of in vivo proliferation of growth-arrested A spermatogonia in the vitamin A-deficient mouse testis. Endocrinology 137:479–485

Gaemers IC, Sonneveld E, van Pelt AM, Schrans BH, Themmen AP, van der Saag PT, de Rooij DG (1998a) The effect of 9-cis-retinoic acid on proliferation and differentiation of a spermatogonia and retinoid receptor gene expression in the vitamin A-deficient mouse testis. Endocrinology 139:4269–4276

Gaemers IC, Van Pelt AM, Themmen AP, De Rooij DG (1998b) Isolation and characterization of all-trans-retinoic acid-responsive genes in the rat testis. Mol Reprod Dev 50:1–6

Gely-Pernot A, Raverdeau M, Celebi C, Dennefeld C, Feret B, Klopfenstein M, Yoshida S, Ghyselinck NB, Mark M (2012) Spermatogonia differentiation requires retinoic acid receptor gamma. Endocrinology 153:438–449

Ghyselinck NB, Bavik C, Sapin V, Mark M, Bonnier D, Hindelang C, Dierich A, Nilsson CB, Hakansson H, Sauvant P, Azais-Braesco V, Frasson M et al (1999) Cellular retinol-binding protein I is essential for vitamin A homeostasis. EMBO J 18:4903–4914

Ghyselinck NB, Vernet N, Dennefeld C, Giese N, Nau H, Chambon P, Viville S, Mark M (2006) Retinoids and spermatogenesis: lessons from mutant mice lacking the plasma retinol binding protein. Dev Dyn 235:1608–1622

Griswold MD (2016) Spermatogenesis: the commitment to meiosis. Physiol Rev 96:1–17

Griswold MD, Bishop PD, Kim KH, Ping R, Siiteri JE, Morales C (1989) Function of vitamin A in normal and synchronized seminiferous tubules. Ann N Y Acad Sci 564:154–172

Heller CG, Moore DJ, Paulsen CA (1961) Suppression of spermatogenesis and chronic toxicity in men by a new series of bis(dichloroacetyl) diamines. Toxicol Appl Pharmacol 3:1–11

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

Hogarth CA, Evanoff R, Mitchell D, Kent T, Small C, Amory JK, Griswold MD (2013a) Turning a spermatogenic wave into a tsunami: synchronizing murine spermatogenesis using WIN 18,446. Biol Reprod 88:40

Hogarth CA, Evanoff R, Mitchell D, Kent T, Small C, Amory JK, Griswold MD (2013b) Turning a spermatogenic wave into a tsunami: synchronizing murine spermatogenesis using WIN 18,446. Biol Reprod 88:40

Hogarth CA, Arnold S, Kent T, Mitchell D, Isoherranen N, Griswold MD (2015) Processive pulses of retinoic acid propel asynchronous and continuous murine sperm production. Biol Reprod 92:37

Houmard B, Small C, Yang L, Naluai-Cecchini T, Cheng E, Hassold T, Griswold M (2009) Global gene expression in the human fetal testis and ovary. Biol Reprod 81:438–443

Ismail N, Morales C, Clermont Y (1990) Role of spermatogonia in the stage-synchronization of the seminiferous epithelium in vitamin-A-deficient rats. Am J Anat 188:57–63

Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC (2006) Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc Natl Acad Sci U S A 103:2474–2479

Lambrot R, Coffigny H, Pairault C, Donnadieu AC, Frydman R, Habert R, Rouiller-Fabre V (2006) Use of organ culture to study the human fetal testis development: effect of retinoic acid. J Clin Endocrinol Metab 91:2696–2703

Leblond CP, Clermont Y (1952) Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N Y Acad Sci 55:548–573

Lee KF, Yeung WS, Chow JF, Shum CK, Luk JM (2004) Different testicular gene expression patterns in the first spermatogenic cycle of postnatal and vitamin A-deficient rat testis. Biol Reprod 70:1010–1017

Li H, Palczewski K, Baehr W, Clagett-Dame M (2011) Vitamin A deficiency results in meiotic failure and accumulation of undifferentiated spermatogonia in prepubertal mouse testis. Biol Reprod 84:336–341

Lindqvist A, He YG, Andersson S (2005) Cell type-specific expression of beta-carotene 9′,10′-monooxygenase in human tissues. J Histochem Cytochem 53:1403–1412

Liu L, Gudas LJ (2005) Disruption of the lecithin:retinol acyltransferase gene makes mice more susceptible to vitamin A deficiency. J Biol Chem 280:40226–40234

Livera G, Rouiller-Fabre V, Pairault C, Levacher C, Habert R (2002) Regulation and perturbation of testicular functions by vitamin A. Reproduction 124:173–180

MacLean G, Li H, Metzger D, Chambon P, Petkovich M (2007) Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. Endocrinology 148:4560–4567

Mark M, Teletin M, Vernet N, Ghyselinck NB (1849) Role of retinoic acid receptor (RAR) signaling in post-natal male germ cell differentiation. Biochim Biophys Acta 2015:84–93

Mark M, Jacobs H, Oulad-Abdelghani M, Dennefeld C, Feret B, Vernet N, Codreanu CA, Chambon P, Ghyselinck NB (2008) STRA8-deficient spermatocytes initiate, but fail to complete, meiosis and undergo premature chromosome condensation. J Cell Sci 121:3233–3242

McLean DJ, Russell LD, Griswold MD (2002a) Biological activity and enrichment of spermatogonial stem cells in vitamin A-deficient and hyperthermia-exposed testes from mice based on colonization following germ cell transplantation. Biol Reprod 66:1374–1379

McLean DJ, Russell LD, Griswold MD (2002b) Biological activity and enrichment of spermatogonial stem cells in vitamin A-deficient and hyperthermia-exposed testes from mice based on colonization following germ cell transplantation. Biol Reprod 66:1374–1379

Mitranond V, Sobhon P, Tosukhowong P, Chindaduangrat W (1979) Cytological changes in the testes of vitamin-A-deficient rats. I. Quantitation of germinal cells in the seminiferous tubules. Acta Anat 103:159–168

Miyamoto T, Sengoku K, Takuma N, Hasuike S, Hayashi H, Yamauchi T, Yamashita T, Ishikawa M (2002) Isolation and expression analysis of the testis-specific gene, STRA8, stimulated by retinoic acid gene 8. J Assist Reprod Genet 19:531–535

Molotkov A, Ghyselinck NB, Chambon P, Duester G (2004) Opposing actions of cellular retinol-binding protein and alcohol dehydrogenase control the balance between retinol storage and degradation. Biochem J 383:295–302

Munson L, Chassy LM, Asa C (2004) Efficacy, safety and reversibility of bisdiamine as a male contraceptive in cats. Theriogenology 62:81–92

Nya-Ngatchou JJ, Arnold SL, Walsh TJ, Muller CH, Page ST, Isoherranen N, Amory JK (2013) Intratesticular 13-cis retinoic acid is lower in men with abnormal semen analyses: a pilot study. Andrology 1:325–331

Oulad-Abdelghani M, Bouillet P, Decimo D, Gansmuller A, Heyberger S, Dolle P, Bronner S, Lutz Y, Chambon P (1996) Characterization of a premeiotic germ cell-specific cytoplasmic protein encoded by Stra8, a novel retinoic acid-responsive gene. J Cell Biol 135:469–477

Paik J, Vogel S, Quadro L, Piantedosi R, Gottesman M, Lai K, Hamberger L, Vieira Mde M, Blaner WS (2004) Vitamin A: overlapping delivery pathways to tissues from the circulation. J Nutr 134:276S–280S

Raverdeau M, Gely-Pernot A, Feret B, Dennefeld C, Benoit G, Davidson I, Chambon P, Mark M, Ghyselinck NB (2012) Retinoic acid induces Sertoli cell paracrine signals for spermatogonia differentiation but cell autonomously drives spermatocyte meiosis. Proc Natl Acad Sci U S A 109:16582–16587

Ren P, Bishop PD (1989) Uptake and metabolism of retinol in isolated cells of germinal epithelium in vitro. J Tongji Med Univ 9:36–43

Sanz E, Yang L, Su T, Morris DR, McKnight GS, Amieux PS (2009) Cell-type-specific isolation of ribosome-associated mRNA from complex tissues. Proc Natl Acad Sci U S A 106:13939–13944

Sanz E, Evanoff R, Quintana A, Evans E, Miller JA, Ko C, Amieux PS, Griswold MD, McKnight GS (2013) RiboTag analysis of actively translated mRNAs in Sertoli and Leydig cells in vivo. PLoS One 8:e66179

Shima JE, McLean DJ, McCarrey JR, Griswold MD (2004) The murine testicular transcriptome: characterizing gene expression in the testis during the progression of spermatogenesis. Biol Reprod 71:319–330

Singh SK, Dominic CJ (1980) Effect of N,N′-bis(dichloroacetyl)-i,8-octamethylenediamine (WIN 18446) on the testis + epididymis of the musk shrew Suncus murinus L. Indian J Exp Biol 18:1217–1220

Singh AK, Dominic CJ (1995) Testicular toxicity of WIN 18446 in the laboratory mouse. Reprod Toxicol 9:475–481

Small CL, Shima JE, Uzumcu M, Skinner MK, Griswold MD (2005) Profiling gene expression during the differentiation and development of the murine embryonic gonad. Biol Reprod 72:492–501

Snyder EM, Small C, Griswold MD (2010) Retinoic acid availability drives the asynchronous initiation of spermatogonial differentiation in the mouse. Biol Reprod 83:783–790

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

Sommer A (2009) The continuing challenge of vitamin A deficiency. Ophthalmic Epidemiol 16(1)

Sugimoto R, Nabeshima Y, Yoshida S (2012) Retinoic acid metabolism links the periodical differentiation of germ cells with the cycle of Sertoli cells in mouse seminiferous epithelium. Mech Dev 128:610–624

Tedesco M, La Sala G, Barbagallo F, De Felici M, Farini D (2009) STRA8 shuttles between nucleus and cytoplasm and displays transcriptional activity. J Biol Chem 284:35781–35793

Tong MH, Yang QE, Davis JC, Griswold MD (2013) Retinol dehydrogenase 10 is indispensible for spermatogenesis in juvenile males. Proc Natl Acad Sci U S A 110:543–548

Trautmann E, Guerquin MJ, Duquenne C, Lahaye JB, Habert R, Livera G (2008) Retinoic acid prevents germ cell mitotic arrest in mouse fetal testes. Cell Cycle 7:656–664

Unni E, Rao MR, Ganguly J (1983) Histological & ultrastructural studies on the effect of vitamin A depletion & subsequent repletion with vitamin A on germ cells & Sertoli cells in rat testis. Indian J Exp Biol 21:180–192

van Pelt AM, de Rooij DG (1990a) Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. Biol Reprod 43:363–367

van Pelt AM, de Rooij DG (1990b) Synchronization of the seminiferous epithelium after vitamin A replacement in vitamin A-deficient mice. Biol Reprod 43:363–367

van Pelt AM, van Dissel-Emiliani FM, Gaemers IC, van der Burg MJ, Tanke HJ, de Rooij DG (1995) Characteristics of A spermatogonia and preleptotene spermatocytes in the vitamin A-deficient rat testis. Biol Reprod 53:570–578

van Pelt AM, Morena AR, van Dissel-Emiliani FM, Boitani C, Gaemers IC, de Rooij DG, Stefanini M (1996) Isolation of the synchronized A spermatogonia from adult vitamin A-deficient rat testes. Biol Reprod 55:439–444

Vernet N, Dennefeld C, Guillou F, Chambon P, Ghyselinck NB, Mark M (2006a) Prepubertal testis development relies on retinoic acid but not rexinoid receptors in Sertoli cells. EMBO J 25:5816–5825

Vernet N, Dennefeld C, Rochette-Egly C, Oulad-Abdelghani M, Chambon P, Ghyselinck NB, Mark M (2006b) Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. Endocrinology 147:96–110

Wang Z, Kim KH (1993) Vitamin A-deficient testis germ cells are arrested at the end of S phase of the cell cycle: a molecular study of the origin of synchronous spermatogenesis in regenerated seminiferous tubules. Biol Reprod 48:1157–1165

Zhou Q, Li Y, Nie R, Friel P, Mitchell D, Evanoff RM, Pouchnik D, Banasik B, McCarrey JR, Small C, Griswold MD (2008a) 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

Zhou Q, Nie R, Li Y, Friel P, Mitchell D, Hess RA, Small C, Griswold MD (2008b) Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine testes. Biol Reprod 79:35–42

Gonadotropin and Steroid Hormone Control of Spermatogonial Differentiation

7

Rod T. Mitchell, Laura O'Hara, and Lee B. Smith

Abstract

Spermatogenesis, the process essential for male fertility, relies on the continuous supply of differentiating germ cells from a pool of spermatogonial stem cells. Spermatogenesis is dependent upon hormonal stimuli that result in a complex pattern of intratesticular signalling pathways. The two main hormones responsible for its control are pituitary follicle stimulating hormone (FSH) and testicular testosterone. Testosterone and FSH act through their somatic cell receptors in the testis to promote the initiation of spermatogenesis, increase in germ cell numbers, development and differentiation. This knowledge has been advanced by animal models, but these models often pose more questions than they answer. However, when all the evidence provided by these studies is taken together, some important conclusions can be drawn. Evidence from animal models confirms that testosterone is required for completion of meiosis and spermiation, and can have a stimulatory effect on spermatogonia numbers in both rodent and primate models of gonadotropin suppression, even in the absence of FSH. FSH alone cannot drive spermatogenesis to completion, but it has a stimulatory effect on both Sertoli cell number and spermatogonia number that results in a higher adult sperm output. Evidence suggests that testosterone and FSH are more important for the development of spermatogonia in primates than they are in rodents. The sum of this knowledge highlights future studies that are required to develop male contraceptives or infertility treatments.

Keywords

Spermatogenesis • Male fertility • Testis • Luteinizing hormone (LH) • Testosterone • Follicle-stimulating hormone (FSH) • Sertoli • Leydig

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7.1 Introduction

Spermatogenesis, the process essential for male fertility, relies on the continuous supply of differentiating germ cells from a pool of spermatogonial stem cells (SSC, see Chap. 1). These cells arise during early postnatal life from a population of gonocytes that develop during fetal life (see Chap. 2). In primates, at least two distinct types of undifferentiated spermatogonia exist. A_{dark} spermatogonia are reserve stem cells that only proliferate during pubertal expansion and following depletion of spermatogonia due to irradiation or toxic exposure. In contrast, A_{pale} spermatogonia are self-renewing progenitors which proliferate regularly during each spermatogenic cycle (reviewed in Schlatt and Ehmcke (2014)). Type A spermatogonia can either self-renew or differentiate into type B spermatogonia that enter into spermatogenesis. In mice, a single (A_s) spermatogonia can divide to self-renew, or to form two paired (A_{pr}) spermatogonia that are joined by cytoplasmic bridges. Both A_s and A_{pr} spermatogonia are undifferentiated. A_{pr} divide to form chains of aligned (A_{al}) spermatogonia which undergo further mitoses before becoming type B spermatogonia (Boitani et al. 2016).

SSC reside in a unique microenvironment or 'niche' and receive a multitude of paracrine and endocrine signals to maintain their self-renewal and differentiation (see Chaps. 3 and 4). Spermatogenesis is dependent upon hormonal stimuli that result in a complex pattern of intratesticular signalling pathways (Fig. 7.1). The two main hormones responsible for its control are pituitary follicle stimulating hormone (FSH) and testosterone, which is produced by the testicular Leydig cells under the influence of pituitary luteinising hormone (LH). The trophic actions of these hormones on spermatogenesis were originally demonstrated through the pioneering work of Greep and Smith in the 1920s and 1930s (Greep et al. 1936; Smith and Engle 1927). Both hormones act through their cognate receptors in the somatic cells of the testis to promote the initiation of spermatogenesis, increase in germ cell numbers, development and differentiation. It is now commonly accepted that testosterone is required for completion of meiosis and spermiation and whilst FSH is not required to complete a specific step of spermatogenesis, it is required for quantitatively normal spermatogenesis in rodents, although the evidence in primates is less clear. Current experimental evidence shows that testosterone and FSH both have effects on the final number of spermatogonia mediated through testicular somatic cells, but that neither is required for their renewal or maturation.

Here, we present the current state of knowledge about the timing and effects of FSH and testosterone on spermatogonial development, comparing analyses of human studies and animal models.

7.2 The HPG Axis

7.2.1 Mechanism

Control of the production of testosterone and FSH is through the hypothalamic pituitary gonadal (HPG) axis, a negative feedback system involving the production of endocrine hormones by these three organs (Fig. 7.1). Gonadotropin-releasing

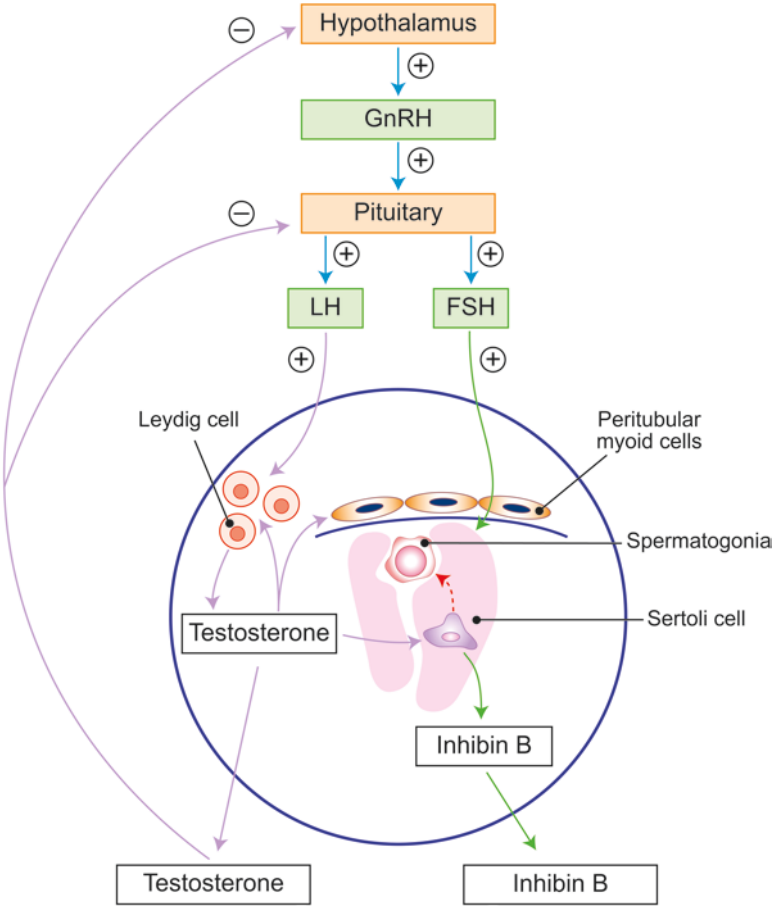

Fig. 7.1 The male HPG axis. Hypothalamic production of Gonadotropin releasing hormone (GnRH) signals at the level of the pituitary to stimulate gonadotropin [luteinizing hormone (LH) and follicle stimulating hormone (FSH)] release into the bloodstream. LH binds to its cognate receptor on Leydig cells to stimulate testosterone production. Testosterone binds to the androgen receptor in somatic cells of the testis to modulate gene expression necessary for support of spermatogenesis. Testosterone released into the bloodstream also signals back to the brain to modulate further release of LH in a negative feedback loop. Pituitary FSH binds its cognate receptor in testicular Sertoli cells, modulating Sertoli cell function to support spermatogenesis. FSH stimulation also leads to release of Inhibin B by Sertoli cells, which also acts at the level of the brain to control FSH secretion in a negative-feedback loop. Together these hormones provide homeostatic support for germ cell development, with the potential to improve support in response to physiological challenges. (+) = trophic stimulation; (−) = negative feedback

hormone (GnRH) is released from parvocellular secretory neurons in the hypothalamus into the hypophysial portal bloodstream at the median eminence where it is transported to the pituitary gland. It then binds to the gonadotropin receptor GnRHR present in pituitary gonadotrophs (Millar 2005) to stimulate the production and release of the gonadotropins FSH and LH (Savage et al. 2003). FSH and LH are glycoprotein hormones that consist of two subunits, the α subunit is common to both hormones (and also thyroid stimulating hormone), whilst the β subunit is specific to each hormone (Ryan et al. 1988). LH and FSH are released into the systemic circulation and transported to the testis.

LH binds to specific, high-affinity receptors (Luteinising hormone/chorionic gonadotropin receptor: LHCGR) on the surface of testicular Leydig cells (Dufau 1988) and stimulates steroidogenesis of testosterone. Binding of LH to LHCGR causes an increase in the production of cAMP in the Leydig cell, resulting in both short-term changes in protein phosphorylation and long-term effects on gene transcription. The long-term effects are the expression and upregulation of steroidogenic enzyme genes including *StAR*, *Hsd3b* subtypes and *Cyp11a1* (Lavoie and King 2009). One particular short-term effect is an increase in the phosphorylation of steroidogenic acute regulatory (StAR) protein which facilitates the transport of cholesterol from the outer to the inner mitochondrial membrane to act as a substrate for the initial stages of steroidogenesis (reviewed in Miller and Bose (2011)). Testosterone produced by Leydig cells acts upon Sertoli and other testicular cells to regulate gene expression and the activation of signalling pathways that are required for supporting spermatogenesis, as well as throughout the body for the development and maintenance of male-specific and other physiological characteristics. FSH acts via a specific G protein coupled receptor (FSHR) present in the Sertoli cells (reviewed in George et al. (2011)). Binding activates at least five different intracellular signalling pathways and results in the transcription of genes that act to stimulate and support steroidogenesis (reviewed in Walker and Cheng (2005)).

Testicular factors are known to negatively regulate the production and release of gonadotropins from the pituitary, forming the HPG feedback loop. Immunisation of rhesus monkeys against testosterone results in consistently high plasma LH levels, and administration of exogenous testosterone causes a decrease in circulating LH and a decrease in Leydig cell synthesis of testosterone (Wickings and Nieschlag 1978). In *Tfm* mice which have a non-functional androgen receptor, levels of serum LH and FSH are increased (Amador et al. 1986) due to a failure of testosterone to limit LH expression and an excess of LH then stimulating more testosterone production. This feedback is thought to act through binding AR in the hypothalamus (Tilbrook and Clarke 2001), but pituitary AR is not required for pituitary feedback (O'Hara et al. 2015). In men, there is some evidence that inhibition of luteinising hormone secretion by testosterone requires aromatisation for its pituitary but not hypothalamic effects (Pitteloud et al. 2008). Inhibin B is a glycoprotein produced by the testes that was originally identified from its ability to inhibit FSH secretion by the pituitary (Burger 1988). Although it is produced by the testis, the location of its production is disputed: most evidence points towards the Sertoli cells but there is some evidence for the Leydig and germ cells (Meachem et al. 2001). Inhibin acts in

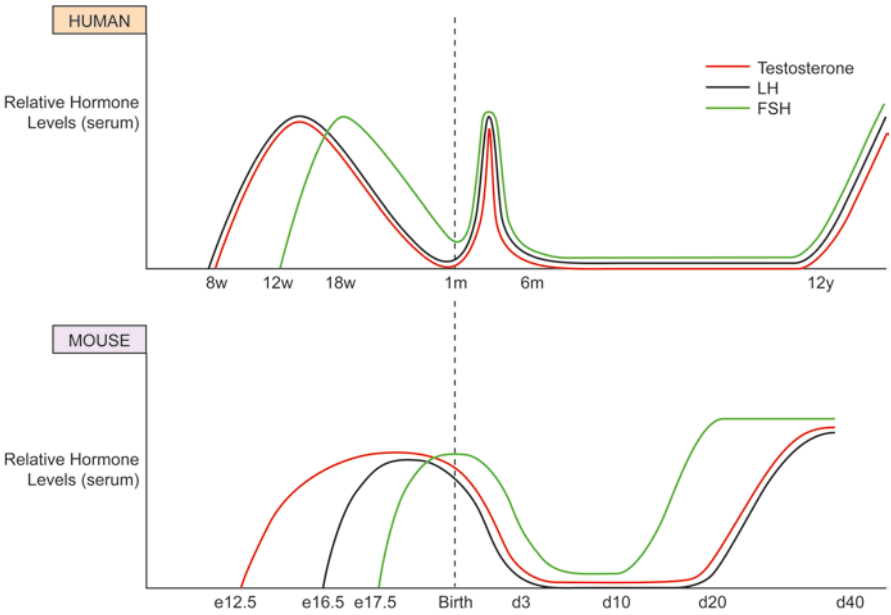

Fig. 7.2 Relative serum hormone profiles of Testosterone, LH and FSH in human and mouse during pre- and postnatal development. In humans Gonadotropins and testosterone peak in early gestation before declining to low levels at birth. A further peak ('mini-puberty') occurs during the neonatal period before returning to baseline at around 6 months (m). Levels remain low until the onset of puberty when they increase from around 12 years (y) to reach adult levels. In mice testosterone increases from embryonic day 12.5 (e12.5) prior to the onset of gonadotropin secretion. Gonadotropins and testosterone peak at birth before declining to low levels by postnatal day (d) 3. FSH increases from around d10, preceding the rise in LH and testosterone which begins around d20 and increases gradually to adult levels. (Y axis, not to scale)

pituitary gonadotropes by binding to betaglycan, which prevents the binding of activin to its receptor and the subsequent activation of the Smad signalling pathway and downstream transcriptional activation (Suresh et al. 2011). Interestingly, many models with chronic low-dose testosterone treatment given to reduce LH levels (discussed in Sect. 4.3.3) also show a dramatic reduction in FSH, probably due to inhibition of GnRH production.

7.2.2 Timing

Prenatal: Production and release of gonadotropins and testosterone fluctuates over the course of fetal development and postnatal life, and differs between humans and the animal models used to study it (Fig. 7.2). The fetal Leydig cells (FLCs) of the human fetal testis begin to produce testosterone shortly after formation at 8 weeks. Production peaks at around 11–14 weeks gestation. Between 17 and 20 weeks

gestation testosterone production begins to decline, and at birth there is no difference between a male and a female fetus (reviewed in Scott et al. (2009)). FSH is detectable in the fetal circulation by week 12 of gestation, increases until mid-pregnancy then declines until birth, presumably as a result of negative feedback from placental estrogen (Clements et al. 1976; Debieve et al. 2000). Significantly higher levels of gonadotropins are found in female fetuses than males, which suggests that the testosterone produced by males is providing additional suppression of the HPG axis. Humans are dependent on stimulation of LHCGR in fetal Leydig cells for the increase in fetal testosterone production that permits masculinisation. Humans with a complete inactivating LHCGR mutation do not undergo fetal masculinisation but humans with an inactivating mutation of LHβ undergo masculinisation of the male reproductive tract due to the presence of placental human chorionic gonadotropin (hCG) which can also activate the LHCGR (O'Shaughnessy et al. 2009). In mice, testosterone production by fetal Leydig cells begins after their formation at e12.5 (Livera et al. 2006). Testosterone levels peak just before birth, after which the cells begin to involute and stop steroidogenesis (Wen et al. 2011). *Lhb* and *Fshb* transcripts are not detected in the murine embryonic pituitary until e16.5 and e17.5 respectively (Japon et al. 1994), so the majority of fetal testosterone production takes place before the commencement of gonadotropin production by the pituitary. Although fetal rat and mouse Leydig cells do express a functional LHCGR (Zhang et al. 1994), their function is not intimately dependent on this stimulus, in contrast to humans. Pituitary gonadotrope development and secretion has not occurred at the time of induction of fetal Leydig cell testosterone production (Savage et al. 2003) and normal masculinisation of the reproductive system occurs in mice with a total failure of pituitary development (Pakarinen et al. 2002) or with genetic ablation of LHβ (Ma et al. 2004) or LHCGR (Lei et al. 2001; Zhang et al. 2001).

Postnatal: Following birth in male humans and some non-human primates there is an initial rise in testosterone and gonadotropins that continues during early infancy (the so-called mini-puberty) (Hadziselimovic 2005) (Fig. 7.2). Immediately after birth the levels of FSH and LH begin to rise, reaching a peak 4–10 weeks post-partum before declining to the prepubertal nadir at around 6 months. LH-induced testosterone increase reaches a peak at around the third month and decreases to the prepubertal levels between 6–9 months (Forest et al. 1974). After this rise, there follows a period of 'quiescence' during which levels of these hormones decrease (Mann and Fraser 1996). Activation of the GnRH pulse generator in the second decade of life results in pulsatile release of gonadotropins, regeneration of the testicular Leydig cell population and production of testosterone resulting in the maturation of the male reproductive system (reviewed in Grumbach (2002)). This pattern of secretion has also been demonstrated in many other primates, including the rhesus macaque (*M. mulatta*) and the common marmoset (*C. jacchus*) (Dixson 1986; Gao et al. 1996). The marmoset has a transient rise in blood testosterone levels in the first 3 months of life, followed by a relatively quiescent, pre-pubertal period up to 6 months of age, with low blood testosterone levels. Puberty begins at about 6–12 months of age, with rapid testicular growth and an increase in blood testosterone levels. Full spermatogenesis is established by 12 months of age (reviewed in Li et al. (2005)). The marmoset is however,

limited as an animal model for reproductive maturation studies by its large variation between individuals in the timing and amount of its hormone production, similar to the variation described in the human, which makes data spread very large and treatment difficult to reproduce (Irfan et al. 2015). The 'mini-puberty' does not occur in rodents. In mice, testosterone production is high at birth, but then decreases during the first days of life and remains low until day 20, when it begins to rise again (Fig. 7.2). The rise is rapid between day 20 and 40, when it peaks, then falls slightly between day 40 and day 90. Levels at day 90 are similar to levels at birth. (Jean-Faucher et al. 1978). Murine FSH declines after postnatal d2 then increases sharply between d10 and d15, where it remains constant throughout adult life (Barakat et al. 2008).

7.3 Models Used for Studying the Effect of Hormones on Spermatogonia

Understanding the role of HPG hormones on the development of spermatogonia requires investigation using relevant models (summarised in Table 7.1). The literature documents human disorders of FSH and testosterone action, and a variety of animal models have been used to investigate the mechanism and timing of their effects. Studies of gonadotropin ablation and selective replacement have shown that testosterone is required for progression through meiosis whereas FSH is required for qualitatively normal spermatogenesis. Nearly all of the models discussed below show that both FSH and testosterone can increase the number of spermatogonia in the testis. Attempts to quantify the relative actions of the two hormones lead to highly heterogeneous results due to differences in the models and the advantages and disadvantages of these models are discussed below. In summary, both FSH and testosterone appear to be stimulatory but not essential for the development of spermatogonia and maintenance of their numbers.

Chemical and surgical models of gonadotropin ablation can be performed at specific times and can potentially be reversed to examine recovery effects, but they often do not entirely remove the hormone stimulus. Studies on models of gonadotropin ablation are complicated by the fact that more recently it has been discovered that luteinising hormone receptor knockout mice can still produce low levels of testosterone (2% of controls) that is enough to reinitiate full spermatogenesis at the age of 12 months (albeit at a reduced level) (Zhang et al. 2003). Moreover, the older hormonal replacement studies often remain inconclusive because of insufficient purity of the biological gonadotropin preparations used (for an example see Purvis et al. (1979)). Modern use of recombinant gonadotropins avoids this issue and it is important that any assessment of the conclusions of a study takes into account whether urinary or recombinant gonadotropins were used. Therefore, although studies with mice that have reduced gonadotropin action developed the early ideas about the hormonal control of spermatogenesis, due to these limitations it is difficult to draw firm conclusions about the relative contribution of testosterone and FSH from these models. In order to completely remove the action of a hormone, its receptor must be genetically ablated.

Table 7.1 Summary of the effects of manipulating the hypothalamo-pituitary-gonadal (hpg) axis and/or downstream receptor signalling on serum levels of FSH and Intra-testicular Testosterone (ITT)

Biological condition	Species	Plasma FSH	ITT
GnRH mutations	Human (HH)	Absent (Dwyer et al. 2013)	Decreased (Dwyer et al. 2013)
	Mouse (hpg)	Absent (Haywood et al. 2003)	Decreased (Haywood et al. 2003)
Hypophysectomy	NHP	Absent (Marshall et al. 1995)	Decreased (Marshall et al. 1986)
	Rat	Absent (Bartlett et al. 1989)	Decreased (Bartlett et al. 1989)
GnRH antagonist	NHP	Absent (Marshall et al. 2005)	Decreased (Zhengwei et al. 1998a; Marshall et al. 2005)
	Rat	Decreased (Bhasin et al. 1987)	Decreased (McLachlan et al. 1995; Hikim and Swerdloff 1995)
Low-dose T	Human	Decreased (McLachlan et al. 2002)	Decreased (McLachlan et al. 2002)
	NHP	Decreased (O'Donnell et al. 2001)	Decreased (O'Donnell et al. 2001)
	Rats	Decreased (McLachlan et al. 1994; Meachem et al. 1997, 1998)	Decreased (McLachlan et al. 1994; Meachem et al. 1997, 1998)
FSH immunoneutralisation	Rats	Decreased (Meachem et al. 1998)	Normal (Meachem et al. 1999)
FSHβ mutations	Human	Absent (Layman et al. 2002)	Normal (Layman et al. 2002)
	Mouse	Absent (Kumar et al. 1997)	Normal (Baker et al. 2003)
FSHR mutations	Human	Increased[a] (Tapanainen et al. 1997)	Normal (Tapanainen et al. 1997)
	Mouse	Increased (Dierich et al. 1998)[a]	Decreased (Baker et al. 2003)
AR mutations	Human (CAIS)	Normal (Bouvattier et al. 2002)	Increased[a] (Bouvattier et al. 2002)
	Mouse (ARKO/Tfm)	Increased (Naik et al. 1984)	Decreased (Naik et al. 1984)[a]

Data derived from animal models and human studies. *NHP* non-human primate, *CAIS* complete androgen insensitivity syndrome, *ARKO* androgen receptor knockout, *Tfm* testicular feminised mouse, *HH* hypogonadotrophic hypogonadism

[a]No receptor activation. Even though levels of the ligand may have changed, the mutation in the receptor means that this is irrelevant as lack of receptor signalling is comparable to absence of ligand

7.3.1 Increasing Circulating FSH Levels

Circulating FSH levels can be increased either by treatment with exogenous FSH, or by unilateral orchiectomy. The increase in FSH in these models is correlated with an increase in sperm output and spermatogonia number when measured.

In many species, unilateral orchiectomy (UO) elicits a compensatory increase in size of and sperm production by the remaining testis, which correlates with an increase in circulating FSH levels brought about by the change in pituitary feedback when one testis is removed. Adult rhesus monkeys (*Macaca mulatta*) have an increase in circulating FSH and the numbers of type B spermatogonia 44 days after orchiectomy, but in contrast to FSH treatment in intact macaques there is no increase in type A_{pale} spermatogonia (Ramaswamy et al. 2000). Bonnet monkeys (*Macaca radiata*) also have an increase in circulating FSH and spermatogonia (as measured by flow cytometry) after UO (Medhamurthy et al. 1993). Humans also have an increase in circulating FSH, testicular volume and sperm count after UO, although these men had undergone orchidectomy for seminomatous testicular germ cell tumour (Selice et al. 2011).

Adult Macaques (*Macaca fascicularis*) treated with FSH for 16 days showed an increase in the number of type A_{pale} spermatogonia to 200% and type B to 160%. There was no increase in A_{dark} (van Alphen et al. 1988). In another study on the rhesus monkey an increase in circulating FSH on the background of physiological LH increased the number of type B, but not type A spermatogonia (Simorangkir et al. 2009a). An increase in circulating LH on the background of normal FSH did not have this effect. This is reminiscent of UO, which leads to a similar increase in B1 spermatogonia in the remaining testis that is exposed to an increase in circulating FSH concentrations.

7.3.2 Gonadotropin Ablation in Genetic Hypogonadotrophic Hypogonadism

Men with hypogonadotrophic hypogonadism (HH) do not produce gonadotropins and have testes in which the only germ cells are primitive type A spermatogonia (Kumar et al. 2006). A mouse model of HH is the *hpg* (hypogonadotrophic) mouse, which carries a naturally occurring point mutation in the *Gnrh1* gene (Mason et al. 1986) and so does not produce LH or FSH. What little spermatogenesis that occurs in these mice does not proceed beyond the pachytene primary spermatocyte stage and adult *hpg* testes have only 27% of control Sertoli cell number and 10% of spermatogonia number (O'Shaughnessy et al. 2012). This is a chronic model of gonadotropin deficiency. It is important to note that the testes of *hpg* mice are cryptorchid and that this contributes to the degeneration of spermatogenesis (Dutta et al. 2013), and that the testis has not been exposed to these hormones during development. The chronically low levels of testosterone, estradiol and other paracrine factors dependent on these hormones will also contribute to the phenotype. Both cryptorchidism and change in hormones may explain why there are fewer

spermatogonia in *hpg* mice compared to hypophysectomised rats. Like the LHRKO mouse, the *hpg* is likely to have residual testicular testosterone levels that are produced by Leydig cells unstimulated by LH, and that this may be affecting the phenotype.

hpg mice expressing transgenic FSH have a twofold increase in the number of Sertoli cells compared to non-transgenic *hpg* mice (Haywood et al. 2003). *hpg* mice given testosterone implants at d21 have a twofold increase in the number of Sertoli cells compared to untreated *hpg* mice. Combined, the transgenic FSH and testosterone further increases the number of Sertoli cells in *hpg* mice, although only to 70% ofcontrol levels. Interestingly, when hpg mice are treated with FSH as adults (as opposed to the lifelong transgene expression above) there is no increase in Sertoli cell number (O'Shaughnessy et al. 2010a). In contrast, the spermatogonial number is increased in *hpg* mice both expressing postnatal transgenic FSH (Haywood et al. 2003) or treated with FSH as adults (O'Shaughnessy et al. 2010a) suggesting that FSH can increase spermatogonia number at any age, but that there is a specific window for its action on Sertoli cell number.

Treatment of *hpg* mice with both testosterone and DHT has been shown to induce an increase in spermatogonial numbers (O'Shaughnessy et al. 2010b; Singh et al. 1995). Another study claims that testosterone implants do not increase spermatogonia numbers in *hpg* mice (Haywood et al. 2003). However, this may also be because intratesticular testosterone (ITT) levels in this model were only restored to ~20% of controls, and this may not be enough to increase spermatogonia numbers.

The response of HH patients to therapy is very variable depending on time of onset and presence or absence of cryptorchidism (Finkel et al. 1985). When 21 men with HH were treated with hCG to stimulate Leydig cell testosterone production the sperm count increased to within the normal range in the 6 in whom hypogonadism had begun after puberty, but in only 1 of the 15 in whom it had begun before puberty. When the remaining 14 men with prepubertal hypogonadism were treated with human menopausal gonadotropin (a mixture of FSH and LH extracted from the urine of postmenopausal women) in addition to hCG, the sperm count increased to normal in 5 of the 7 who had not had cryptorchidism, but in only 1 of the 7 who had. The addition of FSH may be an important factor for improvement in sperm count in these patients. In patients where there may have been a failure of the early postnatal rise in gonadotropins (e.g. those with cryptorchidism ± microphallus) pre-treatment with FSH alone prior to GnRH therapy may increase potential for sperm production by enhancing Sertoli cell proliferation (Dwyer et al. 2013). The response to induction of sperm production may also be affected by the underlying cause of gonadotropin deficiency. In a study comparing 53 men with HH to 22 with congenital combined pituitary hormone deficiency (CCPHD), those with CCPHD had larger testicular volume, serum testosterone and were more likely to demonstrate induction of spermatogenesis following stimulation with hCG/hMG (Mao et al. 2015). The authors conclude that the difference in response between the two groups may be attributable to the different pathogenic genic mutation causing the disorder, with a potential for multiple defects in the different levels of the reproductive axis in the pathogenesis of IHH.

7.3.3 Gonadotropin Ablation by Hypophysectomy

Hypophysectomy is the surgical removal of the pituitary gland. Historical studies on hypophysectomised animals formed the basis of the theory of gonadotropin control of spermatogenesis. P. E. Smith first documented the effects of hypophysectomy on the male adult rat testis in 1930, describing a rapid regression of seminiferous tubules and disappearance of spermatids. In 1955, Clermont and Morgentaler quantified the effects of hypophysectomy on spermatogenesis in adult rats and described a reduction of germ cell number at all stages, with 43% of type A spermatogonia of controls and 25% of type B spermatogonia of controls present 61 days after hypophysectomy (Clermont and Morgentaler 1955). Since there was a reduction in type A and a further reduction in type B spermatogonia, gonadotropins appear to be required for both maintenance of undifferentiated spermatogonia pool and also their progression to differentiation. Treatment of hypophysectomised rats with flutamide to block the effects of the residual testosterone produced by Leydig cells without LH stimulation did not further reduce the number of spermatogonia, suggesting that the small amount of testosterone produced by Leydig cells without LH stimulation is not affecting the number of spermatogonia (Franca et al. 1998). Replacement of FSH or testosterone in hypophysectomised rats can partially but not fully restore spermatogonial number (El Shennawy et al. 1998).

In 1944, Smith noted that the testis of the rhesus monkey also undergoes regression after hypophysectomy, and that exogenous androgen can both maintain and restore qualitatively normal spermatogenesis after hypophysectomy (Smith 1944). Hypophysectomised macaques had no change in type A_{dark} spermatogonia, but a reduction in type A spermatogonia to approximately a third of controls and no type B spermatogonia present 13 weeks after hypophysectomy. ITT concentration was approximately 34% of controls. When supplemented with exogenous testosterone that also increased the ITT levels to at least 50% of the control, A_{pale} spermatogonia numbers were not different to controls, and B type spermatogonia numbers partially recovered (Marshall et al. 1986). Increase of testicular testosterone levels to greater than 100% of control did not further increase this recovery. FSH also partially recovered type B spermatogonia numbers in hypophysectomised rhesus monkeys (Marshall et al. 1995).

These results suggest that circulating gonadotropins are essential for the maintenance of a population of type B spermatogonia in primates, and that residual, non LH-stimulated ITT is not enough to maintain spermatogenesis beyond this cell type in the short term. Supplementation with either testosterone or FSH can increase type B spermatogonia numbers. In rodents, the progression to type B spermatogonia can proceed without circulating FSH or action of ITT, although numbers of both type A and B spermatogonia are reduced.

7.3.4 Gonadotropin Reduction by GnRH Antagonists

Another way of suppressing gonadotropin levels is to use GnRH antagonists (GnRHa), peptides that bind with high affinity to the GnRH receptor in the anterior pituitary.

They are non-functional and compete with the endogenous GnRH for the binding site and provide variable suppression of gonadotropin levels, depending on the model (reviewed in Griesinger et al. (2005)). They are useful for pinpointing temporal effects because they act only for the time they are given to the animal hence their use in developmental studies in marmosets as detailed below in Sect. 4.3.4. However, their reduction of ITT concentrations varies from study to study and it is not known if the variations in residual ITT is enough to contribute to any phenotype seen.

GnRHa have mostly been used to suppress gonadotropin production in primates. In macaques given a GnRHa, ITT was reduced to 21% of controls. There was no reduction in any type A spermatogonia, but reduction in type B spermatogonia to 15% of control. Unfortunately, FSH was not measured so its contribution to this study cannot be assessed (Zhengwei et al. 1998a). Another study using a GnRHa on rhesus monkeys reduced ITT to 9% of controls and FSH under detection limits after 20–33 weeks of treatment (Marshall et al. 2005). Type A_{pale} spermatogonia were not reduced, but type B were completely absent from treated testes. When treated with testosterone implants ITT rose to 71% of controls, when treated with recombinant human FSH ITT rose to 38% of controls, and when treated with both ITT rose to 81% of controls. All treatments induced the testis to start producing type B spermatogonia again, treatment with both was significantly increased over individual treatments. In another study, administration of highly purified FSH to GnRH antagonist-treated monkeys fully maintained spermatogonial numbers and those of spermatocytes and spermatids at 50% of control. ITT was not measured in this study, however since the androgen-critical step of post-meiotic spermatogenesis was observed, the human FSH used may have been contaminated with LH (Weinbauer et al. 1991).

The response of rats to GnRHa is variable. In one study, ITT was reduced to 1.5% of control and type A and B spermatogonia numbers were reduced to approximately 50% of control (McLachlan et al. 1995). In another study, 4 weeks of GnRHa treatment of rats results in only a slight reduction in the number of type B spermatogonia (Hikim and Swerdloff 1995). However, ITT in this treatment model was only reduced to 7.2% of controls, and circulating FSH was not measured. It may be that in the first study the lower ITT was enough to see a more pronounced effect on spermatogonia numbers.

7.3.5 Gonadotropin Reduction by Low-Dose Testosterone

Low-dose exogenous testosterone given as implants or injections can reduce gonadotropin production due to negative feedback on GnRH production by the hypothalamus. It has been trialled as a potential male contraceptive, consequentially there is a relatively large amount of data produced from human trials using this method. In a study of fertile men ($n = 5$), circulating LH was reduced to 0.3% of baseline, circulating FSH was reduced to 1.2% and ITT was reduced to 5.2% of baseline after 12 weeks of testosterone enantate (200 mg; IM once weekly) injections. No decrease in type A_{dark} spermatogonia was seen but type A_{pale} was reduced to 58% of control

and type B was reduced to 40% of controls (McLachlan et al. 2002). Another study by the same group, using the same regimen, treated men for 19–24 weeks and found that type B spermatogonia were reduced to 9% of controls, which may indicate a further degeneration in the number of type B spermatogonia over time (Zhengwei et al. 1998b). In a further study men ($n = 6$ per group) were given a single testosterone implant (800 mg, subcutaneous) and a single depot injection of medroxy progesterone acetate (DMPA; 150 mg, subcutaneous), which suppressed FSH and LH to similar levels and further repressed ITT to 0.5–2% of baseline. This reduced type A_{pale} spermatogonia to 63% of control and type B to 35% of control. Giving back either hCG (1000 IU, subcutaneous, weekly) or FSH (300 IU, subcutaneous, weekly) to these men resulted in no increase in type A spermatogonia, but an increase in type B spermatogonia, with FSH restoring the level to 76% of controls and hCG to 59% of controls (Matthiesson et al. 2006). The authors of these studies noted that a large variation in the suppression of spermatogenesis could be seen between men undergoing the same treatment regimen, and that this would make it difficult to use it for contraceptive purposes. A further study using testicular tissue obtained from gonadotropin suppressed men (McLachlan et al. 2002), concluded that withdrawal of gonadotropin support increased the number of spermatogonia undergoing apoptosis through the intrinsic pathway but did not decrease spermatogonia proliferation, concluding that the mechanism of gonadotropin support is to decrease apoptosis rather than increase proliferation (Ruwanpura et al. 2008a).

Low-dose testosterone implants in primates also reduce levels of endogenous FSH and ITT, though not to the same extent. In macaque monkeys (*M. fascicularis*; $n = 9$) given testosterone implants for 20 weeks, FSH is reduced to 40% of controls and ITT reduced to 11% of controls (O'Donnell et al. 2001). Even without complete ablation this has a profound effect on spermatogonia, with type A spermatogonia reduced to 64% of controls and type B reduced to 31% of controls. Interestingly, there is an initial increase in 'reserve' type A_{dark} spermatogonia 2 weeks after gonadotropin reduction, and an initial decrease in the dividing type A_{pale} and differentiated type B spermatogonia, suggesting that fewer spermatogonia are being pushed down the path towards differentiation. However, after 20 weeks the number of type A_{dark} spermatogonia returns to normal even though the number of type A_{pale} and B remains reduced.

In contrast to primates, implants of low-dose testosterone and estrogen ('TE') in rats suppress pituitary LH production so that it is undetectable in the serum, resulting in testicular testosterone reduction to less than 3% of controls and variable FSH suppression. Spermatogonia numbers are variably reduced in these models but reduction is not proportional to FSH suppression. One study with the largest reduction in spermatogonial number (type A reduced to 44–59% of controls and type B reduced to 49–55% of controls) had one of the smallest reductions in FSH, to 84% of the control (McLachlan et al. 1994). Another study reported an 71% FSH compared to control with spermatogonia reduced to 61% for type A or 77% for type B, with a parallel study in the same paper reporting 51% FSH with spermatogonia reduced to 59% for type A and 68% for type B (Meachem et al. 1997). A further study saw a reduction of FSH to 65% of controls and the reduction in spermatogonia, at 80–85% of controls, was not significant (Meachem et al. 1998).

7.3.6 FSH Suppression by Immunoneutralisation

FSH can be suppressed by injections of an anti-FSH antibody to approximately 10% of circulating control levels. Adult rats acutely immunoneutralised for FSH were shown to have a time dependent decrease in type A and B spermatogonia to approximately 70% of controls and an increase in the number of tubule cross-sections showing apoptosis (Meachem et al. 1999). Apoptosis was shown to be through the intrinsic apoptotic pathway, and reduction of FSH did not result in a reduction of spermatogonial proliferation (Ruwanpura et al. 2008b). In rhesus monkeys, a 50% reduction in testis size and a substantial reduction in sperm output was achieved by active and passive immunisation against FSH (Srinath et al. 1983).

7.3.7 FSH and FSHR Mutations

Follicle stimulating hormone receptor (FSHR) is a G protein coupled receptor found in Sertoli cells (reviewed in George et al. (2011)). Knockout mice have been created both for the FSHβ subunit (Dierich et al. 1998; Kumar et al. 1997), and FSHR (Abel et al. 2000; Dierich et al. 1998). Both types have a decrease in testicular size with reduced Sertoli and germ cell numbers at all stages of spermatogenesis (Wreford et al. 2001; O'Shaughnessy et al. 2012; Johnston et al. 2004; Krishnamurthy et al. 2001a). Despite this, fertility is either normal (Abel et al. 2000) or slightly reduced (Dierich et al. 1998). In FSHβ knockout mice testosterone production and Leydig cell numbers are normal (Wreford et al. 2001) but aged FSHR knockout mice have reduced testosterone production (Krishnamurthy et al. 2001b), Leydig cell numbers and steroidogenic enzyme expression (Baker et al. 2003), suggesting that FSHR signalling in Sertoli cells has a paracrine stimulatory effect on Leydig cells (Haywood et al. 2002). In contrast to mice, men with mutations in the FSHβ gene are azoospermic and infertile (Lindstedt et al. 1998; Phillip et al. 1998; Layman et al. 2002), but those with an inactivating mutation in the FSHR are oligozoospermic: their fertility is severely reduced but some have managed to sire children (Tapanainen et al. 1997). These results suggest that FSH itself is essential for spermatogenesis in men, but can also act through a non-FSHR dependent mechanism to promote testicular development and function.

The disadvantage of using rodent transgenic models is that knockout animals are not exposed to the gene product at any stage in development, and it may have different or essential effects during the embryonic period that result in embryonic lethality or severe developmental problems in complete knockouts. Examination of an adult might present a phenotype that is due to effects that took place during development and that are not representative of the action of the gene product in an adult animal. However the cell-specificity of FSH means that a complete knockout does not have any lethal or non-specific effects, but cannot be used to pinpoint the timing of its action on germ cell development and spermatogenesis.

7.3.8 Androgen Receptor Mutations

The role of testosterone in spermatogenesis has been determined from analysis of human mutations and also animal models. The receptor for testosterone is androgen receptor, which is a nuclear steroid hormone receptor transcription factor expressed in the Sertoli, Leydig and peritubular somatic cells of the testis in both the mouse (Zhou et al. 2002) and human (O'Hara et al. 2011), but is not expressed in germ cells of the adult at any stage of spermatogenesis, so the effects of testosterone on testicular germ cells is mediated through the somatic cells.

Complete androgen insensitivity syndrome (CAIS) is a disorder of sexual development resulting from an inactivating mutation in the single copy of the androgen receptor gene that is present in male cells (Hughes et al. 2012). People with CAIS have an external female phenotype and are assigned the female gender at birth. However, due to XY genotype they have small, undescended testes that do not undergo spermatogenesis, although they produce testosterone and anti-Müllerian hormone (AMH) like fully functional testes. The inability of the developing foetus to respond to testosterone results in the lack of epididymis, seminal vesicles and vas deferens derived from the Wolffian duct. The production of AMH results in a lack of uterus, oviducts and the upper portion of the vagina. CAIS is often diagnosed in childhood due to hernias caused by the undescended testes, or when menstruation does not occur at puberty. Histological analysis of the testis shows that normal spermatogenesis does not occur. Some tubules in the testis lack germ cells completely and the others contain a few spermatogonia, but no post-meiotic germ cells (Hannema et al. 2006), analogous to the phenotype seen in 'testicular feminisation' (Tfm) mouse and androgen receptor knockout (ARKO) mice.

Rodent models of complete androgen insensitivity include the naturally occurring (Tfm) mouse which has a point mutation in the androgen receptor (Charest et al. 1991; Gaspar et al. 1991), and the transgenic (ARKO) mouse (Yeh et al. 2002). Both ARKO and *Tfm* mice have small, inguinal testes, feminisation of external genitalia and lack of Wolffian duct-derived structures. Histological analysis of the testis shows that normal spermatogenesis does not occur. Some tubules in the testis lack germ cells completely and the others contain spermatogonia, but no post-meiotic germ cells (Yeh et al. 2002). A qualitative assessment of the tubules of Tfm mice determined that they have germ cell differentiation in 90% of the tubules, with spermatocytes present in 86% and B spermatogonia in the other 4% (Shetty et al. 2006). The effects of a complete body-wide lack of functional AR on spermatogenesis complicates the phenotype seen because the testes are unable to descend without the action of testosterone on the gubernaculum, so remain in the abdomen experiencing a higher temperature than the scrotum. In both mice (Dutta et al. 2013) and men (Hedinger 1982) this has detrimental effects on spermatogenesis that cannot be separated from ablation of androgen receptor signalling-specific effects.

To circumvent this problem, the Cre-loxP transgenic system has been used to create cell-specific ARKO mice. Mice with a specific ablation of AR in Sertoli cells (SCARKO) have a post-meiotic block, with no germ cells more advanced that pachytene spermatocytes (De Gendt et al. 2004; Chang et al. 2004). However,

spermatogonia (De Gendt et al. 2004) and Sertoli cell numbers do not differ between SCARKOs and controls (Tan et al. 2005). Peritubular myoid (PTM)-ARKO mice have a reduction of germ cell numbers at all stages, including spermatogonia (Welsh et al. 2009), suggesting that maintenance of spermatogonia numbers is controlled by PTM cell AR. The reduction is progressive: it is 80% of controls at d21, 75% at d50 and 38% at d100. It is possible that this effect is due to direct signalling between PTM cells and spermatogonia since spermatogonia are juxtaposed to the basement membrane and are in intimate contact with the PTM cells. PTM cells in the PTM-ARKO progressively downregulate expression of desmin and smooth muscle actin (SMA) indicating that they are losing their smooth muscle phenotype, and a disruption of laminin expression indicates problems with the basement membrane of the seminiferous tubules. These disruptions may impair attachment and signalling between PTM cells and spermatogonia and disrupt the niche (Mayerhofer 2013). It is also possible that the decrease in spermatogonia in the PTM-ARKO is mediated through changes in Sertoli cells induced by disruption to AR signalling in PTM cells. Since Sertoli cell number is not reduced in PTM-ARKO mice, this does not cause the decrease in spermatogonia number. However, Sertoli cell-specific transcripts are reduced in PTM-ARKO testes, suggesting that AR signalling in PTM cells has an effect on the transcriptome and function of their neighbouring Sertoli cells and this may contribute to the reduction in germ cell number.

7.3.9 SCARKO/FSHRKO Double Knockouts

To investigate the synergistic effects of FSH and androgens, mice with double knockouts for Sertoli cell AR and FSHR were generated (Abel et al. 2008). The double knockout had an additive phenotype, with the block at meiosis present in SCARKO mice but also a reduction in Sertoli cells and spermatogonia present in FSHRKO mice. Spermatogonia were reduced to 60% of controls. Since there is no reduction of spermatogonia in the SCARKO mouse it is not surprising that this is not significantly different to spermatogonia number in single FSHRKO mice. Since Sertoli AR is not thought to contribute to spermatogonia development but PTM AR is, it would be informative to generate a PTM-ARKO FSHRKO double knockout mouse and see if there is an even further reduction in spermatogonia, or a complete block in spermatogonia development.

7.4 Developmental Effects of FSH and T on Spermatogonia and Sertoli Cell Number

Some of the models discussed above to address the adult effects of testosterone and FSH can also be modified to investigate their effects during development. This is important because the acquisition of a final cell population number often depends on the environment that it is exposed to during development. Although this section will focus on the effects of FSH and testosterone on gonocytes/spermatogonia at

different ages, we will also discuss data on their effects on the development of Sertoli cells, as final Sertoli cell number is proportional to germ cell number. Figure 7.2 shows the relative hormone profiles of testosterone, LH and FSH in human and mouse at different stages of pre- and postnatal development.

7.4.1 Fetal

Hormones and receptors: Both testosterone and FSH are present in the fetal circulation of rodents and primates. In the mouse, testicular androgen receptors are not present in Sertoli cells but are found in peritubular interstitial cells from 3 months in humans (Rey et al. 2009) and e15.5 in mice (Merlet et al. 2007) (and potentially before) AR is also found to be expressed in fetal gonocytes from e15.5 to e17.5 (Merlet et al. 2007).

FSHR is present at low levels in rat testis from e16.5 and can be localised in the basal compartment of seminiferous tubules by in situ hybridisation at e18.5 (Rannikki et al. 1995). Specific FSH binding to rat testicular homogenates can be first detected on day e17.5 (Warren et al. 1984). FSHR protein is present in 8–16wk human testes and in late gestation rhesus monkey testes but FSH stimulation does not cause cAMP production, suggesting that they may not be able to signal through the classical FSHR signalling pathway (Huhtaniemi et al. 1987). There is currently no documentation of the onset of FSHR expression in fetal mouse testis, and it is commonly assumed to switch on in Sertoli cells in late gestation, as in the rat.

Germ cells: There is no significant difference in germ cell number at day 0 in mice lacking FSHR, AR or both (Migrenne et al. 2012; O'Shaughnessy et al. 2012) or in hpg mice (Baker and O'Shaughnessy 2001). In mice, the total number of gonocytes is already determined by 15.5 dpc, when they enter the quiescent period (Vergouwen et al. 1991), and thus before the onset of FSH secretion (Japon et al. 1994). Murine gonocytes stain for AR from e15.5 to e17.5 and it has been noted that Tfm mice have more gonocytes at e17.5 (Merlet et al. 2007) so absence of AR signalling during this period may cause a temporary increase in gonocytes that is corrected by birth.

Sertoli cells: There is conflicting evidence about whether FSHKO mice have a reduction in fetal Sertoli cell number, with some investigators reporting that they do (Migrenne et al. 2012), and some that they do not. Decapitated or FSH immunoneutralised rat fetuses have been shown to have fewer dividing Sertoli cells (Orth 1986; Sasaki et al. 2000) although analysis of absolute Sertoli cell number has not been conducted.

Summary: There is no evidence that fetal germ or Sertoli cell number is affected by changes in FSH or AR signalling, however this does not rule out a programming effect on number or function that may only manifest itself in later life.

7.4.2 Early Postnatal

Hormones and receptors: In rodents, levels of both circulating testosterone and FSH are high at birth but then rapidly decline. In primates, levels of testosterone and FSH

are low at birth but then increase rapidly during the post-birth 'mini-puberty' then decline into a period of childhood quiescence. A similar hormonal environment of briefly high testosterone exists for both rodents and humans in the early postnatal period. AR begins to be expressed in a few Sertoli cells at d4 in mice and is expressed in all from d5, as well as interstitial and peritubular cells (Willems et al. 2009). In humans, AR is not found in Sertoli cells at 3 months of age but begins to be expressed in some Sertoli cells towards the end of the first year of life, and is strongly expressed by 8 years (Rey et al. 2009; Chemes et al. 2008). The onset of Sertoli cell AR expression in both species occurs as the perinatal testosterone rise begins to decline.

Gonocytes: The early postnatal phase (up to day 5 in the mouse and 3 months in the human (Berensztein et al. 2002)) is marked by a rapid proliferation of gonocytes, their maturation into spermatogonia and continued proliferation as type A spermatogonia. This increase in cell number seems to be mediated by decreased rate of apoptosis (Berensztein et al. 2002). There is strong evidence to suggest that exposure to androgens and FSH in this early postnatal period is required for maintenance of gonocyte number during this process in rodents. *hpg* mice, FSHRKO and ARKO mice all have fewer gonocytes than their controls at d5 (Baker and O'Shaughnessy 2001; O'Shaughnessy et al. 2012). In primates the evidence is not so clear. When neonatal marmosets are administered a GnRH antagonist for the first 14 weeks of life it ablates the postnatal circulating testosterone rise that occur during this period (Lunn et al. 1994). At 18–24 weeks germ cell numbers in 4 out of 5 of the injected marmosets is reduced compared with their fraternal twin controls, although this was not statistically significant when the data was analysed together (Sharpe et al. 2003). Treatment in the neonatal period seemed to have no long-lasting effects on germ cells as there was no difference between germ cell volume per Sertoli cell in neonatal GnRHa treated animals compared to controls when they reached adulthood (Sharpe et al. 2000). The lack of notable effects using GnRHa in the marmoset may be because either ITT or circulating FSH or both were not reduced to a low enough level to affect testicular maturation. Neither hormone level has been reported in these studies; FSH because there was no suitable assay and ITT because it was perhaps not realised at the time how low a concentration of testosterone is actually required for the testis to continue spermatogenesis.

In humans with CAIS, the number of germ cells is normal until approximately 6 months followed by a subsequent rapid depletion (Hannema et al. 2006), however the germ cells that are present appear to have a delay in their maturation from gonocyte to spermatogonia (Cools et al. 2005). This is also found in patients who have undescended testicles without a formal diagnosis of AIS, so it is unclear whether the delay in maturation is a direct result of lack of androgen signalling, or degeneration as a result of the higher temperature in the abdomen to which the testes of cryptorchid patients are subjected (Hadziselimovic and Herzog 2001; Huff et al. 2001).

Sertoli cells: Between birth and d5 in the mouse Sertoli cells are also dividing (Auharek and de Franca 2010). In humans, Sertoli cells are dividing through childhood and puberty (Cortes et al. 1987). However, *hpg* and ARKO mice both have fewer Sertoli cells than their controls do at this age (O'Shaughnessy et al. 2012; Johnston et al. 2004; Baker and O'Shaughnessy 2001)), whereas FSHRKO mice do

not (Baker and O'Shaughnessy 2001; O'Shaughnessy et al. 2012). The potential effects of testosterone on Sertoli cell number is likely to be mediated by other somatic cells, as SCARKO mice do not have fewer Sertoli cells (O'Shaughnessy et al. 2012; Johnston et al. 2004) and AR is not expressed in Sertoli cells until d5 (Willems et al. 2009). Treatment of neonatal marmosets in the first 14 weeks of life with GnRHa during the neonatal period also results in a temporary decrease in Sertoli cell number that recovers by adulthood (Sharpe et al. 2000). However, the effect is permanent when rats are given the equivalent treatment. This may be because the FSH levels are confirmed to have been suppressed in rats by the GnRHa used (Sharpe et al. 1999) but might not be in marmosets, or potentially because FSH levels remained low throughout the entire period of Sertoli cell proliferation in rats but not in marmosets.

Summary: There is evidence to suggest that both FSH and testosterone act in the early postnatal period to maintain spermatogonia number in rodents. However in primates the evidence is that FSH and testosterone act to maintain numbers is inconclusive, possibly because the studies have not been conducted under low enough ITT or circulating FSH levels to show a true effect. The completion of gonocyte transformation into spermatogonia has been suggested as being promoted by intratesticular androgens in the human in the neonatal period. FSH has no impact on Sertoli cell number in the early postnatal period in rodents, but testosterone does, through non-Sertoli mechanisms that have not yet been elucidated.

7.4.3 Juvenile

7.4.3.1 Summary

Hormones and receptors: Between the end of the first year of life and the reactivation of the HPG axis at puberty there is a period of hormonal quiescence where gonadotropin and testosterone levels are low. In rodents this period is extremely short, but in humans it lasts for nearly a decade. Even though average circulating gonadotropin levels in humans during this period are so low as to often be undetectable, there is evidence that there is spontaneous pulsatile release of small amounts of gonadotropins by the pituitary during childhood (Wu et al. 1991, 1996).

Spermatogonia: Type A spermatogonia proliferate throughout postnatal life, and the first emergence of type B spermatogonia is at 4–5 years in the human (Paniagua and Nistal 1984). Approximately 11% of human germ cells are dividing at any point when observed in testis sections from 1 to 6 year olds (Berensztein et al. 2002). In rhesus monkeys type A spermatogonia proliferate at the same rate during neonatal high-gonadotropin conditions as they do during juvenile low-gonadotropin conditions (Simorangkir et al. 2009b), so it appears that both the proliferation of type A spermatogonia and the maturation of type B spermatogonia require limited gonadotropin support.

GnRH antagonists have also been used to study the role of the HPG axis in the juvenile marmoset testis (Choong et al. 1997). One twin of four pairs of marmoset twins was treated from 25 to 35 weeks of postnatal age and the others left as a control.

Three out of four of the pairs had fewer spermatogonia in the treated testis than the control, but this was not statistically significant when the data were analysed together. Type A_{pale}, (but not A_{dark} or B) spermatogonia numbers were increased in rhesus monkeys given exogenous FSH and/or testosterone at an age when endogenous levels are usually low, so the testis could be responsive to these hormones even if they are not usually present (Arslan et al. 1993).

7.4.4 Pubertal

Hormones and receptors: The first wave of spermatogenesis takes place in a hormonal milieu very different from subsequent rounds of spermatogenesis. It straddles the initiation of endocrine puberty with the initial spermatogonial differentiation taking place before the onset of pubertal testosterone and FSH production. In mice, the adult generation of Leydig cells do not produce testosterone until d20 and even then the levels are very low and take several weeks to reach maximum adult testosterone output (Jean-Faucher et al. 1978). Murine FSH is low after d2 then again increases sevenfold between d10 and d15 (Barakat et al. 2008).

In humans, FSH is low after minipuberty but begins to increase in males at around age 11. This increase is only approximately 2.5-fold which is modest compared to the mouse (Sizonenko and Paunier 1975; August et al. 1972). Testosterone begins to rise at the same time and rises between 25 and 50-fold between ages 5 and 15 (Sizonenko and Paunier 1975; August et al. 1972).

Spermatogonia: Type B spermatogonia start to develop at day 8 in the mouse (Bellve et al. 1977) then the first wave of spermatogenesis continues until first spermiation at around d35 (Vergouwen et al. 1993). Type B spermatogonia start to develop at 4–5 years in the human (Paniagua and Nistal 1984). This initiation of spermatogenesis takes place in an environment of low circulating testosterone and FSH levels. As the first wave continues in the rodent, FSH levels begin to rise but testosterone levels remain low. In normal animals there is a large amount of germ cell apoptosis during the first wave of spermatogenesis. Inhibition of this apoptotic event in spermatogonia either by overexpression of anti-apoptotic genes Bcl2 or Bclx (Furuchi et al. 1996; Rodriguez et al. 1997) or by knockout of the pro-apoptotic Bax (Russell et al. 2002; Knudson et al. 1995) causes a massive increase in immature germ cells and subsequent degeneration of the seminiferous epithelium. Germ cell apoptosis during the first wave of spermatogenesis appears to be limited by FSH that is rapidly rising during this time. When rats were passively immunised against FSH from d14 to d18 there was a corresponding increase in germ cell apoptosis (250% of control) which reduced spermatocyte number to 75% of control (Meachem et al. 2005). Hypophysectomy of rats in later puberty at d25 after onset of androgen production led to a decrease in spermatogonia numbers that was further decreased with flutamide treatment to remove any residual androgen action, so it is likely that androgens also affect the number of pubertal spermatogonia (Russell et al. 1998). In the same study, supplementary FSH and residual androgen post hypophysectomy is enough to maintain type A and B spermatogonia at close to control levels during puberty.

Sertoli cells: Between d5 and 15 in the mouse, Sertoli cells are dividing rapidly, they achieve their final adult number at around d15–d20 (Auharek and de Franca 2010; Baker and O'Shaughnessy 2001). Interestingly, this is before the onset of testosterone production by adult Leydig cells, but coincident with the rise in postnatal FSH. FSHRKO and FSHBKO have Sertoli cell number comparable to control at d5 but a significant decrease at d20, suggesting FSH is a critical influence on Sertoli cell number during early puberty (Johnston et al. 2004).

Summary: The role of FSH in pubertal spermatogonia maintenance appears to be limiting the apoptosis that takes place around this time. It also has a stimulatory effect on pubertal Sertoli cell numbers. Androgens may also have a stimulatory effect on spermatogonia in later puberty as their levels begin to rise. Due to the limited amount of data available in the primate it is not known if FSH has the same role, although it is interesting to note that FSH and testosterone rises occur at the same time in primates but FSH rises earlier in puberty in rodents.

7.5 Molecular Mechanisms of FSH and T Effects on Spermatogonia

From the observations detailed above, it is clear that both FSH and testosterone have an impact on spermatogonia, but knowledge of the mechanisms by which these hormones signal through testicular somatic cells to exert their effects on developing spermatogonia is hitherto incomplete.

7.5.1 FSH

Spermatogonia are replenished throughout adult life from a pool of spermatogonial stem cells (SSC). These cells divide to produce both more SSC and cells that differentiate and pass into the spermatogenic lineage. There is evidence that FSH acts to both maintain the population of undifferentiated SSC and promote the differentiation of spermatogonia through its actions on testicular Sertoli cells.

Maintenance of an optimal population of undifferentiated SSC requires and is sensitive to levels of the Sertoli cell product glial cell line-derived neurotrophic factor (GDNF) (Meng et al. 2000). GDNF levels in rodent testes are present in the fetus from e14 (Golden et al. 1999) and increase during the first week postnatally, but then decline over week 2 and 3 (Ding et al. 2011) to lower (Fouchecourt et al. 2006; Trupp et al. 1995) or undetectable (Golden et al. 1999) levels during adulthood. FSH (but not T) has been reported to stimulate expression of GDNF in Sertoli cell culture (Tadokoro et al. 2002; Simon et al. 2007) and injection of week-old mice with recombinant FSH causes an increase in testicular GDNF (Ding et al. 2011).

FSH is also thought to promote differentiation of spermatogonia through its upregulation of the Sertoli cell product KIT ligand (KL) also known as stem cell factor (SCF), mast cell growth factor (MGF) or Steel factor (SLF). The membrane-bound splice variant of KL increases in expression from d6 of postnatal life, and is

required for proliferation and apoptosis prevention of differentiating type A1–A4 spermatogonia (reviewed in Mauduit et al. (1999)). FSH and its downstream cellular messenger cAMP both increase the expression of KL in cultured primary mouse Sertoli cells (Rossi et al. 1991; Rossi et al. 1993). FSH also stimulated seminiferous tubule fragments to upregulate Kitl mRNA expression, this effect was not seen with testosterone (Yan et al. 1999). FSH stimulation of Sertoli cell factors that stimulate both SSC replenishment and SSC differentiation may initially appear contradictory, but since the maintenance of an optimal population is influenced by many testicular signalling pathways, it may just result in a finely tuned balance.

7.5.2 Testosterone

It is clear from the evidence presented above that testosterone influences spermatogonial numbers, but the molecular pathways that it influences in the spermatogonial niche have not yet been fully investigated.

7.6 Conclusion

Testosterone and FSH act through their somatic cell receptors in the testis to promote the initiation of spermatogenesis, increase in germ cell numbers, development and differentiation. As the testis develops throughout fetal and neonatal life the levels of gonadotropins rise and fall. Both FSH and testosterone act to maintain the population of both gonocytes and spermatogonia in different time windows during development. The mechanism of testosterone and FSH support of spermatogonia throughout development appears to be primarily in limiting apoptosis rather than promoting proliferation.

This knowledge has been advanced by animal models, but the models are often imperfect: genetic ablation of hormone receptors results in developmental phenotypes like cryptorchidism that complicate the adult phenotype, and chemical or surgical ablation models often incompletely suppress hormone production or suppress both testosterone and FSH so the contributions of the individual hormones can't be unravelled from the phenotype. However, when all the evidence provided by these studies is taken together, some important conclusions can be drawn.

Evidence from animal models strongly backs the hypothesis that testosterone is required for completion of meiosis and spermiation. There is also evidence that testosterone can have a stimulatory effect on spermatogonia numbers in both rodent and primate models of gonadotropin suppression, even in the absence of FSH. FSH alone cannot drive spermatogenesis to completion, but it has a stimulatory effect on both Sertoli cell number and spermatogonia number that results in a higher adult sperm output. Evidence suggests that testosterone and FSH are more important for the development of spermatogonia in primates than they are in rodents. Reduction of FSH and testosterone by gonadotropin suppression has a more severe effect on the numbers of type B spermatogonia in primates than it does in rodents, with many

models demonstrating a complete loss of type B spermatogonia; rodents still have a reduction in type B spermatogonia, although it is not as severe. Supplementation of either testosterone or FSH is generally enough to partially recover spermatogonia number, but even supplementation of both rarely returns the number to normal, perhaps indicating a specific dose-dependence or a lag in recovery time. Interestingly, many models of gonadotropin suppression do not completely eliminate ITT due to the ability of Leydig cells to produce low levels of testosterone without LH stimulation. This low level of ITT may be having an impact on the phenotype seen after gonadotropin ablation, although on the one occasion where the study was designed with flutamide to block residual androgens this did not further reduce spermatogonia numbers, but this study was done in a rodent model.

The sum of this knowledge highlights future studies that are required to develop male contraceptives or infertility treatments. Although the roles of FSH and testosterone are now relatively well-defined through experiments with animal models there is still some uncertainty about whether human spermatogenesis is more sensitive to levels of these two hormones. Up until now male contraception has focussed on suppression of androgens to reduce spermatogenesis, but data suggests that reduction of FSH, or both FSH and testosterone could be more efficacious and with potentially fewer side effects. Further research to better define the roles of these two hormones in spermatogenesis and their potential for future therapy is needed.

References

Abel MH, Wootton AN, Wilkins V, Huhtaniemi I, Knight PG, Charlton HM (2000) The effect of a null mutation in the follicle-stimulating hormone receptor gene on mouse reproduction. Endocrinology 141(5):1795–1803

Abel MH, Baker PJ, Charlton HM, Monteiro A, Verhoeven G, De Gendt K, Guillou F, O'Shaughnessy PJ (2008) Spermatogenesis and sertoli cell activity in mice lacking sertoli cell receptors for follicle-stimulating hormone and androgen. Endocrinology 149(7):3279–3285. https://doi.org/10.1210/en.2008-0086

Amador AG, Parkening TA, Beamer WG, Bartke A, Collins TJ (1986) Testicular LH receptors and circulating hormone levels in three mouse models for inherited diseases (Tfm/y, lit/lit and hyt/hyt). Endocrinol Exp 20(4):349–358

Arslan M, Weinbauer GF, Schlatt S, Shahab M, Nieschlag E (1993) FSH and testosterone, alone or in combination, initiate testicular growth and increase the number of spermatogonia and Sertoli cells in a juvenile non-human primate (Macaca mulatta). J Endocrinol 136(2):235–243

August GP, Grumbach MM, Kaplan SL (1972) Hormonal changes in puberty. 3. Correlation of plasma testosterone, LH, FSH, testicular size, and bone age with male pubertal development. J Clin Endocrinol Metab 34(2):319–326. https://doi.org/10.1210/jcem-34-2-319

Auharek SA, de Franca LR (2010) Postnatal testis development, Sertoli cell proliferation and number of different spermatogonial types in C57BL/6J mice made transiently hypo- and hyperthyroidic during the neonatal period. J Anat 216(5):577–588. https://doi.org/10.1111/j.1469-7580.2010.01219.x

Baker PJ, O'Shaughnessy PJ (2001) Role of gonadotrophins in regulating numbers of Leydig and Sertoli cells during fetal and postnatal development in mice. Reproduction 122(2):227–234

Baker PJ, Pakarinen P, Huhtaniemi IT, Abel MH, Charlton HM, Kumar TR, O'Shaughnessy PJ (2003) Failure of normal Leydig cell development in follicle-stimulating hormone (FSH) receptor-deficient mice, but not FSHbeta-deficient mice: role for constitutive FSH receptor activity. Endocrinology 144(1):138–145

Barakat B, O'Connor AE, Gold E, de Kretser DM, Loveland KL (2008) Inhibin, activin, follistatin and FSH serum levels and testicular production are highly modulated during the first spermatogenic wave in mice. Reproduction 136(3):345–359. https://doi.org/10.1530/REP-08-0140

Bartlett JM, Weinbauer GF, Nieschlag E (1989) Differential effects of FSH and testosterone on the maintenance of spermatogenesis in the adult hypophysectomized rat. J Endocrinol 121(1):49–58

Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M (1977) Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol 74(1):68–85

Berensztein EB, Sciara MI, Rivarola MA, Belgorosky A (2002) Apoptosis and proliferation of human testicular somatic and germ cells during prepuberty: high rate of testicular growth in newborns mediated by decreased apoptosis. J Clin Endocrinol Metab 87(11):5113–5118. https://doi.org/10.1210/jc.2002-020032

Bhasin S, Fielder TJ, Swerdloff RS (1987) Testosterone selectively increases serum follicle-stimulating hormonal (FSH) but not luteinizing hormone (LH) in gonadotropin-releasing hormone antagonist-treated male rats: evidence for differential regulation of LH and FSH secretion. Biol Reprod 37(1):55–59

Boitani C, Di Persio S, Esposito V, Vicini E (2016) Spermatogonial cells: mouse, monkey and man comparison. Semin Cell Dev Biol. https://doi.org/10.1016/j.semcdb.2016.03.002

Bouvattier C, Carel JC, Lecointre C, David A, Sultan C, Bertrand AM, Morel Y, Chaussain JL (2002) Postnatal changes of T, LH, and FSH in 46,XY infants with mutations in the AR gene. J Clin Endocrinol Metab 87(1):29–32. https://doi.org/10.1210/jcem.87.1.7923

Burger HG (1988) Inhibin: definition and nomenclature, including related substances. J Endocrinol 117(2):159–160

Chang C, Chen YT, Yeh SD, Xu Q, Wang RS, Guillou F, Lardy H, Yeh S (2004) Infertility with defective spermatogenesis and hypotestosteronemia in male mice lacking the androgen receptor in Sertoli cells. Proc Natl Acad Sci U S A 101(18):6876–6881. https://doi.org/10.1073/pnas.0307306101

Charest NJ, Zhou ZX, Lubahn DB, Olsen KL, Wilson EM, French FS (1991) A frameshift mutation destabilizes androgen receptor messenger RNA in the Tfm mouse. Mol Endocrinol 5(4):573–581

Chemes HE, Rey RA, Nistal M, Regadera J, Musse M, Gonzalez-Peramato P, Serrano A (2008) Physiological androgen insensitivity of the fetal, neonatal, and early infantile testis is explained by the ontogeny of the androgen receptor expression in Sertoli cells. J Clin Endocrinol Metab 93(11):4408–4412. https://doi.org/10.1210/jc.2008-0915

Choong CS, Sturm MJ, Strophair JA, McCulloch RK, Hurley DM (1997) Reduced expression and normal nucleotide sequence of androgen receptor gene coding and promoter regions in a family with partial androgen insensitivity syndrome. Clin Endocrinol 46(3):281–288

Clements JA, Reyes FI, Winter JS, Faiman C (1976) Studies on human sexual development. III. Fetal pituitary and serum, and amniotic fluid concentrations of LH, CG, and FSH. J Clin Endocrinol Metab 42(1):9–19. https://doi.org/10.1210/jcem-42-1-9

Clermont Y, Morgentaler H (1955) Quantitative study of spermatogenesis in the hypophysectomized rat. Endocrinology 57(3):369–382. https://doi.org/10.1210/endo-57-3-369

Cools M, van Aerde K, Kersemaekers AM, Boter M, Drop SL, Wolffenbuttel KP, Steyerberg EW, Oosterhuis JW, Looijenga LH (2005) Morphological and immunohistochemical differences between gonadal maturation delay and early germ cell neoplasia in patients with undervirilization syndromes. J Clin Endocrinol Metab 90(9):5295–5303. https://doi.org/10.1210/jc.2005-0139

Cortes D, Muller J, Skakkebaek NE (1987) Proliferation of Sertoli cells during development of the human testis assessed by stereological methods. Int J Androl 10(4):589–596

De Gendt K, Swinnen JV, Saunders PT, Schoonjans L, Dewerchin M, Devos A, Tan K, Atanassova N, Claessens F, Lecureuil C, Heyns W, Carmeliet P, Guillou F, Sharpe RM, Verhoeven G (2004) A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc Natl Acad Sci U S A 101(5):1327–1332

Debieve F, Beerlandt S, Hubinont C, Thomas K (2000) Gonadotropins, prolactin, inhibin A, inhibin B, and activin A in human fetal serum from midpregnancy and term pregnancy. J Clin Endocrinol Metab 85(1):270–274. https://doi.org/10.1210/jcem.85.1.6249

Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P (1998) Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci U S A 95(23):13612–13617

Ding LJ, Yan GJ, Ge QY, Yu F, Zhao X, Diao ZY, Wang ZQ, Yang ZZ, Sun HX, YL H (2011) FSH acts on the proliferation of type A spermatogonia via Nur77 that increases GDNF expression in the Sertoli cells. FEBS Lett 585(15):2437–2444. https://doi.org/10.1016/j.febslet.2011.06.013

Dixson AF (1986) Plasma testosterone concentrations during postnatal development in the male common marmoset. Folia Primatol (Basel) 47(4):166–170

Dufau ML (1988) Endocrine regulation and communicating functions of the Leydig cell. Annu Rev Physiol 50:483–508. https://doi.org/10.1146/annurev.ph.50.030188.002411

Dutta S, Joshi KR, Sengupta P, Bhattacharya K (2013) Unilateral and bilateral cryptorchidism and its effect on the testicular morphology, histology, accessory sex organs, and sperm count in laboratory mice. J Hum Reprod Sci 6(2):106–110. https://doi.org/10.4103/0974-1208.117172

Dwyer AA, Sykiotis GP, Hayes FJ, Boepple PA, Lee H, Loughlin KR, Dym M, Sluss PM, Crowley WF Jr, Pitteloud N (2013) Trial of recombinant follicle-stimulating hormone pretreatment for GnRH-induced fertility in patients with congenital hypogonadotropic hypogonadism. J Clin Endocrinol Metab 98(11):E1790–E1795. https://doi.org/10.1210/jc.2013-2518

El Shennawy A, Gates RJ, Russell LD (1998) Hormonal regulation of spermatogenesis in the hypophysectomized rat: cell viability after hormonal replacement in adults after intermediate periods of hypophysectomy. J Androl 19(3):320–334; discussion 341–322

Finkel DM, Phillips JL, Snyder PJ (1985) Stimulation of spermatogenesis by gonadotropins in men with hypogonadotropic hypogonadism. N Engl J Med 313(11):651–655. https://doi.org/10.1056/NEJM198509123131102

Forest MG, Sizonenko PC, Cathiard AM, Bertrand J (1974) Hypophyso-gonadal function in humans during the first year of life. 1. Evidence for testicular activity in early infancy. J Clin Invest 53(3):819–828. https://doi.org/10.1172/JCI107621

Fouchecourt S, Godet M, Sabido O, Durand P (2006) Glial cell-line-derived neurotropic factor and its receptors are expressed by germinal and somatic cells of the rat testis. J Endocrinol 190(1):59–71. https://doi.org/10.1677/joe.1.06699

Franca LR, Parreira GG, Gates RJ, Russell LD (1998) Hormonal regulation of spermatogenesis in the hypophysectomized rat: quantitation of germ-cell population and effect of elimination of residual testosterone after long-term hypophysectomy. J Androl 19(3):335–340; discussion 341–332

Furuchi T, Masuko K, Nishimune Y, Obinata M, Matsui Y (1996) Inhibition of testicular germ cell apoptosis and differentiation in mice misexpressing Bcl-2 in spermatogonia. Development 122(6):1703–1709

Gao T, Marcelli M, McPhaul MJ (1996) Transcriptional activation and transient expression of the human androgen receptor. J Steroid Biochem Mol Biol 59(1):9–20

Gaspar ML, Meo T, Bourgarel P, Guenet JL, Tosi M (1991) A single base deletion in the Tfm androgen receptor gene creates a short-lived messenger RNA that directs internal translation initiation. Proc Natl Acad Sci U S A 88(19):8606–8610

George JW, Dille EA, Heckert LL (2011) Current concepts of follicle-stimulating hormone receptor gene regulation. Biol Reprod 84(1):7–17. https://doi.org/10.1095/biolreprod.110.085043

Golden JP, DeMaro JA, Osborne PA, Milbrandt J, Johnson EM Jr (1999) Expression of neurturin, GDNF, and GDNF family-receptor mRNA in the developing and mature mouse. Exp Neurol 158(2):504–528. https://doi.org/10.1006/exnr.1999.7127

Greep RO, Fevold HL, Hisaw FL (1936) Effects of two hypophyseal gonadotrophic hormones on the reproductive system of the male rat. Anat Rec 65:261–270

Griesinger G, Felberbaum R, Diedrich K (2005) GnRH-antagonists in reproductive medicine. Arch Gynecol Obstet 273(2):71–78. https://doi.org/10.1007/s00404-005-0021-2

Grumbach MM (2002) The neuroendocrinology of human puberty revisited. Horm Res 57(Suppl 2):2–14
Hadziselimovic F (2005) For debate: is mini puberty the right terminology? Pediatr Endocrinol Rev 2(4):644
Hadziselimovic F, Herzog B (2001) Importance of early postnatal germ cell maturation for fertility of cryptorchid males. Horm Res 55(1):6–10
Hannema SE, Scott IS, Rajpert-De Meyts E, Skakkebaek NE, Coleman N, Hughes IA (2006) Testicular development in the complete androgen insensitivity syndrome. J Pathol 208(4):518–527. https://doi.org/10.1002/path.1890
Haywood M, Tymchenko N, Spaliviero J, Koch A, Jimenez M, Gromoll J, Simoni M, Nordhoff V, Handelsman DJ, Allan CM (2002) An activated human follicle-stimulating hormone (FSH) receptor stimulates FSH-like activity in gonadotropin-deficient transgenic mice. Mol Endocrinol 16(11):2582–2591
Haywood M, Spaliviero J, Jimemez M, King NJ, Handelsman DJ, Allan CM (2003) Sertoli and germ cell development in hypogonadal (hpg) mice expressing transgenic follicle-stimulating hormone alone or in combination with testosterone. Endocrinology 144(2):509–517
Hedinger E (1982) Histopathology of undescended testes. Eur J Pediatr 139(4):266–271
Hikim AP, Swerdloff RS (1995) Temporal and stage-specific effects of recombinant human follicle-stimulating hormone on the maintenance of spermatogenesis in gonadotropin-releasing hormone antagonist-treated rat. Endocrinology 136(1):253–261. https://doi.org/10.1210/endo.136.1.7828538
Huff DS, Fenig DM, Canning DA, Carr MG, Zderic SA, Snyder HM 3rd (2001) Abnormal germ cell development in cryptorchidism. Horm Res 55(1):11–17
Hughes IA, Davies JD, Bunch TI, Pasterski V, Mastroyannopoulou K, MacDougall J (2012) Androgen insensitivity syndrome. Lancet 380(9851):1419–1428. https://doi.org/10.1016/S0140-6736(12)60071-3
Huhtaniemi IT, Yamamoto M, Ranta T, Jalkanen J, Jaffe RB (1987) Follicle-stimulating hormone receptors appear earlier in the primate fetal testis than in the ovary. J Clin Endocrinol Metab 65(6):1210–1214. https://doi.org/10.1210/jcem-65-6-1210
Irfan S, Wistuba J, Ehmcke J, Shahab M, Schlatt S (2015) Pubertal and testicular development in the common marmoset (Callithrix jacchus) shows high individual variation. Primate Biol 2:1–8. https://doi.org/10.5194/pb-2-1-2015
Japon MA, Rubinstein M, Low MJ (1994) In situ hybridization analysis of anterior pituitary hormone gene expression during fetal mouse development. J Histochem Cytochem 42(8):1117–1125
Jean-Faucher C, Berger M, de Turckheim M, Veyssiere G, Jean C (1978) Developmental patterns of plasma and testicular testosterone in mice from birth to adulthood. Acta Endocrinol 89(4):780–788
Johnston H, Baker PJ, Abel M, Charlton HM, Jackson G, Fleming L, Kumar TR, O'Shaughnessy PJ (2004) Regulation of Sertoli cell number and activity by follicle-stimulating hormone and androgen during postnatal development in the mouse. Endocrinology 145(1):318–329. https://doi.org/10.1210/en.2003-1055
Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. Science 270(5233):96–99
Krishnamurthy H, Babu PS, Morales CR, Sairam MR (2001a) Delay in sexual maturity of the follicle-stimulating hormone receptor knockout male mouse. Biol Reprod 65(2):522–531
Krishnamurthy H, Kats R, Danilovich N, Javeshghani D, Sairam MR (2001b) Intercellular communication between Sertoli cells and Leydig cells in the absence of follicle-stimulating hormone-receptor signaling. Biol Reprod 65(4):1201–1207
Kumar TR, Wang Y, Lu N, Matzuk MM (1997) Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15(2):201–204. https://doi.org/10.1038/ng0297-201
Kumar PA, Pitteloud N, Andrews PA, Dwyer A, Hayes F, Crowley WF Jr, Dym M (2006) Testis morphology in patients with idiopathic hypogonadotropic hypogonadism. Hum Reprod 21(4):1033–1040. https://doi.org/10.1093/humrep/dei444

Lavoie HA, King SR (2009) Transcriptional regulation of steroidogenic genes: STARD1, CYP11A1 and HSD3B. Exp Biol Med (Maywood) 234(8):880–907. https://doi.org/10.3181/0903-MR-97

Layman LC, Porto AL, Xie J, da Motta LA, da Motta LD, Weiser W, Sluss PM (2002) FSH beta gene mutations in a female with partial breast development and a male sibling with normal puberty and azoospermia. J Clin Endocrinol Metab 87(8):3702–3707

Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV (2001) Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. Mol Endocrinol 15(1):184–200

Li LH, Donald JM, Golub MS (2005) Review on testicular development, structure, function, and regulation in common marmoset. Birth Defects Res B Dev Reprod Toxicol 74(5):450–469. https://doi.org/10.1002/bdrb.20057

Lindstedt G, Nystrom E, Matthews C, Ernest I, Janson PO, Chatterjee K (1998) Follitropin (FSH) deficiency in an infertile male due to FSHbeta gene mutation. A syndrome of normal puberty and virilization but underdeveloped testicles with azoospermia, low FSH but high lutropin and normal serum testosterone concentrations. Clin Chem Lab Med 36(8):663–665. https://doi.org/10.1515/CCLM.1998.118

Livera G, Delbes G, Pairault C, Rouiller-Fabre V, Habert R (2006) Organotypic culture, a powerful model for studying rat and mouse fetal testis development. Cell Tissue Res 324(3):507–521. https://doi.org/10.1007/s00441-006-0167-7

Lunn SF, Recio R, Morris K, Fraser HM (1994) Blockade of the neonatal rise in testosterone by a gonadotrophin-releasing hormone antagonist: effects on timing of puberty and sexual behaviour in the male marmoset monkey. J Endocrinol 141(3):439–447

Ma X, Dong Y, Matzuk MM, Kumar TR (2004) Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility. Proc Natl Acad Sci U S A 101(49):17294–17299. https://doi.org/10.1073/pnas.0404743101

Mann DR, Fraser HM (1996) The neonatal period: a critical interval in male primate development. J Endocrinol 149(2):191–197

Mao J, Xu H, Wang X, Huang B, Liu Z, Zhen J, Nie M, Min L, Wu X (2015) Congenital combined pituitary hormone deficiency patients have better responses to gonadotrophin-induced spermatogenesis than idiopathic hypogonadotropic hypogonadism patients. Hum Reprod 30(9):2031–2037. https://doi.org/10.1093/humrep/dev158

Marshall GR, Jockenhovel F, Ludecke D, Nieschlag E (1986) Maintenance of complete but quantitatively reduced spermatogenesis in hypophysectomized monkeys by testosterone alone. Acta Endocrinol 113(3):424–431

Marshall GR, Zorub DS, Plant TM (1995) Follicle-stimulating hormone amplifies the population of differentiated spermatogonia in the hypophysectomized testosterone-replaced adult rhesus monkey (Macaca mulatta). Endocrinology 136(8):3504–3511. https://doi.org/10.1210/endo.136.8.7628387

Marshall GR, Ramaswamy S, Plant TM (2005) Gonadotropin-independent proliferation of the pale type A spermatogonia in the adult rhesus monkey (Macaca mulatta). Biol Reprod 73(2):222–229. https://doi.org/10.1095/biolreprod.104.038968

Mason AJ, Hayflick JS, Zoeller RT, Young WS 3rd, Phillips HS, Nikolics K, Seeburg PH (1986) A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the hpg mouse. Science 234(4782):1366–1371

Matthiesson KL, McLachlan RI, O'Donnell L, Frydenberg M, Robertson DM, Stanton PG, Meachem SJ (2006) The relative roles of follicle-stimulating hormone and luteinizing hormone in maintaining spermatogonial maturation and spermiation in normal men. J Clin Endocrinol Metab 91(10):3962–3969. https://doi.org/10.1210/jc.2006-1145

Mauduit C, Hamamah S, Benahmed M (1999) Stem cell factor/c-kit system in spermatogenesis. Hum Reprod Update 5(5):535–545

Mayerhofer A (2013) Human testicular peritubular cells: more than meets the eye. Reproduction 145(5):R107–R116. https://doi.org/10.1530/REP-12-0497

McLachlan RI, Wreford NG, Meachem SJ, De Kretser DM, Robertson DM (1994) Effects of testosterone on spermatogenic cell populations in the adult rat. Biol Reprod 51(5):945–955

McLachlan RI, Wreford NG, de Kretser DM, Robertson DM (1995) The effects of recombinant follicle-stimulating hormone on the restoration of spermatogenesis in the gonadotropin-releasing

hormone-immunized adult rat. Endocrinology 136(9):4035–4043. https://doi.org/10.1210/endo.136.9.7649112
McLachlan RI, O'Donnell L, Stanton PG, Balourdos G, Frydenberg M, de Kretser DM, Robertson DM (2002) Effects of testosterone plus medroxyprogesterone acetate on semen quality, reproductive hormones, and germ cell populations in normal young men. J Clin Endocrinol Metab 87(2):546–556. https://doi.org/10.1210/jcem.87.2.8231
Meachem SJ, Wreford NG, Robertson DM, McLachlan RI (1997) Androgen action on the restoration of spermatogenesis in adult rats: effects of human chorionic gonadotrophin, testosterone and flutamide administration on germ cell number. Int J Androl 20(2):70–79
Meachem SJ, Wreford NG, Stanton PG, Robertson DM, McLachlan RI (1998) Follicle-stimulating hormone is required for the initial phase of spermatogenic restoration in adult rats following gonadotropin suppression. J Androl 19(6):725–735
Meachem SJ, McLachlan RI, Stanton PG, Robertson DM, Wreford NG (1999) FSH immunoneutralization acutely impairs spermatogonial development in normal adult rats. J Androl 20(6):756–762; discussion 755
Meachem SJ, Nieschlag E, Simoni M (2001) Inhibin B in male reproduction: pathophysiology and clinical relevance. Eur J Endocrinol 145(5):561–571
Meachem SJ, Ruwanpura SM, Ziolkowski J, Ague JM, Skinner MK, Loveland KL (2005) Developmentally distinct in vivo effects of FSH on proliferation and apoptosis during testis maturation. J Endocrinol 186(3):429–446. https://doi.org/10.1677/joe.1.06121
Medhamurthy R, Aravindan GR, Moudgal NR (1993) Hemiorchidectomy leads to dramatic and immediate alterations in pituitary follicle-stimulating hormone secretion and the functional activity of the remaining testis in the adult male bonnet monkey (Macaca radiata). Biol Reprod 49(4):743–749
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, Saarma M, Sariola H (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287(5457):1489–1493
Merlet J, Racine C, Moreau E, Moreno SG, Habert R (2007) Male fetal germ cells are targets for androgens that physiologically inhibit their proliferation. Proc Natl Acad Sci U S A 104(9):3615–3620. https://doi.org/10.1073/pnas.0611421104
Migrenne S, Moreau E, Pakarinen P, Dierich A, Merlet J, Habert R, Racine C (2012) Mouse testis development and function are differently regulated by follicle-stimulating hormone receptors signaling during fetal and prepubertal life. PLoS One 7(12):e53257. https://doi.org/10.1371/journal.pone.0053257
Millar RP (2005) GnRHs and GnRH receptors. Anim Reprod Sci 88(1–2):5–28. https://doi.org/10.1016/j.anireprosci.2005.05.032
Miller WL, Bose HS (2011) Early steps in steroidogenesis: intracellular cholesterol trafficking. J Lipid Res 52(12):2111–2135. https://doi.org/10.1194/jlr.R016675
Naik SI, Young LS, Charlton HM, Clayton RN (1984) Pituitary gonadotropin-releasing hormone receptor regulation in mice. I: males. Endocrinology 115(1):106–113. https://doi.org/10.1210/endo-115-1-106
O'Donnell L, Narula A, Balourdos G, YQ G, Wreford NG, Robertson DM, Bremner WJ, McLachlan RI (2001) Impairment of spermatogonial development and spermiation after testosterone-induced gonadotropin suppression in adult monkeys (Macaca fascicularis). J Clin Endocrinol Metab 86(4):1814–1822. https://doi.org/10.1210/jcem.86.4.7400
O'Hara L, Welsh M, Saunders PT, Smith LB (2011) Androgen receptor expression in the caput epididymal epithelium is essential for development of the initial segment and epididymal spermatozoa transit. Endocrinology 152(2):718–729. https://doi.org/10.1210/en.2010-0928
O'Hara L, Curley M, Tedim Ferreira M, Cruickshanks L, Milne L, Smith LB (2015) Pituitary androgen receptor signalling regulates prolactin but not gonadotrophins in the male mouse. PLoS One 10(3):e0121657. https://doi.org/10.1371/journal.pone.0121657
O'Shaughnessy PJ, Morris ID, Huhtaniemi I, Baker PJ, Abel MH (2009) Role of androgen and gonadotrophins in the development and function of the Sertoli cells and Leydig cells: data from mutant and genetically modified mice. Mol Cell Endocrinol 306(1–2):2–8. https://doi.org/10.1016/j.mce.2008.11.005

O'Shaughnessy PJ, Monteiro A, Verhoeven G, De Gendt K, Abel MH (2010a) Effect of FSH on testicular morphology and spermatogenesis in gonadotrophin-deficient hypogonadal mice lacking androgen receptors. Reproduction 139(1):177–184. https://doi.org/10.1530/REP-09-0377

O'Shaughnessy PJ, Verhoeven G, De Gendt K, Monteiro A, Abel MH (2010b) Direct action through the sertoli cells is essential for androgen stimulation of spermatogenesis. Endocrinology 151(5):2343–2348. https://doi.org/10.1210/en.2009-1333

O'Shaughnessy PJ, Monteiro A, Abel M (2012) Testicular development in mice lacking receptors for follicle stimulating hormone and androgen. PLoS One 7(4):e35136. https://doi.org/10.1371/journal.pone.0035136

Orth JM (1986) FSH-induced Sertoli cell proliferation in the developing rat is modified by beta-endorphin produced in the testis. Endocrinology 119(4):1876–1878. https://doi.org/10.1210/endo-119-4-1876

Pakarinen P, Kimura S, El-Gehani F, Pelliniemi LJ, Huhtaniemi I (2002) Pituitary hormones are not required for sexual differentiation of male mice: phenotype of the T/ebp/Nkx2.1 null mutant mice. Endocrinology 143(11):4477–4482

Paniagua R, Nistal M (1984) Morphological and histometric study of human spermatogonia from birth to the onset of puberty. J Anat 139(Pt 3):535–552

Phillip M, Arbelle JE, Segev Y, Parvari R (1998) Male hypogonadism due to a mutation in the gene for the beta-subunit of follicle-stimulating hormone. N Engl J Med 338(24):1729–1732. https://doi.org/10.1056/NEJM199806113382404

Pitteloud N, Dwyer AA, DeCruz S, Lee H, Boepple PA, Crowley WF Jr, Hayes FJ (2008) Inhibition of luteinizing hormone secretion by testosterone in men requires aromatization for its pituitary but not its hypothalamic effects: evidence from the tandem study of normal and gonadotropin-releasing hormone-deficient men. J Clin Endocrinol Metab 93(3):784–791. https://doi.org/10.1210/jc.2007-2156

Purvis K, Clausen OP, Hansson V (1979) LH contamination may explain FSH effects on rat Leydig cells. J Reprod Fertil 56(2):657–665

Ramaswamy S, Marshall GR, McNeilly AS, Plant TM (2000) Dynamics of the follicle-stimulating hormone (FSH)-inhibin B feedback loop and its role in regulating spermatogenesis in the adult male rhesus monkey (Macaca mulatta) as revealed by unilateral orchidectomy. Endocrinology 141(1):18–27. https://doi.org/10.1210/endo.141.1.7276

Rannikki AS, Zhang FP, Huhtaniemi IT (1995) Ontogeny of follicle-stimulating hormone receptor gene expression in the rat testis and ovary. Mol Cell Endocrinol 107(2):199–208

Rey RA, Musse M, Venara M, Chemes HE (2009) Ontogeny of the androgen receptor expression in the fetal and postnatal testis: its relevance on Sertoli cell maturation and the onset of adult spermatogenesis. Microsc Res Tech 72(11):787–795. https://doi.org/10.1002/jemt.20754

Rodriguez I, Ody C, Araki K, Garcia I, Vassalli P (1997) An early and massive wave of germinal cell apoptosis is required for the development of functional spermatogenesis. EMBO J 16(9):2262–2270. https://doi.org/10.1093/emboj/16.9.2262

Rossi P, Albanesi C, Grimaldi P, Geremia R (1991) Expression of the mRNA for the ligand of c-kit in mouse Sertoli cells. Biochem Biophys Res Commun 176(2):910–914

Rossi P, Dolci S, Albanesi C, Grimaldi P, Ricca R, Geremia R (1993) Follicle-stimulating hormone induction of steel factor (SLF) mRNA in mouse Sertoli cells and stimulation of DNA synthesis in spermatogonia by soluble SLF. Dev Biol 155(1):68–74. https://doi.org/10.1006/dbio.1993.1007

Russell LD, Kershaw M, Borg KE, El Shennawy A, Rulli SS, Gates RJ, Calandra RS (1998) Hormonal regulation of spermatogenesis in the hypophysectomized rat: FSH maintenance of cellular viability during pubertal spermatogenesis. J Androl 19(3):308–319; discussion 341–302

Russell LD, Chiarini-Garcia H, Korsmeyer SJ, Knudson CM (2002) Bax-dependent spermatogonia apoptosis is required for testicular development and spermatogenesis. Biol Reprod 66(4):950–958

Ruwanpura SM, McLachlan RI, Matthiesson KL, Meachem SJ (2008a) Gonadotrophins regulate germ cell survival, not proliferation, in normal adult men. Hum Reprod 23(2):403–411. https://doi.org/10.1093/humrep/dem376

Ruwanpura SM, McLachlan RI, Stanton PG, Loveland KL, Meachem SJ (2008b) Pathways involved in testicular germ cell apoptosis in immature rats after FSH suppression. J Endocrinol 197(1):35–43. https://doi.org/10.1677/JOE-07-0637

Ryan RJ, Charlesworth MC, McCormick DJ, Milius RP, Keutmann HT (1988) The glycoprotein hormones: recent studies of structure-function relationships. FASEB J 2(11):2661–2669

Sasaki M, Yamamoto M, Arishima K, Eguchi Y (2000) Effect of follicle-stimulating hormone on sertoli cell division in cultures of fetal rat testes. Biol Neonate 78(1):48–52

Savage JJ, Yaden BC, Kiratipranon P, Rhodes SJ (2003) Transcriptional control during mammalian anterior pituitary development. Gene 319:1–19. https://doi.org/10.1016/S0378-1119(03)00804-7

Schlatt S, Ehmcke J (2014) Regulation of spermatogenesis: an evolutionary biologist's perspective. Semin Cell Dev Biol 29:2–16. https://doi.org/10.1016/j.semcdb.2014.03.007

Scott HM, Mason JI, Sharpe RM (2009) Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds. Endocr Rev 30(7):883–925. https://doi.org/10.1210/er.2009-0016

Selice R, Ferlin A, Garolla A, Caretta N, Foresta C (2011) Effects of endogenous FSH on normal human spermatogenesis in adults. Int J Androl 34(6 Pt 2):e511–e517. https://doi.org/10.1111/j.1365-2605.2010.01134.x

Sharpe RM, Turner KJ, McKinnell C, Groome NP, Atanassova N, Millar MR, Buchanan DL, Cooke PS (1999) Inhibin B levels in plasma of the male rat from birth to adulthood: effect of experimental manipulation of Sertoli cell number. J Androl 20(1):94–101

Sharpe RM, Walker M, Millar MR, Atanassova N, Morris K, McKinnell C, Saunders PT, Fraser HM (2000) Effect of neonatal gonadotropin-releasing hormone antagonist administration on sertoli cell number and testicular development in the marmoset: comparison with the rat. Biol Reprod 62(6):1685–1693

Sharpe RM, Fraser HM, Brougham MF, McKinnell C, Morris KD, Kelnar CJ, Wallace WH, Walker M (2003) Role of the neonatal period of pituitary-testicular activity in germ cell proliferation and differentiation in the primate testis. Hum Reprod 18(10):2110–2117

Shetty G, Weng CC, Porter KL, Zhang Z, Pakarinen P, Kumar TR, Meistrich ML (2006) Spermatogonial differentiation in juvenile spermatogonial depletion (jsd) mice with androgen receptor or follicle-stimulating hormone mutations. Endocrinology 147(7):3563–3570

Simon L, Ekman GC, Tyagi G, Hess RA, Murphy KM, Cooke PS (2007) Common and distinct factors regulate expression of mRNA for ETV5 and GDNF, Sertoli cell proteins essential for spermatogonial stem cell maintenance. Exp Cell Res 313(14):3090–3099. https://doi.org/10.1016/j.yexcr.2007.05.002

Simorangkir DR, Ramaswamy S, Marshall GR, Pohl CR, Plant TM (2009a) A selective monotropic elevation of FSH, but not that of LH, amplifies the proliferation and differentiation of spermatogonia in the adult rhesus monkey (Macaca mulatta). Hum Reprod 24(7):1584–1595. https://doi.org/10.1093/humrep/dep052

Simorangkir DR, Marshall GR, Plant TM (2009b) A re-examination of proliferation and differentiation of type A spermatogonia in the adult rhesus monkey (Macaca mulatta). Hum Reprod 24(7):1596–1604. https://doi.org/10.1093/humrep/dep051

Singh J, O'Neill C, Handelsman DJ (1995) Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. Endocrinology 136(12):5311–5321

Sizonenko PC, Paunier L (1975) Hormonal changes in puberty III: correlation of plasma dehydroepiandrosterone, testosterone, FSH, and LH with stages of puberty and bone age in normal boys and girls and in patients with Addison's disease or hypogonadism or with premature or late adrenarche. J Clin Endocrinol Metab 41(5):894–904. https://doi.org/10.1210/jcem-41-5-894

Smith PE (1944) Maintenance and restoration of spermatogenesis in hypophysectomized rhesus monkeys by androgen administration. Yale J Biol Med 17(1):281–287

Smith PE, Engle ET (1927) Experimental evidence regarding the role of the anterior pituitary in development and regulation of the genital system. Am J Anatomy 40:159–168

Srinath BR, Wickings EJ, Witting C, Nieschlag E (1983) Active immunization with follicle-stimulating hormone for fertility control: a 4 1/2-year study in male rhesus monkeys. Fertil Steril 40(1):110–117

Suresh PS, Rajan T, Tsutsumi R (2011) New targets for old hormones: inhibins clinical role revisited. Endocr J 58(4):223–235

Tadokoro Y, Yomogida K, Ohta H, Tohda A, Nishimune Y (2002) Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. Mech Dev 113(1):29–39

Tan KA, De Gendt K, Atanassova N, Walker M, Sharpe RM, Saunders PT, Denolet E, Verhoeven G (2005) The role of androgens in sertoli cell proliferation and functional maturation: studies in mice with total or Sertoli cell-selective ablation of the androgen receptor. Endocrinology 146(6):2674–2683

Tapanainen JS, Aittomaki K, Min J, Vaskivuo T, Huhtaniemi IT (1997) Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. Nat Genet 15(2):205–206. https://doi.org/10.1038/ng0297-205

Tilbrook AJ, Clarke IJ (2001) Negative feedback regulation of the secretion and actions of gonadotropin-releasing hormone in males. Biol Reprod 64(3):735–742

Trupp M, Ryden M, Jornvall H, Funakoshi H, Timmusk T, Arenas E, Ibanez CF (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J Cell Biol 130(1):137–148

van Alphen MM, van de Kant HJ, de Rooij DG (1988) Follicle-stimulating hormone stimulates spermatogenesis in the adult monkey. Endocrinology 123(3):1449–1455. https://doi.org/10.1210/endo-123-3-1449

Vergouwen RP, Jacobs SG, Huiskamp R, Davids JA, de Rooij DG (1991) Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J Reprod Fertil 93(1):233–243

Vergouwen RP, Huiskamp R, Bas RJ, Roepers-Gajadien HL, Davids JA, de Rooij DG (1993) Postnatal development of testicular cell populations in mice. J Reprod Fertil 99(2):479–485

Walker WH, Cheng J (2005) FSH and testosterone signaling in Sertoli cells. Reproduction 130(1):15–28. https://doi.org/10.1530/rep.1.00358

Warren DW, Huhtaniemi IT, Tapanainen J, Dufau ML, Catt KJ (1984) Ontogeny of gonadotropin receptors in the fetal and neonatal rat testis. Endocrinology 114(2):470–476. https://doi.org/10.1210/endo-114-2-470

Weinbauer GF, Behre HM, Fingscheidt U, Nieschlag E (1991) Human follicle-stimulating hormone exerts a stimulatory effect on spermatogenesis, testicular size, and serum inhibin levels in the gonadotropin-releasing hormone antagonist-treated nonhuman primate (Macaca fascicularis). Endocrinology 129(4):1831–1839. https://doi.org/10.1210/endo-129-4-1831

Welsh M, Saunders PT, Atanassova N, Sharpe RM, Smith LB (2009) Androgen action via testicular peritubular myoid cells is essential for male fertility. FASEB J 23(12):4218–4230. https://doi.org/10.1096/fj.09-138347

Wen Q, Liu Y, Gao F (2011) Fate determination of fetal Leydig cells. Front Biol 6(1):12–18. https://doi.org/10.1007/s11515-011-1100-3

Wickings EJ, Nieschlag E (1978) The effects of active immunization with testosterone on pituitary-gonadal feedback in the male rhesus monkey (Macaca mulatta). Biol Reprod 18(4):602–607

Willems A, De Gendt K, Allemeersch J, Smith LB, Welsh M, Swinnen JV, Verhoeven G (2009) Early effects of Sertoli cell-selective androgen receptor ablation on testicular gene expression. Int J Androl 33(3):507–517. https://doi.org/10.1111/j.1365-2605.2009.00964.x

Wreford NG, Rajendra Kumar T, Matzuk MM, de Kretser DM (2001) Analysis of the testicular phenotype of the follicle-stimulating hormone beta-subunit knockout and the activin type II receptor knockout mice by stereological analysis. Endocrinology 142(7):2916–2920

Wu FC, Butler GE, Kelnar CJ, Stirling HF, Huhtaniemi I (1991) Patterns of pulsatile luteinizing hormone and follicle-stimulating hormone secretion in prepubertal (midchildhood) boys and girls and patients with idiopathic hypogonadotropic hypogonadism (Kallmann's syndrome): a study using an ultrasensitive time-resolved immunofluorometric assay. J Clin Endocrinol Metab 72(6):1229–1237. https://doi.org/10.1210/jcem-72-6-1229

Wu FC, Butler GE, Kelnar CJ, Huhtaniemi I, Veldhuis JD (1996) Ontogeny of pulsatile gonadotropin releasing hormone secretion from midchildhood, through puberty, to adulthood in the

human male: a study using deconvolution analysis and an ultrasensitive immunofluorometric assay. J Clin Endocrinol Metab 81(5):1798–1805. https://doi.org/10.1210/jcem.81.5.8626838

Yan W, Linderborg J, Suominen J, Toppari J (1999) Stage-specific regulation of stem cell factor gene expression in the rat seminiferous epithelium. Endocrinology 140(3):1499–1504. https://doi.org/10.1210/endo.140.3.6590

Yeh S, Tsai MY, Xu Q, XM M, Lardy H, Huang KE, Lin H, Yeh SD, Altuwaijri S, Zhou X, Xing L, Boyce BF, Hung MC, Zhang S, Gan L, Chang C (2002) Generation and characterization of androgen receptor knockout (ARKO) mice: an in vivo model for the study of androgen functions in selective tissues. Proc Natl Acad Sci U S A 99(21):13498–13503

Zhang FP, Hamalainen T, Kaipia A, Pakarinen P, Huhtaniemi I (1994) Ontogeny of luteinizing hormone receptor gene expression in the rat testis. Endocrinology 134(5):2206–2213

Zhang FP, Poutanen M, Wilbertz J, Huhtaniemi I (2001) Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. Mol Endocrinol 15(1):172–183

Zhang FP, Pakarainen T, Poutanen M, Toppari J, Huhtaniemi I (2003) The low gonadotropin-independent constitutive production of testicular testosterone is sufficient to maintain spermatogenesis. Proc Natl Acad Sci U S A 100(23):13692–13697. https://doi.org/10.1073/pnas.2232815100

Zhengwei Y, Wreford NG, Schlatt S, Weinbauer GF, Nieschlag E, McLachlan RI (1998a) Acute and specific impairment of spermatogonial development by GnRH antagonist-induced gonadotrophin withdrawal in the adult macaque (Macaca fascicularis). J Reprod Fertil 112(1):139–147

Zhengwei Y, Wreford NG, Royce P, de Kretser DM, McLachlan RI (1998b) Stereological evaluation of human spermatogenesis after suppression by testosterone treatment: heterogeneous pattern of spermatogenic impairment. J Clin Endocrinol Metab 83(4):1284–1291. https://doi.org/10.1210/jcem.83.4.4724

Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS, Hess RA (2002) Localization of androgen and estrogen receptors in adult male mouse reproductive tract. J Androl 23(6):870–881

Part V

Genome Integrity of Spermatogonia

Frequency of Human Disease Mutations and Spermatogonial Stem Cell Function

8

Norman Arnheim and Peter Calabrese

Abstract

Some human disease mutations enter the human population each generation as a result of de novo germline base substitutions that immediately affect children born to normal parents. In some cases the frequency of these mutations exceeds the well-documented germline mutation rate 100–1000 fold. Recent technologies have made it possible to estimate the frequency of single base disease mutations in both sperm and testes from normal men. The evidence confirms that, although unaffected, the men have high enough frequencies of these mutations in semen and testis to explain the high sporadic disease incidence. The explanation for the high frequency initially was ascribed to the idea that the affected nucleotide site was a mutation hot spot with a mutation rate per cell division at that site far greater than the rate at other sites. Recent evidence rules out this hot spot model. An alternative model suggests that any of these types of rare disease mutations can confer upon a single testis stem cell a selective advantage. Over time, a disproportionate increase of mutant stem cells over the wild-type stem cells occurs that increases the disease mutation frequency in sperm. The evidence against the hot spot model and for the selection model is reviewed and the functional consequences of these disease mutations on testis stem cell proliferation is also summarized. Finally, the consequence of these mutations is considered within the context of the paternal age effect and the human genetic load.

Keywords

Spermatogonia • Stem cell • SSC • Mutation • Human • Germline • Selection • RAMP • Paternal age effect • Testis

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8.1 Introduction

In the early twentieth century Wilhelm Weinberg observed that children with normal parents who were affected by the condition achondroplasia appeared to be among the youngest in their respective (and large) families (Weinberg 1912). Weinberg took this to mean that this condition was due to new mutations and was more likely to arise as the parents aged. Weinberg was able define these cases as new mutations because achondroplasia is inherited as an autosomal dominant Mendelian disease and the children he identified as having inherited a new mutation had unaffected parents. It took many years and additional epidemiological data to discover that the aging normal fathers were primarily responsible for transmitting these new mutations to the next generation (Penrose 1955).

Statistical analysis of the epidemiological data documenting this paternal age effect (PAE) for many additional Mendelian diseases was published in 1987 (Risch et al. 1987). The term PAE has been expanded to include any type of condition whose incidence increases as a function of the age of the father regardless of the type of inheritance involved (including multigenic phenotypes and could also include a combination of age- and male-specific environmental effects). Here, we restrict ourselves to single human gene Mendelian disease mutations that arise de novo.

The most common explanation for the PAE involves replication of the spermatogonial stem cells (SSC) throughout a man's life (see Vogel and Motulsky (1997)). This results in the gradual accumulation of new germ-line mutations at any particular nucleotide site thereby continually increasing the frequency of sperm carrying the specific disease mutation over time.

8.2 Spermatogenesis

A vast literature has arisen on mouse spermatogenesis based on molecular, genetic, and cellular tools that provide increasingly refined information about the population of spermatogonia (see Chaps. 1 and 3 in this book). While the same level of detailed information in human or nonhuman primates is currently not available (due to technical and ethical experimental boundaries) it is obvious from histological analysis that there are both similarities and differences. In both mouse and human there are essentially two types of spermatogonia: undifferentiated and differentiating. However, the cellular complexity of these two species' compartments differs dramatically. In mice, one model is the undifferentiated compartment consists of a stem cell (A_s) and four additional undifferentiated spermatogonial stages (A_{pr}, $A_{al\text{-}4}$, $A_{al\text{-}8}$, and $A_{al\text{-}16}$) that succeed each other, in the order as written, on the way to produce spermatogonia (A1) that are irreversibly committed to produce sperm. Note that this idea of a one-way street between A_s and the intermediate undifferentiated spermatogonial stages has recently been challenged by a series of convincing experiments (Yoshida et al. 2007; Hara et al. 2014; Zhang et al. 2016).

In humans the situation is different with fewer spermatogonial types identified. Until very recently it has been based on nuclear staining (Clermont 1966) that identifies two undifferentiated spermatogonial types: A_p and A_d. A_p divide every 16 days (Heller and Clermont 1963) and are thought to be the active SSC. A_d are considered reserve stem cells with minimal cycling behavior. Evidence exists in nonhuman primates (van Alphen et al. 1988) using autoradiography that A_d divide infrequently but proliferate in response to induced radiation damage that radically reduces the number of cycling A_p; routine replacement of A_p by A_d might be difficult to detect. The B spermatogonia represent the differentiating spermatogonia (analogous to A1 in mice).

8.3 The PAE and Stem Cell Divisions

The PAE depends on the cell divisions of the active A_p SSC in the post-pubertal man. There are two main stem cell division strategies, asymmetric and symmetric division (the latter has been called "population asymmetry"(Klein and Simons 2011)). With the onset of puberty an asymmetric division of a SSC would produce a daughter SSC (self-renewal) and a daughter that is committed to differentiation; each individual SSC originates its own stem cell lineage. On the other hand, a symmetric division scheme (Fig. 8.1) provides the SSC with two different options at any particular division. It can divide to produce two new SSC or it can undergo differentiation into an A1 (mouse) or B spermatogonium (human). These alternative events must be balanced in number to maintain fertility. Note that in the symmetric model many new SSC lineages can arise from one initial SSC but these lineages can also be reduced in number by differentiation events. Regardless of the differences between these schemes the linear increase in cell divisions with age (Fig. 8.2) should be reflected in a linear accumulation of mutations as men age. Data from whole genome next generation sequencing (NGS) of parents and their offspring supports the linear PAE model (Kong et al. 2012; Segurel and Quintana-Murci 2014; Segurel et al. 2014).

8.4 Human Germline Mutation Rates

The average human germline mutation rate per generation at any nucleotide site (or frequency of sperm mutant at that site in a semen sample) has been estimated in a variety of different ways based on direct DNA sequencing (reviewed in Arnheim and Calabrese (2009), Campbell and Eichler (2013), and Segurel and Quintana-Murci (2014)). Each way provides a very similar genome-*average* nucleotide substitution frequency: at CpG sites approximately 10^{-7} and at other sites between 10^{-8} and 10^{-9} (note: the term mutation frequency is used interchangeably with "mutation rate per generation", often shortened to "mutation rate"; these equivalent meanings must be distinguished from "mutation rate per cell division"). See Walter et al. (2004) and Masumura et al. (2016) for references to studies of mouse germline mutation rates.

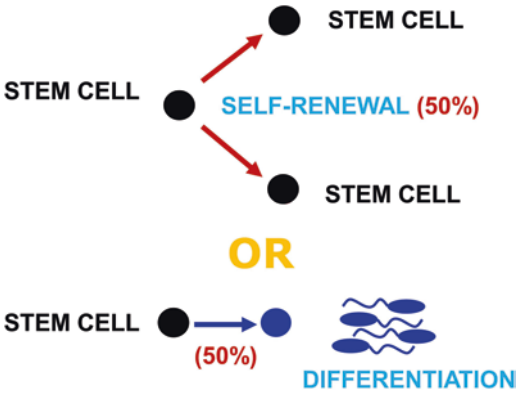

Fig. 8.1 Symmetric stem cell division scheme. Spermatogonial stem cells (SSC) are shown in *black* and differentiated cells in *blue*. Each SSC has two choices: produce two daughters or undergo differentiation. Life-long fertility requires an equal probability of the two events

8.5 Unusual Disease Mutations with Common Features

There is one class of nucleotide sites that has a unique set of common features. New mutations at these sites cause sporadic cases of a number of different Mendelian genetic diseases. Relevant examples include Apert syndrome (mapped to chromosome 10, McKusick, MIM 101200) and characterized by prematurely fused cranial sutures and fused fingers and toes; achondroplasia (chromosome 4, McKusick, MIM 100800), the most common form of long bone dwarfism; multiple endocrine neoplasia 2B (MEN2B, mapped to chromosome 10, McKusick, MIM162300), an aggressive childhood thyroid cancer; and Noonan syndrome (the most common form on chromosome 12, McKusick, MIM163950), characterized by craniofacial abnormalities, short stature, heart defects, intellectual disability and delay, and a predisposition to certain cancers.

These disease mutations share the following important features. First, the spontaneous mutation rate for the causal mutations exceed by 100–1000 fold the highest known genome-average rate (Qin et al. 2007; Choi et al. 2008, 2012; Arnheim and Calabrese 2009; Goriely and Wilkie 2012; Shinde et al. 2013; Yoon et al. 2013). Historically, certain genes were observed to have much higher disease mutation frequencies than other genes (Vogel and Motulsky 1997). It was assumed that genetic diseases with a high de novo frequency resulted from mutations at a relatively large number of different nucleotide sites in the gene. We know now that this is not necessarily true for all highly mutated genes. For example, new dominantly transmitted mutations in the fibroblast growth factor receptor 3 gene (*FGFR3*) produce offspring with sporadic achondroplasia at a birth frequency close to 10^{-4}. The average germline mutation frequency per nucleotide site in humans is ~10^{-8} (reviewed in (Arnheim and Calabrese (2009), Campbell and Eichler (2013), and Segurel and Quintana-Murci (2014)) or 10^{3}–10^{4} fold greater than the expected average suggesting many sites in the gene were targets for disease-causing mutations. The first notable exception was discovered when virtually all of the sporadic cases of achondroplasia were found to occur by independent recurrent mutation at the same *FGFR3* nucleotide (c.1138G>A, G380R). Similarly, virtually all Apert

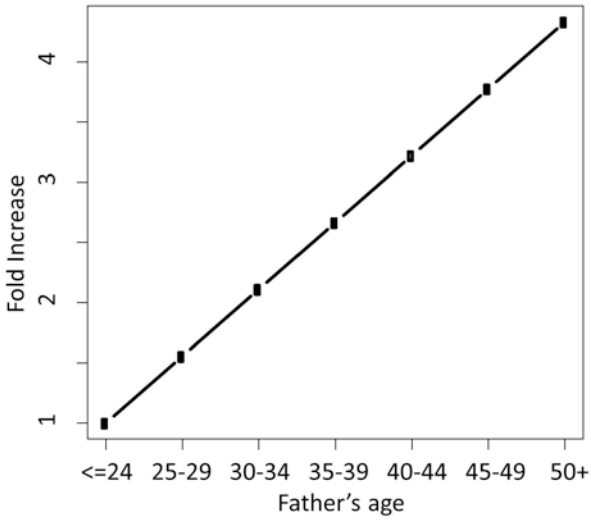

Fig. 8.2 The classical paternal age effect. The theoretical fold-increase in the number of SSC cell divisions between the age of puberty and the age a father conceived a child. Calculations can be found in (Crow 2000). The fold increase is proportional to the chance that a father of a particular age will pass on a new germline mutation to his offspring

syndrome cases are caused by a mutation at either one of two sites in *FGFR2* (c.755C>G, S308W and c.758C>G, P253R). Almost all MEN2B cases are caused by a mutation at only one site in *RET* (c.2943T>C, M918T). Noonan syndrome can be caused by many mutations in *PTPN11* (and a few other genes). The most frequent Noonan mutation is in *PTPN11* (c.922A>G, N308D) and occurs at a rate thousands of times higher than the average.

Second, only a single copy of this class of mutations is needed to cause the disease phenotype (autosomal or X-linked dominant transmission). Third, the spontaneous mutations virtually always occur in the father's germline (discussed in Risch et al. (1987), Crow (1997, 2000), Glaser and Jabs (2004), Arnheim and Calabrese (2009), and Goriely and Wilkie (2012)). Since there are more germline divisions in men than women, more point mutations are expected to originate in the male than the female (Drost and Lee 1995; Ellegren 2007; Arnheim and Calabrese 2009; Sayres and Makova 2011). Still, the measured magnitude of the male bias for the diseases mentioned above is exceptionally high relative to the expected difference (Segurel et al. 2014). Fourth, older fathers are at greater risk for having affected children with these conditions than younger fathers. The term RAMP, an acronym for *R*ecurrent, *A*utosomal dominant, *M*ale-biased, and *P*aternal age effect has been introduced for disease mutations with these characteristics (Yoon et al. 2013).

8.6 Explaining the Enhanced RAMP Mutation Rate

The most intuitive explanation is that these RAMP nucleotide sites must be mutation "hot spots" where the mutation rate *per cell division* is significantly greater than the genome average. A test of the hot spot model based on the "molecular anatomy" of de novo RAMP mutations in the testes of normal men was carried out (Qin et al.

2007) under the assumption that every SSC replication would be expected to have the same increased chance to produce a RAMP disease mutation at the site.

Assuming that human SSC are uniformly distributed throughout the testis any new RAMP mutation should also be uniformly distributed. Using microscopic analysis of mouse seminiferous tubule cross-sections it has been shown that undifferentiated spermatogonia appear nonuniformly distributed at the level of resolution (~1.0×10^{-4} mm^3) of individual seminiferous tubules (Chiarini-Garcia et al. 2001; Yoshida et al. 2007). However, the human testis pieces studied were ~90 mm^3. The resolution difference is almost 10^6. Even though human SSC may be nonuniformly distributed within a seminiferous tubule at the same resolution as in mouse, the human SSC are effectively distributed uniformly with respect to each human testis piece.

8.7 Testis Dissection and RAMP Mutation Detection

To test whether any particular RAMP mutation is uniformly distributed the postmortem testes from men ranging in age from 19 to 80 years old were acquired. Men who had received radiation, chemotherapy, taken drugs or received treatments that might affect fertility were not considered for donations. Each testis was dissected into 192 pieces with an address so the 3-D distribution of mutations could be reconstructed (Fig. 8.3).

A modified PCR-like assay (*p*yrophosphorolysis-*a*ctivated *P*CR (Liu and Sommer 2004)), called PAP, was used as the mutation detection method. PAP is an ultra-sensitive mutation/allele-specific version of PCR that allows only the mutation to be amplified. This assay was modified to identify a single template molecule with a RAMP disease mutation among 25,000 normal templates (Qin et al. 2007); 40 such assays were carried out for each testis piece. Limiting dilutions of DNA from each testis piece were examined at single molecule sensitivity allowing a direct digital counting of the number of mutants after Poisson correction to estimate the mutation frequency. In every experiment both negative and positive controls were included. The false positive rate of the assay was 10^{-6}–10^{-7}.

8.8 Studies on Apert Syndrome

The most common of the two Apert syndrome mutations is c.755C>G. Figure 8.4 shows the mutation frequency data in a 62-year-old testis. The average mutation frequency for this particular testis is 3.8×10^{-4}. Notice however that the vast majority (88%) of pieces have mutation frequencies less than 5×10^{-5}. In contrast, the testis piece colored burgundy in the lower left corner of the second slice from the left has a mutation frequency of 0.027 (mutation cluster). This piece is part of a mutation super-cluster of several pieces colored red (0.005–0.01) and orange (0.0025–0.005) in the first and second slices. For this testis, 95% of the mutants are

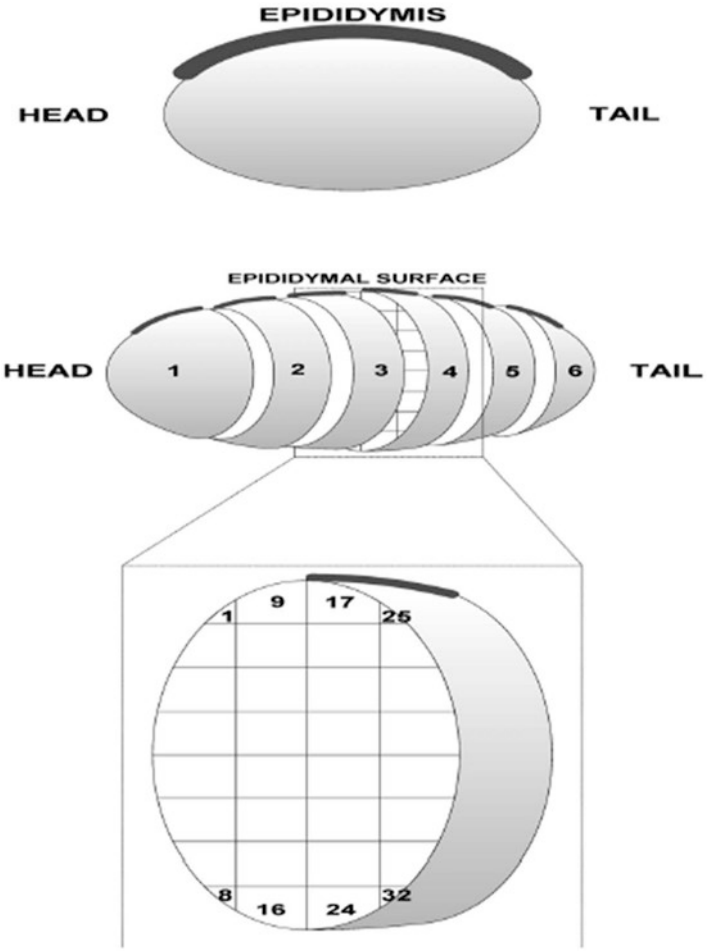

Fig. 8.3 Testis dissection strategy (Qin et al. 2007). After removing the epididymis, the testis is cut into six approximately equal size slices at right angles to the testis' long axis. Each slice is divided into 32 approximately equal size pieces (a total of 192 pieces) of four columns and eight rows. For each slice, piece #1 is in the upper left hand corner, pieces #2–8 are down the first column, and pieces #9, #17, and #25 are across the top row

found in only 5.7% of the testis. As we will show later, this clustered distribution is incompatible with the hot spot model.

Figure 8.5 shows the c.755C>G data on 14 additional normal testes ranging in age from 19 to 80 years. In each of the middle age and older testes (36–80) the mutations are similarly clustered. In contrast, for the youngest individuals (19–23 years) mutations are exceedingly rare compared to the 36–80-year-old men indicating that the mutation clusters grow in the adult.

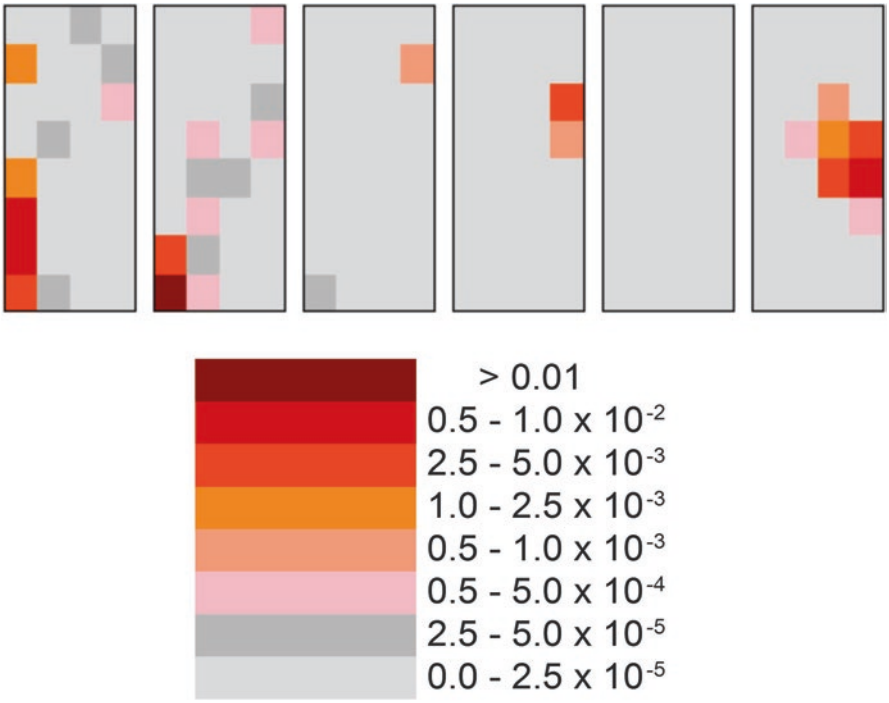

Fig. 8.4 Data on c.755C>G Apert mutations (Qin et al. 2007) present in a testis donated by a 62-year-old normal man (374-1). The heat map indicates the mutation frequency of each of the 192 pieces. The estimate of the total testis mutation frequency is the average of the frequencies of the pieces each weighted by the number of genomes in those pieces

Two testes from the same individual (374-1 and 374-2) were also examined for mutations at the C of a CpG nucleotide site unassociated with a RAMP mutation and on a different chromosome (Qin et al. 2007). All the testis pieces' mutation frequencies at this site were less than 2.5×10^{-5}, arguing that mutation clusters (and super-clusters) are unusual occurrences.

8.9 Analysis of Four Additional RAMP Mutations

The analysis of the second Apert mutation (c.758C>G) was carried out on six testis samples (Choi et al. 2008). The mutation in the *RET* gene (c.2943T>C) causing MEN2B and the Noonan syndrome mutation in *PTPN11* (c.922A>G) were studied in 14 testes (Choi et al. 2012) and 15 testes (Yoon et al. 2013), respectively. Finally, the achondroplasia mutation in *FGFR3* (c.1138G>A) was studied in a testis from one individual (Shinde et al. 2013). The results in all cases were very similar to the data collected on the c.755C>G Apert mutation discussed above in terms of the nonuniform distribution of the disease mutations (clusters) and extreme variation in mutation frequency among the testis pieces in any one testis.

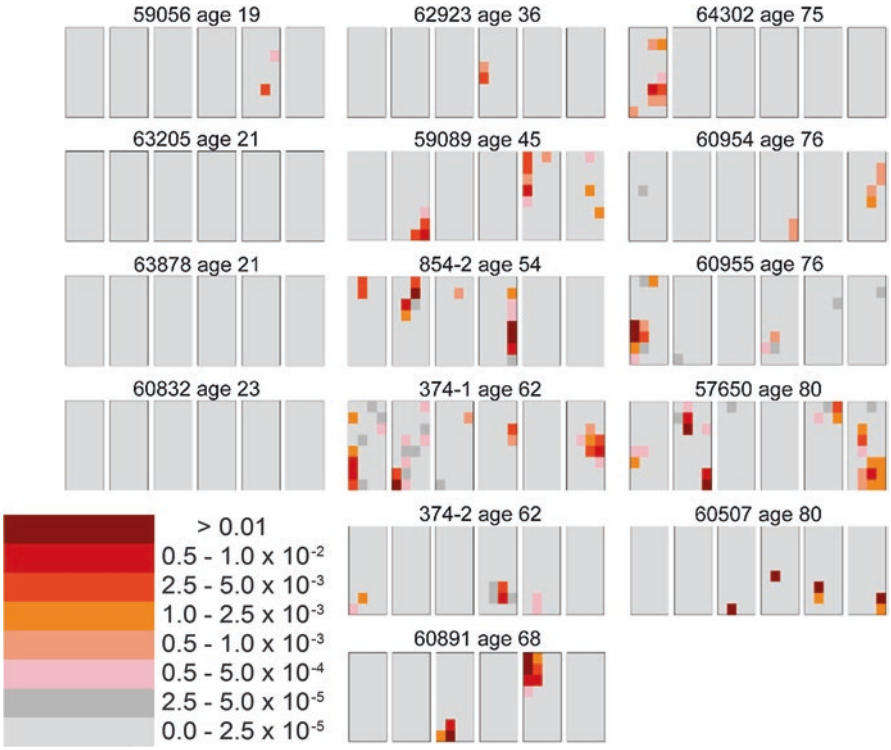

Fig. 8.5 Data on c.755C>G Apert mutations observed in 15 different testes donated by normal men of different ages (Qin et al. 2007; Choi et al. 2012). Young men are shown in the left column, middle aged in the middle, and old men on the right. The mutation frequency of each piece is coded in a heat map

8.9.1 Are the RAMP Mutation Data Consistent with a Hot Spot Model?

The clustered mutation data in the testes appears to reject the hot spot model. Several computational models have been proposed to statistically test the hot spot model. These models are based on what is known about testis development and adult spermatogenesis including the stem cell division scheme (Qin et al. 2007; Choi et al. 2008, 2012; Arnheim and Calabrese 2009; Yoon et al. 2009, 2013; Shinde et al. 2013). Next we will describe the symmetric division hot spot model (Choi et al. 2012; Yoon et al. 2013); there is also a model based on an asymmetric division scheme that is not discussed here but has been considered in other testis studies (Qin et al. 2007; Choi et al. 2008, 2012; Shinde et al. 2013; Yoon et al. 2013). Later in the manuscript some variants to the models are considered.

All of the models have two phases. In the first phase from zygote formation to puberty (growth phase), the germ cells divide symmetrically and increase in number exponentially. Since mutations are inherited, mutation early in this phase can lead

to a "mutation jackpot" similar to those in bacteria (Luria and Delbruck 1943). The primordial germ cells migrate to the site of gonad formation and form the seminiferous cords early in fetal development (Nistal and Paniagua 1984; Muller and Skakkebaek 1992) and since germ cells are expected to remain physically close to their ancestors once the chords are formed, early rare mutations can result in mutation clusters. The germ cells originating during the growth phase eventually form the adult self-renewing A_p spermatogonial stem cells (SrA_p). After spermatogenesis begins at puberty the SrA_p cycle throughout a man's life (approximately every 16 days (Heller and Clermont 1963)) and many opportunities for new mutations arise. In this second phase (adult phase), randomly half of the SrA_p divide symmetrically to produce two SrA_p while the other half form differentiated cells (and these stem cell lineages are terminated). Thus in the adult phase the number of SrA_p remains constant and there is a constant production of differentiated cells. If a mutation is followed by several symmetric divisions a mutation cluster can grow in the adult. However, if some or all of these mutated SrA_p then differentiate the cluster can decrease or even disappear. Incorporating both phases is critical to the modeling.

8.9.2 Testing the Hot Spot Model

In order to quantify the mutation clustering in each testis, several summary statistics have been introduced. We will only discuss the Max/Ave statistic that is the ratio of the highest testis piece mutation frequency to the average mutation frequency for the testis (for other statistics see Qin et al. (2007) and Choi et al. (2012)). If the mutants were uniformly distributed we would expect this ratio to be near 1, but for the RAMP mutations in the middle-age testes this ratio is much greater. For example, for the Noonan syndrome mutation in testis 374-1 this ratio is 129.

The hotspot model has one free parameter: the mutation rate per cell division. Separately for each testis, this parameter is fit to match the average mutation frequency for the testis. The model is simulated with this parameter value so that there are many simulations with average mutation frequencies near that observed in that testis. The clustering statistics for these simulations are then compared to those for the testis (Qin et al. 2007; Choi et al. 2008, 2012; Shinde et al. 2013; Yoon et al. 2013). For example, Fig. 8.6 shows the simulated Max/Ave statistics for the Noonan syndrome mutation in testis 374-1. For 95% of the simulations this statistic is less than 4.8. A red X at 129 marks the Max/Ave statistic observed in this testis. In one million simulations, the simulated Max/Ave values were always less than the value observed in the testis. Consequently, the symmetric hot spot model is strongly rejected with p-value $< 10^{-6}$ for the MEN2B mutation in testis 374-1.

The same testing procedure was performed with the Max/Ave and two other clustering statistics, on all testes and RAMP mutations described above, with similar results (Qin et al. 2007; Choi et al. 2008, 2012; Shinde et al. 2013; Yoon et al. 2013). Again, similar results were also obtained for the hotspot model based on the asymmetric division scheme (Qin et al. 2007; Choi et al. 2008, 2012; Shinde et al. 2013; Yoon et al. 2013).

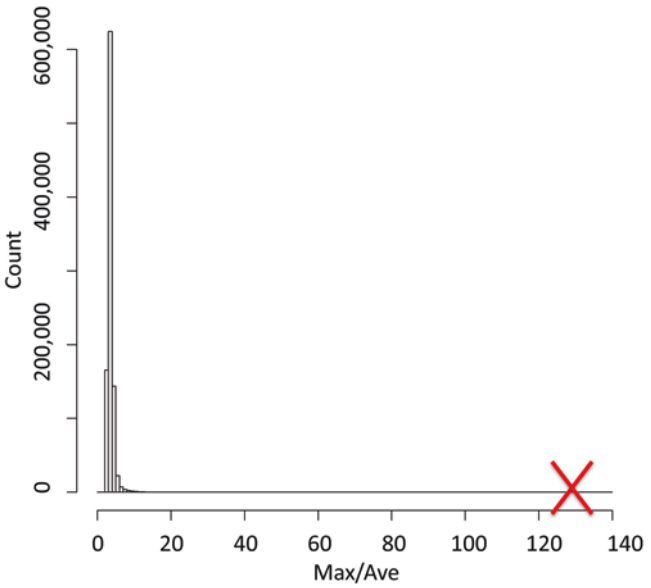

Fig. 8.6 Histogram of simulations of the symmetric hot spot model using data on the Noonan syndrome mutation from testis 374-1(Yoon et al. 2013). The X-axis shows the Max/Ave summary statistic and the Y-axis the number (count) of simulations that gave that value. The large *red X* indicates the Max/Ave value observed in the testis. Since this value is greater than one million simulated values, the symmetric hot spot model is rejected with p-value $< 10^{-6}$

The symmetric hot spot model (unlike the asymmetric hot spot model) allows for the possibility for mutation clusters to form in the adult. However, the mutation clusters simulated in the symmetric hot spot model are not nearly as extreme as those observed in the testes. Therefore, the symmetric hot spot model (like the asymmetric hot spot model) is strongly rejected. Nonetheless, rejecting just the idea that an elevated mutation rate per cell division can explain both the high mutation frequency and the mutation clusters observed in the testes is a strong conclusion. Consequently, in addition to considering symmetric and asymmetric division schemes, in the next section, other variations to the hot spot model are also addressed.

8.9.3 Variations to the Hot Spot Model

A fundamental assumption of the model is that mutations are replication-dependent, but there are also replication-independent mutation whose rate depends on time or environmental circumstances; for example a deamination mutation at a 5-methyl cytosine. With this assumption the hot spot model was also rejected (Qin et al. 2007). Another critical modeling assumption is the estimate that adult SrA_p divide every 16 days (Heller and Clermont 1963). If incorrect, it would affect our inference of the mutation rate per cell division, but would not alter the mutation clustering

(Qin et al. 2007). The same lack of effect on clustering is true if the SrA_p cell division rate changes as men age. Similarly, the mutation rate per cell division is assumed to be constant, although this rate might increase as men age, due to less efficient DNA repair. Although this could help to explain the PAE it would not produce mutation clusters. A similar alternative is that the mutation rate per cell division is greater in the growth phase than the adult phase thereby producing mutation clusters during the growth phase (zygote formation to puberty) but this contradicts the substantially lower mutation frequencies and absence of clusters in young men. We have not included SrA_p cell migration in the hot spot models since any cluster of mutant cells able to form would be reduced by such migration. Finally, the idea that mutations in early germ cell development are in some way precluded from getting into the adult stem cell population (reviewed in Hamra (2015)) would reduce the impact of early mutations on any hot spot model. In conclusion, under biologically realistic assumptions such alternative hot spot models cannot explain the testis mutation cluster data.

8.9.4 An Alternative Approach to Test the Hot Spot Model

Studies on the c.755C>G Apert syndrome mutation (Goriely et al. 2003) and the Costello syndrome H-RAS c.34G>A mutation (Giannoulatou et al. 2013) were made using sperm samples from normal men of different ages. Here, a single nucleotide polymorphism closely linked to the mutation site was used to ask if the high frequency of new mutations occurred with equal frequency on the two chromosomes as would be expected for the hot spot model. This did not happen and both studies argued this unexpected observation resulted from a selective advantage.

However, when one data set (Goriely et al. 2003) was modeled by another group (Qin et al. 2007) it was pointed out that the stochastic nature of the hot spot model made the observed deviation from expectation insufficient to exclude the model.

8.9.5 If Not a Hotspot then What?

The population geneticist Ian Hastings considered in theoretical studies (Hastings 1989, 1991) that a new gain of function mutation with a germline selective advantage will more likely be transmitted to the next generation because the effective mutation frequency is elevated beyond the level that can be achieved by the mutation process alone. Hastings also realized that a mutation that confers a selective advantage in the germline may also be deleterious if transmitted to an offspring. Are RAMP mutations an example of this process?

The germline selection model is a modification to the hotspot model. In the adult phase, the mutated SrA_p (but not the wild-type SrA_p) favor self-renewal divisions over differentiation events so that the mutation clusters are more likely to grow and persist (Yoon et al. 2013). Figure 8.7 shows that the symmetric selection model is consistent with the Max/Ave clustering statistic for the 374-1 testis and Noonan

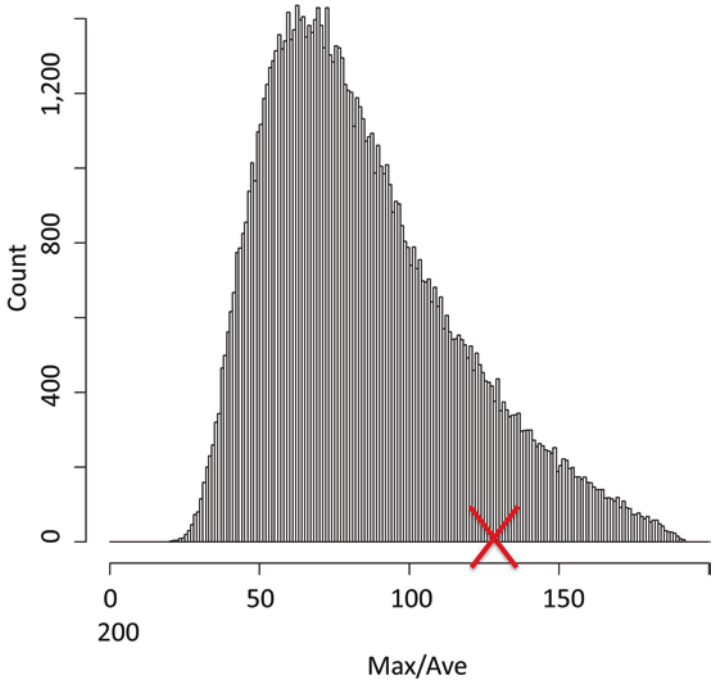

Fig. 8.7 Histogram of simulations of the symmetric selection model using the same data in Fig. 8.6. The X-axis shows the Mx/Ave summary statistic and the Y-axis the number (count) of simulations that gave that value. The large *red X* indicates the Max/Ave value observed in the testis. Since this value is not in the extremes of the distribution of simulated values, the symmetric selection model cannot be rejected

syndrome mutation. This same testing procedure was repeated for multiple clustering statistics, and for all testes and RAMP mutations, with similar results (Shinde et al. 2013; Yoon et al. 2013).

The germline selection model has two parameters: the mutation rate per cell division and the selection parameter. Calculating the inferred value of the mutation rate per cell division gave the genome average mutation rate, implying that the disease mutations do not arise more frequently than expected but that selection increases these mutations' frequencies. Moreover the selective advantage required is relatively small, the frequency of self-renewal divisions compared to differentiation events is about 51% to 49%. (The model based on the asymmetric division scheme has a similarly simple modification incorporating germline selection that is also consistent with the testis data (Qin et al. 2007; Choi et al. 2008, 2012; Shinde et al. 2013; Yoon et al. 2013).)

8.9.6 Cytological Evidence for Human SSC Mutation Clusters?

In a search for a histological analog of mutation clusters, immunocytochemical analyses on a small number of sections cut from several blocks of formalin-fixed human testes tissue were carried out (Lim et al. 2012). The authors assumed that

SrA_p proliferation would mimic early oncogenesis. A search was made for histological features reminiscent of the early stages of cancer, especially with regard to irregular immunostaining with antibodies against proteins and enzymes known to be overexpressed in a rare form of late onset testicular cancer known as spermatocytic seminoma (but excluding the RAMP mutations discussed here). In a small number of histological sections they found a few examples of what they call "immunopositive tubules" that were consistent with their expectations of SrA_p mutation-carrying clusters. As discussed above, an SrA_p cell with a mutation, such as a neutral mutation, that does not confer a selective advantage also has a chance to form a small cluster composed of its descendants (Klein et al. 2010; Hara et al. 2014). Finally, one of the antibody targets used in the Lim et al. study is FGFR3 that they suggest may be a hallmark of cells with a selective advantage. However, FGFR3 has been shown to be an excellent marker for Ad spermatogonia when found in association with an absence of the proliferation-associated antigen Ki-67 (von Kopylow et al. 2012a, b), a feature that seems to be in conflict with the idea that strong FGFR3 expression is a hallmark only of cells with a selective advantage.

This work has been extended (Maher et al. 2016a) by performing DNA sequencing on laser capture microdissected portions of a small number of cut sections containing formalin fixed immunopositive tubules. Over 100 genes that might be candidates for germline selection were studied. A total of 11 mutations (from five genes) were discovered; one example was a FGFR2 c.758C>G RAMP mutation (as described above). The authors argued that all 11 mutations caused selfish clonal expansions. However, the authors did not count the number of normal and mutated spermatogonia in the immunopositive tubules making it difficult to interpret their data within the context of the mutation cluster frequencies determined in whole testis analysis. Further, we should note that there is some controversy over this work as another group has offered a different interpretation (Pohl et al. 2016), while the original group has disagreed with this interpretation (Maher et al. 2016b) and argued that their original interpretation (Maher et al. 2016a) is correct.

8.10 Function of RAMP Mutations in Spermatogonia

According to the selection model, SSC carrying a RAMP mutation must have acquired some functional alteration to give the cell a selective advantage. It is interesting that the RAMP genes, originally chosen for study because of their common genetic features, are all directly involved in normal SSC proliferation, differentiation and survival.

8.10.1 RET

The RET protein is a receptor tyrosine kinase with a central role in many organ systems. Its normal biochemical properties are well known including interactions with adapter or signaling proteins that initiate a variety of downstream signaling

pathways including Ras/MAPK, SFK, and PI3K/AKT among others (reviewed in Arighi et al. (2005) and Mulligan (2014)). RET signaling is also critical for SSC self-renewal, survival and differentiation in the mouse (Oatley and Brinster 2008; Zhou and Griswold 2008; Ebata et al. 2011; Yoshida 2012; Griswold and Oatley 2013; Kanatsu-Shinohara and Shinohara 2013; Yang and Oatley 2014).

The germline effect of the *RET* c.2943T>C, M918T MEN2B mutation must be subtle given that a mouse model of MEN2B (Smith-Hicks et al. 2000) showed normal sperm production in both homozygous and heterozygous animals. Also, men that inherited the MEN2B mutation can father children with the same disease (Carlson et al. 1994) and are not subject to an increased risk of germ cell tumor formation. Similarly, study of the most common type of human testis cancers (seminomas) as well as rare spermatocytic seminomas both failed to find tumors carrying the MEN2B mutation (Goriely et al. 2009; Chevalier et al. 2010). All together the functional properties of the MEN2B protein seem consistent with normal spermatogenesis and spermiogenesis in vivo.

The biochemical consequences of the human M918T mutation on RET function is not known in the human germline unlike the affected organ systems where the mutant protein can alter its own pattern of tyrosine autophosphorylation and increase or decrease signaling in many downstream signaling pathways (reviewed in Arighi et al. (2005), Runeberg-Roos and Saarma (2007), Wells and Santoro (2009), and Lemmon and Schlessinger (2010)). The details of how the MEN2B mutation might alter the signaling to confer a germline selective advantage to mutated SrA_p cells are yet to be elucidated.

8.10.2 SHP-2

The *PTPN11* gene codes for the SHP-2 protein tyrosine phosphatase and is expressed ubiquitously and critical for normal animal development (Mohi and Neel 2007; Dance et al. 2008; Grossmann et al. 2010). SHP-2 is activated by binding to and dephosphorylating phosphotyrosines Tyr (P) on receptor tyrosine kinases and other proteins. Such events can lead to enhancement and/or inhibition of different signaling pathways. Most relevant here, SHP-2 can positively regulate RAS/MAPK, PI3K/AKT and SFK signaling and negatively regulate STAT3 signaling (Dance et al. 2008; Neel et al. 2010) stimulating cell proliferation, survival, differentiation and migration depending on cell type and expression pattern. SHP-2 function is required for SSC to proliferate or survive and also controls SSC potential to produce progenitor cells (Puri et al. 2014). Data on a mouse model carrying a knock-in mutation of the *SHP-2* N308D RAMP mutation are fertile in the heterozygous state (Araki et al. 2009).

One property of the SHP-2 RAMP mutation may provide a clue to the mechanism of germline selection. Studies of non-germline adult mouse cells grown in vitro indicate SHP-2 protein negatively regulates STAT3 signaling (discussed in Zhang et al. (2009)). Importantly, relative to wild-type SHP-2, the hyperactivated phosphatase activity of the SHP-2 RAMP mutation enhanced SHP-2's negative

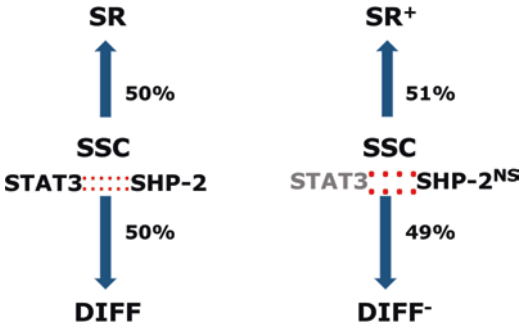

Fig. 8.8 Model for how the Noonan c.922G>A mutation contributes to germline selection. (*Left panel*) SSC require an almost equal probability of self-renewal (SR) and differentiation (DIFF) to maintain fertility. Without sufficient STAT3 activity this balance is destroyed leading a loss of differentiation and a slight increase in SSC proliferation (Oatley et al. 2010; Kaucher et al. 2012). Non-germline data show that STAT3 is naturally downregulated by interaction with SHP-2 (Zhang et al. 2009). The speculated STAT3-SHP-2 interaction in the germline is shown by *small red circles*. The same non-germline data show that the SHP-2 with the Noonan syndrome c.922G>A (N308D) mutation is a more efficient inhibitor of STAT3 than wild-type SHP-2. (*Right panel*) Translating this information to the human germline predicts a scenario where a small decrease (*gray*) in normal STAT3 activity resulting from more intense interactions with the mutant SHP-2 (*large red circles*) could provide a subtle increase in SSC proliferation leading to a 51%:49% ratio of self-renewal to differentiation. This altered ratio is predicted from the calculated selective advantage that can lead to the Noonan syndrome testis data (Yoon et al. 2013)

regulation of STAT3 by removing Tyr (P) from STAT3 (Zhang et al. 2009). STAT3 signaling plays an important role in differentiation of mouse SSC (Oatley et al. 2010; Kaucher et al. 2012). When STAT3 activity in mouse SSC cultures is knocked down and the cells subsequently transplanted into germ cell-free testes, they retained their capacity to proliferate (perhaps slightly better than the control SSC) but did not to produce the normal differentiated stages.

Based on the specific interaction between STAT3 and SHP-2 Fig. 8.8 shows a potential mechanism whereby SrA_p cells carrying a new c.922A>G mutation could experience lower STAT3 activity providing a subtle bias of SSC self-renewal over SSC differentiation.

8.10.3 FGFR2 and FGFR3

These proteins are members of the FGFR family of receptor tyrosine kinases and act in many organs and cell types. They are activated on the cell surface by fibroblast growth factors (FGFs, e.g. FGF2/bFGF) associated with heparin sulfate proteoglycans (Zhang et al. 2006) and can influence cell proliferation, cell survival, differentiation and a myriad of other cell functions through stimulation of the PI3K/AKT, SFK (src family kinases), RAS/MAPK as well as other pathways (Eswarakumar et al. 2002; Itoh and Ornitz 2004; L'Hote and Knowles 2005; Thisse and Thisse 2005; Gotoh 2008; Zhou and Griswold 2008; Lee et al. 2009; Caires et al. 2010;

Phillips et al. 2010; Yoshida 2010). The effect of mutant FGFRs depend upon the specific nature of their amino acid substitution (Schlessinger 2000; Hart et al. 2001; Mohammadi et al. 2005; Ahmed et al. 2008; Lew et al. 2009).

The fertility of transgenic achondroplasia male mice (c.1138G>A, G380R) has not been reported in detail but the implication was that they were poor breeders (Wang et al. 1999). Human males with the condition can reproduce (Pauli 1993). Mice with Apert syndrome are more complex. For the c.755C>G Apert mutation different laboratories have different insights. Adult mice heterozygous for the RAMP mutation reported some features of human Apert syndrome and fertility (Chen et al. 2003) while another reported death 2–3 days after birth (Wang et al. 2005) preventing adult fertility assessment.

The evidence for FGFR2 and FGFR3 functioning in SSC proliferation is indirect and inferred from wild-type SSC cultures grown in chemically defined media where the addition of the fibroblast growth factor FGF2/bFGF enhances SSC self-renewal. A complex relationship between RET signaling (through GDNF and GFRA1) and the signaling due to FGF2 binding to either FGFR2 and/or FGFR3 (or another FGFR) is likely. Because FGFRs can each bind many of the same FGFs (such as FGF2) it has been difficult to distinguish between the relative importance of FGFRs in SSC function. In a recent experiment a small fraction of SSC from mice deficient in Ret but grown in FGF2 were capable of proliferation and germline transmission although less efficiently than SSC that retained a functioning Ret gene (Takashima et al. 2015). Additional studies will be needed before we fully understand the important functions of the different FGFs and FGFRs in normal adult spermatogenesis.

The model (Fig. 8.8) presented to explain how the SHP-2 Noonan mutation might promote excessive inhibition of STAT3 leading to a small proliferative advantage might also apply to SSC with the Apert syndrome and achondroplasia mutations (Yoon et al. 2013). When wild-type mouse SSC cultures were stimulated by a cocktail of Epidermal growth factor, FGF2 and GDNF (Lee et al. 2009) increased levels of Cyclin D1 (G1/S-specific cyclin D1, CCND1) were found. In non-germline cells (Germain and Frank 2007) CCND1 can represses the synthesis of STAT3 as well as bind to STAT3 and inhibit its activity. Thus, subtle inhibition of STAT3 by this mechanism might also lead to a slight increase in SSC proliferation. Note this suggestion is currently not consistent with the ideas of Lee et al. (2009) who speculated that increased expression of CCND1, though unclear, may be involved in differentiation in their experimental system. However, the idea that a selective advantage may be acquired by repression of stem cell differentiation pathways (Yoon et al. 2013) rather than simply activation of positive self-renewal signals, as has been previously suggested (Choi et al. 2012; Goriely and Wilkie 2012; Maher et al. 2014), is worth further testing.

8.10.4 Direct Germline Cell Competition Experiments in Mice

A recent study (Martin et al. 2014) looked at competition between wild-type mouse SSC carrying either a human wild-type FGFR2 or a human version of the *FGFR2* gene with the Apert c.755C>G RAMP mutation, each with a different fluorescent

marker. They transplanted mixtures of the mutant and wild-type SSC (in equal cell numbers) into germ cell-free mouse testes in vivo to directly test SSC cell competition. Two months later a statistically significant greater number of distinct testis colonies, each originating from a single SSC, were observed to have been derived from a single mutant rather than a single wild-type SSC. This paper is the first attempt to directly test for germline selection in vivo but has several technical issues. First, the authors created wild-type and mutant SSC by transfection using lentivirus with a promotor (PGK) different from the normal mouse FGFR2 promotor to drive the human *FGFR2* gene expression. Thus, aspects of normal *FGFR2* transcriptional regulation in SSC would be missing possibly resulting in overexpression and excess proliferation. Secondly, injection into germ cell-free testes does not speak directly to the competition between mutant and wild-type SSC in an overwhelming wild-type spermatogenic environment as would be the case when a single new mutation arises. Finally, it was not determined whether the testes produce mutant sperm leaving open the idea that the mutant cells might be expressing some oncogenic potential that could explain their enhanced proliferation. As a result, the significance of these pioneering experiments to germline selection must wait for additional studies.

8.11 Population Consequences of Germline Selection

8.11.1 PAE

The germline selection model can explain both the paternal age effect, since the disease mutation clusters grow as men age, as well as the male mutation bias, since these clusters are only in the male germline. However, for Apert syndrome the incidence does not increase monotonically with the father's age (Risch et al. 1987) as would be expected under the germline selection model, rather there is a dip as is shown in Fig. 8.9. There is also a dip in the Apert syndrome mutation frequencies measured in sperm donors (Yoon et al. 2009), and in the birth data for several other paternal age effect diseases (Risch et al. 1987). Moreover, for MEN2B some of the testes from older donors (75–80 years) had very few mutations and appeared like the testes from younger donors (Choi et al. 2012).

Both of these observations can be explained by incorporating cell death into the germline selection models. As men age, the number of SrA_p decreases (Nistal et al. 1987). Further, there are A-dark spermatogonia (A_d) that appear quiescent until there has been sufficient SrA_p death at which point A_d start dividing regularly and provide new SrA_p (van Alphen et al. 1988). Since A_d had been dividing only rarely they likely are not mutated. A general increase in spermatogonial cell death, including both wild-type and mutant SrA_p, with replacement by wild-type A_d would lead to a relative decrease in the mutation frequency. These new SrA_p will start dividing, acquire mutations and the mutation frequency will increase again. Similarly, cell death can cause the elimination of mutation clusters in some older individuals who presumably had mutation clusters previously. The details of incorporating cell death into the models can be found in the following references (Yoon et al. 2009; Choi et al. 2012).

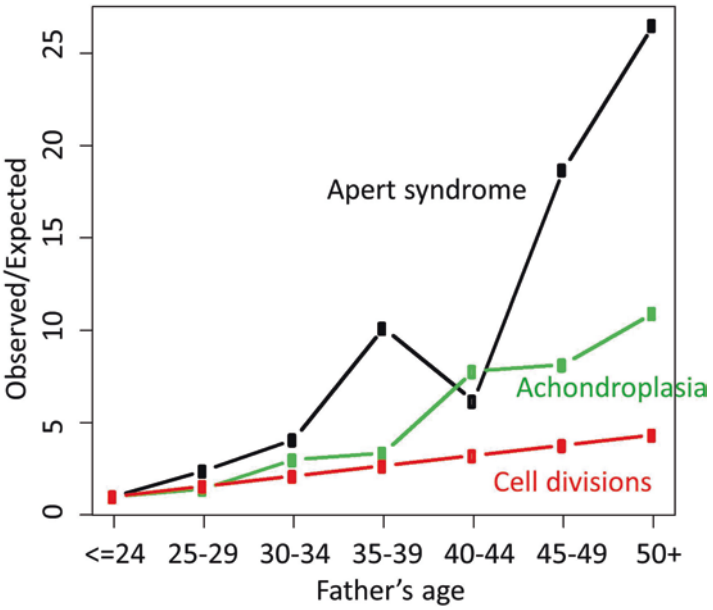

Fig. 8.9 PAE for the achondroplasia (*green*) and Apert syndrome (*black*) births due to new mutations. The X-axis shows the age of the father (binned into 5 year groups) at the time his affected child was conceived. The Y-axis is the ratio of the observed number of affected children in a particular age group of fathers to the expected number of fathers in that age group based on census data, normalized to be one for the youngest age group. For both conditions there is a nonlinear increase in this relationship (Risch et al. 1987). As a comparison, the calculated increase in cell divisions with a man's age is also shown (*red*)

8.11.2 Human Mutational Load

Given our data on all five RAMP disease sites it is possible to determine the anatomical distribution of all the different mutations within the same testes. Figure 8.10 shows one testis (374-2) from an individual where every testis piece was studied for the c.755C>G Apert, c.2943T>C MEN2B, and c.922A>G Noonan mutations. Mutation clusters can be found for each of the three diseases in this 62-year-old man's testis. Notice also that the anatomical location of the highest frequencies at one mutation site do not overlap with the highest mutation frequencies at the other two mutation sites (we have calculated that any overlap is due purely to chance). This of course would be expected for independently arising mutations.

An important conclusion from these data is that every normal man appears to be accumulating different RAMP disease mutations as he ages. The accumulation of RAMP mutation types drastically increases the probability of having a child affected with a RAMP mutation as men age, relative to the chance of a non-RAMP mutation. An open question is how many additional RAMP mutations there are. Future research will likely search for new candidates. One can imagine that the testis of a man gradually takes on the form of a mosaic with an ever increasing complexity as

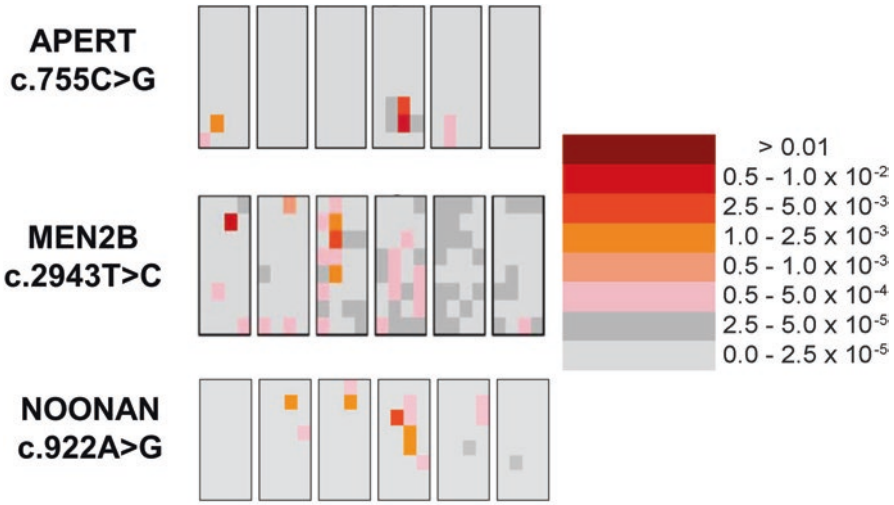

Fig. 8.10 Mutation data on three different RAMP mutations from a single testis of donor (374-2). Three aliquots from each testis piece were each examined for the Apert c.755C>G, MEN2B c.2943T>C and the Noonan *PTPN11* c.922A>G mutations in all pieces (Qin et al. 2007; Choi et al. 2012; Yoon et al. 2013)

new de novo mutations arise at random positions throughout life. Most will be neutral mutations and their birth and death in the testis will be subject to random forces. Some will provide the cells with an advantage that could lead to testicular cancer but oncogenesis would prevent transmission to the next generation. Other advantageous mutations could be heritable and of the RAMP type that reach very high frequencies, while others may fall in frequency and may be more difficult to identify because the mutation in stem cells leads to a disadvantage. The stochastic nature of these processes makes more specific advice with respect to age impossible, at least at this time, but all these processes occur throughout a man's life suggesting, as Jim Crow did many years ago, that in order to decrease the transmission of de novo disease mutations men should father children earlier rather than later in life.

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References

Ahmed Z, Schuller AC, Suhling K, Tregidgo C, Ladbury JE (2008) Extracellular point mutations in FGFR2 elicit unexpected changes in intracellular signalling. Biochem J 413:37–49

Araki T, Chan G, Newbigging S, Morikawa L, Bronson RT et al (2009) Noonan syndrome cardiac defects are caused by PTPN11 acting in endocardium to enhance endocardial-mesenchymal transformation. Proc Natl Acad Sci U S A 106:4736–4741

Arighi E, Borrello MG, Sariola H (2005) RET tyrosine kinase signaling in development and cancer. Cytokine Growth Factor Rev 16:441–467
Arnheim N, Calabrese P (2009) Understanding what determines the frequency and pattern of human germline mutations. Nat Rev Genet 10:478–488
Caires K, Broady J, McLean D (2010) Maintaining the male germline: regulation of spermatogonial stem cells. J Endocrinol 205:133–145
Campbell CD, Eichler EE (2013) Properties and rates of germline mutations in humans. Trends Genet 29:575–584
Carlson KM, Bracamontes J, Jackson CE, Clark R, Lacroix A et al (1994) Parent-of-origin effects in multiple endocrine neoplasia type 2B. Am J Hum Genet 55:1076–1082
Chen L, Li D, Li C, Engel A, Deng CX (2003) A Ser252Trp [corrected] substitution in mouse fibroblast growth factor receptor 2 (Fgfr2) results in craniosynostosis. Bone 33:169–178
Chevalier N, Barlier A, Roche C, Mograbi B, Camparo P et al (2010) RET gene mutations are not involved in the origin of human testicular seminoma. Int J Androl 33:848–852
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
Choi SK, Yoon SR, Calabrese P, Arnheim N (2008) A germ-line-selective advantage rather than an increased mutation rate can explain some unexpectedly common human disease mutations. Proc Natl Acad Sci U S A 105:10143–10148
Choi SK, Yoon SR, Calabrese P, Arnheim N (2012) Positive selection for new disease mutations in the human germline: evidence from the heritable cancer syndrome multiple endocrine neoplasia type 2B. PLoS Genet 8:e1002420
Clermont Y (1966) Renewal of spermatogonia in man. Am J Anat 118:509–524
Crow JF (1997) The high spontaneous mutation rate: is it a health risk? Proc Natl Acad Sci U S A 94:8380–8386
Crow JF (2000) The origins, patterns and implications of human spontaneous mutation. Nat Rev Genet 1:40–47
Dance M, Montagner A, Salles JP, Yart A, Raynal P (2008) The molecular functions of Shp2 in the Ras/Mitogen-activated protein kinase (ERK1/2) pathway. Cell Signal 20:453–459
Drost JB, Lee WR (1995) Biological basis of germline mutation: comparisons of spontaneous germline mutation rates among drosophila, mouse, and human. Environ Mol Mutagen 25:48–64
Ebata KT, Yeh JR, Zhang X, Nagano MC (2011) Soluble growth factors stimulate spermatogonial stem cell divisions that maintain a stem cell pool and produce progenitors in vitro. Exp Cell Res 317:1319–1329
Ellegren H (2007) Characteristics, causes and evolutionary consequences of male-biased mutation. Proc Biol Sci 274:1–10
Eswarakumar VP, Monsonego-Ornan E, Pines M, Antonopoulou I, Morriss-Kay GM et al (2002) The IIIc alternative of Fgfr2 is a positive regulator of bone formation. Development 129:3783–3793
Germain D, Frank DA (2007) Targeting the cytoplasmic and nuclear functions of signal transducers and activators of transcription 3 for cancer therapy. Clin Cancer Res 13:5665–5669
Giannoulatou E, McVean G, Taylor IB, McGowan SJ, Maher GJ et al (2013) Contributions of intrinsic mutation rate and selfish selection to levels of de novo HRAS mutations in the paternal germline. Proc Natl Acad Sci U S A 110:20152–20157
Glaser RL, Jabs EW (2004) Dear old dad. Sci Aging Knowl Environ 2004:re1
Goriely A, Hansen RM, Taylor IB, Olesen IA, Jacobsen GK et al (2009) Activating mutations in FGFR3 and HRAS reveal a shared genetic origin for congenital disorders and testicular tumors. Nat Genet 41:1247–1252
Goriely A, McVean GA, Rojmyr M, Ingemarsson B, Wilkie AO (2003) Evidence for selective advantage of pathogenic FGFR2 mutations in the male germ line. Science 301:643–646
Goriely A, Wilkie AO (2012) Paternal age effect mutations and selfish spermatogonial selection: causes and consequences for human disease. Am J Hum Genet 90:175–200
Gotoh N (2008) Regulation of growth factor signaling by FRS2 family docking/scaffold adaptor proteins. Cancer Sci 99:1319–1325

Griswold MD, Oatley JM (2013) Concise review: defining characteristics of mammalian spermatogenic stem cells. Stem Cells 31:8–11
Grossmann KS, Rosario M, Birchmeier C, Birchmeier W (2010) The tyrosine phosphatase Shp2 in development and cancer. Adv Cancer Res 106:53–89
Hamra FK (2015) Diagnosing spermatogonial stemness. Biol Reprod 92:119
Hara K, Nakagawa T, Enomoto H, Suzuki M, Yamamoto M et al (2014) Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. Cell Stem Cell 14:658–672
Hart KC, Robertson SC, Donoghue DJ (2001) Identification of tyrosine residues in constitutively activated fibroblast growth factor receptor 3 involved in mitogenesis, Stat activation, and phosphatidylinositol 3-kinase activation. Mol Biol Cell 12:931–942
Hastings IM (1989) Potential germline competition in animals and its evolutionary implications. Genetics 123:191–197
Hastings IM (1991) Germline selection: population genetic aspects of the sexual/asexual life cycle. Genetics 129:1167–1176
Heller CG, Clermont Y (1963) Spermatogenesis in man: an estimate of its duration. Science 140:184–186
Itoh N, Ornitz DM (2004) Evolution of the Fgf and Fgfr gene families. Trends Genet 20:563–569
Kanatsu-Shinohara M, Shinohara T (2013) Spermatogonial stem cell self-renewal and development. Annu Rev Cell Dev Biol 29:163–187
Kaucher AV, Oatley MJ, Oatley JM (2012) NEUROG3 is a critical downstream effector for STAT3-regulated differentiation of mammalian stem and progenitor spermatogonia. Biol Reprod 86(164):161–111
Klein AM, Nakagawa T, Ichikawa R, Yoshida S, Simons BD (2010) Mouse germ line stem cells undergo rapid and stochastic turnover. Cell Stem Cell 7:214–224
Klein AM, Simons BD (2011) Universal patterns of stem cell fate in cycling adult tissues. Development 138:3103–3111
Kong A, Frigge ML, Masson G, Besenbacher S, Sulem P et al (2012) Rate of de novo mutations and the importance of father's age to disease risk. Nature 488:471–475
L'Hote CG, Knowles MA (2005) Cell responses to FGFR3 signalling: growth, differentiation and apoptosis. Exp Cell Res 304:417–431
Lee J, Kanatsu-Shinohara M, Morimoto H, Kazuki Y, Takashima S et al (2009) Genetic reconstruction of mouse spermatogonial stem cell self-renewal in vitro by Ras-cyclin D2 activation. Cell Stem Cell 5:76–86
Lemmon MA, Schlessinger J (2010) Cell signaling by receptor tyrosine kinases. Cell 141:1117–1134
Lew ED, Furdui CM, Anderson KS, Schlessinger J (2009) The precise sequence of FGF receptor autophosphorylation is kinetically driven and is disrupted by oncogenic mutations. Sci Signal 2:ra6
Lim J, Maher GJ, Turner GD, Dudka-Ruszkowska W, Taylor S et al (2012) Selfish spermatogonial selection: evidence from an immunohistochemical screen in testes of elderly men. PLoS One 7:e42382
Liu Q, Sommer SS (2004) Detection of extremely rare alleles by bidirectional pyrophosphorolysis-activated polymerization allele-specific amplification (Bi-PAP-A): measurement of mutation load in mammalian tissues. BioTechniques 36:156–166
Luria SE, Delbruck M (1943) Mutations of bacteria from virus sensitivity to virus resistance. Genetics 28:491–511
Maher GJ, Goriely A, Wilkie AO (2014) Cellular evidence for selfish spermatogonial selection in aged human testes. Andrology 2:304–314
Maher GJ, McGowan SJ, Giannoulatou E, Verrill C, Goriely A et al (2016a) Visualizing the origins of selfish de novo mutations in individual seminiferous tubules of human testes. Proc Natl Acad Sci U S A 113:2454–2459
Maher GJ, Rajpert-De Meyts E, Goriely A, Wilkie AO (2016b) Cellular correlates of selfish spermatogonial selection. Andrology 4:550–553

Martin LA, Assif N, Gilbert M, Wijewarnasuriya D, Seandel M (2014) Enhanced fitness of adult spermatogonial stem cells bearing a paternal age-associated FGFR2 mutation. Stem Cell Rep 3:219–226

Masumura K, Toyoda-Hokaiwado N, Ukai A, Gondo Y, Honma M et al (2016) Estimation of the frequency of inherited germline mutations by whole exome sequencing in ethyl nitrosourea-treated and untreated gpt delta mice. Genes Environ 38:10

Mohammadi M, Olsen SK, Ibrahimi OA (2005) Structural basis for fibroblast growth factor receptor activation. Cytokine Growth Factor Rev 16:107–137

Mohi MG, Neel BG (2007) The role of Shp2 (PTPN11) in cancer. Curr Opin Genet Dev 17:23–30

Muller J, Skakkebaek NE (1992) The prenatal and postnatal development of the testis. Bailliere Clin Endocrinol Metab 6:251–271

Mulligan LM (2014) RET revisited: expanding the oncogenic portfolio. Nat Rev Cancer 14:173–186

Neel BG, Chan G, Dhanji S (2010) SH2 domain-containing protein-tyrosine phosphatases. In: Bradshaw RA, Dennis EA (eds) Handbook of cell signaling, 2nd edn. Academic Press, Amsterdam; San Diego, CA, pp 771–810

Nistal M, Codesal J, Paniagua R, Santamaria L (1987) Decrease in the number of human Ap and Ad spermatogonia and in the Ap/Ad ratio with advancing age. New data on the spermatogonial stem cell. J Androl 8:64–68

Nistal M, Paniagua R (1984) Testicular and epididymal pathology. Thieme-Stratton, New York

Oatley JM, Brinster RL (2008) Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 24:263–286

Oatley JM, Kaucher AV, Avarbock MR, Brinster RL (2010) Regulation of mouse spermatogonial stem cell differentiation by STAT3 signaling. Biol Reprod 83:427–433

Pauli RM (1993) Achondroplasia. In: Pagon RA, Adam MP, Ardinger HH, Wallace SE, Amemiya A et al (eds) GeneReviews(R). University of Washington, Seattle, WA

Penrose L (1955) Paternal age and mutation. Lancet 269:312–313

Phillips BT, Gassei K, Orwig KE (2010) Spermatogonial stem cell regulation and spermatogenesis. Philos Trans R Soc Lond Ser B Biol Sci 365:1663–1678

Pohl E, Gromoll J, Kliesch S, Wistuba J (2016) An alternative interpretation of cellular 'selfish spermatogonial selection'-clusters in the human testis indicates the need for 3-D-analyses. Andrology 4:213–217

Puri P, Phillips BT, Suzuki H, Orwig KE, Rajkovic A et al (2014) The transition from stem cell to progenitor spermatogonia and male fertility requires the SHP2 protein tyrosine phosphatase. Stem Cells 32:741–753

Qin J, Calabrese P, Tiemann-Boege I, Shinde DN, Yoon SR et al (2007) The molecular anatomy of spontaneous germline mutations in human testes. PLoS Biol e224:5

Risch N, Reich EW, Wishnick MM, McCarthy JG (1987) Spontaneous mutation and parental age in humans. Am J Hum Genet 41:218–248

Runeberg-Roos P, Saarma M (2007) Neurotrophic factor receptor RET: structure, cell biology, and inherited diseases. Ann Med 39:572–580

Sayres MA, Makova KD (2011) Genome analyses substantiate male mutation bias in many species. BioEssays 33:938–945

Schlessinger J (2000) Cell signaling by receptor tyrosine kinases. Cell 103:211–225

Segurel L, Quintana-Murci L (2014) Preserving immune diversity through ancient inheritance and admixture. Curr Opin Immunol 30:79–84

Segurel L, Wyman MJ, Przeworski M (2014) Determinants of mutation rate variation in the human germline. Annu Rev Genomics Hum Genet 15:47–70

Shinde DN, Elmer DP, Calabrese P, Boulanger J, Arnheim N et al (2013) New evidence for positive selection helps explain the paternal age effect observed in achondroplasia. Hum Mol Genet 22(20):4117–4126

Smith-Hicks CL, Sizer KC, Powers JF, Tischler AS, Costantini F (2000) C-cell hyperplasia, pheochromocytoma and sympathoadrenal malformation in a mouse model of multiple endocrine neoplasia type 2B. EMBO J 19:612–622

Takashima S, Kanatsu-Shinohara M, Tanaka T, Morimoto H, Inoue K et al (2015) Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. Stem Cell Rep 4:489–502

Thisse B, Thisse C (2005) Functions and regulations of fibroblast growth factor signaling during embryonic development. Dev Biol 287:390–402

van Alphen MM, van de Kant HJ, de Rooij DG (1988) Depletion of the spermatogonia from the seminiferous epithelium of the rhesus monkey after X irradiation. Radiat Res 113:473–486

Vogel F, Motulsky AG (1997) Human genetics: problems and approaches. Springer, Berlin; New York

von Kopylow K, Staege H, Schulze W, Will H, Kirchhoff C (2012a) Fibroblast growth factor receptor 3 is highly expressed in rarely dividing human type A spermatogonia. Histochem Cell Biol 138:759–772

von Kopylow K, Staege H, Spiess AN, Schulze W, Will H et al (2012b) Differential marker protein expression specifies rarefaction zone-containing human Adark spermatogonia. Reproduction 143:45–57

Walter CA, Intano GW, McMahan CA, Kelner K, McCarrey JR et al (2004) Mutation spectral changes in spermatogenic cells obtained from old mice. DNA Repair (Amst) 3:495–504

Wang Y, Spatz MK, Kannan K, Hayk H, Avivi A et al (1999) A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. Proc Natl Acad Sci U S A 96:4455–4460

Wang Y, Xiao R, Yang F, Karim BO, Iacovelli AJ et al (2005) Abnormalities in cartilage and bone development in the Apert syndrome FGFR2+/S252W mouse. Development 132:3537–3548

Weinberg W (1912) Zur Verebung des Zwergwuches. Archiv Rassen-und Gesellschafts-Hygie Biolne 9:710–718

Wells SA Jr, Santoro M (2009) Targeting the RET pathway in thyroid cancer. Clin Cancer Res 15:7119–7123

Yang QE, Oatley JM (2014) Spermatogonial stem cell functions in physiological and pathological conditions. Curr Top Dev Biol 107:235–267

Yoon SR, Choi SK, Eboreime J, Gelb BD, Calabrese P et al (2013) Age-dependent germline mosaicism of the most common noonan syndrome mutation shows the signature of germline selection. Am J Hum Genet 92:917–926

Yoon SR, Qin J, Glaser RL, Jabs EW, Wexler NS et al (2009) The ups and downs of mutation frequencies during aging can account for the Apert syndrome paternal age effect. PLoS Genet 5:e1000558

Yoshida S (2010) Stem cells in mammalian spermatogenesis. Develop Growth Differ 52:311–317

Yoshida S (2012) Elucidating the identity and behavior of spermatogenic stem cells in the mouse testis. Reproduction 144:293–302

Yoshida S, Sukeno M, Nabeshima Y (2007) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317:1722–1726

Zhang T, Oatley J, Bardwell VJ, Zarkower D (2016) DMRT1 is required for mouse spermatogonial stem cell maintenance and replenishment. PLoS Genet 12:e1006293

Zhang W, Chan RJ, Chen H, Yang Z, He Y et al (2009) Negative regulation of Stat3 by activating PTPN11 mutants contributes to the pathogenesis of Noonan syndrome and juvenile myelomonocytic leukemia. J Biol Chem 284:22353–22363

Zhang X, Ibrahimi OA, Olsen SK, Umemori H, Mohammadi M et al (2006) Receptor specificity of the fibroblast growth factor family. J Biol Chem 281:15694–15700

Zhou QG, Griswold MD (2008) Regulation of spermatogonia. In: T. S. R. Community (ed) StemBook

The Spermatogonial Stem Cell and the Environment

9

Tegan S.A. Horan, Caroline V. Sartain, and Patricia A. Hunt

Abstract

In the span of a single generation, we have witnessed a revolution in the treatment of human infertility. The first baby conceived through in vitro fertilization (IVF) was born in 1978, and the nearly four decades since have produced an ever-increasing variety of treatment options available to men and women with impaired fertility. The development of treatments for patients with complete gametogenic failure represents the next frontier in the treatment of infertility, and recent technical advances in induced pluripotent stem (iPS) cell technology and in vitro culture suggest that this is within our grasp. Our understanding, however, of the environmental cues that shape the development of normal gametes and embryos remains woefully inadequate. In developing treatments for male infertility, an understanding of the normal testicular environment and how perturbations to it affect the programming of the spermatogonial stem cell (SSC) and its descendants is essential. This knowledge will drive the development of in vitro systems both for the culture of SSCs and the support of spermatogenesis ex vivo, and is essential in assessing the risk to male reproductive health posed by exposure to common environmental pollutants. In this chapter, we review current knowledge of naturally occurring environmental influences, focusing on the stages of male germ cell development they affect. Using this context, we summarize the best-characterized effects of exogenous exposures to endocrine disrupting chemicals (EDCs) on the male germline and questions currently driving the field.

Keywords

SSC • Environment • EDCs • Transgenerational • Epigenetic

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9.1 Germ Cell Fate Is Environmentally Determined, but Genotype Matters

Germ cell development in mammals is characterized by numerous sex-specific differences (Fig. 9.1). Not only is the time of meiotic onset different in the testis and ovary (postnatal in males, prenatal in females), but the outcome (4 sperm vs. a single egg) and duration (weeks vs. years, in humans) of gametogenesis are sex-specific. In addition, there are intriguing differences in the meiotic process, including the activity of the sex chromosomes (two active Xs in females and meiotic sex chromosome inactivation (MSCI) in males), the number and placement of meiotic recombination sites, and the response to errors during both meiotic prophase and metaphase. These differences, although key to understanding environmental impacts on mammalian gametogenesis, have been extensively reviewed (e.g., Saitou and Yamaji 2012; Nagaoka et al. 2012; Turner 2015). Thus, we have chosen to limit this discussion to the male, focusing on how the normal testis environment and exogenous signals influence male germ cell development.

Primordial germ cells (PGCs), the precursors of spermatocytes and oocytes, actively migrate to the developing genital ridge, where one of life's most important decisions is made. Gonad development occurs independently of the germ cells, as evidenced by the fact that the development of the genital ridge into a testis or ovary is initiated even if germ cells fail to arrive (reviewed in Maatouk et al. 2012). In contrast to the somatic component of the developing gonad, however, the fate of the germ cell is dictated by the environment; migration into a testis triggers the formation of prospermatogonia, whereas germ cells that migrate into an ovary enter meiosis and embark upon oogenesis.

Although a germ cell can be coopted to enter either the male or female pathway of development, successful gamete production requires a match between gonadal sex and germ cell genotype. Early studies of germ cell development in the mouse demonstrated that germ cells that fail to reach the gonad in a developing male fetus enter meiosis on a female schedule (Mclaren 1983). As a result, the female pathway of development was considered the default pathway for many years. However, with increased understanding of the complex signals involved in the specification of germ cells, the maintenance of pluripotency, and the elaborate epigenetic reprogramming that occurs during germ cell development, it has become apparent that nothing about germ cell development occurs by default (Saitou et al. 2012). Further, as detailed in Chapter 6, we now know that the onset of meiosis in germ cells that populate the developing testis is actively prevented by the expression of *Cyp26b1*, which attenuates retinoic acid (RA) signaling. In lieu of meiotic entry, male germ cell development is initiated by a series of mitotic divisions, followed by the designation of a small population of spermatogonial stem cells or SSCs. These stem cells divide slowly, their descendants amplify, undergo meiosis, and acquire the appropriate morphology, motility, and ability to fertilize during spermiogenesis and subsequent transit through the epididymis.

Successful navigation of the spermatogenic pathway requires an appropriate XY germ cell genotype; inappropriate (XX) or abnormal (XXY, XYY, XO) sex

chromosome constitutions pose problems that result in different types of spermatogenic impairment. The presence of more than a single X chromosome is a catastrophic germ cell genotype in the testis. XXY or Klinefelter syndrome affects 1 in 500 to 1 in 1000 liveborn males (Lanfranco et al. 2004). Although these males have mild somatic anomalies, they are sterile with small, azoospermatic testes. Experimental studies of XXY mice suggest that the presence of two X chromosomes causes a subtle reduction in germ cell proliferation during prenatal development but, intriguingly, the demise of XXY germ cells coincides with the formation of the spermatogonial stem cell pool during the first several days after birth (Hunt et al. 1998). Because X-reactivation occurs in XX or XXY germ cells when they reach the genital ridge regardless of the sex of the developing gonad they enter (Mroz et al. 1999), germ cell demise in the XXY male suggests that proper X gene dosage is essential for SSC formation.

Despite the clear evidence that two X chromosomes are not compatible with germ cell development in the testis, sperm production in XXY men has been reported, and it has been argued that XXY cells can undergo meiosis. Does this mean that the presence of two X chromosomes is less deleterious in the human? Based on studies of the XXY male mouse, it seems more likely that sperm production in the human XXY testis is a lucky mistake. Male mice with two X chromosomes are invariably infertile, but breakthrough patches of spermatogenesis are a common feature of the XXY testis. Studies of these rare surviving germ cells, however, demonstrate that they are exclusively XY (Mroz et al. 1999). Thus, rare mitotic nondisjunction events that give rise to clones of XY SSCs provides the most likely explanation for the low levels of sperm produced in the mouse and human XXY testis. Although Klinefelter syndrome is the leading genetic cause of male infertility, the advent of testicular sperm extraction and intracytoplasmic sperm injection (TESE-ICSI) has made biological paternity possible for XXY men who produce small numbers of sperm. A recent review of pregnancy outcomes provides further support that sperm in XXY men are produced from SSCs that have lost the second X chromosome; i.e., the sex ratio of offspring conceived following TESE-ICSI is normal and the frequency of sex chromosome aneuploidy is low (Plotton et al. 2014). Thus, the available data from studies in both mice and men strongly suggest that two X chromosomes are incompatible with the formation of SSCs and/or their survival.

In addition to X chromosome imbalance, the lack or presence of multiple copies of the Y chromosome elicits problems in the orchestration of meiotic events during spermatogenesis. The effects have been reviewed extensively (Turner 2015), but, in essence, an additional copy (or copies) of the Y chromosome cause mechanical problems in the pairing, synapsis, and recombination of sex chromosomes during prophase and in their segregation at the first meiotic division. The phenotype of males with two Y chromosomes is variable in both mice and men. As in XXY males, loss of the additional Y chromosome often occurs in XYY men (Shi and Martin 2000). In the mouse, failure of all three sex chromosomes to undergo synapsis during meiotic prophase results in failure of sex chromosome inactivation, leading to arrest and death of the spermatocyte at pachytene (Royo et al. 2010); XYY spermatocytes in the human testis presumably are at a similar disadvantage.

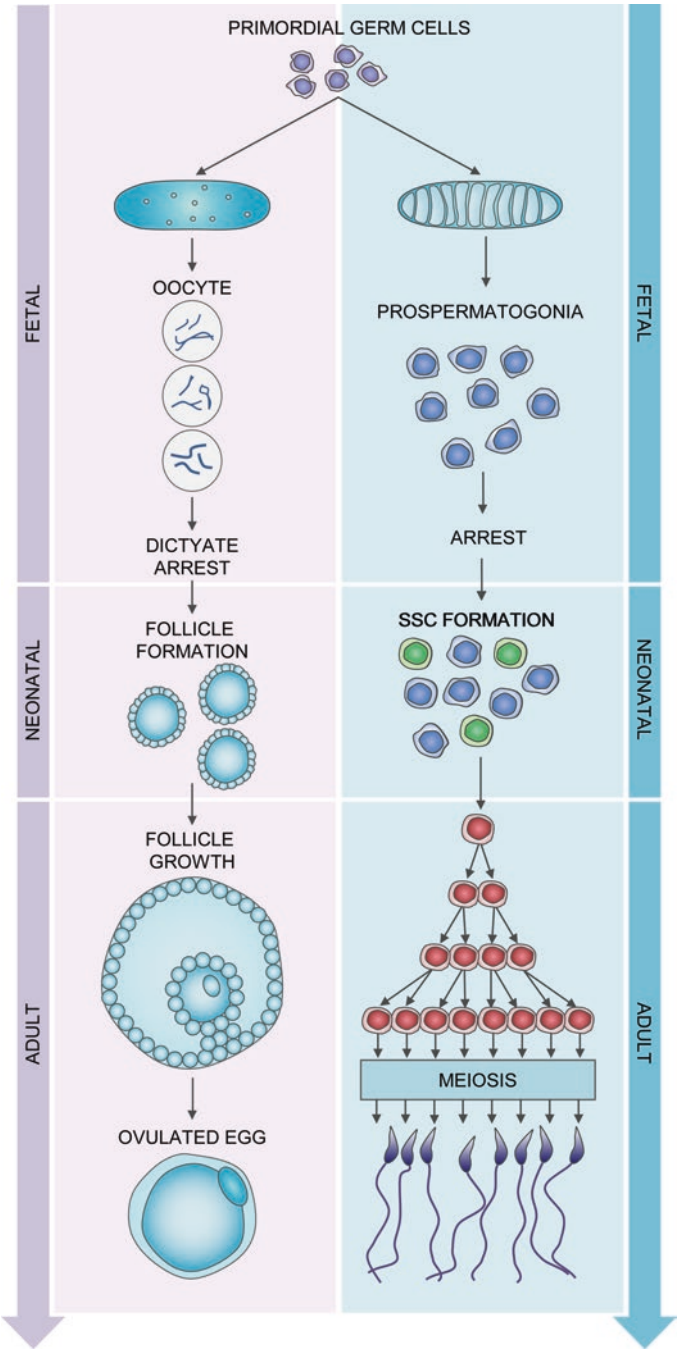


Fig. 9.1 Timeline of male and female germ cell development. *Primordial germ cells* (*PGCs*) migrate to the developing genital ridge, and the decision to embark upon oogenesis or spermatogenesis

9.2 The Changing Testicular Environment: The Effect of Paternal Age

In addition to dictating germ cell fate, a normal testicular environment is essential for spermatogenesis, and subtle changes in the environment have the potential to adversely impact sperm production. In females—especially the human female—the devastating effect of advancing maternal age on reproduction has been well characterized (reviewed in Nagaoka et al. 2012). Given that fertility in the male is maintained throughout adult life, the effect of age on spermatogenesis is mild by comparison. Nevertheless, there is ample evidence that spermatogenesis declines with age. Ageing is associated with a decrease in testicular volume, a decline in testicular function, changes in reproductive hormones, and reduced fertility (reviewed in Paul and Robaire 2013 and Sharma et al. 2015). Intriguingly, experimental data suggest that the SSC lineage is adversely affected by both an age-related decline in SSC number and changes in the somatic environment that supports their development (Ryu et al. 2006; Zhang et al. 2006). Specifically, a reduction in the expression of glial cell-derived neurotrophic factor (GDNF) in the niche has been postulated to cause a decrease in SSC numbers with age (Ryu et al. 2006); however, SSC function also appears to diminish with age, since transplantation of SSCs from aged testes produces fewer, smaller colonies (Zhang et al. 2006). Consistent with this, expression profiles of SSC-enriched populations from aged testes suggest downregulation of genes associated with stem cell maintenance, GDNF signaling, and upregulation in DNA repair pathways (Kokkinaki et al. 2010; Paul et al. 2013). Additionally, in humans, advanced paternal age is associated with modest increases in both the number of aneuploid sperm (Griffin et al. 1995) and paternally inherited de novo gene mutations (reviewed in Paul and Robaire 2013; Arnheim and Calabrese 2016).

Although the data suggesting a decline in spermatogenesis with advancing paternal age are compelling, the mechanisms responsible for this decline remain unclear. The suggestion that both the quality and quantity of SSCs declines with age raises important questions about the malleability of these cells (both epigenetically and

Fig. 9.1 (continued) is dictated by sex determination events in the somatic cells of the developing gonad. In the developing ovary (*left panel*), germ cells enter meiosis, and the resultant *oocytes* proceed through the prophase events of synapsis and recombination, and enter a protracted period of arrest, termed *dictyate*. Immediately after birth in the mouse and in the third trimester of pregnancy in humans, arrested oocytes become surrounded by somatic cells, forming *primordial follicles*. In the sexually mature female, groups of primordial follicles initiate an extended period of growth that typically culminates in the maturation and ovulation of one (human) or several (mouse) mature *eggs* each cycle. In contrast to the female, primordial germ cells in the fetal testis (*right panel*) undergo mitotic proliferation, followed by a period of quiescence during which these *prospermatogonia* remain in mitotic arrest until after birth. In the mouse, mitotic proliferation of prospermatogonia after birth is accompanied by establishment of the *spermatogonial stem cell* (*SSC*) population. In the adult male, SSCs slowly divide to give rise to an expanded population of differentiated *spermatogonia* that ultimately give rise to primary spermatocytes that enter meiosis. Following meiosis, round spermatids undergo spermiogenesis, a differentiation process that produces mature spermatozoa

transcriptionally) in response to changes in their environment. Age-related changes could simply reflect the number of divisions an SSC has undergone, which would have important implications for the long-term maintenance of these cells in culture. Alternatively, age-induced effects on SSCs could result from changes in the somatic environment that influence the paracrine and endocrine signals received by SSCs. Importantly, in the adult testis, SSCs reside outside the blood–testis barrier, and thus may be vulnerable to the effects of any contaminant that enters the body. Given their ability to survive and regenerate spermatogenesis following geno- and cytotoxic insults, SSCs are thought to be more resistant to environmental stress than are other germ cell stages (Van der Meer et al. 1992; Forand et al. 2009; Aloisio et al. 2014); however, surviving SSCs may have mutations or epigenetic alterations that can be transmitted to progeny. Thus, apparent age-related effects could also be a reflection of damage in adult SSCs accumulated over time as a result of environmental exposure. To date, effects on the SSC of adult exposure to endocrine disrupting chemicals (EDCs) have not been examined. As detailed below, however, both epidemiological and experimental data suggest that exposures to environmental contaminants at several different developmental stages (including the adult) can impair male fertility.

9.3 The Estrogen Hypothesis and Beyond

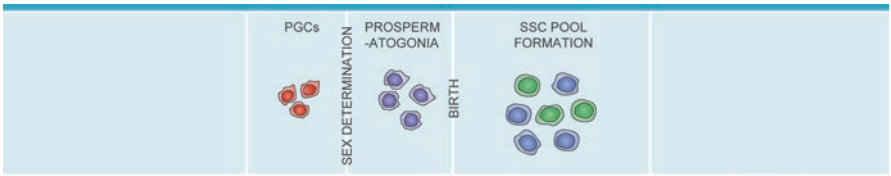
Although initially considered controversial, data from several developed countries have provided compelling evidence of a decline in human sperm counts over the last several decades (Jorgensen et al. 2001, 2002, 2011; Iwamoto et al. 2013; Mendiola et al. 2013; reviewed by Levine et al. 2017). These findings have coincided with growing documentation of an increased incidence of morphological anomalies of the male reproductive tract—including hypospadias, undescended testes, and testicular cancer (Sharpe 2003). This spectrum of reproductive disorders has been termed testicular dysgenesis syndrome (TDS). In 1993, Richard Sharpe and Niels Skakkebaek proposed that TDS results from exposure of the developing testis to either maternally- or environmentally derived estrogens (Sharpe and Skakkebaek 1993). The implication of this "estrogen hypothesis" is monumental: It suggests that twenty-first century exposures are adversely affecting human male reproductive health.

Both correlative human studies and experimental studies in animal models support the estrogen hypothesis. Diethylstilbestrol (DES) exposure provides the most compelling example of the effect in humans of estrogenic endocrine disruption on the developing testis. DES is a synthetic estrogen prescribed to millions of pregnant women from the late 1940s through the early 1970s in an attempt to prevent miscarriage. Disastrously, not only did DES not safeguard pregnancy but also it caused reproductive disorders and cancers in the offspring of women to whom it was prescribed: DES sons had increased levels of cryptorchidism, underdeveloped testes, testicular cancer, decreased sperm counts, and diminished sperm quality (reviewed in Reed and Fenton 2013 and Harris and Waring 2012).

In the U.S., the FDA withdrew approval for the use of DES during pregnancy in the 1970s (Harris and Waring 2012). However, the post-WWII influx of chemicals

into everyday life has resulted in a vast and ever-increasing variety of EDCs in our environment. Although studies of the effects of developmental exposures in humans are limited, epidemiological data have implicated environmental factors in the etiology of testicular germ cell cancers, abnormalities thought to have their origin during early germ cell development (reviewed in Skakkebaek et al. 2016). Compelling support for the estrogen hypothesis also comes from studies in model organisms, where feminizing effects of environmental estrogens have been reported in a broad range of species. For example, it is well established that estrogenic effluents affect wild fish, causing expression in the testis of the vitellogenin gene, a gene that encodes a protein synthesized during oocyte maturation (reviewed in Ortiz-Zarragoitia et al. 2014). Further, data from both field and laboratory studies provide evidence that not only estrogenic but also anti-androgenic EDCs and EDCs with other modes of action can impact the developing testis. For example, exposure to the common pesticide atrazine has been reported to cause severe morphological and functional reproductive anomalies, including complete feminization, ovotestis formation, testicular lesions, hormonal dysregulation, and reduced germ cell numbers in fish, amphibians, reptiles, and mammals (Desesso et al. 2014 and reviewed in Hayes et al. 2011).

Figure 9.2 summarizes data from experimental studies in rodents that provide evidence of germ cell effects as a result of exposure to different classes of EDCs—"estrogens," "anti-androgens," or "other/mixed" for EDCs with unknown or multiple modes of action—during either prenatal or neonatal development. It is important, however, to note the limitation of these labels, since EDCs frequently have complex mechanisms of action that are dependent upon both developmental stage and tissue (e.g. BPA can have estrogenic and anti-androgenic activities, with effects depending upon organ system, age, and sex, (reviewed in Richter et al. 2007). The rapid publication of new studies precludes complete coverage, as does the rate of introduction of new chemicals and variations of existing ones. Thus, this is not intended as a comprehensive summary. Because differences among studies in experimental endpoints makes weighing evidence of germ cell effects difficult, we only included studies reporting significant differences between treated and control groups in the number of prospermatogonia in late fetal or neonatal testes, or in measurements of sperm production in adult males. We excluded studies using SSC primary cell cultures or cell lines (e.g. Lucas et al. 2012), since effects of the culture environment on the SSC remain unknown. Although the species, strains, doses, and endpoints examined vary widely among studies, several points emerge. First, exposure to the developing testis either during fetal development or in the early postnatal period elicits detectable effects on developing germ cells. These exposures target different periods of germ cell development (Fig. 9.2), and suggest both prospermatogonia and the developing SSC pool and/or undifferentiated spermatogonia may be vulnerable to the effects of EDC exposure. Second, the different classes of EDCs appear to elicit similar effects on the testis; i.e. reductions in the number of neonatal prospermatogonia and/or reduced sperm counts are a feature of all three types of EDCs. Finally, although the data in Fig. 9.2 are limited to rodent models, there is evidence that germ cell effects—at least those induced by prenatal exposure—are not limited



		Exposure Window			End Point	
	Model	Prenatal	Pre- + Postnatal	Postnatal	Neonatal prospermatogonia #	Adult sperm production
Estrogenic						
DES						
Lassurguère et al. 2003	rat	*in vitro*			↓[1]	
Atanassova et al. 1999	rat			X		↓[1]
EE						
Porro et al. 2015	mouse		X			↓
Howdeshell et al. 2008	rat		X			↓
Lassurguère et al. 2003	rat	*in vitro*			↓[1]	
Thayer et al. 2001	mice	X				↓[1]
Atanassova et al. 1999	rat			X		↓[1]
Anti-Androgenic						
DBP						
Van den Driesche et al. 2015	rat	X	X		↓[2]	
Mahood et al. 2007	rat	X			[2]	
Gaido et al. 2007	mouse	X			[2]	
Ferrara et al. 2006	rat	X			↓[1,2]	↓
MEHP						
Lehraiki et al. 2009	mouse	*in vitro*			↓[1,2]	
Chauvigné et al. 2009	rat	*in vitro*			↓[1]	
Other						
Atrazine						
DeSesso et al. 2014	rat	X				[3]
BPA						
Salian et al. 2009a	rat			X		↓
Salian et al. 2009b	rat			X		↓
Okada and Kai 2008	mouse		X			↓
vom Saal et al. 1998	mouse	X				↓

Fig. 9.2 Summary of rodent studies providing evidence of EDC-induced germline effects. Studies using rat and mouse models and providing evidence of a significant reduction in either prospermatogonia number in the neonatal testis or in sperm production in the adult male are subdivided by type of EDC exposure, i.e., estrogenic, anti-androgenic and other (for chemicals with unknown or mixed modes of action). Adult sperm production is inclusive of observed sperm counts and germ cell volume. For each study, the window of exposure (prenatal, postnatal, or both pre- and postnatal) is denoted by an 'X' or by "in vitro," for studies utilizing ex vivo exposure to test effects on the fetal testis. Superscript numbers indicate additional observations, i.e. *1* germ cell apoptosis; *2* multinucleated prospermatogonia; and *3* abnormal sperm. As denoted in the cartoon at the top of the figure, prenatal exposure coincides with the transition of primordial germ cells to prospermatogonia, and postnatal exposure with the establishment of the spermatogonial stem cell (SSC) pool

to rodents. Specifically, evidence of an effect of prenatal phthalate exposure on early germ cell development has been reported in numerous studies in rat and mice (Ferrara et al. 2006; Mahood et al. 2007; Gaido et al. 2007; Lehraiki et al. 2009; Chauvigne et al. 2009), while similar exposure-induced reductions in prospermatogonia numbers have also been reported in analyses of human fetal testis xenographs (Van den Driesche et al. 2015; Spade et al. 2014).

Importantly, a decline in sperm production in the adult testis could be a secondary effect resulting from changes in somatic lineages. However, the reports of exposure-associated reductions in prospermatogonia number in the neonatal testis (Fig. 9.2) suggest a direct effect on the germline. In addition, a separate report provides the first evidence that estrogenic exposure affects the SSC in mice (Vrooman et al. 2015). Specifically, Vrooman et al. found that postnatal estrogenic exposure coinciding with the time of SSC formation was sufficient to permanently alter the meiotic profiles of adult male spermatocytes. Transplantation experiments demonstrated that the SSCs themselves were permanently altered by the exposure, since the phenotype of descendant spermatocytes persisted following SSC transplantation to an unexposed testicular environment. Together with the data summarized in Fig. 9.2, this suggests that several different stages of early germline development in the male rodent are vulnerable to the effects of exogenous endocrine disruptors.

It is also important to consider the possibility that, because they reside outside the blood–testis barrier, SSCs in the adult remain vulnerable to EDCs. Environmental or occupational exposure to pesticides has been implicated in declining sperm parameters in adult men (reviewed in Roeleveld and Bretveld 2008; Martenies and Perry 2013; Mehrpour et al. 2014; and Perry 2008), while exposure to plasticizers has been correlated with a variety of negative outcomes, including abnormalities in semen quality and sperm motility (reviewed in Lagos-Cabre and Moreno 2012; Manfo et al. 2014; and Peretz et al. 2014). The interpretation of these data is complicated by geographic and demographic variation in the populations under investigation. Thus, at present, multiple studies indicate a negative association between male reproductive health and these exposures, but there is no evidence of a direct effect of exposure on the SSC.

Taken together, data from human DES exposure and epidemiological studies, as well as evidence from studies of model organisms, provide compelling evidence of adverse effects of EDC exposure on male reproductive health, and specifically on the male germline. Further, at least with regard to germ cells, the effects are not limited to those induced by estrogenic activity. Thus, it seems prudent to broaden the "estrogen hypothesis" to include other effectors, i.e., the "endocrine disrupting hypothesis." Although the mechanisms underlying the effects on the germline remain unclear, growing evidence suggests that both prospermatogonia and the forming SSC pool can be affected by exposure to exogenous EDCs. This raises important questions about the sensitivity of the early germ cell and SSC epigenome to environmental influences and, because SSC descendants ultimately give rise to the next generation, the transmission to future generations of changes induced in SSCs.

9.4 Multi- and Transgenerational Effects Induced by EDCs

The transmission of EDC-induced effects across generations, including both "multigenerational" and "transgenerational" effects, has been reported in numerous studies (reviewed in Xin et al. 2015). By definition, transmission requires changes to the

germline of an exposed parent and could result from either maternal or paternal exposure, but the distinction between multi- and transgenerational effects is an important one: An effect can only be considered transgenerational if it is transmitted to an *unexposed* generation, and the number of generations required for this depends on the time of exposure and the sex of the exposed parent. Exposure during pregnancy has the potential to simultaneously induce effects in three generations: The mother (F0), her gestating fetus (F1), and the germ cells developing in the fetal gonad that will give rise to her grandchildren (F2). Thus, a maternal exposure during pregnancy can produce multigenerational effects in children and grandchildren, but can only be considered transgenerational if effects are evident in the first unexposed generation, the F3 or great grandchildren. In contrast, a non-pregnant maternal exposure or paternal exposure can simultaneously induce effects in only two generations. In males, the exposed individual and the SSCs and developing spermatocytes in his testes that will give rise to his children are directly exposed. Thus, transmission to the F2 is sufficient to demonstrate a paternal transgenerational effect.

Obtaining evidence of transgenerational effects in humans is obviously difficult. DES, the best-documented example of human developmental EDC exposure, provides evidence of effects in daughters and granddaughters of exposed women (Hatch et al. 2011; Hoover et al. 2011), but data on effects in great granddaughters are not yet available. Further, while effects have been reported in DES sons (Troisi et al. 2013; Palmer et al. 2009), data on their fertility have been comparatively sparse. In experimental studies, however, a variety of transgenerational phenotypes have been reported to result from developmental exposures to EDCs (reviewed in Martos et al. 2015 and Xin et al. 2015). In rodents, TDS-like spermatogenic defects have been reportedly transmitted to F3 males following gestational exposure to anti-androgenic (e.g. vinclozolin (Guerrero-Bosagna et al. 2012; Guerrero-Bosagna et al. 2013; Anway et al. 2005; Skinner and Anway 2005) and phthalates (Doyle et al. 2013; Quinnies et al. 2015)); estrogenic (e.g. methoxychlor (Anway et al. 2005; Manikkam et al. 2014; Skinner and Anway 2005), and dioxin, TCDD (Manikkam et al. 2012a)); or mixtures of EDCs or EDCs with mixed modes of action (e.g. BPA (Wolstenholme et al. 2013), BPA and phthalates (Manikkam et al. 2013) and DEET (Manikkam et al. 2012b)). Because the high incidence and reproducibility of effects within a study makes an EDC-induced gene mutation unlikely, these exposure effects are thought to result from induced alterations to the germline epigenome that are not erased in the next generation. Epigenetic transgenerational effects as a result of environmental exposure add a new and concerning dimension to the estrogen hypothesis (Sharpe and Skakkebaek 1993), and, if supported by sound experimental data demonstrating alterations in SSCs that are transmitted across generations, provide a sobering outlook for male fertility. Importantly, most experimental studies have assessed the effects of exposure to a single chemical; however, humans are exposed to a wide and ever-increasing range of chemicals. Thus, efforts to understand the effects of multiple exposures and exposures that span several generations are urgently needed. In addition, given the complexity of human exposure (e.g., air, water, food and beverage packaging, personal care

products, and cleaners and disinfectants), the development of new methods of screening chemicals before they are used commercially and of monitoring human exposure levels are essential.

9.5 Epigenetic Mechanisms of Transgenerational Inheritance

Identifying alterations in the SSC epigenome and tracking their transmission across generations is essential in understanding when and how spurious signals from environmental contaminants can induce multi- and transgenerational effects. This is, in fact, the key to—as well as the most intriguing feature of—transgenerational effects; transmission to an unexposed generation not only necessitates the induction of epimutations, but also their escape from sex-specific reprogramming in the germline. It is the absence of compelling documentation of this that has stirred controversy about epigenetic transgenerational inheritance in mammals (Martos et al. 2015; Heard and Martienssen 2014).

That EDC exposure can induce epigenetic changes is not in dispute. Ample evidence of epigenetic changes in somatic tissues exists (e.g., the viable yellow agouti (A^{vy}) mouse (Dolinoy et al. 2007), differential methylation at imprinted regions (Susiarjo et al. 2013) and repetitive elements in somatic tissues (Nahar et al. 2015)), with perhaps the best-characterized involving changes in the cerebral cortex, where prenatal exposure to BPA induces changes in both DNA methyltansferase expression and methylation of the ERα promotor, causing behavioral alterations in those animals (Kundakovic and Champagne 2011; Kundakovic et al. 2013, 2015; Yaoi et al. 2008). However, most reports of transgenerational studies have focused on tracing the inheritance of an induced phenotype across generations, rather than identifying an associated persistent epimutation. Further, the results of the few studies that have focused on the germline are far from definitive. Skinner and colleagues (Skinner et al. 2013) observed DNA methylation changes in PGCs and prospermatogonia of male descendants of pregnant female rats exposed to the agricultural fungicide, vinclozolin. However, analysis was limited to the first unexposed generation (F3), so it was not possible to trace the penetrance of the phenotype across generations, nor were specific changes in PGCs that persisted in prospermatogonia identified (Skinner et al. 2013). Stouder and Paoloni-Giacobino reported alterations in the differentially methylated regions of five imprinted genes in F1 offspring resulting from prenatal exposure to methoxychlor in mice. Analysis of subsequent generations revealed that effects persisted through the F3 generation but diminished with each successive generation and were not clearly correlated with TDS phenotypes (Stouder and Paoloni-Giacobino 2010). Thus, although the available data provide a clear link between EDC-induced epigenetic changes and exposure-induced phenotypes, evidence of the transmission of specific epimutations through the germline is currently lacking.

Unravelling the mechanisms of epimutation induction and transmission requires an understanding of the timing and the nature of effects induced in the germline. If

we assume that transgenerational effects result from EDC-induced changes to the germline epigenome, it seems likely that the most vulnerable stages of germ cell development would be those associated with major epigenetic events. Epigenetic reprogramming in the mammalian germline is complex, with significant epigenetic modifications characterizing several stages of male germ cell development (reviewed in Ly et al. 2015): genome-wide erasure of DNA methylation occurs as PGCs migrate to and populate the gonad (~E8.0–E13.5 in mice), and male-specific reprogramming occurs in prospermatogonia (~E14–birth in mice) in the developing testis. Although the majority of the genome is remethylated during the time of imprint establishment, a handful of retrotransposons are silenced in a subsequent piRNA-dependent wave of DNA methylation (~E17.5–P2 after birth in mice), and a subtle increase in DNA methylation corresponding with the establishment of the SSC pool occurs neonatally (reviewed in Ly et al. 2015).

Importantly, changes in DNA methylation do not occur in isolation but are generally coupled with histone modifications. Around the time of sex determination (~E12.5 in mice) a set of up to 4300 genes in the germline exist in a "poised" state, with promoters simultaneously containing histone modifications associated with activation (H3K4me3) and repression (H3K27me3). A subset remain poised in the SSC, pachytene spermatocyte, and round spermatid, and it has been suggested that these genes are critical after fertilization in the transition to a totipotent program (Lesch et al. 2013; and reviewed in Lesch and Page 2014). Histone modifications are not limited to poised promoters, however, and are dynamic throughout germ cell development: Global levels of H3K9me2, H3K27me3, and H3K4me3 are in flux in PGCs starting as early as E7.5 and continuing through late fetal development (reviewed in Ly et al. 2015). Although less well-characterized, postnatal histone modifications, including acetylation, methylation, and phosphorylation have been identified on H3 and H4 in germ cells during spermatogenesis (reviewed in Ly et al. 2015). Importantly, after the completion of meiosis, a major change in sperm chromatin occurs during spermiogenesis, with the replacement of the majority of histones by protamines (reviewed in Ly et al. 2015).

Most studies of EDC-induced epigenetic changes have focused on DNA methylation patterns, but recent data provide compelling evidence of the importance of considering other types of epimutations. Overexpression of a histone demethylase during spermatogenesis in mice was reported to result in transmission of reduced histone methylation, with specific loss of H3K4me2 at over 2300 genes in sperm for two subsequent generations (Siklenka et al. 2015). This study provides evidence that histone modifications can be transmitted and maintained in subsequent generations, challenging the traditional notion that, aside from specific incidences of H3K27me3 marks in heterochromatic regions, DNA methylation is the only type of heritable epigenetic mark. Together with data demonstrating that EDCs can affect the expression and activity of histone modifying enzymes (e.g., EZH2 downstream of estrogen receptor (Bhan et al. 2014; Bredfeldt et al. 2010; Doherty et al. 2010)), evidence linking histone modifications with EDC-induced epigenetic transgenerational effects is gaining respectability.

The extensive epigenetic modifications that are essential for the production of functional sperm would suggest that there are multiple windows—both pre- and postnatal—during which the male germ line is particularly vulnerable to disturbances in its environment. Defining these windows, understanding the epigenetic processes and cell types affected by exposure, and determining the types of exogenous signals that affect germline epigenetic programs is essential in understanding the transmission of epimutations. The results of published studies of the effects of developmental exposure to EDCs demonstrate that these essential characterization studies will be far from simple. Most studies have examined the effects of a single exposure window but, importantly, comparable results have not always been obtained even when an equivalent window and dose of exposure has been used. These studies provide ample evidence that subtle differences, including dosing strategy, strain or species of animal used, and even animal feed can influence the outcome of studies of the effects of EDCs (Mclaren 1983; Hunt et al. 2009; Gioiosa et al. 2013; Vandenberg et al. 2014). Determining if and how epigenetic changes elude erasure and reprogramming in the germ line in subsequent generations are critical pieces of information needed to validate putative epigenetic transgenerational effects. However, essential to this endeavor is a thorough understanding of the normal germ cell epigenome at different stages of fetal and postnatal development. Only with this road map can we comprehensively determine the types of epigenetic changes induced, the stages of germ cell developmental that are vulnerable, and the generations that will be affected by EDC exposure.

9.6 Conclusions

In this chapter we have attempted to illustrate some of the ways that the SSC and its descendants are influenced by the environment. Environmental effects can be mediated by both endogenous and exogenous signals. The effect of endogenous signals from the somatic cells of the testis is demonstrated by sex chromosome abnormalities, where genetic determinants (e.g., X chromosome dosage) affect the ability of SSCs to develop and survive in the testis. On a more global scale, appropriate endocrine and paracrine signals are essential for the development of the male reproductive tract, and spurious signals from exogenous sources can impact the development of male reproductive tissues, including the testis. Indeed, data from human and experimental studies demonstrate that exposure during fetal development to exogenous endocrine disrupting chemicals, whether through pharmaceuticals (e.g., DES or oral contraceptives) or environmental contaminants (e.g., phthalates and BPA), has the potential to adversely affect the reproductive capacity of the adult male. Although most experimental studies have focused on the effects of individual chemicals with endocrine disrupting properties, the additive effects of exposure to multiple contaminants more closely model human daily exposure, making an understanding of the effects of chemical mixtures essential. Further, beyond simply understanding the effects of exposure on an individual, a growing body of evidence suggesting that effects can be transmitted to subsequent generations (e.g., epigenetic transgenerational effects) raises new

concerns about the potential accumulation of genetic and epigenetic abnormalities across generations. Taken together, the currently available evidence underscores the urgency of characterizing the windows of male germ cell development (e.g., fetal/neonatal vs. adult exposure) that are vulnerable to environmental effects and understanding the mechanism(s) through which effects on the germline are induced and transmitted. The recent development of rapid toxicity screening in *C. elegans* has allowed for high-throughput analysis of chemical effects on germline differentiation, dysfunction, apoptosis, and epigenetic regulation (Lundby et al. 2016; Parodi et al. 2015). Given the rapid production and diversification of EDCs, both rapid screening methodology and insight gained from carefully designed animal studies will be instrumental in guiding the development of new treatments for male infertility. Importantly, recent data suggest that the spermatogonial stem cell itself may be a key target of estrogenic EDC exposures (Vrooman et al. 2015). Thus, given the current interest in the potential of clinical therapies involving SSCs (see Chapter 14), it is important to consider that the ex vivo handling and transplantation of these cells provide opportunities to introduce epimutations. From the standpoint of understanding the risk to male fertility posed by environmental contaminants, the ability to successfully maintain SSCs in vitro for extended time provides a unique and powerful approach to assess effects of individual chemicals and of chemical mixtures. Similarly, SSC transplantation can facilitate experimental studies to assess reprogramming in the male germline to understand the transmission of paternally induced epimutations. Thus, working to understand if and how epimutations occur during the collection, culture, and transplantation of SSCs is a critical step in discovering the environmental origins of—and developing improved treatments for—male infertility.

References

Aloisio GM, Nakada Y, Saatcioglu HD, Pena CG, Baker MD, Tarnawa ED, Mukherjee J, Manjunath H, Bugde A, Sengupta AL, Amatruda JF, Cuevas I, Hamra FK, Castrillon DH (2014) PAX7 expression defines germline stem cells in the adult testis. J Clin Invest 124:3929–3944

Anway MD, Cupp AS, Uzumcu M, Skinner MK (2005) Epigenetic transgenerational actions of endocrine disruptors and male fertility. Science 308:1466–1469

Arnheim N, Calabrese P (2016) Germline stem cell competition, mutation hot spots, genetic disorders, and older fathers. Annu Rev Genomics Hum Genet 17:219–243

Atanassova N, McKinnell C, Walker M, Turner KJ, Fisher JS, Morley M, Millar MR, Groome NP, Sharpe RM (1999) Permanent effects of neonatal estrogen exposure in rats on reproductive hormone levels, Sertoli cell number, and the efficiency of spermatogenesis in adulthood. Endocrinology 140:5364–5373

Bhan A, Hussain I, Ansari KI, Bobzean SA, Perrotti LI, Mandal SS (2014) Histone methyltransferase EZH2 is transcriptionally induced by estradiol as well as estrogenic endocrine disruptors bisphenol-A and diethylstilbestrol. J Mol Biol 426:3426–3441

Bredfeldt TG, Greathouse KL, Safe SH, Hung MC, Bedford MT, Walker CL (2010) Xenoestrogen-induced regulation of EZH2 and histone methylation via estrogen receptor signaling to PI3K/AKT. Mol Endocrinol 24:993–1006

Chauvigne F, Menuet A, Lesne L, Chagnon MC, Chevrier C, Regnier JF, Angerer J, Jegou B (2009) Time- and dose-related effects of di-(2-ethylhexyl) phthalate and its main metabolites on the function of the rat fetal testis in vitro. Environ Health Perspect 117:515–521

Desesso JM, Scialli AR, White TE, Breckenridge CB (2014) Multigeneration reproduction and male developmental toxicity studies on atrazine in rats. Birth Defects Res B Dev Reprod Toxicol 101:237–253

Doherty LF, Bromer JG, Zhou Y, Aldad TS, Taylor HS (2010) In utero exposure to diethylstilbestrol (DES) or bisphenol-A (BPA) increases EZH2 expression in the mammary gland: an epigenetic mechanism linking endocrine disruptors to breast cancer. Horm Cancer 1:146–155

Dolinoy DC, Huang D, Jirtle RL (2007) Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. Proc Natl Acad Sci U S A 104:13056–13061

Doyle TJ, Bowman JL, Windell VL, Mclean DJ, Kim KH (2013) Transgenerational effects of di-(2-ethylhexyl) phthalate on testicular germ cell associations and spermatogonial stem cells in mice. Biol Reprod 88:112

Ferrara D, Hallmark N, Scott H, Brown R, Mckinnell C, Mahood IK, Sharpe RM (2006) Acute and long-term effects of in utero exposure of rats to di(n-butyl) phthalate on testicular germ cell development and proliferation. Endocrinology 147:5352–5362

Forand A, Messiaen S, Habert R, Bernardino-Sgherri J (2009) Exposure of the mouse perinatal testis to radiation leads to hypospermia at sexual maturity. Reproduction 137:487–495

Gaido KW, Hensley JB, Liu D, Wallace DG, Borghoff S, Johnson KJ, Hall SJ, Boekelheide K (2007) Fetal mouse phthalate exposure shows that Gonocyte multinucleation is not associated with decreased testicular testosterone. Toxicol Sci 97:491–503

Gioiosa L, Parmigiani S, Vom Saal FS, Palanza P (2013) The effects of bisphenol A on emotional behavior depend upon the timing of exposure, age and gender in mice. Horm Behav 63:598–605

Griffin DK, Abruzzo MA, Millie EA, Sheean LA, Feingold E, Sherman SL, Hassold TJ (1995) Non-disjunction in human sperm: evidence for an effect of increasing paternal age. Hum Mol Genet 4:2227–2232

Guerrero-Bosagna C, Covert TR, Haque MM, Settles M, Nilsson EE, Anway MD, Skinner MK (2012) Epigenetic transgenerational inheritance of vinclozolin induced mouse adult onset disease and associated sperm epigenome biomarkers. Reprod Toxicol 34:694–707

Guerrero-Bosagna C, Savenkova M, Haque MM, Nilsson E, Skinner MK (2013) Environmentally induced epigenetic transgenerational inheritance of altered Sertoli cell transcriptome and epigenome: molecular etiology of male infertility. PLoS One 8:e59922

Harris RM, Waring RH (2012) Diethylstilboestrol—a long-term legacy. Maturitas 72:108–112

Hatch EE, Troisi R, Wise LA, Titus-Ernstoff L, Hyer M, Palmer JR, Strohsnitter WC, Robboy SJ, Anderson D, Kaufman R, Adam E, Hoover RN (2011) Preterm birth, fetal growth, and age at menarche among women exposed prenatally to diethylstilbestrol (DES). Reprod Toxicol 31:151–157

Hayes TB, Anderson LL, Beasley VR, de Solla SR, Iguchi T, Ingraham H, Kestemont P, Kniewald J, Kniewald Z, Langlois VS, Luque EH, Mccoy KA, Munoz-de-Toro M, Oka T, Oliveira CA, Orton F, Ruby S, Suzawa M, Tavera-Mendoza LE, Trudeau VL, Victor-Costa AB, Willingham E (2011) Demasculinization and feminization of male gonads by atrazine: consistent effects across vertebrate classes. J Steroid Biochem Mol Biol 127:64–73

Heard E, Martienssen RA (2014) Transgenerational epigenetic inheritance: myths and mechanisms. Cell 157:95–109

Hoover RN, Hyer M, Pfeiffer RM, Adam E, Bond B, Cheville AL, Colton T, Hartge P, Hatch EE, Herbst AL, Karlan BY, Kaufman R, Noller KL, Palmer JR, Robboy SJ, Saal RC, Strohsnitter W, Titus-Ernstoff L, Troisi R (2011) Adverse health outcomes in women exposed in utero to diethylstilbestrol. N Engl J Med 365:1304–1314

Howdeshell KL, Furr J, Lambright CR, Wilson VS, Ryan BC, Gray LE (2008) Gestational and lactational exposure to ethinyl estradiol, but not bisphenol A, decreases androgen-dependent reproductive organ weights and epididymal sperm abundance in the male long evans hooded rat. Toxicol Sci 102:371–382

Hunt PA, Susiarjo M, Rubio C, Hassold TJ (2009) The bisphenol A experience: a primer for the analysis of environmental effects on mammalian reproduction. Biol Reprod 81:807–813

Hunt PA, Worthman C, Levinson H, Stallings J, Lemaire R, Mroz K, Park C, Handel MA (1998) Germ cell loss in the XXY male mouse: altered X-chromosome dosage affects prenatal development. Mol Reprod Dev 49:101–111

Iwamoto T, Nozawa S, Mieno MN, Yamakawa K, Baba K, Yoshiike M, Namiki M, Koh E, Kanaya J, Okuyama A, Matsumiya K, Tsujimura A, Kanetake H, Eguchi J, Skakkebaek NE, Vierula M, Toppari J, Jorgensen N (2013) Semen quality of 1559 young men from four cities in Japan: a cross-sectional population-based study. BMJ Open 3

Jorgensen N, Andersen AG, Eustache F, Irvine DS, Suominen J, Petersen JH, Andersen AN, Auger J, Cawood EH, Horte A, Jensen TK, Jouannet P, Keiding N, Vierula M, Toppari J, Skakkebaek NE (2001) Regional differences in semen quality in Europe. Hum Reprod 16:1012–1019

Jorgensen N, Carlsen E, Nermoen I, Punab M, Suominen J, Andersen AG, Andersson AM, Haugen TB, Horte A, Jensen TK, Magnus O, Petersen JH, Vierula M, Toppari J, Skakkebaek NE (2002) East-west gradient in semen quality in the Nordic-Baltic area: a study of men from the general population in Denmark, Norway, Estonia and Finland. Hum Reprod 17:2199–2208

Jorgensen N, Vierula M, Jacobsen R, Pukkala E, Perheentupa A, Virtanen HE, Skakkebaek NE, Toppari J (2011) Recent adverse trends in semen quality and testis cancer incidence among Finnish men. Int J Androl 34:e37–e48

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

Kundakovic M, Champagne FA (2011) Epigenetic perspective on the developmental effects of bisphenol A. Brain Behav Immun 25:1084–1093

Kundakovic M, Gudsnuk K, Franks B, Madrid J, Miller RL, Perera FP, Champagne FA (2013) Sex-specific epigenetic disruption and behavioral changes following low-dose in utero bisphenol A exposure. Proc Natl Acad Sci U S A 110:9956–9961

Kundakovic M, Gudsnuk K, Herbstman JB, Tang D, Perera FP, Champagne FA (2015) DNA methylation of BDNF as a biomarker of early-life adversity. Proc Natl Acad Sci U S A 112:6807–6813

Lagos-Cabre R, Moreno RD (2012) Contribution of environmental pollutants to male infertily: a working model of germ cell apoptosis induced by plasticizers. Biol Res 45:5–14

Lanfranco F, Kamischke A, Zitzmann M, Nieschlag E (2004) Klinefelter's syndrome. Lancet 364:273–283

Lassurguere J, Livera G, Habert R, Jegou B (2003) Time- and dose-related effects of estradiol and diethylstilbestrol on the morphology and function of the fetal rat testis in culture. Toxicol Sci 73:160–169

Lehraiki A, Racine C, Krust A, Habert R, Levacher C (2009) Phthalates impair germ cell number in the mouse fetal testis by an androgen- and estrogen-independent mechanism. Toxicol Sci 111:372–382

Lesch BJ, Dokshin GA, Young RA, Mccarrey JR, Page DC (2013) A set of genes critical to development is epigenetically poised in mouse germ cells from fetal stages through completion of meiosis. Proc Natl Acad Sci U S A 110:16061–16066

Lesch BJ, Page DC (2014) Poised chromatin in the mammalian germ line. Development 141:3619–3626

Levine H, Jørgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, Pinotti R, Swan SH (2017) Temporal trends in sperm count: a systematic review and meta-regression analysis. Hum Reprod Update 1–14

Lucas BE, Fields C, Joshi N, Hofmann MC (2012) Mono-(2-ethylhexyl)-phthalate (MEHP) affects ERK-dependent GDNF signalling in mouse stem-progenitor spermatogonia. Toxicology 299:10–19

Lundby Z, Camacho J, Allard P (2016) Fast functional germline and epigenetic assays in the nematode caenorhabditis elegans. Methods Mol Biol 1473:99–107

Ly L, Chan D, Trasler JM (2015) Developmental windows of susceptibility for epigenetic inheritance through the male germline. Semin Cell Dev Biol 43:96–105

Maatouk DM, Mork L, Hinson A, Kobayashi A, Mcmahon AP, Capel B (2012) Germ cells are not required to establish the female pathway in mouse fetal gonads. PLoS One 7:e47238
Mahood IK, Scott HM, Brown R, Hallmark N, Walker M, Sharpe RM (2007) In utero exposure to di(n-butyl) phthalate and testicular dysgenesis: comparison of fetal and adult end points and their dose sensitivity. Environ Health Perspect 115(Suppl 1):55–61
Manfo FP, Jubendradass R, Nantia EA, Moundipa PF, Mathur PP (2014) Adverse effects of bisphenol A on male reproductive function. Rev Environ Contam Toxicol 228:57–82
Manikkam M, Haque MM, Guerrero-Bosagna C, Nilsson EE, Skinner MK (2014) Pesticide methoxychlor promotes the epigenetic transgenerational inheritance of adult-onset disease through the female germline. PLoS One 9:e102091
Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK (2012a) Dioxin (TCDD) induces epigenetic transgenerational inheritance of adult onset disease and sperm epimutations. PLoS One 7:e46249
Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK (2012b) Pesticide and insect repellent mixture (permethrin and DEET) induces epigenetic transgenerational inheritance of disease and sperm epimutations. Reprod Toxicol 34:708–719
Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK (2013) Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. PLoS One 8:e55387
Martenies SE, Perry MJ (2013) Environmental and occupational pesticide exposure and human sperm parameters: a systematic review. Toxicology 307:66–73
Martos SN, Tang WY, Wang Z (2015) Elusive inheritance: Transgenerational effects and epigenetic inheritance in human environmental disease. Prog Biophys Mol Biol 118:44–54
Mclaren A (1983) Studies on mouse germ cells inside and outside the gonad. J Exp Zool 228:167–171
Mehrpour O, Karrari P, Zamani N, Tsatsakis AM, Abdollahi M (2014) Occupational exposure to pesticides and consequences on male semen and fertility: a review. Toxicol Lett 230:146–156
Mendiola J, Jorgensen N, Minguez-Alarcon L, Sarabia-Cos L, Lopez-Espin JJ, Vivero-Salmeron G, Ruiz-Ruiz KJ, Fernandez MF, Olea N, Swan SH, Torres-Cantero AM (2013) Sperm counts may have declined in young university students in Southern Spain. Andrology 1:408–413
Mroz K, Carrel L, Hunt PA (1999) Germ cell development in the XXY mouse: evidence that X chromosome reactivation is independent of sexual differentiation. Dev Biol 207:229–238
Nagaoka SI, Hassold TJ, Hunt PA (2012) Human aneuploidy: mechanisms and new insights into an age-old problem. Nat Rev Genet 13:493–504
Nahar MS, Liao C, Kannan K, Harris C, Dolinoy DC (2015) In utero bisphenol A concentration, metabolism, and global DNA methylation across matched placenta, kidney, and liver in the human fetus. Chemosphere 124:54–60
Okada A, Kai O (2008) Effects of estradiol-17beta and bisphenol A administered chronically to mice throughout pregnancy and lactation on the male pups' reproductive system. Asian J Androl 10:271–276
Ortiz-Zarragoitia M, Bizarro C, Rojo-Bartolome I, de Cerio OD, Cajaraville MP, Cancio I (2014) Mugilid fish are sentinels of exposure to endocrine disrupting compounds in coastal and estuarine environments. Mar Drugs 12:4756–4782
Palmer JR, Herbst AL, Noller KL, Boggs DA, Troisi R, Titus-Ernstoff L, Hatch EE, Wise LA, Strohsnitter WC, Hoover RN (2009) Urogenital abnormalities in men exposed to diethylstilbestrol in utero: a cohort study. Environ Health 8:37
Parodi DA, Damoiseaux R, Allard P (2015) Comprehensive assessment of germline chemical toxicity using the nematode Caenorhabditis elegans. J Vis Exp (96):52445
Paul C, Nagano M, Robaire B (2013) Aging results in molecular changes in an enriched population of undifferentiated rat spermatogonia. Biol Reprod 89:147
Paul C, Robaire B (2013) Ageing of the male germ line. Nat Rev Urol 10:227–234
Peretz J, Vrooman L, Ricke WA, Hunt PA, Ehrlich S, Hauser R, Padmanabhan V, Taylor HS, Swan SH, Vandevoort CA, Flaws JA (2014) Bisphenol a and reproductive health: update of experimental and human evidence, 2007–2013. Environ Health Perspect 122:775–786

Perry MJ (2008) Effects of environmental and occupational pesticide exposure on human sperm: a systematic review. Hum Reprod Update 14:233–242
Plotton I, Brosse A, Cuzin B, Lejeune H (2014) Klinefelter syndrome and TESE-ICSI. Ann Endocrinol (Paris) 75:118–125
Porro V, Pagotto R, Harreguy MB, Ramirez S, Crispo M, Santamaria C, Luque EH, Rodriguez HA, Bollati-Fogolin M (2015) Characterization of Oct4-GFP transgenic mice as a model to study the effect of environmental estrogens on the maturation of male germ cells by using flow cytometry. J Steroid Biochem Mol Biol 154:53–61
Quinnies KM, Doyle TJ, Kim KH, Rissman EF (2015) Transgenerational effects of Di-(2-Ethylhexyl) phthalate (DEHP) on stress hormones and behavior. Endocrinology 156:3077–3083
Reed CE, Fenton SE (2013) Exposure to diethylstilbestrol during sensitive life stages: a legacy of heritable health effects. Birth Defects Res C Embryo Today 99:134–146
Richter CA, Birnbaum LS, Farabollini F, Newbold RR, Rubin BS, Talsness CE, Vandenbergh JG, Walser-Kuntz DR, vom Saal FS (2007) In vivo effects of bisphenol A in laboratory rodent studies. Reprod Toxicol 24:199–224
Roeleveld N, Bretveld R (2008) The impact of pesticides on male fertility. Curr Opin Obstet Gynecol 20:229–233
Royo H, Polikiewicz G, Mahadevaiah SK, Prosser H, Mitchell M, Bradley A, de Rooij DG, Burgoyne PS, Turner JM (2010) Evidence that meiotic sex chromosome inactivation is essential for male fertility. Curr Biol 20:2117–2123
Ryu BY, Orwig KE, Oatley JM, Avarbock MR, Brinster RL (2006) Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. Stem Cells 24:1505–1511
Saitou M, Kagiwada S, Kurimoto K (2012) Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development 139:15–31
Saitou M, Yamaji M (2012) Primordial germ cells in mice. Cold Spring Harb Perspect Biol 4(11)
Salian S, Doshi T, Vanage G (2009a) Neonatal exposure of male rats to Bisphenol A impairs fertility and expression of sertoli cell junctional proteins in the testis. Toxicology 265:56–67
Salian S, Doshi T, Vanage G (2009b) Perinatal exposure of rats to Bisphenol A affects the fertility of male offspring. Life Sci 85:742–752
Sharma R, Agarwal A, Rohra VK, Assidi M, Abu-Elmagd M, Turki RF (2015) Effects of increased paternal age on sperm quality, reproductive outcome and associated epigenetic risks to offspring. Reprod Biol Endocrinol 13:35
Sharpe RM (2003) The 'oestrogen hypothesis'—where do we stand now? Int J Androl 26:2–15
Sharpe RM, Skakkebaek NE (1993) Are oestrogens involved in falling sperm counts and disorders of the male reproductive tract? Lancet 341:1392–1395
Shi Q, Martin RH (2000) Multicolor fluorescence in situ hybridization analysis of meiotic chromosome segregation in a 47,XYY male and a review of the literature. Am J Med Genet 93:40–46
Siklenka K, Erkek S, Godmann M, Lambrot R, Mcgraw S, Lafleur C, Cohen T, Xia J, Suderman M, Hallett M, Trasler J, Peters AH, Kimmins S (2015) Disruption of histone methylation in developing sperm impairs offspring health transgenerationally. Science 350:aab2006
Skakkebaek NE, Rajpert-de Meyts E, Buck Louis GM, Toppari J, Andersson AM, Eisenberg ML, Jensen TK, Jorgensen N, Swan SH, Sapra KJ, Ziebe S, Priskorn L, Juul A (2016) Male reproductive disorders and fertility trends: influences of environment and genetic susceptibility. Physiol Rev 96:55–97
Skinner MK, Anway MD (2005) Seminiferous cord formation and germ-cell programming: epigenetic transgenerational actions of endocrine disruptors. Ann N Y Acad Sci 1061:18–32
Skinner MK, Guerrero-Bosagna C, Haque M, Nilsson E, Bhandari R, Mccarrey JR (2013) Environmentally induced transgenerational epigenetic reprogramming of primordial germ cells and the subsequent germ line. PLoS One 8:e66318
Spade DJ, Hall SJ, Saffarini CM, Huse SM, Mcdonnell EV, Boekelheide K (2014) Differential response to abiraterone acetate and di-n-butyl phthalate in an androgen-sensitive human fetal testis xenograft bioassay. Toxicol Sci 138:148–160
Stouder C, Paoloni-Giacobino A (2010) Transgenerational effects of the endocrine disruptor vinclozolin on the methylation pattern of imprinted genes in the mouse sperm. Reproduction 139:373–379

Susiarjo M, Sasson I, Mesaros C, Bartolomei MS (2013) Bisphenol a exposure disrupts genomic imprinting in the mouse. PLoS Genet 9:e1003401

Thayer KA, Ruhlen RL, Howdeshell KL, Buchanan DL, Cooke PS, Preziosi D, Welshons WV, Haseman J, vom Saal FS (2001) Altered prostate growth and daily sperm production in male mice exposed prenatally to subclinical doses of 17alpha-ethinyl oestradiol. Hum Reprod 16:988–996

Troisi R, Hyer M, Hatch EE, Titus-Ernstoff L, Palmer JR, Strohsnitter WC, Herbst AL, Adam E, Hoover RN (2013) Medical conditions among adult offspring prenatally exposed to diethylstilbestrol. Epidemiology 24:430–438

Turner JM (2015) Meiotic silencing in mammals. Annu Rev Genet 49:395–412

Van den Driesche S, Mckinnell C, Calarrao A, Kennedy L, Hutchison GR, Hrabalkova L, Jobling MS, Macpherson S, Anderson RA, Sharpe RM, Mitchell RT (2015) Comparative effects of di(n-butyl) phthalate exposure on fetal germ cell development in the rat and in human fetal testis xenografts. Environ Health Perspect 123:223–230

Van der Meer Y, Huiskramp R, Davids JAG, Van der Tweel I, de Rooij DG (1992) The sensitivity to X rays of mouse spermatogonia that are committed to differentiate and of differentiating spermatogonia. Radiat Res 130:296–302

Vandenberg LN, Welshons WV, Vom Saal FS, Toutain PL, Myers JP (2014) Should oral gavage be abandoned in toxicity testing of endocrine disruptors? Environ Health 13:46

vom Saal FS, Cooke PS, Buchanan DL, Palanza P, Thayer KA, Nagel SC, Parmigiani S, Welshons WV (1998) A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. Toxicol Ind Health 14:239–260

Vrooman LA, Oatley JM, Griswold JE, Hassold TJ, Hunt PA (2015) Estrogenic exposure alters the spermatogonial stem cells in the developing testis, permanently reducing crossover levels in the adult. PLoS Genet 11:e1004949

Wolstenholme JT, Goldsby JA, Rissman EF (2013) Transgenerational effects of prenatal bisphenol A on social recognition. Horm Behav 64:833–839

Xin F, Susiarjo M, Bartolomei MS (2015) Multigenerational and transgenerational effects of endocrine disrupting chemicals: a role for altered epigenetic regulation? Semin Cell Dev Biol 43:66–75

Yaoi T, Itoh K, Nakamura K, Ogi H, Fujiwara Y, Fushiki S (2008) Genome-wide analysis of epigenomic alterations in fetal mouse forebrain after exposure to low doses of bisphenol A. Biochem Biophys Res Commun 376:563–567

Zhang X, Ebata KT, Robaire B, Nagano MC (2006) Aging of male germ line stem cells in mice. Biol Reprod 74:119–124

Testicular Germ Cell Tumors and Teratomas

10

Denise G. Lanza and Jason D. Heaney

Abstract

Testicular germ cell tumors (TGCTs) lie at the intersection of cancer and developmental biology. These tumors arise from defects in germ cell development, pluripotent primordial germ cells that fail to develop into normal male gametes. To understand the developmental defects that allow these tumors to form, we must study the developmental biology surrounding embryonic germ cell development, specifically during sex specification. Fortunately, excellent mouse models are available that recapitulate the pathology of the human disease. In this chapter, we focus on what has been learned by studying embryonic germ cell development in the 129/Sv inbred mouse model, and how this model is contributing to the study of human TGCTs.

Keywords

Teratoma • Germ cell tumor • 129 mice • Embryonal carcinoma • Germ cell neoplasia in situ

10.1 Pathogenesis of Human Testicular Germ Cell Tumors

TGCTs represent three of the five types of germ cell tumors, as described by Oosterhuis and Looijenga (2005) and recognized by the World Health Organization (Ulbright et al. 2016): (Type I) the teratomas and yolk-sac tumors of newborns and infants; (Type II) the seminomatous and non-seminomatous tumors of adolescents

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and young adults; and (Type III) the spermatocytic seminomas of the elderly (Oosterhuis et al. 1997; Looijenga and Oosterhuis 2002). These three types of TGCTs are classified based on chromosomal constitution and genomic imprinting, in addition to epidemiology and clinical presentation. Type I infantile germ cell tumors and Type III spermatocytic tumors are rare, with incidences of 0.2–0.3 per million and 0.4 per million individuals for each respective age group (Carriere et al. 2007; Ulbright et al. 2016). Type II TGCTs are the most frequent type of solid tumor diagnosed in Caucasian males 20–40 years of age in industrialized nations, with incidences in the range of 6–11 per 100,000 individuals; however, Type II TGCT incidences are much lower among non-whites in developing countries (Ulbright et al. 2016).

10.1.1 Cell of Origin and Pathology of Human TGCTs

All type I germ cell tumors are proposed to arise from primordial germ cells (PGCs) that have undergone immediate transformation into pluripotent embryonal carcinoma cells (ECCs) and clinically manifest before puberty. These germ cell tumors most often arise in the gonads but can also develop extragonadally, and are histologically classified as teratomas, yolk sac tumors, embryonal carcinomas, and mixed tumors, such as teratocarcinomas (teratomas with ECC elements; Fig. 10.1) (Oosterhuis et al. 1997; Oosterhuis and Looijenga 2005). In teratomas all three germinal layers are present (endoderm, mesoderm, and ectoderm). Additionally, teratomas may be composed exclusively of well differentiated, mature tissues that are typically benign, or have immature, fetal-like tissues that can be highly malignant (Ulbright et al. 2016). The most common testicular neoplasm in children are yolk sac tumors, which contain tissues that recapitulate the yolk sac, allantois, and other extra-embryonic lineages (Kaplan et al. 1988).

Type II TGCTs arise from a precursor lesion, termed germ cell neoplasia in situ (GCNIS) of the seminiferous tubules (Skakkebaek 1978; Ulbright et al. 2016) (Fig. 10.1). GCNIS cells are positive for the kit receptor (KIT) (Rajpert-De Meyts and Skakkebaek 1994) and the pluripotency factor POU5F1 (OCT4) (Palumbo et al. 2002). However, GCNIS cells appear to not express all factors necessary to establish a pluripotent state.

Although GCNIS originates during embryogenesis, Type II TGCTs clinically manifest at or after puberty and are histologically subclassified as seminomas, non-seminomas, or tumors of mixed seminoma and non-seminomas components. The default pathway of Type II TGCTs is hypothesized to be from GCNIS towards the development of a seminoma, which consists of undifferentiated, KIT/OCT4-positive cells morphologically similar to GCNIS. The development of a non-seminoma requires activation (reprogramming) of pluripotency in either a GCNIS cell or a seminoma cell to establish ECCs (Oosterhuis and Looijenga 2005). Non-seminomas are found as pure tumor types (teratomas, embryonal carcinomas, yolk sac tumors, and choriocarcinomas) or as mixed tumor types, either as mixed non-seminoma (including teratocarcinomas) or as mixed seminoma and non-seminoma

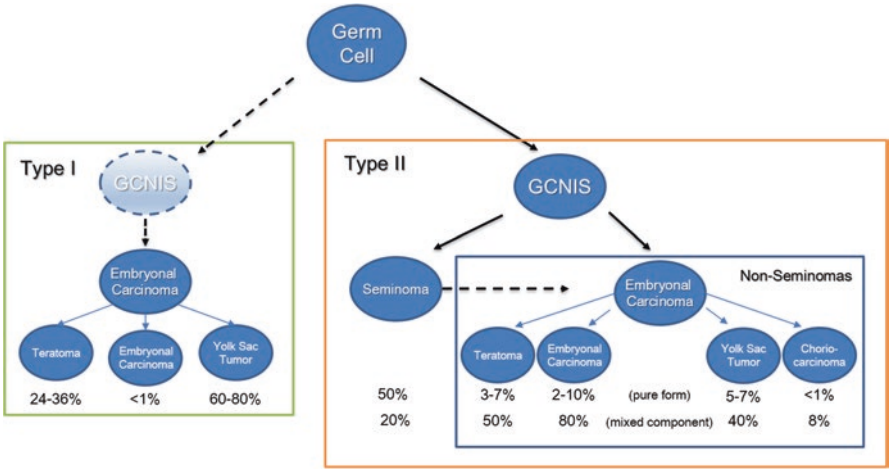

Fig. 10.1 Cells of origin and pathogenesis of Type I and II testicular germ cell tumors (TGCTs). Both Type I and Type II TGCTS arise during embryogenesis from primordial germ cells (PGCs). In Type I TGCTs, embryonic germ cells are proposed to directly transform into pluripotent embryonal carcinoma cells (ECCs) , which form pure ECC tumors or differentiate to form teratomas or yolk sac tumors. Testicular teratomas in 129 inbred mice are also proposed to arise from gonocytes that directly transform into ECCs. However, evidence from both human and mouse studies suggest that a transient germ cell neoplasia in situ (GCNIS)-like state may occur prior to ECC formation. In Type II TGCTs, GCNIS precursor cells have been identified as the cell-of-origin. These cells give rise to either seminomatous or non-seminomatous tumors. Non-seminomas are the result of reprogramming of GCNIS, or potentially seminomatous tumor cells, into pluripotent ECCs, which form pure ECC tumors or differentiate to form teratoma, yolk sac tumor, or choriocarcinoma. Non-seminomas are generally found in mixed tumors (i.e. seminoma and non-seminoma components), however, pure forms are also observed. Of the type II TGCTs, 50% are pure seminomas and 30% are non-seminomas, with the remaining percentage of tumors a mix of seminoma and non-seminoma (Horwich et al. 2006). Percentages for Type I TGCTs and Type II Non-seminomas were collected from several references (Krag Jacobsen et al. 1984; Mostofi et al. 1988; Howlader et al. 2012; Ulbright et al. 2016)

(Bahrami et al. 2007). Pure form embryonal carcinomas comprise only 2–10%, while more than 80% of mixed tumors have embryonal carcinoma as a component (Mostofi et al. 1988). Yolk sac tumors in adults are more often seen in mixed non-seminomas, occurring in about 40% of non-seminomas (Ulbright et al. 2016). Pure choriocarcinoma represents less than 1% (0.19%) of TGCTs; choriocarcinoma is also found mixed with other germ cell tumor elements in 8% of TGCTs (Krag Jacobsen et al. 1984).

Genomic imprinting studies suggest Type I and Type II TGCTs originate from PGCs at different stages of development (van Gurp et al. 1994; Ross et al. 1999; Bussey et al. 2001; Schneider et al. 2001). Type I teratomas and yolk sac tumors of infants show a slightly different pattern of genomic imprinting (Ross et al. 1999; Schneider et al. 2001), supporting the model that these tumors originate from an earlier stage of germ cell development than Type II TGCTs. Based on genomic imprinting patterns Type I teratomas and yolk sac tumors have been postulated to

originate from an early PGC that has retained biparental epigenetic marks (Oosterhuis et al. 1997; Oosterhuis and Looijenga 2005). Type II TGCTs are most likely derived from a PGC blocked or delayed in maturation and with erased genomic imprinting (Oosterhuis and Looijenga 2005). Changes in DNA methylation is a hallmark of most cancers, and like developing PGCs, changes in DNA methylation status may contribute to genome instability to promote transformation to ECCs. Interestingly, a significant difference in genome methylation has been reported between seminomas (hypomethylated) and non-seminomas (hypermethylated) (Gillis et al. 1997; Smiraglia et al. 2002). The difference in methylation status could reflect the pluripotent potential of each subtype and the capacity of the non-seminomas to mimic embryonal and extra-embryonal development. Gene expression patterns of OCT4 and X-inactivation status in TGCTs support this theory (Looijenga et al. 1997; Palumbo et al. 2002).

There is conflicting data as to whether GCNIS exists in both Type I and Type II TGCTs. Several groups have reported that GCNIS is not observed in Type I TGCTs and therefore is not a precursor of these tumors; transformed PGCs progress directly to ECCs (Koide et al. 1987; Manivel et al. 1988, 1989; Soosay et al. 1991). Other studies provide evidence that TGCTs of infants and young men share a common precursor, and have documented GCNIS in Type I infantile teratomas (Stamp et al. 1993; Stamp and Jacobsen 1995). It is possible that GCNIS is a transient state of tumor progression in Type I TGCTs and is more difficult to observe clinically in fully developed tumors (Fig. 10.1). The transient state model is similar to that proposed for transition of seminomas to non-seminomas, where a clinically manifested seminoma stage may not be observed (de Jong et al. 1990).

Type III spermatocytic tumors display paternal patterning of genomic imprinting, and therefore most likely do not have an embryonic origin but instead develop from spermatocytes (Looijenga et al. 2006, 2007). This chapter focuses on the pathogenesis and genetics of Type I and Type II TGCTs, which initiate during embryogenesis. A review on Type III spermatocytic tumors is available (Looijenga et al. 1994).

10.1.2 Comparisons of Cell of Origin and Pathology of TGCTs in 129 Inbred Mice to Human TGCTs

Considering the embryonic origins of Type I and II of TGCTs, animal models are critical for the study of tumor initiation and pathogenesis. The 129/Sv inbred strain of mice has a spontaneous TGCT incidence between 5 and 10%. These spontaneous tumors closely resemble human Type I infantile teratomas and share many pathological characteristics with adult Type II non-seminomas. However, an animal model that fully recapitulates all of Type II TGCT pathology has not been established. In this chapter, we focus on what has been learned by studying embryonic germ cell development in the 129/Sv inbred mouse model, and how this model is contributing to the study of human Type I and II TGCTs.

TGCTs in 129/Sv mice are first evident microscopically at E15 as foci of EC cells, and macroscopically at 3–4 weeks after birth. (Stevens 1962, 1967a, b; Vos et al. 1990; Rodriguez et al. 1992; Looijenga et al. 1998). Tumors evolve in tissue type from being mainly comprised of EC cells in embryonic and neonatal mice. Shortly after birth, tumors will contain both differentiated and embryonal tissues, comprising a teratocarcinoma (Pierce et al. 1967; Matin et al. 1998). Most adult germ cell tumors in mice are benign teratomas, however ECCs can persist, as evident by the ability to transplant primary tumor cells to the testis of adult mice to form new tumors (Stevens 1958, 1981). Seminal studies from Leroy Stevens demonstrated the transplantability of genital ridges from E12.5129/Sv mice into adult testes to form teratomas, demonstrating the PGC as the originating cell of teratomas in mice (Stevens 1967b). Curiously, ECCs morphologically resemble totipotent cells of normal embryos (Pierce et al. 1967) and have similar developmental potential (Kleinsmith and Pierce 1964). ECCs are also similar to ES cells obtained from the inner cell mass of blastocysts of normal preimplantation mouse embryos; depending on the microenvironment ECCs can participate in normal mouse development (Martin 1981). Blastocysts injected with in vivo passaged ECCs give rise to chimeric offspring, demonstrating that ECCs can revert to aspects of normal development (Martin 1981; Rossant and Papaioannou 1984; Chadalavada et al. 2007).

It has been suggested that GCNIS is not the TGCT precursor lesion in 129/Sv mice, and similar to Type I infantile teratomas, TGCTs in mice also develop directly from the transformation of PGCs to ECCs (around E15) (Walt et al. 1993). However, as suggested from the human Type I TGCTs data, GCNIS in mice may be a transient stage that is not observed in developed tumors. Curiously, atypical gonocytes resembling GCNIS of humans have been observed in mice (Stevens and Bunker 1964; Walt et al. 1993). These abnormal cells have been disregarded as GCNIS because they are present in both TGCT susceptible (129/Sv) and nonsusceptible mouse strains, and in the nontumorigenic grafts of experimentally induced TGCT studies. However, the pro-survival, anti-apoptotic environment of the 129/Sv background may be required for tumor progression beyond GCNIS, which will be discussed in detail later.

10.2 Human TGCT Chemoresistance

TGCTs are highly treatable (>95% cure rate) by surgery, radiation, and platinum-based (e.g. cisplatin) chemotherapy, which induces apoptosis through DNA damage (Bosl and Motzer 1997; Horwich et al. 2006). However, there are limited treatment options for patients that demonstrate platinum resistance, a group for whom the long-term survival rate decreases to 10–15% (Mayer et al. 2003; Horwich et al. 2006; Nitzsche et al. 2012). Several hypotheses have been proposed to explain both the exceptionally high sensitivity of TGCTs to platinum-based therapy and the evolution of resistance in a small subset of tumors (Litchfield et al. 2016). One of the most convincing models for TGCT hypersensitivity to platinum is active (or even upregulated) TP53 mediating apoptotic responses to DNA damage (Gutekunst et al. 2011).

Unlike most solid tumors, mutations in *TP53* are extremely rare in TGCTs (Litchfield et al. 2016; Taylor-Weiner et al. 2016). The mechanisms driving platinum resistance remain unclear and are likely to involve genetic and epigenetic changes. Targeted analysis of mutational hotspots have identified chemoresistant-specific mutations in *PIK3CA*, *AKT1*, *RAS*, and *FGFR3* in a subset of tumors (Feldman et al. 2014). Moreover, whole exome sequencing of two treatment resistant TGCTs identified mutations in the DNA repair gene *XRCC2*, suggesting that activation of DNA repair pathways and the corresponding suppression of apoptosis induced by DNA damage may induce chemoresistance (Litchfield et al. 2015c). Finally, *CCND1* overexpression in TGCTs, as well as other tumor types, has been associated with cisplatin resistance (Noel et al. 2010). The role of CCND1 in promoting cell cycle progression and suppression of apoptosis has been proposed to mediate resistance (Zhou et al. 2009).

Curiously, a recent analysis of teratomas and transformed carcinomas that developed chemoresistance revealed a loss of pluripotency marker expression (*NANOG* and *POU5F1*), suggesting that tumor differentiation drives resistance (Taylor-Weiner et al. 2016). In agreement with these genetic findings, in vitro studies employing human EC cell lines demonstrated that retinoic acid-induced differentiation, and the resulting loss of *NANOG* and *POU5F1* expression, increased cisplatin resistance (Abada and Howell 2014). Moreover, cisplatin alone was sufficient to reduce pluripotency gene expression and induce resistance to itself. Importantly, this same study demonstrated that enforced expression of *NANOG* can suppress cisplatin resistance. How differentiation facilitates chemoresistance has not been directly tested. However, the link between differentiation and resistance to apoptosis in pluripotent cell types has been proposed as the underlying mechanism for resistance (Abada and Howell 2014). Importantly, these findings may explain the overall sensitivity of undifferentiated seminomas and resistance of differentiated non-seminomas to systemic therapy (Oosterhuis and Looijenga 2005).

10.3 Genetic Contributions to TGCTs

10.3.1 Genetic Susceptibility and Human Genome-Wide Association Studies

There is a strong genetic component of human TGCTs, indicated by a high familial index (Lindelof and Eklund 2001) and significantly elevated relative risk of sons and brothers of affected individuals (Heimdal et al. 1996; Bromen et al. 2004; Chia et al. 2009). The heritability of TGCTs is third highest among all cancers, with genetic effects accounting for nearly 50% of risk (Heimdal et al. 1997; Czene et al. 2002; Litchfield et al. 2015d). Traditional genetic studies of candidate gene approaches and linkage analysis have been hampered by the genetic complexity of TGCT development and the lack of multigenerational pedigrees with affected individuals (Rapley et al. 2000; Nathanson et al. 2005; Crockford et al. 2006). In contrast, recent genome-wide association studies (GWAS) have identified approximately 25 genomic intervals (loci) associated with TGCT risk (Kanetsky et al. 2009, 2011;

Rapley et al. 2009; Turnbull et al. 2010; Kratz et al. 2011; Poynter et al. 2012; Andreassen et al. 2013; Chung et al. 2013; Ruark et al. 2013; Litchfield et al. 2015a). These loci have provided considerable new insights into testicular germ cell tumorigenesis, implicating genes involved in PGC specification and/or sex differentiation (*DAZL*, *PRDM14*, *HPGDS DMRT1,* and *ZFPM1*), including the KIT-KITLG signaling pathway (*KITLG*, *SPRY4*, *BAK1*, *GAB2*), and genes involved in microtubule assembly (*TEX14*, *CENPE*, *PMF1,* and *MAD1L1*), DNA repair (*RAD51C* and *RFWD3*), apoptosis (*BAK1*, *CLPTM1L*, and *GSPT1*) and telomerase regulation (*TERT*, *ATF7IP*, and *PITX1*) (Litchfield et al. 2015a). The strong genetic component to TGCTs is underlined by the per-allele odds ratios (ORs) for TGCT susceptibility loci, which are often in excess of 2.5, among the highest reported in GWAS of any cancer type (Chanock 2009). The TGCT-associated SNP rs995030 (in *KITLG)* has the strongest effect of all common SNPs for which a statistically significant association with a cancer phenotype has been reported (Welter et al. 2014). Notably, TGCT susceptibility loci interact in an additive rather than epistatic manner and are predominantly dominant; SNP variants identified often represent the common allele in the population.

Historically, none of the loci identified in GWAS show significant differences in effect on tumor risk when comparing Type II seminomas and non-seminomas (Kanetsky et al. 2009; Rapley et al. 2009; Rapley and Nathanson 2010; Turnbull et al. 2010; Ruark et al. 2013; Litchfield et al. 2015b). The absence of a difference between seminoma and non-seminoma is rather remarkable, as additional GWAS are conducted, sample sizes are now sufficiently powered to detect a difference in these two subgroups (Litchfield et al. 2015b). Follow-up studies investigating risk alleles have not yet identified associations with histological subtype (Karlsson et al. 2013). However, the absence of difference between seminoma and non-seminoma for assessing risk with GWAS TGCT loci is not surprising, considering both tumor types arise from the same cell of origin. Additionally, at least 10–15% of TGCT tumors identified are of mixed pathology (Gori et al. 2005; Horwich et al. 2006) and bilateral and familial cases do not show evidence of clustering within histological subtype or display histological similarity greater than that expected by chance (Forman et al. 1992; Mai et al. 2010). Despite the diversity in Type II TGCT subtypes, these findings provide further evidence that there is relative uniformity and complexity in the genetics of susceptibility. The genetic similarity between TGCT histological subtypes can most likely be attributed to the germ cell origin of TGCTs and early pathogenesis of the disease.

10.3.2 Genetic Susceptibility in 129/Sv Mice

The development of spontaneous TGCTs in 129/Sv mice but not in other inbred mouse strains denotes the complex genetic component of TGCT susceptibility. Classic genetic approaches, such as segregating crosses between 129/Sv and other strains, have failed to identify susceptibility loci in 129 mice, due to the complex genetic interactions required for tumor initiation (Matin et al. 1999; Muller et al. 2000;

Table 10.1 Published genetic variants that affect TGCT susceptibility in 129/Sv mice

Gene/locus	Function in wild-type	Mutation	TGCT (%)	Reference
129/Sv	Unknown	Control	3–10	Stevens and Hummel (1957)
Trp53	Cell cycle, apoptosis	Knockout	15, 35[a]	Harvey et al. (1993)
M19	SF1 deficiency; unknown	CSS	24, 80[a]	Matin et al. (1999) and Zhu et al. (2010)
Pten	Lipid phosphatase	Knockout	100[b]	Kimura et al. (2003)
Dnd1	RNA binding and editing	Nonsense	17, 94[a]	Youngren et al. (2005) and Cook et al. (2011)
Kitl	KIT receptor ligand	Deletion	14[c]	Heaney et al. (2008)
Dmrt1	Transcription factor	Knockout	4, 90[a]	Krentz et al. 2009)
Eif2s2	Translation initiation	Deletion	36[c,d]	Heaney et al. (2009)
M18	Unknown	CSS	0[b]	Anderson et al. (2009)
Apobec1	RNA binding and editing	Deletion	4[b]	Nelson et al. (2012)
Nanos3	RNA binding and editing	Deletion	45[c]	Schemmer et al. (2013)
Tfap2c	Transcription factor	Deletion	82[c]	Schemmer et al. (2013)
Ago2	RNA interference	Knockout	1–4[c]	Carouge et al. (2016)
A1cf	APOBEC1 complementation factor	Knockout	2–5[c]	Carouge et al. (2016)
Ccnd1	Cell cycle regulator	Knockout	65, 29[a,d]	Lanza et al. (2016)

CSS, chromosome substitution strain
[a]Heterozygotes and homozygotes, respectively
[b]Homozygotes
[c]Heterozygotes
[d]Allele surveyed on M19 CSS

Anderson et al. 2009; Zhu and Matin 2014). In segregating crosses between 129/Sv and other strains, only 1 affected male was found among more than 11,000 progeny tested, which is consistent with as many as 15 different genes that interact to control TGCT susceptibility (Stevens and Mackensen 1961; Stevens 1967a, 1981; Matin et al. 1998, 1999; Jiang and Nadeau 2001). The low frequency of affected males (0.01%) in the segregating population precludes analysis of TGCT susceptibility with standard genetic approaches. However, specific genetic mutations introduced on the 129/Sv background have been shown to modify TGCT susceptibility (Table 10.1). A modifier gene, unlike a susceptibility gene, is not required or sufficient to induce a phenotype, but instead interacts with susceptibility genes to alter the penetrance of a phenotype (Heaney and Nadeau 2008). All of the genetic variants listed are at least partially dependent on the 129/Sv background to modify TGCT incidence, and will not cause TGCTs when congenic on other inbred mouse backgrounds. Modifier genes allow researchers to explore the genetic basis for susceptibility and provide avenues to characterize the genes and pathways involved in tumorigenesis.

The 129/Sv inbred strain has been used as a model of Type I TGCTs, considering the similarities in tumor emergence, pathology, and the seeming lack of GCNIS. However, parallels can be drawn between the spontaneous tumors observed in mice and Type II non-seminomas. It is interesting to consider the susceptibility genes *KITLG* and *DMRT1* identified in human GWAS susceptibility loci were first

discovered to contribute to TGCT susceptibility in 129/Sv mice (Heaney et al. 2008; Krentz et al. 2009). Additional studies in mice might be able to shed light to the initial commonality and the eventual dichotomy in the evolution of Type I and Type II TGCTs.

10.3.3 Chromosomal Abnormalities, Fusion Genes, and Single Nucleotide Variants in Type I and Type II TGCTs

As mentioned previously, human Type II TGCT GWAS have identified several susceptibility loci that harbor genes with roles in microtubule assembly, attachment of chromosomes to spindle microtubules, and alignment of chromosomes at the metaphase plate (Litchfield et al. 2015b). Telomerase function and DNA damage repair genes have also been identified in susceptibility loci of human TGCT GWAS (Turnbull et al. 2010; Kanetsky et al. 2011; Chung et al. 2013; Ruark et al. 2013). Together these observations implicate correct chromosomal segregation and destabilization of the genome in TGCT pathogenesis and may explain the karyotype evolution characteristic of TGCT progression.

A number of chromosomal abnormalities have been identified in different Type I and Type II TGCTs (Kraggerud et al. 2002; von Eyben 2004). Intriguingly, a pattern begins to emerge while studying the different abnormalities identified between TGCT types and subtypes. Foremost, Type I infantile teratomas (and coincidently teratomas in 129 mice) are nearly diploid (Kommoss et al. 1990; Hoffner et al. 1994; Silver et al. 1994; Stock et al. 1994; Bussey et al. 1999, 2001; Mostert et al. 2000; Schneider et al. 2001). However, Type I yolk sac tumors are aneuploid and have chromosomal abnormalities distinct from Type II TGCTs (Hoffner et al. 1994; Silver et al. 1994; Stock et al. 1994; Bussey et al. 1999; Mostert et al. 2000). Chromosomal aberrations often seen specifically in Type I yolk sac tumors include overrepresentation of regions of chromosomes 1, 12, 20, and 22, and an underrepresentation of parts of chromosomes 1, 4, and 6 (Mostert et al. 2000; Schneider et al. 2001). However, the contribution of these chromosomal abnormalities to tumor initiation and progression are not known.

Ploidy of Type II seminomas and non-seminomas progresses from tetraploid (in GCNIS), to hypertriploid (in seminomas) and finally hypotriploid (in non-seminomas) (Oosterhuis et al. 1989; de Jong et al. 1990; Vos et al. 1990; de Graaff et al. 1992; Bosl and Motzer 1997; von Eyben 2004). This karyotype evolution is consistent with a model of multipolar cell division starting from a tetraploid tumor stem cell population (Frigyesi et al. 2004). In both seminomas and non-seminomas, loss of chromosomes 4, 5, 11, 13, 18, and Y, and gain of chromosomes 7, 8, 12, and X are observed (Castedo et al. 1989; Rodriguez et al. 1993; van Echten 1995; Ottesen et al. 1997; Summersgill et al. 1998; Looijenga et al. 2000; Kraggerud et al. 2002). However, gains in chromosomes 15 and 22 are more specific to seminomas, whereas gain of chromosome 17 and loss of chromosome 10 have been more closely associated with non-seminomas, suggesting that particular chromosome losses or gains may be involved in establishing Type II TGCT subtypes

(Kraggerud et al. 2002). Of all the chromosomal abnormalities observed in Type II TGCTs, gains in a region of chromosome 12 (12p) may be the most important to TGCT progression, with at least one study showing that ~70% of TGCTs harbor 12p amplifications (Litchfield et al. 2015c; Taylor-Weiner et al. 2016). The vast majority of these amplifications are due to one or more copies of isochromosome 12p (i(12p)) (Atkin and Baker 1983; Looijenga et al. 2003c). Importantly, most studies have demonstrated that premalignant GCNIS with no adjacent invasive tumor does not harbor 12p amplifications, in particular i(12p); however, malignant GCNIS has been identified with 12p amplifications (Summersgill et al. 2001; Ottesen et al. 2003). Therefore, i(12p) is not required for TGCT initiation, but plays an important role in the transition from a noninvasive to invasive phenotype. Of note, 12p harbors several candidate genes (e.g. *KRAS*, *NANOG*, and *STELLAR*) whose roles as oncogenes and pluripotency/stem cell regulators may promote tumorigenesis when overrepresented (Oosterhuis and Looijenga 2005).

Evidence for smaller somatic mutations such as fusion genes or transcripts, single nucleotide variants (SNVs), and interval deletions contributing to TGCT pathogenesis is beginning to emerge. Hoff et al. used next-generation RNA sequencing to analyzed human EC cell lines and nonmalignant ES cell line controls for fusion genes or aberrant fusion transcripts (Hoff et al. 2016). Eight novel fusion transcripts and one gene with alternative promoter usage were identified in the EC cell lines. Intriguingly, four of the nine transcripts were found to be recurrently expressed in primary Type II TGCTs, including GCNIS and EC tumor stem cells, suggesting putative roles as driver mutations. However, whether these fusions contribute to germ cell transformation (tumor initiation), disease progression/metastasis, or both remains to be determined. Litchfield et al. (2015c) employed whole-exome sequencing of 42 Type II TGCTs and matched normal blood samples to identify somatic SNV and copy number driver mutations. Reoccurring SNV mutations were observed in only two genes (*KIT*, 14% of TGCTs and *CDC27*, 11.9% of TGCTs). KIT mutations were concentrated in seminomas (31%), which has been previously reported in studies utilizing targeted sequencing (Kemmer et al. 2004; McIntyre et al. 2005). Overall, nonsynonymous mutation rates were found to be low compared to other cancers. Two previously undescribed, reoccurring amplifications involving *FSIP2* and a region of the X chromosome were also discovered with both occurring in 15% of TGCTs. However, copy number gain in chromosome region 12p was by far the most common amplification observed (71% of TGCTs). This dataset was subsequently used to determine whether somatic mutations reoccur in genes within four TGCT susceptibility loci associated with inherited risk (Litchfield et al. 2015a). Only reoccurring events within the susceptibility locus on chromosome 11 (deletions encompassing *GAB2* and *USP35*) were observed (7% of the TGCTs). Therefore, even though TGCT susceptibility loci are important determinants of inherited risk, somatic mutations within these loci appear to be rare.

Importantly, the whole-exome sequencing studies by Litchfield et al. were powered to detect recurrent mutations having a tumor-associated frequency greater than 15% (84% power) (Litchfield et al. 2015c). Therefore, it is unlikely that additional high frequency driver mutations exist in TGCTs other than *KIT* SNVs or

chromosome 12p amplifications. Two additional whole-exome sequencing studies (Brabrand et al. 2015; Cutcutache et al. 2015), using smaller sample sizes, also found a low incidence of nonsynonymous mutations in TGCTs with little evidence for high frequency driver mutations. Therefore, most somatic mutations observed in TGCTs are either passengers of the tumorigenic process or oncogenic factors in only a small subset of TGCTs. Importantly, these mutations are likely to be drivers of TGCT progression rather than initiation, as they most likely occur after the tumor stem cell population is established. TGCT data are currently under analysis for The Cancer Genome Atlas (TCGA) project (Chin et al. 2011). Once analysis of whole-exome, whole-genome, copy number variation (CNV), and microarray data from a larger cohort of patient tumor and control samples is complete, the mutational landscape of TGCTs will become more apparent.

Based on the strong heritable component, the evolving genome, and the low somatic mutation rates observed in TGCTs, a model of tumor susceptibility (initiation) and progression is beginning to emerge. TGCT initiation appears to be primarily caused by genetic factors inherited through the germline (i.e. common SNP variants) working in combination possibly with environmental factors, which predisposes embryonic germ cell transformation into ECCs. Once ECCs are established, the TGCT genome can evolve over time, gaining or losing chromosomal components and accumulating SNVs and gene/transcript fusions. Together these somatic mutations influence disease progression (e.g. development of a specific tumor subtype) and metastasis. Such a model can explain the higher incidence of aneuploidy and somatic mutations observed in post-pubertal Type II TGCTs compared to infantile Type I TGCTs. Type II TGCTs may simply accumulate more genetic abnormalities during the latency period between tumor stem cell development in the embryo and tumor expansion after puberty. Importantly, inherited genetic factors within TGCT susceptibility loci harbor genes associated with chromosome segregation and DNA repair. These risk alleles may be the cause of the evolving karyotypes and infrequent somatic mutations of TGCTs. Importantly, this tumor initiation/progression model contrasts those for spontaneous cancers in other tissues, such as the colon (Davies et al. 2005), in which accumulation of somatic mutations are the primary drivers of tumor initiation and progression, and inherited genetic factors modulate disease risk and severity.

The evolving model of tumor susceptibility has been largely supported by data uncovered in GWAS. These studies provide ample opportunity for identifying risk loci, but fail to provide an avenue for validation. As mentioned previously, two genes first characterized as modifiers of TGCT incidence in 129/Sv mice, *Kitl* and *Dmrt1* (Heaney et al. 2008; Krentz et al. 2009), were also later identified as susceptibility loci in human GWAS (Kanetsky et al. 2009, 2011). This overlap highlights the similarity between mouse and human TGCT genetic susceptibility and pathogenesis, and suggests that additional genes in susceptibility loci identified in human TGCT GWAS may also be modeled as modifiers of TGCT incidence in mice. The ultimate goal of mouse models of human disease is to translate genetic alterations in mice to identical alterations in humans. To study tumor development in mice, a basic understanding of the normal developmental biology is critical to place in

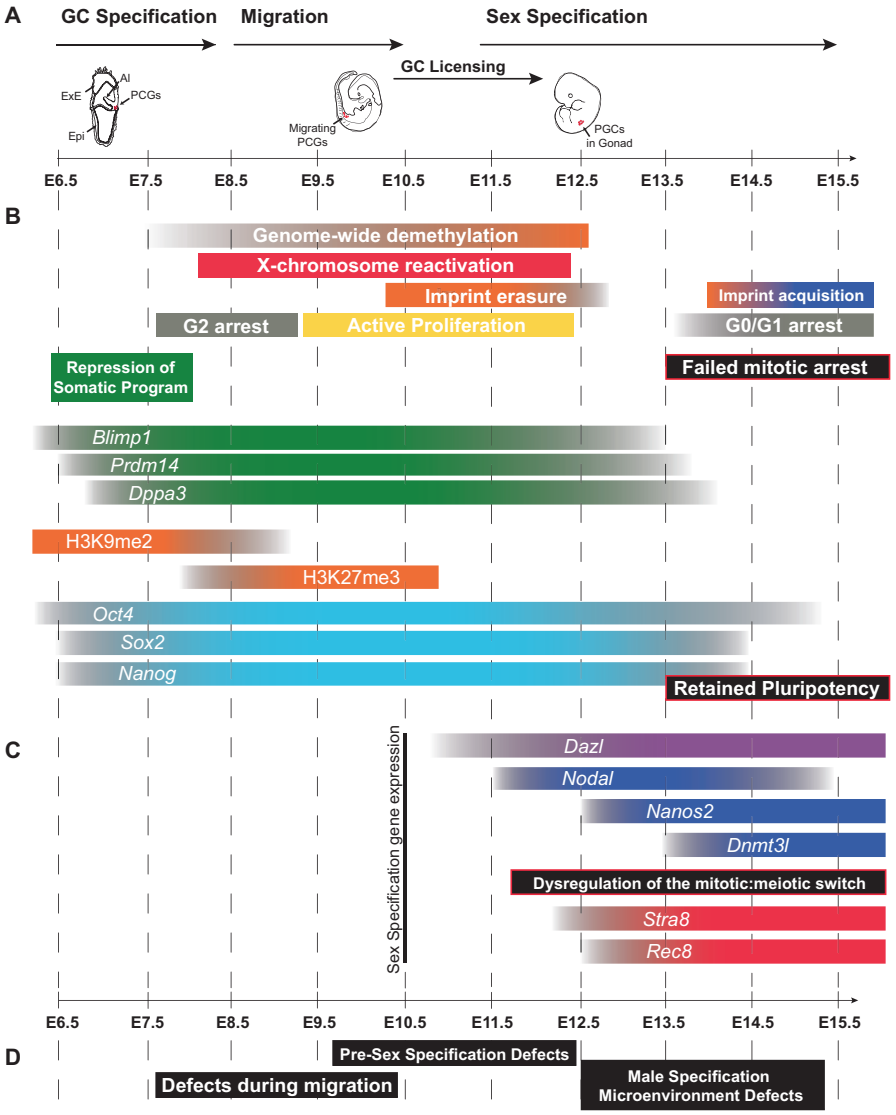

Fig. 10.2 (**a**) Schematic of the development of mouse PGCs through the four major phases: Specification, migration, licensing, and sex specification. ExE, extra-embryonic ectoderm; Epi, epiblast; Al, allantois. (**b**) Temporal expression patterns of key genes and developmental events involved in specification and epigenetic reprogramming of mouse PGCs. The *green bars* represent the expression of indicated genes associated with PGC specification; *orange bars* represent the expression of the chromatin modifiers and methylation status; *light blue bars* represent the expression of pluripotency genes; *pink and dark blue bars* indicate female and male specific events, respectively. Figure adapted from several references (Saitou et al. 2012; Bustamante-Marin et al. 2013; Moshfegh et al. 2016; Saitou and Miyauchi 2016), data as revealed by immunohistochemistry and other methods (Seki et al. 2005, 2007; Hajkova et al. 2008; Popp et al. 2010). Extensive remodeling of additional histone modifications occurs in the genital ridges at around E11.5, during

context the defects leading to tumorigenesis. We will present an overview of germ cell development, and then discuss the key disruptions in male germ cell development that contribute to testicular germ cell tumorigenesis.

10.4 Mouse Germ Cell Development and the Embryonic Origins of Tumorigenesis

For reference, Fig. 10.2 summarizes the main developmental time points and key gene expression patterns associated with mouse germ cell development. Additionally, time points associated with published deficiencies associated with tumor formation and the potential formative windows for TGCT development are also illustrated.

10.4.1 Primordial Germ Cells

PGCs originate from the proximal epiblast cells of the mouse at embryonic day (E) 6.5, in response to bone morphogenetic protein (BMP)4 signaling from the extra-embryonic ectoderm at ~E6.0 (Lawson et al. 1999; McLaren 2000; Surani 2001). BMP4 pathway signaling induces the expression of *Fragilis*, defining the portion of

Fig. 10.2 (continued) the rapid genome demethylation (Hajkova et al. 2008; Hajkova et al. 2010). Interestingly, *Oct4* (*Pou5f1*) has continuous RNA expression even beyond E15.5, while *Sox2* and *Nanog* are reactivated during GC specification and downregulated again starting at E14.5. However, OCT4 protein is not detected by immunofluorescence in gonocytes by E15.5 (Western et al. 2010). *Blimp1* (*Prdm1*), PR domain containing 1, with ZNF domain; *Prdm14*, PR domain containing 14; *Dppa3* (*Stella*), developmental pluripotency-associated 3. (**c**) Expression patterns of key genes in XY germ cells during male sex specification. *Purple bars* indicates concomitant expression in both sexes, *dark blue bars*, male, and *pink bars*, female. Licensing of PGCs by *Dazl* is a key event (Lin and Page 2005; Gill et al. 2011) for upregulation of genes involved in the male specification pathway, such as Nanos2 (Tsuda et al. 2003; Suzuki and Saga 2008) and Dnmt3l (Bourc'his et al. 2001), or female specification pathway (Stra8, Rec8) (Menke et al. 2003; Koubova et al. 2014). Other factors produced from somatic cells serve to initiate expression of key genes for sex specification, such as *Fgf9* expression in males and *Wnt4* in females, starting at E11.5 (Lin and Capel 2015). Somatic signaling from the Tgfβ pathway, including Activin, serves to initiate mitotic arrest and induces male fate in XY germ cells through p38 MAPK and SMAD2 (Miles et al. 2012; Wu et al. 2013, 2015). The *black boxes with red outline* indicate published defects observed in TGCT-susceptible gonocytes during sex specification (Kimura et al. 2003; Krentz et al. 2009; Cook et al. 2011; Heaney et al. 2012; Lanza et al. 2016); see text for in-depth discussion. (**d**) Postulated windows during which defects could accumulate to initiate tumorigenesis at E15.5. For example, defects during migration are known to occur, through the presence of extragonadal tumors observed in human neonates and children. *Kit* and *Kitl* are critical components to the migration of PGCs from the primary streak through the hindgut to the genital ridge (Mahakali Zama et al. 2005; Kunwar et al. 2006). GWAS have implicated several variants in or around *KITLG* (Kanetsky et al. 2009; Rapley et al. 2009; Kratz et al. 2011). Epigenetic abnormalities could occur during PGC migration or pre-sex specification, which could result in the misexpression of genes during the wrong developmental window. Altered microenvironments within the gonad during male specification, such as inappropriate exposure to meiosis-promoting factors or insufficient expression of meiosis-inhibiting factors, could also provide avenues for TGCT initiating events

embryonic mesoderm with germ cell competence (Saitou et al. 2002). During PGC specification, the majority of epiblast cells are being pushed towards somatic fates and losing pluripotency (Kurimoto et al. 2008). High *Fragilis*-expressing cells induce *Stella*, to repress Homeobox gene expression and differentiate themselves from their somatic neighbors (Saitou et al. 2002). However, subsequent studies have demonstrated that neither *Stella* nor *Fragilis* are required for PGC specification (Payer et al. 2003; Lange et al. 2008; Saitou 2009).

BMP4 signaling also induces the expression of two transcriptional regulators, *Prdm1* (also known as *Blimp1*, PR domain containing 1, with ZNF domain) and *Prdm14*, in the most proximal epiblasts at ~E6.25 and E6.5, respectively. BLIMP1- and PRDM14-positive cells progress to form a cluster of ~40 alkaline phosphatase (AP)-positive PGCs at the base of the incipient allantois at ~E7.25 (Ginsburg et al. 1990; Ohinata et al. 2005, 2009; Vincent et al. 2005; Yamaji et al. 2008). *Blimp1* is exclusively expressed in founder PGCs and required for PGC specification (Ohinata et al. 2005; Vincent et al. 2005). BLIMP1 and PRDM14 work in concert to achieve repression of the somatic program, genome-wide epigenetic reprograming, and re-acquisition of potential pluripotency in PGCs (Saitou et al. 2008; Yamaji et al. 2008; Saitou 2009).

Epigenetic reprogramming in newly specified PGCs goes beyond suppression of the somatic program by BLIMP1 and PRDM14. In early PGCs, histone methylation markers and de novo methylases *Dnmt3b*, *Dnmt3a* and *Uhrf1* are transcriptionally repressed (Kurimoto et al. 2008; Sasaki and Matsui 2008). Changes in genome-wide DNA methylation, removal of histone H3 lysine9 dimethylation (H3K9me2), and acquisition of high levels of tri-methylation of H3K27 (H3K27me3) occur just prior and continue throughout PGC migration, processes that might be crucial for the maintenance of potency in the germline (Seki et al. 2005). During migration, PGC methylation sites at imprinted loci are maintained (Hajkova et al. 2002; Lee et al. 2002), however, other studies have identified a heterogeneous "reprogramming" in a cell-by-cell manner (Hajkova et al. 2002; Lee et al. 2002; Lane et al. 2003; Seki et al. 2007; Hajkova et al. 2008). After arriving at the genital ridge PGCs undergo rapid genome-wide demethylation to cause reactivation of the inactivated X-chromosome in females, imprinted loci no longer retain methylation marks, and most transposable elements are demethylated by E13.5 (Surani 2001; Hajkova et al. 2002; Li 2002; McLaren 2003; Hayashi and Surani 2009). Extensive reviews on epigenetic reprogramming in PGC specification and maintenance have been published (Surani et al. 2007; Sasaki and Matsui 2008; Saitou et al. 2012).

Pluripotency is maintained in PGCs through approximately E13.5 (Yamaguchi et al. 2005; Western et al. 2010), which is evident from the ability to generate embryonal germ cells (EGCs) and ES cells from PGCs in vitro (Matsui et al. 1992; Pesce and Scholer 2000). At E12.5 PGCs can be cultured to generate alkaline phosphatase-positive, specific embryonic antigen 1 (SSEA1)-positive cells resembling undifferentiated embryonic stem cells that can be transplanted to form teratomas in nude mice (Matsui et al. 1992). Critical to the maintenance of pluripotency PGCs express the gene *Pou5f1* (*Oct4*), which is expressed exclusively in PGCs starting at E7.5 through spermatogenesis up to the onset of spermatogenic

differentiation (Scholer 1991; Pesce et al. 1998; Pesce and Scholer 2000). BMP4 signaling also controls the activity of the *Oct4* distal enhancer in founder PGCs during germ cell specification (Yeom et al. 1996; McLaren 1999; Pesce and Scholer 2000). *Oct4* expression is also necessary for survival of migrating PGCs in later stage embryos (Kehler et al. 2004).

10.4.2 PGC Migration

Beginning around E10.5–E11.5, PGCs complete their migration along the midline through the hindgut to arrive at the genital ridge (McLaren 2000). While migrating, the PGCs greatly increase in number by proliferating, relying on the c-kit/stem cell factor signal transduction pathway for continued proliferation and migratory guidance to the genital ridge (Matsui et al. 1990; Sutton 2000). Correspondingly, embryos homozygous for mutations in genes coding for either the receptor (*W*) or the ligand (*Steel*) are deficient in PGCs (McLaren 2000). By E11.5, the genital ridge is clearly defined from the mesonephros, thereby preventing any subsequent migration.

10.4.3 Sex Specification and the Mitotic: Meiotic Switch

After arriving in the genital ridge, PGCs continue proliferating for the next 2–3 days to expand the germ cell pool, going from tens to hundreds at their inception in the primary streak to over 12,000 PGCs per colonized gonad at E13.5 (Mintz and Russell 1957; Tam and Snow 1981). It is during this time period that germ cells, now termed gonocytes or oogonia, commit to either a male or female fate (respectively) and enter G1/G0 mitotic arrest or initiate meiosis (the mitotic:meiotic switch), respectively (McLaren 1984). These sex-specific developmental events are controlled by cues from the somatic environment. In normal development, *Sry* expression in somatic cells at E10.5 causes the upregulation of *Fgf9* and *Sox9* to signal XY PCGs to begin suppression of the female pathway (Kim et al. 2006; Sekido and Lovell-Badge 2008). Somatic cells increase proliferation during this peak of *Sry* expression (Hacker et al. 1995). Blocking this somatic proliferation disrupts the male pathway of development (Schmahl et al. 2000; Schmahl and Capel 2003) and inhibits the survival of the XY PGCs by preventing the eventual enclosure of germ cells and somatic cells in specific germ cell compartments (Byskov 1986). This chapter does not go into the differentiation of supporting somatic cells outside the context of male germ cell specification. For more information, excellent reviews have been published on cell fate commitment during sex determination (Park and Jameson 2005; Lin and Capel 2015).

FGF9 signaling from somatic cells induces the upregulation of the Nodal/Activin pathway in PGCs around E12.5 (Spiller et al. 2012; Wu et al. 2013). Activins and Nodal are members of the transforming growth factor β (TGFβ) superfamily of morphogens (Oshimori and Fuchs 2012). TGFβ family members play important

roles in gonadal development in both sexes (Munsterberg and Lovell-Badge 1991; Yi et al. 2001; Nicholls et al. 2009; Moreno et al. 2010; Mendis et al. 2011). Activins and Nodal, with co-receptor CRIPTO, can signal through the same receptors and effectors to regulate transcription (Pauklin and Vallier 2015). In gonocytes co-expression of *Cripto* and *Nodal* generate a positive feedback loop to sustain NODAL signaling and expression of downstream targets *Lefty1* and *Lefty2*, which display peak expression at E13.5 (Spiller et al. 2013). Expression of *Nodal* and *Cripto* at this time serves to maintain pluripotency in the XY germ cell population, and in the absence of NODAL/CRIPTO signaling, pluripotency potential of the gonocytes is reduced (Spiller et al. 2012). FGF9 signaling from Sertoli cells to gonocytes also helps to transiently maintain expression of pluripotency genes *Oct4* and *Sox2,* prior to mitotic arrest (Bowles et al. 2010). *Nodal* has previously been shown to maintain *Nanog* expression in early embryos and pluripotent cells (Mesnard et al. 2006; Vallier et al. 2009). Recent studies have also indicated a role of NODAL signaling, and its activation of SMAD2 and p38 MAPK pathways, in promoting male differentiation through the induction of *Nanos2* expression (Wu et al. 2013). In the absence of NODAL/Activin signaling, XY germ cells will enter meiosis (Souquet et al. 2012; Miles et al. 2013). TGFβ and Activin signaling are required to ensure correct mitotic arrest in XY germ cells (Moreno et al. 2010; Mendis et al. 2011; Miles et al. 2013; Wu et al. 2015).

Germ cells activate the G1-S phase cell cycle checkpoint in a gradual and unsynchronized manner due to a shift in the expression of positive and negative regulators of the G1-S phase transition. Prior to entry into mitotic arrest, germ cells express cyclins E1 and E2 (CCNE1/2) and cyclin D3 (CCND3), which form complexes with cyclin dependent kinases 2 (CDK2) and 4 or 6 (CDK4/6), respectively (Western et al. 2008). These cyclin-CDK complexes hyperphosphorylate (inactivate) retinoblastoma protein 1 (pRB1), leading to de-repression of E2F transcription factors and activation of genes required for progression into S phase (Deshpande et al. 2005; Western et al. 2008; Spiller et al. 2010). Cyclin D-CDK4/6 complexes also promote the G1-S transition through the sequestration of cyclin E-CDK2 inhibitors $p27^{KIP1}$ (CDKN1B) and $p21^{CIP1}$ (CDKN1A) (Deshpande et al. 2005). Mitotic arrest is initiated around E13.5 through decreases in expression of CCNE1/2 and CCND3 and increases in expression of $p27^{KIP1}$ and cyclin D-CDK4/6 inhibitors $p15^{INK4B}$ (CDKN2B) and $p16^{INK4A}$ (CDKN2A), which result in hypophosphorylation (activation) of pRB1, suppression of E2F transcriptional activity, and gonocyte transition into G1/G0 arrest (Western et al. 2008). These negative regulators of the cell cycle, in addition to TGFβ/Activin signaling and Prostaglandin D_2 signaling (Moniot et al. 2014) ensure the proper mitotic arrest in male gonocytes. Additional factors or a master regulator may be in control of the switch between mitotic arrest and meiosis (Adamah et al. 2006; Feng et al. 2014).

In addition to proper signaling to initiate mitotic arrest, checks and balances exist in the male gonad to prevent premature meiosis. Sertoli cell differentiation and the establishment of testis cords are critical to prevent XY germ cells from entering meiosis (Byskov 1978). If a male genital ridge is disaggregated and reaggregated at E11.5, testis cords do not develop and all the PGCs enter the oogenesis pathway

(McLaren and Southee 1997; Adams and McLaren 2002). These studies investigating sex specification in the mouse ovary and testis postulated a "meiosis inducing substance" to be responsible for the initiation of meiosis, which was subsequently blocked in the testis around E12.5–E13.5, when germ cells commit to a sex specific pathway (McLaren and Southee 1997). Subsequent studies would identify this meiosis-inducing substance as retinoic acid, which normally induces genes such as *Stra8* to initiate meiosis in the XX fetal ovary (Bowles et al. 2006; Koubova et al. 2006; Koubova et al. 2014) and is sufficient to initiate meiosis in the fetal testis ex vivo (Trautmann et al. 2008). An extensive review on the influence of retinoic acid in sex specification of embryonic germ cells was presented by Bowles and Koopman (2010).

Exposure to retinoic acid is a tightly regulated process in the developing mouse gonad, critical for the mitotic:meiotic switch in gonocytes between E13.5 and E15.5. During this time period, female oogonia are exposed to a wave of retinoic acid to induce expression of *Stra8*, which mirrors the anterior-to-posterior wave of retinoic acid and initiates meiotic differentiation that lasts for 4 days (E12.5–E16.5) (Menke et al. 2003). During the same developmental period in male gonocytes, CYP26B1 degrades retinoic acid, thereby blocking STRA8 expression to prevent the initiation of meiosis (Menke and Page 2002; Bowles et al. 2006; Koubova et al. 2006; Vernet et al. 2006; MacLean et al. 2007). To further inhibit *Stra8* expression as CYP26B1 levels begin to decline and to promote the male germ cell differentiation program, *Nanos2* expression is activated in male gonocytes at E14.5 (Suzuki and Saga 2008; Barrios et al. 2010; Bowles et al. 2010). NANOS2 represses meiosis and the female differentiation pathway in embryonic male germ cells independent of the expression of *Nanos3* (Suzuki et al. 2007; Suzuki and Saga 2008; Barrios et al. 2010). NANOS proteins are evolutionary conserved RNA-binding proteins, involved in post-transcriptional RNA metabolism via their binding to target mRNAs in germ cells (Kadyrova et al. 2007). Interestingly, *Nanos2* is required for normal male germ cell differentiation, as evident by the lack of rescue of male differentiation gene expression at E15.5 in *Nanos2/Stra8* double knockout mice (Saba et al. 2014a). These data suggest that *Nanos2* plays larger role in male germ cell development than inhibiting retinoic acid.

By E15.5 male gonocytes should have committed to mitotic arrest and should be expressing genes associated with male germ cell differentiation (e.g., *Nanos2*, *Dnmt3l*, *Piwil4/Miwi2*, *Tdrd9*, and *Mili)* (Shovlin et al. 2007; Aravin et al. 2008; Shoji et al. 2009). Following initiation of mitotic arrest at E13.5, male gonocytes normally downregulate expression of pluripotency factors (e.g. *Oct4*, *Nanog*, and *Sox2*) (Pesce et al. 1998; Avilion et al. 2003; Yamaguchi et al. 2005; Western et al. 2010). Accordingly, downregulation of Nodal expression is observed starting at E14.5 (Spiller et al. 2012), which supports the role of Nodal expression in transiently maintaining pluripotency. From the misregulation of mitotic arrest and the failure to downregulate pluripotency, male gonocytes in the 129 mouse strain are susceptible to develop into TGCTs. To note, misregulation of mitotic arrest and retention of pluripotency have also been proposed to cause TGCTs in humans (Palumbo et al. 2002; Looijenga et al. 2003b; Rajpert-De Meyts et al. 2004; Spiller et al. 2012).

10.5 TGC Tumorigenesis in the 129 Inbred Strain of Mice: Dysfunctional Germ Cell Development

10.5.1 Failure to Enter into Mitotic Arrest

Delayed entry into G1/G0 mitotic arrest has been linked with susceptibility to teratoma formation in the 129/Sv inbred strain of mice (Stevens 1964, 1967a; Noguchi and Stevens 1982; Matin et al. 1998). Many of the genetic modifiers that increase 129/Sv tumor incidence of 129/Sv characterize this failure to enter mitotic arrest as sustained proliferation through the mitotic:meiotic switch to E15.5: Noguchi and Stevens identified in genital ridge grafting experiments that sub-strains of 129/Sv with increased incidence of teratoma formation also exhibited a longer period of proliferation compared to 129/Sv sub-strains with lower teratoma frequency (Noguchi and Stevens 1982). Populations of germ cells in males of the 129-Chr19$^{MOLF/Ei}$ (M19) chromosome substitution strain, which has a tenfold tumor incidence compared to wild-type 129/Sv mice (Matin et al. 1999), still proliferate at E15.5, as evident by KI67 expression (Heaney et al. 2012). Mice with homozygous deletions of *Trp53* and *Pten* also exhibit increased TGCT incidence, which underlies the role of cell cycle control in TGCT tumorigenesis (Harvey et al. 1993; Kimura et al. 2003; Western 2009). Importantly, several other genes that regulate male germ cell entry into mitotic arrest may influence TGCT susceptibility. Mice deficient for *Dazl*, a gene critical for germ cell development and survival of XY germ cells, have a few surviving germ cells that display sustained proliferation and retained pluripotency (Lin and Page 2005). Of note, *DAZL* is located near one of the TGCT risk loci identified in human TGCT GWAS. Mice deficient for genes involved in prostaglandin D_2 synthesis, *Ptgds* and *Hpgds*, show increased proliferation of gonocytes at E13.5 through E15.5, compared to wild-type controls (Moniot et al. 2014). *HPDGS* is also located near a human TGCT GWAS risk locus. Therefore, in both mice and humans, a pro-proliferative germ cell program appears to be a central component of TGCT initiation.

Germ cell entry into G1/G0 mitotic arrest during the mitotic:meiotic switch is dependent on coordinated alterations in the expression of positive and negative regulators of the G1-S phase transition (Western et al. 2008). During embryogenesis, D-type cyclin expression is primarily restricted to CCND3 in male germ cells (Beumer et al. 2000; Western et al. 2008). Curiously, even though its expression decreases during the mitotic:meiotic switch, CCND3 protein persisted in FVB gonocytes through at least E17.5, a time point at which these cells are quiescent. Thus, expression of negative regulators of the G1-S transition must be sufficient to counteract residual D-type cyclin expression to induce G1/S mitotic arrest (Beumer et al. 2000). A sufficient increase in the ratio of positive to negative regulators of G1-S cell cycle progression during the mitotic:meiotic switch might tip the balance toward proliferation rather than mitotic arrest. It has been previously demonstrated that *Ccnd1* expression levels are significantly higher in TGCT-susceptible gonocytes (Heaney et al. 2012); *Ccnd1* expression is normally restricted to differentiating

postnatal spermatogonia (Beumer et al. 2000). Furthermore, more recent data has revealed that *Ccnd1* is the only G1-S phase cyclin upregulated in TGCT-susceptible gonocytes at E15.5, the time point at which ECCs are first evident (Lanza et al. 2016). *Ccnd1*-deficiency permitted TGCT-susceptible gonocytes to activate the G1-S cell cycle checkpoint and induce G1/G0 mitotic arrest in a more developmental stage-appropriate manner, as evident by phospho-pRB1 and KI67 immunostaining of E14.5 and E15.5 gonocytes. Thus, cyclin D1 appears to be the G1-S phase cyclin delaying mitotic arrest of TGCT-susceptible gonocytes. While *Ccnd1*-deficiency was not sufficient to prevent tumor initiation in TGCT-susceptible gonocytes, the misexpression of CCND1 in embryonic germ cells represents a severe consequence of the larger developmental defect present in 129/Sv mice, which permits spontaneous germ cell tumorigenesis.

Mutations of other genetic modifiers of the 129/Sv background, in addition to delayed mitotic entry, also display alterations in genes controlling the G1-S checkpoint. $Dnd1^{Ter/Ter}$ mutants on a 129/Sv background fail to express the negative regulators of the cell cycle $p27^{KIP1}$ and $p21^{CIP1}$ at E14.5, while expression of both proteins is detected in wild-type littermates (Western et al. 2008, 2011; Cook et al. 2011). DND1 has been postulated to promote translation of $P27^{KIP1}$, in addition to NANOS2, which would directly link *Dnd1* to male differentiation and cell cycle control (Western 2009; Cook et al. 2011). *Dmrt1* null mutants on the 129/Sv background have decreased expression of the negative regulators of the cell cycle, $p18^{INK4c}$ and $p19^{iNK4d}$, and DMRT1 has been shown to bind to the promoter of $p19^{iNK4d}$ in E13.5 testes (Krentz et al. 2009). Therefore, misregulation of entry into mitotic arrest is affected in multiple modifiers of TGCT incidence, highlighting this checkpoint as a critical step in tumorigenesis.

10.5.2 Failure to Repress Pluripotency

Retention of pluripotency has been shown to play an important role in TGCT initiation. In normal PGCs of both TGCT-resistant and susceptible mice, pluripotency is maintained through E13.5, as evident by the expression of NANOG and other pluripotency genes (Heaney et al. 2012). In TGCT-susceptible mice, gonocytes that fail to enter mitotic arrest continue to express pluripotency factors through the transition to ECCs (Kimura et al. 2003; Krentz et al. 2009; Cook et al. 2011). TGCT-susceptible gonocytes have significantly increased expression levels of NANOG at E15.5 (Heaney et al. 2012). Importantly, signaling pathways involving OCT4 and NANOG have been implicated in TGCT initiation in humans (Looijenga et al. 2003a; Clark et al. 2004; Oosterhuis and Looijenga 2005).

The germ cell specification gene *DAZL* has been shown to regulate pluripotency in both mouse and human. Forced overexpression of *Dazl* in ES cells, in the absense of LIF, promotes germ cell differentation and in germ cells meiotic induction; *Dazl* deficiency results in germ cell apoptosis and infertility (Lin and Page 2005; Kee et al. 2009; Yu et al. 2009; Medrano et al. 2012). Another gene involved in regulating

pluripotency in mouse embryonic germ cells, *PRDM14*, has also been shown to regulate the expression of *OCT4, NANOG*, and *SOX2* in human embryonic stem cells (Tsuneyoshi et al. 2008; Chia et al. 2010). Both *DAZL* and *PRDM14* have been identified in LD with risk loci from human TGCT GWAS (Ruark et al. 2013).

The maintenance of pluripotency in germ cells has also been linked to tumor susceptibility in mice. The transcription factor *Dmrt1* (doublesex and mab-3 related) has been shown to control pluripotency by regulating transcription of several genes, including *Sox2* (Krentz et al. 2013), and its deficiency is sufficient to induce tumors on the 129/Sv inbred background (Krentz et al. 2009). Studies have also demonstrated a direct relationship of the maintenance of pluripotency to active NODAL/CRIPTO signaling, and an overexpression of Nodal signaling components in TGCTs (Spiller et al. 2012, 2013). In normal development, NODAL/Activin signaling directly affects pluripotency, male differentiation, and entry into meiosis in XY germ cells (Mendis et al. 2011; Souquet et al. 2012; Spiller et al. 2012; Miles et al. 2013; Wu et al. 2013). Decreased NODAL/CRIPTO signaling leads to significant increases expression of male differentiation makers, such as $p15^{INK4b}$ and *Dnmt3l* (Spiller et al. 2012). Notably, CRIPTO was first identified in a human EC cell line (Ciccodicola et al. 1989), and NODAL signaling components are overexpressed in human TGCTs (Spiller et al. 2012).

It has not been determined whether retention of pluripotency is regulated independently of failed mitotic arrest in TGCT-susceptible gonocytes. The tumor initiation capacity of ECCs is dependent upon their pluripotent capacity (Gidekel et al. 2003). Retention of both proliferation and pluripotency therefore appear to be necessary for germ cell transformation into ECCs. There are varying data in normal gonocytes as to whether mitotic arrest and downregulation of pluripotency in gonocytes are linked or independently regulated. Miles et al. previously demonstrated that Activin signaling from somatic cells and autocrine NODAL signaling induces gonocytes to enter into mitotic arrest and transiently maintain pluripotency, respectively (Miles et al. 2013). Separate studies showed that subsequent loss of NODAL expression by gonocytes facilitates suppression of pluripotency (Spiller et al. 2012). Thus, aspects of mitotic arrest and suppression of pluripotency are independently regulated.

Importantly, retention of pluripotency is, at least in part, dependent on the misexpression of genes that promote G1-S cell cycle progression, such as *Ccnd1*. *Ccnd1*-deficiency suppressed TGCT-susceptible gonocyte pluripotency during the mitotic:meiotic switch (Lanza et al. 2016). In both ES and ECCs, rapid transition through G1 into S phase facilitates the maintenance of pluripotency (Filipczyk et al. 2007; Singh and Dalton 2009). A short G1 and long S phase promotes the euchromatic state of chromatin and suppresses differentiation, which preferentially occurs during the G1 phase in pluripotent cells (Mummery et al. 1987; Jonk et al. 1992; Herrera et al. 1996). Moreover, recent data demonstrate that pRB1 directly binds to the regulatory regions the core components of pluripotency (*Oct4*, *Nanog*, *Sox2*) and suppresses their expression (Kareta et al. 2015). Therefore, regulation of the cell cycle may contribute to retention of pluripotency observed in TGCT-susceptible gonocytes.

10.5.3 Pro-survival, Anti-apoptotic Microenvironment for Aberrant Germ Cell Proliferation

By E15.5 all male gonocytes should have entered mitotic quiescence, to eventually reinitiate proliferation and differentiate to form the spermatogonial lineage after birth (McLaren 1984). As previously discussed, TGCT susceptibility is only observed in mice on the 129 inbred background. To date, it is unclear as to why TGC tumorigenesis is possible on the 129 strain, but not other inbred mouse strains. This sensitivity issue has been addressed using the *Ter* mutation in *Dnd1 (dead end homolog 1)* on both 129/Sv and non-TGCT susceptible strains. The increase in the occurrence of teratomas in 129 mice caused by the *Ter* mutation was first reported in 1973 (Stevens 1973). Intriguingly, the *Ter* mutation also causes a dramatic loss of germ cells in both sexes in all genetic backgrounds, which led eventually researchers to identify *Ter* as a nonsense mutation in the gene *Dnd1* (Asada et al. 1994; Youngren et al. 2005). The loss of germ cells in $Dnd1^{Ter/Ter}$ mice is consistent with the early defect in germ cell specification (Noguchi et al. 1996).

It has been postulated that more efficient cell death pathways might protect certain strains by eliminating errant germ cells prior to tumor initiation (Bustamante-Marin et al. 2013). Apoptosis of fetal germ cells through a BAX-dependent mechanism has been postulated in the absence of teratomas in C57BL/6 mice with mutations in known 129 susceptibility genes (Cook et al. 2009). To test this hypothesis, a mutation in the pro-apoptotic gene *Bax* was introduced into mice of several genetic backgrounds carrying the $Dnd1^{Ter}$ mutation. *Bax*-deficient mice had partial rescue of the germ cell loss phenotype in all strains (Cook et al. 2009) and a high incidence of teratomas was detected in double mutant $Dnd1^{Ter/Ter}$, $Bax^{-/-}$ and $Dnd1^{Ter/Ter}$, $Bax^{-/+}$ mice on mixed genetic backgrounds, where teratomas were not seen in the absence of the Bax mutation. However, on a pure C57BL/6 background where ~50% of germ cells were rescued, no teratomas were seen, even in double mutants (Cook et al. 2011). These data underlie the complex control needed in the regulation of apoptosis in male germ cell development.

The anti-apoptosis phenotype is involved in the pathology of human TGCTs as well. Human GWAS have identified a susceptibility locus in humans that falls within an intron of the gene *BAK1* (BCL2-antagonist/killer 1). BAK1 promotes apoptosis by antagonizing the apoptosis repressor activity of BCL2 and other anti-apoptotic proteins (Yan et al. 2000; Rapley et al. 2009). Therefore, a direct link between TGCT susceptibility in mouse and humans can be established by the apparent need to establish a pro-survival environment for transformed germ cells to evolve into TGCTs.

Additional studies in mice sampling gene expression differences in E14.5 XY gonocytes identified cell cycle regulators, apoptotic pathways, and tumor suppressors to be among the genes enriched in C57BL/6 compared to 129/Sv (Cook et al. 2011). These findings suggest that increased expression of factors that promote cell cycle arrest or apoptotic pathways prior to mitotic arrest in gonocytes may be sufficient to prevent teratoma formation, even in the presence of mutations that promote the transformation of germ cells. A better understanding of the genetic basis

for the pro-survival phenotype in 129/Sv tumor susceptibility versus the apoptosis-driven C57BL/6 TGCT resistance could lead to the identification of additional genetic factors/modifiers that contribute to the developmental defects in 129/Sv mice that permit testicular germ cell tumorigenesis.

10.5.4 Altered Epigenetic States

As previously described, *Blimp1*, *Prdm14*, and a third transcriptional regulator, *Tfap2c* (also known as AP2γ or *Tcfap2c*), specify PGCs by inducing DNA demethylation and histone remodeling, repressing the somatic cell program, and establishing a naïve pluripotent expression profile (Saitou and Yamaji 2010). Expression of *Blimp1* ceases at E11.0 as PGCs migrate into the gonad, whereas expression of *Prdm14* and *Tfap2c* continues through the mitotic:meiotic switch (E13.5–14.5) (Yamaji et al. 2008; Weber et al. 2010). *Blimp1*, *Prdm14,* and *Tfap2c* expression is observed during comparable developmental periods in humans (Saitou and Yamaji 2010). Genetic experiments indicate that each factor is essential for germ cell specification (Saitou et al. 2003; Yamaji et al. 2008; Weber et al. 2010). Additionally, *Prdm14* suppress early somatic tissue specification genes (e.g. *Fgfr1* and *Fgfr2*), DNA methyltransferases (e.g. *Dnmt3a* and *Dnmrt3b*), and mediators of G1-S phase transition (e.g. *Ccnd1*), and activates germ cell specification genes (e.g. *Nanos3, Dmrt1,* & *Tfap2c*) and mitotic arrest factors (e.g. *Dnd1*) (Yamaji et al. 2008; Grabole et al. 2013; Magnusdottir et al. 2013; Yamaji et al. 2013). *PRDM14* has also been identified in linkage disequilibrium with risk loci from human TGCT GWAS (Ruark et al. 2013). A recent study showed that *Tfap2c* haploinsufficiency increases TGCT incidence in 129/Sv mice by tenfold (Schemmer et al. 2013). Historically, *Tfap2c* haploinsufficiency does not cause TGCTs in C57Bl/6 mice (Werling and Schorle 2002; Weber et al. 2010). These results suggest that haploinsufficiency for *Tfap2c* has phenotypic consequences only in the context of 129 developmental defects that cause TGCT initiation.

Genome-wide demethylation of 5-methylcytosine sites occurs during normal PGC development. Interestingly, expression of de novo *Dnmt3A* and *Dnmt3L* methyltransferases are required for germ cell viability; Male mice that lack *Dnmt3L* are viable but sterile, with a complete absence of germ cells in adult males (Bourc'his et al. 2001). Additionally, *Dnmt3L* is required for normal imprinting of male germ cells, and normal male meiosis, but is not expressed in spermatocytes (Bourc'his and Bestor 2004). There is some evidence that there are strain-specific differences in the establishment of new methylation imprints, 129/Sv mice have been shown to establish new imprints more slowly than C57BL/6 mice (Davis et al. 2000; Durcova-Hills et al. 2006). While it remains to be elucidated whether the methylation states merely reflects the pluripotency of TGCTs or are part of the changes leading to tumorigenesis, it is interesting to consider epigenetic changes contributing to TGCT formation.

10.6 Remaining Questions

TGCTs are highly treatable with platinum-based chemotherapy. However, current treatment regimens cause long-term side effects including hearing loss, cardiovascular disease, cognitive impairment, and infertility (Horwich et al. 2006; Oldenburg et al. 2007b; Kraggerud et al. 2013). Moreover, long-term prognosis markedly worsens as the disease progresses with the potential for metastasis and there are limited alternative treatment options for patients that demonstrate platinum resistance. Thus, improvements in risk assessment, screening, and alternative treatment options remain important and the social, emotional, and medical costs remain high. Over the last decade, significant improvements in our understanding of the developmental origins, inherited risk factors, and somatic mutations that contribute to TGCT initiation and progression have been made. However, despite these advances several important questions remain to be answered regarding TGCT pathogenesis. The answers to these questions will not only provide us with a clearer understanding of the basic biology and genetics of TGCTs, but also may provide targets for more efficacious screening and treatment paradigms.

What are the genetic risk factors for chemoresistance and the morbidities of platinum treatment? As previously discussed, a subset of TGCTs are resistant to platinum-based chemotherapy agents and the long-term outlook for individuals with these tumors is bleak. By contrast, for those patients whose tumors do respond to treatment, there are significant long-term survivorship issues (associated morbidities) resulting from platinum-based treatment (Singhera et al. 2012; Bujan et al. 2013; de Haas et al. 2013). Although evidence is beginning to emerge for inherited genetic risk factors and somatic mutations that determine chemoresistance (Litchfield et al. 2016; Taylor-Weiner et al. 2016) and predisposition to treatment morbidities (Peters et al. 2000; Oldenburg et al. 2007a), much still remains to be learned.

How does genomic instability start? Although the karyotypes of developed TGCTs have been well characterized, the origin and progression of chromosomal abnormalities is unclear. What promotes nuclear instability, when do chromosomal abnormalities first evolve, and how do karyotypic abnormalities contribute to tumor progression remain unanswered questions. Unfortunately, it is difficult to study human TGCTs during the early stages of tumorigenesis; such studies are possible in mice.

Observations from investigations using both human tissue samples from TGCTs and ECC lines have led researchers to suggest that the polyploidization observed in GCNIS and TGCT (Atkin and Baker 1983; Kraggerud et al. 2002; Skotheim et al. 2002; Adamah et al. 2006; Rajpert-De Meyts 2006; Rajpert-de Meyts and Hoei-Hansen 2007) might be a result of confused meiosis signaling (Adamah et al. 2006; Jorgensen et al. 2013). Additionally, Jorgensen et al. hypothesize that germ cells with highly expressed genes located on 12p and 17q (human) that are frequently amplified in TGCT, especially in non-seminomas, could be among the genetic abnormalities that escape normal DNA repair checkpoints. GCNIS cells and TGCT cells do not complete meiosis, which could be the result of concurrent expression of

CYP26B1 and NANOS2 in addition to the high expression of pluripotency factors. Again, germ cells may be responding to the conflicting signals present in the surrounding microenvironment after ensuing genomic instability, as postulated in the mouse (Bustamante-Marin et al. 2013).

Sexual disorders in humans, conditions that often blur morphological differences between testis and ovary (Hughes et al. 2006), increase the risk of an individual developing TGCTs (Muller et al. 1985; Looijenga et al. 2010; Pleskacova et al. 2010; Cools et al. 2011; Jorgensen et al. 2015). In the gonads of patients with sexual development disorders, immature germ cells persist as the supporting niche is not able to provide the appropriate environment for germ cell development. Based on evidence in mice, under-development of the somatic niche in the testis has also been associated with increased frequency of GCNIS (Skakkebaek et al. 2001; Hoei-Hansen et al. 2003).

The dysregulation of the mitotic:meiotic switch and inappropriate exposure of male gonocytes to retinoic acid has influenced researchers to screen for GCNIS. These studies utilize human tissue samples from adult testes and testicular tumors, and diagnostic biopsies from young boys with sex chromosome aneuploidy to monitor for GCNIS. Several human studies have also demonstrated the role of retinoic acid and its contribution to the disruption of meiosis regulation in the progress towards TGCT development (Childs et al. 2011; Jorgensen et al. 2012, 2013, 2015).

How do defects at the mitotic:meiotic switch interplay to promote tumorigenesis? The concurrent timing of aberrant proliferation, the mitotic:meiotic switch, and sex specification may provide clues to what signals are dysregulating the mitotic:meiotic switch and driving TGCT initiation in 129 mice. It has been previously shown that oogonia in both TGCT-resistant and susceptible mouse strains transiently express *Ccnd1* from E12.5 to E15.5, just prior to initiating meiosis. *Ccnd1* misexpression in TGCT-susceptible gonocytes occurs at the same developmental time-points that *Ccnd1* is normally expressed in pre-meiotic oogonia (Heaney et al. 2012). These observations suggest that either a signal normally restricted to the developing ovary is aberrantly active or that activation of genes important to male gonocyte specification is delayed in the TGCT-susceptible testis. In the ovary, oogonia expression of *Ccnd1* coincides with RA induction of *Stra8* expression and the meiotic program from E13.5 to E15.5 (Koubova et al. 2006). In the embryonic testis RA is normally degraded by CYP26B1 expressed by Sertoli cells, prevents *Stra8* induction and inhibits meiosis in gonocytes. However, recent evidence from *Cyp26b1* and *Stra8* double knockout mice, in which RA signaling is constitutively active in the embryonic testis but meiosis cannot be initiated, demonstrated that RA also has a Stra8-independent, pro-proliferative influence on gonocytes (Saba et al. 2014b). Importantly, this same study revealed that RA induced the expression of several genes normally restricted to pre-meiotic oogonia and adult spermatogonia, including *Ccnd1*, *Ngn3*, and *Stra8*. It has been previously demonstrated that these same genes are misexpressed by TGCT-susceptible gonocytes (Heaney et al. 2012). Furthermore, $p15^{INK4b}$, which is downregulated in TGCT-susceptible gonocytes, was found to be inhibited in the gonocytes of *Cyp26b1* and

Stra8 double knockout mice (Cook et al. 2011; Saba et al. 2014b). Thus, an aberrant RA signal could be altering the expression of positive and negative regulators of the G1-S transition and delay the mitotic arrest of gonocytes in 129/Sv testes.

Interestingly, *Cyp26b1*/*Stra8* double knockout mice have rescued initiation of male differentiation, but still fail to enter mitotic arrest, similar to *Cyp26b1* single knockout mice (Saba et al. 2014b). Therefore, the failure to enter mitotic arrest is not linked to an alternate pathway to initiate meiosis through the activation of *Stra8*. Intrinsic male gonocyte differentiation factors acting together with aberrant RA may be sufficient in the *Cyp26b1*/*Stra8* double knockout mice to cause a failure of mitotic arrest. Convincingly, overexpression of NANOS2 in XX germ cells is sufficient to suppress meiosis and induce male specification (Suzuki and Saga 2008). In XY germ cells that are deficient for both *Nanos2* and *Stra8*, normal male germ cell development is not rescued (Saba et al. 2014a), suggesting a possible role for *Nanos2* in several aspects of sex specification in male gonocytes, in addition to the suppression of meiosis.

As alluded earlier, sex specification is intimately related with the developmental defects that contribute to the initiation of TGCTs in mice. The male or female specification pathways direct signaling from the microenvironment to control PGC specification, from E10.5, through the mitotic:meiotic switch starting at E13.5 to E15.5. The antagonism between signaling from female pathways for the induction of meiosis and normal male differentiation has been suggested for several decades (Wai-Sum and Baker 1976; McLaren 1984; Vigier et al. 1987; Yao et al. 2003; Kim et al. 2006). Additionally, studies in the mouse have postulated that decreased efficiency of intercellular somatic-germ cell signaling may lead to decreased activation of the male specification pathway and escape from induction of apoptosis. This hypothesis is exemplified in the reported differences in morphology of testis cords between TGCT-susceptible and resistant mouse strains; the 129 testes displayed large testis cords containing numerous germ cells, while C57Bl/6 testes had significantly smaller testis cords and fewer germ cells per cord (Western et al. 2011). Ideally, germ cells that are exposed to any aberrant signal are most likely removed by apoptosis. However, if the timing and the environment are just right, perhaps these mixed-up gonocytes initiate TGCTs.

As previously discussed, FGF9 signaling is also essential for XY germ cell survival and commitment to male specification (DiNapoli et al. 2006). *Fgf9*-null mice undergo sex reversal (Colvin et al. 2001; Schmahl et al. 2004; Bowles et al. 2010), but normal male differentiation can be rescued by deleting *Wnt4* (Colvin et al. 2001; Kim et al. 2006). Conversely, loss of *Wnt4* creates a partial sex reversal in XX gonads, and is not rescued by deleting *Fgf9*, indicating that FGF signaling is not necessary for the partial male characteristics developed in XX *Wnt4*-null gonads (Jameson et al. 2012). Thus, the pathways are not simply in opposition of each other, secondary components act in the male lineage to downregulate female pathways and promote male differentiation. This may provide insight as to how the "just right" condition comes about to promote tumor development in the face of preexisting developmental abnormalities.

Why has there been such an increase in TGCTs in the last 50 years? In this chapter, there has been a lengthy discussion of the genetic and developmental defects that give rise to TGCTs. But is there an environmental component that increases the prevalence of TGCTs? Studies have also found increased male reproductive health risks of undescended testis and hypospadias, and deteriorating semen quality in certain demographics and geographical regions (Skakkebaek et al. 2001). Xenoestrogens and other endocrine disrupting compounds may be to blame. Furthermore, the development of TGCTs has been linked to several disorders of gonadal development and sexual differentiation, which hints back to dysregulation of germ cell development. With a steady increase in incidence of Type II TGCTs in the last 40 years (Bernstein et al. 1999), in utero exposure to environmental factors may be interacting with genetic predispositions to disrupt normal germ cell development. (For more information on the discussion of the environment and hormone disruptors in spermatogonial development, refer to the chapter by Pat Hunt). Considering the large genetic component of developing TGCTs and the limited number of studies available to elucidate environmental risk, one could speculate that even a low risk of genetic susceptibility to developing TGCTs could interact with increased exposure to environmental triggers to increase rates of TGCTs.

How does epigenetics contribute to tumorigenesis? One of the areas that remains to be explored in the mouse model is the contribution of epigenetic changes to the genomic instability interacting with the developmental defects in the 129 background. There is evidence to reduced methylation contributing to increased malignancy of tumors in mice (Fraga et al. 2004), which may be the result of chromosomal instability. Reduced methylation has been shown to result in chromosomal instability in human glioblastomas (Cadieux et al. 2006) and colon cancers (Rodriguez et al. 2006). Hypomethylation of specific DNA sites is also associated with an erased (removed) pattern of genomic imprinting, found to be able to induce cancers in mice, related to the TRP53 and TGFβ pathway (Holm et al. 2005). Curiously, a few of the genes in susceptibility loci identified by GWAS, *Dmrt1* and *Prdm14*, function to maintain epigenetic states in the developing PGC. DMRT1 functions as a DNA methylase (Krentz et al. 2009) and PRDM14 maintains a naïve pluripotent state by regulating DNA methylation (Leitch et al. 2013; Okashita et al. 2014). Considering the genome-wide demethylation that occurs during PGC specification, changes in methylation may be the initial instability that goes on to interact with the developmental defects, ultimately giving rise to TGCTs in 129 mice.

Germ cells that transform into ECCs, what switch is thrown? As PGCs begin sex specification around E11.5, these pluripotent cells have an increased capacity for teratoma formation. Seminal studies from Leroy Stevens demonstrated the high success rate (75–80%) in grafting genital ridges from E11.5 or E12.5129 mice into adult testes to form teratomas. As development progresses, however, the incidence of teratoma formation steeply declines as the age of the genital ridge increases. Grafted genital ridges from E13.5 embryos results in 16% teratoma incidence, and incidence falls to <10% from E14.5 or later (Stevens 1966). What signals can be received by nearly all E11.5 PGCs to transform these cells to ECCs, but can only transform a fraction of E13.5 gonocytes? From Steven's studies, the window for

exposing germ cells with developmental defects to the adult male signaling environment to induce tumor formation occurs prior to E13.5; starting at E13.5 gonocytes are refractory to ECCs transformation in an altered signaling environment. The timing of the mitotic:meiotic switch in this same developmental window has lead researchers to explore altered male and female germ cell specification as clues to inducing tumorigenesis.

10.7 Conclusion

The 129 inbred mouse model has provided a generous amount of insight to the pathology and etiology of TGCTs. There has been debate as to the applicability of this mouse model to the most common cancer in adolescent and young adult human males, considering the relatively young age of tumor presentation in mice compared to the activation of dormant GCNIS at puberty in humans. However, considering that the same genes in susceptibility loci induce tumors and that the first two loci were first identified as susceptibility genes on the 129/Sv background, the pathology of the disease may progress similarly between mouse and Type II TGCTs. The technology is available to understand the genetic susceptibility in humans and validate these findings in a suitable model. From human GWAS to the interplay between genetics and environmental exposures, future studies using mouse models are strategically poised to begin deconvoluting the tough questions at the intersection of cancer and developmental biology.

References

Abada PB, Howell SB (2014) Cisplatin induces resistance by triggering differentiation of testicular embryonal carcinoma cells. PLoS One 9(1):e87444

Adamah DJ, Gokhale PJ, Eastwood DJ, Rajpert De-Meyts E, Goepel J, Walsh JR, Moore HD, Andrews PW (2006) Dysfunction of the mitotic:meiotic switch as a potential cause of neoplastic conversion of primordial germ cells. Int J Androl 29(1):219–227

Adams IR, McLaren A (2002) Sexually dimorphic development of mouse primordial germ cells: switching from oogenesis to spermatogenesis. Development 129(5):1155–1164

Anderson PD, Nelson VR, Tesar PJ, Nadeau JH (2009) Genetic factors on mouse chromosome 18 affecting susceptibility to testicular germ cell tumors and permissiveness to embryonic stem cell derivation. Cancer Res 69(23):9112–9117

Andreassen KE, Kristiansen W, Karlsson R, Aschim EL, Dahl O, Fossa SD, Adami HO, Wiklund F, Haugen TB, Grotmol T (2013) Genetic variation in AKT1, PTEN and the 8q24 locus, and the risk of testicular germ cell tumor. Hum Reprod 28(7):1995–2002

Aravin AA, Sachidanandam R, Bourc'his D, Schaefer C, Pezic D, Toth KF, Bestor T, Hannon GJ (2008) A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. Mol Cell 31(6):785–799

Asada Y, Varnum DS, Frankel WN, Nadeau JH (1994) A mutation in the Ter gene causing increased susceptibility to testicular teratomas maps to mouse chromosome 18. Nat Genet 6(4):363–368

Atkin NB, Baker MC (1983) i(12p): specific chromosomal marker in seminoma and malignant teratoma of the testis? Cancer Genet Cytogenet 10(2):199–204

Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R (2003) Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 17(1):126–140
Bahrami A, Ro JY, Ayala AG (2007) An overview of testicular germ cell tumors. Arch Pathol Lab Med 131(8):1267–1280
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(Pt 6):871–880
Bernstein L, Smith MA, Lihua L, Deapen D, Friedman DL (1999) Germ cell, trophoblastic and other gonadal neoplasms. In: Ries LAG, Smith MA, Gurney JC et al (eds) Cancer incidence and survival among childrem and adolescents: United States SEER program 1975–1995. National Cancer Institute, Bethesda, MD
Beumer TL, Roepers-Gajadien HL, Gademan IS, Kal HB, de Rooij DG (2000) Involvement of the D-type cyclins in germ cell proliferation and differentiation in the mouse. Biol Reprod 63(6):1893–1898
Bosl GJ, Motzer RJ (1997) Testicular germ-cell cancer. N Engl J Med 337(4):242–253
Bourc'his D, Bestor TH (2004) Meiotic catastrophe and retrotransposon reactivation in male germ cells lacking Dnmt3L. Nature 431(7004):96–99
Bourc'his D, Xu GL, Lin CS, Bollman B, Bestor TH (2001) Dnmt3L and the establishment of maternal genomic imprints. Science 294(5551):2536–2539
Bowles J, Feng CW, Spiller C, Davidson TL, Jackson A, Koopman P (2010) FGF9 suppresses meiosis and promotes male germ cell fate in mice. Dev Cell 19(3):440–449
Bowles J, Knight D, Smith C, Wilhelm D, Richman J, Mamiya S, Yashiro K, Chawengsaksophak K, Wilson MJ, Rossant J, Hamada H, Koopman P (2006) Retinoid signaling determines germ cell fate in mice. Science 312(5773):596–600
Bowles J, Koopman P (2010) Sex determination in mammalian germ cells: extrinsic versus intrinsic factors. Reproduction 139(6):943–958
Brabrand S, Johannessen B, Axcrona U, Kraggerud SM, Berg KG, Bakken AC, Bruun J, Fossa SD, Lothe RA, Lehne G, Skotheim RI (2015) Exome sequencing of bilateral testicular germ cell tumors suggests independent development lineages. Neoplasia 17(2):167–174
Bromen K, Stang A, Baumgardt-Elms C, Stegmaier C, Ahrens W, Metz KA, Jockel KH (2004) Testicular, other genital, and breast cancers in first-degree relatives of testicular cancer patients and controls. Cancer Epidemiol Biomark Prev 13(8):1316–1324
Bujan L, Walschaerts M, Moinard N, Hennebicq S, Saias J, Brugnon F, Auger J, Berthaut I, Szerman E, Daudin M, Rives N (2013) Impact of chemotherapy and radiotherapy for testicular germ cell tumors on spermatogenesis and sperm DNA: a multicenter prospective study from the CECOS network. Fertil Steril 100(3):673–680
Bussey KJ, Lawce HJ, Himoe E, Shu XO, Heerema NA, Perlman EJ, Olson SB, Magenis RE (2001) SNRPN methylation patterns in germ cell tumors as a reflection of primordial germ cell development. Genes Chromosomes Cancer 32(4):342–352
Bussey KJ, Lawce HJ, Olson SB, Arthur DC, Kalousek DK, Krailo M, Giller R, Heifetz S, Womer R, Magenis RE (1999) Chromosome abnormalities of eighty-one pediatric germ cell tumors: sex-, age-, site-, and histopathology-related differences—a Children's Cancer Group study. Genes Chromosomes Cancer 25(2):134–146
Bustamante-Marin X, Garness JA, Capel B (2013) Testicular teratomas: an intersection of pluripotency, differentiation and cancer biology. Int J Dev Biol 57(2–4):201–210
Byskov AG (1978) The anatomy and ultrastructure of the rete system in the fetal mouse ovary. Biol Reprod 19(4):720–735
Byskov AG (1986) Differentiation of mammalian embryonic gonad. Physiol Rev 66(1):71–117
Cadieux B, Ching TT, VandenBerg SR, Costello JF (2006) Genome-wide hypomethylation in human glioblastomas associated with specific copy number alteration, methylenetetrahydrofolate reductase allele status, and increased proliferation. Cancer Res 66(17):8469–8476
Carouge D, Blanc V, Knoblaugh SE, Hunter RJ, Davidson NO, Nadeau JH (2016) Parent-of-origin effects of A1CF and AGO2 on testicular germ-cell tumors, testicular abnormalities, and fertilization bias. Proc Natl Acad Sci U S A 113(37):E5425–E5433

Carriere P, Baade P, Fritschi L (2007) Population based incidence and age distribution of spermatocytic seminoma. J Urol 178(1):125–128

Castedo SM, de Jong B, Oosterhuis JW, Seruca R, te Meerman GJ, Dam A, Schraffordt Koops H (1989) Cytogenetic analysis of ten human seminomas. Cancer Res 49(2):439–443

Chadalavada RS, Korkola JE, Houldsworth J, Olshen AB, Bosl GJ, Studer L, Chaganti RS (2007) Constitutive gene expression predisposes morphogen-mediated cell fate responses of NT2/D1 and 27X-1 human embryonal carcinoma cells. Stem Cells 25(3):771–778

Chanock S (2009) High marks for GWAS. Nat Genet 41(7):765–766

Chia NY, Chan YS, Feng B, Lu X, Orlov YL, Moreau D, Kumar P, Yang L, Jiang J, Lau MS, Huss M, Soh BS, Kraus P, Li P, Lufkin T, Lim B, Clarke ND, Bard F, Ng HH (2010) A genome-wide RNAi screen reveals determinants of human embryonic stem cell identity. Nature 468(7321):316–320

Chia, V. M., Y. Li, L. R. Goldin, B. I. Graubard, M. H. Greene, L. Korde, M. V. Rubertone, R. L. Erickson and K. A. McGlynn (2009). "Risk of cancer in first- and second-degree relatives of testicular germ cell tumor cases and controls." Int J Cancer 124(4): 952–957

Childs AJ, Cowan G, Kinnell HL, Anderson RA, Saunders PT (2011) Retinoic Acid signalling and the control of meiotic entry in the human fetal gonad. PLoS One 6(6):e20249

Chin L, Andersen JN, Futreal PA (2011) Cancer genomics: from discovery science to personalized medicine. Nat Med 17(3):297–303

Chung CC, Kanetsky PA, Wang Z, Hildebrandt MA, Koster R, Skotheim RI, Kratz CP, Turnbull C, Cortessis VK, Bakken AC, Bishop DT, Cook MB, Erickson RL, Fossa SD, Jacobs KB, Korde LA, Kraggerud SM, Lothe RA, Loud JT, Rahman N, Skinner EC, Thomas DC, Wu X, Yeager M, Schumacher FR, Greene MH, Schwartz SM, McGlynn KA, Chanock SJ, Nathanson KL (2013) Meta-analysis identifies four new loci associated with testicular germ cell tumor. Nat Genet 45(6):680–685

Ciccodicola A, Dono R, Obici S, Simeone A, Zollo M, Persico MG (1989) Molecular characterization of a gene of the 'EGF family' expressed in undifferentiated human NTERA2 teratocarcinoma cells. EMBO J 8(7):1987–1991

Clark AT, Rodriguez RT, Bodnar MS, Abeyta MJ, Cedars MI, Turek PJ, Firpo MT, Reijo Pera RA (2004) Human STELLAR, NANOG, and GDF3 genes are expressed in pluripotent cells and map to chromosome 12p13, a hotspot for teratocarcinoma. Stem Cells 22(2):169–179

Colvin JS, Green RP, Schmahl J, Capel B, Ornitz DM (2001) Male-to-female sex reversal in mice lacking fibroblast growth factor 9. Cell 104(6):875–889

Cook MS, Coveney D, Batchvarov I, Nadeau JH, Capel B (2009) BAX-mediated cell death affects early germ cell loss and incidence of testicular teratomas in Dnd1(Ter/Ter) mice. Dev Biol 328(2):377–383

Cook MS, Munger SC, Nadeau JH, Capel B (2011) Regulation of male germ cell cycle arrest and differentiation by DND1 is modulated by genetic background. Development 138(1):23–32

Cools M, Wolffenbuttel KP, Drop SL, Oosterhuis JW, Looijenga LH (2011) Gonadal development and tumor formation at the crossroads of male and female sex determination. Sex Dev 5(4):167–180

Crockford GP, Linger R, Hockley S, Dudakia D, Johnson L, Huddart R, Tucker K, Friedlander M, Phillips KA, Hogg D, Jewett MA, Lohynska R, Daugaard G, Richard S, Chompret A, Bonaiti-Pellie C, Heidenreich A, Albers P, Olah E, Geczi L, Bodrogi I, Ormiston WJ, Daly PA, Guilford P, Fossa SD, Heimdal K, Tjulandin SA, Liubchenko L, Stoll H, Weber W, Forman D, Oliver T, Einhorn L, McMaster M, Kramer J, Greene MH, Weber BL, Nathanson KL, Cortessis V, Easton DF, Bishop DT, Stratton MR, Rapley EA (2006) Genome-wide linkage screen for testicular germ cell tumour susceptibility loci. Hum Mol Genet 15(3):443–451

Cutcutache I, Suzuki Y, Tan IB, Ramgopal S, Zhang S, Ramnarayanan K, Gan A, Lee HH, Tay ST, Ooi A, Ong CK, Bolthouse JT, Lane BR, Anema JG, Kahnoski RJ, Tan P, Teh BT, Rozen SG (2015) Exome-wide sequencing shows low mutation rates and identifies novel mutated genes in seminomas. Eur Urol 68(1):77–83

Czene K, Lichtenstein P, Hemminki K (2002) Environmental and heritable causes of cancer among 9.6 million individuals in the Swedish Family-Cancer Database. Int J Cancer 99(2):260–266

Davies RJ, Miller R, Coleman N (2005) Colorectal cancer screening: prospects for molecular stool analysis. Nat Rev Cancer 5(3):199–209

Davis TL, Yang GJ, McCarrey JR, Bartolomei MS (2000) The H19 methylation imprint is erased and re-established differentially on the parental alleles during male germ cell development. Hum Mol Genet 9(19):2885–2894

de Graaff WE, Oosterhuis JW, de JB, Dam A, van Putten WL, Castedo SM, Sleijfer DT, Schraffordt KH (1992) Ploidy of testicular carcinoma in situ. Lab Investig 66(2):166–168

de Haas EC, Altena R, Boezen HM, Zwart N, Smit AJ, Bakker SJ, van Roon AM, Postma A, Wolffenbuttel BH, Hoekstra HJ, van Leeuwen FE, Sleijfer DT, Gietema JA (2013) Early development of the metabolic syndrome after chemotherapy for testicular cancer. Ann Oncol 24(3):749–755

de Jong B, Oosterhuis JW, Castedo SM, Vos A, te Meerman GJ (1990) Pathogenesis of adult testicular germ cell tumors. A cytogenetic model. Cancer Genet Cytogenet 48(2):143–167

Deshpande A, Sicinski P, Hinds PW (2005) Cyclins and cdks in development and cancer: a perspective. Oncogene 24(17):2909–2915

DiNapoli L, Batchvarov J, Capel B (2006) FGF9 promotes survival of germ cells in the fetal testis. Development 133(8):1519–1527

Durcova-Hills G, Adams IR, Barton SC, Surani MA, McLaren A (2006) The role of exogenous fibroblast growth factor-2 on the reprogramming of primordial germ cells into pluripotent stem cells. Stem Cells 24(6):1441–1449

Feldman DR, Iyer G, Van Alstine L, Patil S, Al-Ahmadie H, Reuter VE, Bosl GJ, Chaganti RS, Solit DB (2014) Presence of somatic mutations within PIK3CA, AKT, RAS, and FGFR3 but not BRAF in cisplatin-resistant germ cell tumors. Clin Cancer Res 20(14):3712–3720

Feng CW, Bowles J, Koopman P (2014) Control of mammalian germ cell entry into meiosis. Mol Cell Endocrinol 382(1):488–497

Filipczyk AA, Laslett AL, Mummery C, Pera MF (2007) Differentiation is coupled to changes in the cell cycle regulatory apparatus of human embryonic stem cells. Stem Cell Res 1(1): 45–60

Forman D, Oliver RT, Brett AR, Marsh SG, Moses JH, Bodmer JG, Chilvers CE, Pike MC (1992) Familial testicular cancer: a report of the UK family register, estimation of risk and an HLA class 1 sib-pair analysis. Br J Cancer 65(2):255–262

Fraga MF, Herranz M, Espada J, Ballestar E, Paz MF, Ropero S, Erkek E, Bozdogan O, Peinado H, Niveleau A, Mao JH, Balmain A, Cano A, Esteller M (2004) A mouse skin multistage carcinogenesis model reflects the aberrant DNA methylation patterns of human tumors. Cancer Res 64(16):5527–5534

Frigyesi A, Gisselsson D, Hansen GB, Soller M, Mitelman F, Hoglund M (2004) A model for karyotypic evolution in testicular germ cell tumors. Genes Chromosomes Cancer 40(3):172–178

Gidekel S, Pizov G, Bergman Y, Pikarsky E (2003) Oct-3/4 is a dose-dependent oncogenic fate determinant. Cancer Cell 4(5):361–370

Gill ME, Hu YC, Lin Y, Page DC (2011) Licensing of gametogenesis, dependent on RNA binding protein DAZL, as a gateway to sexual differentiation of fetal germ cells. Proc Natl Acad Sci U S A 108(18):7443–7448

Gillis AJ, Verkerk AJ, Dekker MC, van Gurp RJ, Oosterhuis JW, Looijenga LH (1997) Methylation similarities of two CpG sites within exon 5 of human H19 between normal tissues and testicular germ cell tumours of adolescents and adults, without correlation with allelic and total level of expression. Br J Cancer 76(6):725–733

Ginsburg M, Snow MH, McLaren A (1990) Primordial germ cells in the mouse embryo during gastrulation. Development 110(2):521–528

Gori S, Porrozzi S, Roila F, Gatta G, De Giorgi U, Marangolo M (2005) Germ cell tumours of the testis. Crit Rev Oncol Hematol 53(2):141–164

Grabole N, Tischler J, Hackett JA, Kim S, Tang F, Leitch HG, Magnusdottir E, Surani MA (2013) Prdm14 promotes germline fate and naive pluripotency by repressing FGF signalling and DNA methylation. EMBO Rep 14(7):629–637

Gutekunst M, Oren M, Weilbacher A, Dengler MA, Markwardt C, Thomale J, Aulitzky WE, van der Kuip H (2011) p53 hypersensitivity is the predominant mechanism of the unique responsiveness of testicular germ cell tumor (TGCT) cells to cisplatin. PLoS One 6(4):e19198

Hacker A, Capel B, Goodfellow P, Lovell-Badge R (1995) Expression of Sry, the mouse sex determining gene. Development 121(6):1603–1614

Hajkova P, Ancelin K, Waldmann T, Lacoste N, Lange UC, Cesari F, Lee C, Almouzni G, Schneider R, Surani MA (2008) Chromatin dynamics during epigenetic reprogramming in the mouse germ line. Nature 452(7189):877–881

Hajkova P, Erhardt S, Lane N, Haaf T, El-Maarri O, Reik W, Walter J, Surani MA (2002) Epigenetic reprogramming in mouse primordial germ cells. Mech Dev 117(1–2):15–23

Hajkova P, Jeffries SJ, Lee C, Miller N, Jackson SP, Surani MA (2010) Genome-wide reprogramming in the mouse germ line entails the base excision repair pathway. Science 329(5987):78–82

Harvey M, McArthur MJ, Montgomery CA Jr, Bradley A, Donehower LA (1993) Genetic background alters the spectrum of tumors that develop in p53-deficient mice. FASEB J 7(10):938–943

Hayashi K, Surani MA (2009) Self-renewing epiblast stem cells exhibit continual delineation of germ cells with epigenetic reprogramming in vitro. Development 136(21):3549–3556

Heaney JD, Anderson EL, Michelson MV, Zechel JL, Conrad PA, Page DC, Nadeau JH (2012) Germ cell pluripotency, premature differentiation and susceptibility to testicular teratomas in mice. Development 139(9):1577–1586

Heaney JD, Lam MY, Michelson MV, Nadeau JH (2008) Loss of the transmembrane but not the soluble kit ligand isoform increases testicular germ cell tumor susceptibility in mice. Cancer Res 68(13):5193–5197

Heaney JD, Michelson MV, Youngren KK, Lam MY, Nadeau JH (2009) Deletion of eIF2beta suppresses testicular cancer incidence and causes recessive lethality in agouti-yellow mice. Hum Mol Genet 18(8):1395–1404

Heaney JD, Nadeau JH (2008) Testicular germ cell tumors in mice: new ways to study a genetically complex trait. Methods Mol Biol 450:211–231

Heimdal K, Olsson H, Tretli S, Flodgren P, Borresen AL, Fossa SD (1996) Risk of cancer in relatives of testicular cancer patients. Br J Cancer 73(7):970–973

Heimdal K, Olsson H, Tretli S, Fossa SD, Borresen AL, Bishop DT (1997) A segregation analysis of testicular cancer based on Norwegian and Swedish families. Br J Cancer 75(7):1084–1087

Herrera RE, Chen F, Weinberg RA (1996) Increased histone H1 phosphorylation and relaxed chromatin structure in Rb-deficient fibroblasts. Proc Natl Acad Sci U S A 93(21):11510–11515

Hoei-Hansen CE, Holm M, Rajpert-De Meyts E, Skakkebaek NE (2003) Histological evidence of testicular dysgenesis in contralateral biopsies from 218 patients with testicular germ cell cancer. J Pathol 200(3):370–374

Hoff AM, Alagaratnam S, Zhao S, Bruun J, Andrews PW, Lothe RA, Skotheim RI (2016) Identification of novel fusion genes in testicular germ cell tumors. Cancer Res 76(1):108–116

Hoffner L, Deka R, Chakravarti A, Surti U (1994) Cytogenetics and origins of pediatric germ cell tumors. Cancer Genet Cytogenet 74(1):54–58

Holm TM, Jackson-Grusby L, Brambrink T, Yamada Y, Rideout WM 3rd, Jaenisch R (2005) Global loss of imprinting leads to widespread tumorigenesis in adult mice. Cancer Cell 8(4):275–285

Horwich A, Shipley J, Huddart R (2006) Testicular germ-cell cancer. Lancet 367(9512):754–765

Howlader N, Noone AM, Krapcho M, Neyman N, Aminou R, Altekruse SF, Korsay CL, Ruhl J, Tatalovich Z, Cho H, Mariotto A, Eisner MP, Lewis DR, Chen HS, Feuer EJ, Cronin KA (2012) SEER cancer statistics review, 1975–2009 (Vintage 2009 Populations). National Cancer Institute, Bethesda, MD

Hughes IA, Houk C, Ahmed SF, Lee PA, Lawson Wilkins Pediatric Endocrine Society/European Society for Paediatric Endocrinology Consensus Group (2006) Consensus statement on management of intersex disorders. J Pediatr Urol 2(3):148–162

Jameson SA, Lin YT, Capel B (2012) Testis development requires the repression of Wnt4 by Fgf signaling. Dev Biol 370(1):24–32

Jiang LI, Nadeau JH (2001) 129/Sv mice—a model system for studying germ cell biology and testicular cancer. Mamm Genome 12(2):89–94

Jonk LJ, de Jonge ME, Kruyt FA, Mummery CL, van der Saag PT, Kruijer W (1992) Aggregation and cell cycle dependent retinoic acid receptor mRNA expression in P19 embryonal carcinoma cells. Mech Dev 36(3):165–172

Jorgensen A, Lindhardt Johansen M, Juul A, Skakkebaek NE, Main KM, Rajpert-De Meyts E (2015) Pathogenesis of germ cell neoplasia in testicular dysgenesis and disorders of sex development. Semin Cell Dev Biol 5:124–137

Jorgensen A, Nielsen JE, Almstrup K, Toft BG, Petersen BL, Rajpert-De ME (2013) Dysregulation of the mitosis-meiosis switch in testicular carcinoma in situ. J Pathol 229(4):588–598

Jorgensen A, Nielsen JE, Blomberg Jensen M, Graem N, Rajpert-De Meyts E (2012) Analysis of meiosis regulators in human gonads: a sexually dimorphic spatio-temporal expression pattern suggests involvement of DMRT1 in meiotic entry. Mol Hum Reprod 18(11):523–534

Kadyrova LY, Habara Y, Lee TH, Wharton RP (2007) Translational control of maternal Cyclin B mRNA by Nanos in the Drosophila germline. Development 134(8):1519–1527

Kanetsky PA, Mitra N, Vardhanabhuti S, Li M, Vaughn DJ, Letrero R, Ciosek SL, Doody DR, Smith LM, Weaver J, Albano A, Chen C, Starr JR, Rader DJ, Godwin AK, Reilly MP, Hakonarson H, Schwartz SM, Nathanson KL (2009) Common variation in KITLG and at 5q31.3 predisposes to testicular germ cell cancer. Nat Genet 41(7):811–815

Kanetsky PA, Mitra N, Vardhanabhuti S, Vaughn DJ, Li M, Ciosek SL, Letrero R, D'Andrea K, Vaddi M, Doody DR, Weaver J, Chen C, Starr JR, Hakonarson H, Rader DJ, Godwin AK, Reilly MP, Schwartz SM, Nathanson KL (2011) A second independent locus within DMRT1 is associated with testicular germ cell tumor susceptibility. Hum Mol Genet 20(15):3109–3117

Kaplan GW, Cromie WC, Kelalis PP, Silber I, Tank ES Jr (1988) Prepubertal yolk sac testicular tumors—report of the testicular tumor registry. J Urol 140(5 Pt 2):1109–1112

Kareta MS, Gorges LL, Hafeez S, Benayoun BA, Marro S, Zmoos AF, Cecchini MJ, Spacek D, Batista LF, O'Brien M, Ng YH, Ang CE, Vaka D, Artandi SE, Dick FA, Brunet A, Sage J, Wernig M (2015) Inhibition of pluripotency networks by the Rb tumor suppressor restricts reprogramming and tumorigenesis. Cell Stem Cell 16(1):39–50

Karlsson R, Andreassen KE, Kristiansen W, Aschim EL, Bremnes RM, Dahl O, Fossa SD, Klepp O, Langberg CW, Solberg A, Tretli S, Magnusson PK, Adami HO, Haugen TB, Grotmol T, Wiklund F (2013) Investigation of six testicular germ cell tumor susceptibility genes suggests a parent-of-origin effect in SPRY4. Hum Mol Genet 22(16):3373–3380

Kee K, Angeles VT, Flores M, Nguyen HN, Reijo Pera RA (2009) Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. Nature 462(7270):222–225

Kehler J, Tolkunova E, Koschorz B, Pesce M, Gentile L, Boiani M, Lomeli H, Nagy A, McLaughlin KJ, Scholer HR, Tomilin A (2004) Oct4 is required for primordial germ cell survival. EMBO Rep 5(11):1078–1083

Kemmer K, Corless CL, Fletcher JA, McGreevey L, Haley A, Griffith D, Cummings OW, Wait C, Town A, Heinrich MC (2004) KIT mutations are common in testicular seminomas. Am J Pathol 164(1):305–313

Kim Y, Kobayashi A, Sekido R, DiNapoli L, Brennan J, Chaboissier MC, Poulat F, Behringer RR, Lovell-Badge R, Capel B (2006) Fgf9 and Wnt4 act as antagonistic signals to regulate mammalian sex determination. PLoS Biol 4(6):e187

Kimura T, Suzuki A, Fujita Y, Yomogida K, Lomeli H, Asada N, Ikeuchi M, Nagy A, Mak TW, Nakano T (2003) Conditional loss of PTEN leads to testicular teratoma and enhances embryonic germ cell production. Development 130(8):1691–1700

Kleinsmith LJ, Pierce GB Jr (1964) Multipotentiality of single embryonal carcinoma cells. Cancer Res 24:1544–1551

Koide O, Iwai S, Baba K, Iri H (1987) Identification of testicular atypical germ cells by an immunohistochemical technique for placental alkaline phosphatase. Cancer 60(6):1325–1330

Kommoss F, Bibbo M, Talerman A (1990) Nuclear deoxyribonucleic acid content (ploidy) of endodermal sinus (yolk sac) tumor. Lab Investig 62(2):223–231

Koubova J, Hu YC, Bhattacharyya T, Soh YQ, Gill ME, Goodheart ML, Hogarth CA, Griswold MD, Page DC (2014) Retinoic acid activates two pathways required for meiosis in mice. PLoS Genet 10(8):e1004541

Koubova J, Menke DB, Zhou Q, Capel B, Griswold MD, Page DC (2006) Retinoic acid regulates sex-specific timing of meiotic initiation in mice. Proc Natl Acad Sci U S A 103(8):2474–2479

Krag Jacobsen G, Barlebo H, Olsen J, Schultz HP, Starklint H, Sogaard H, Vaeth M (1984) Testicular germ cell tumours in Denmark 1976-1980. Pathology of 1058 consecutive cases. Acta Radiol Oncol 23(4):239–247

Kraggerud SM, Hoei-Hansen CE, Alagaratnam S, Skotheim RI, Abeler VM, Rajpert-de ME, Lothe RA (2013) Molecular characteristics of malignant ovarian germ cell tumors and comparison with testicular counterparts: implications for pathogenesis. Endocr Rev 34(3):339–376

Kraggerud SM, Skotheim RI, Szymanska J, Eknaes M, Fossa SD, Stenwig AE, Peltomaki P, Lothe RA (2002) Genome profiles of familial/bilateral and sporadic testicular germ cell tumors. Genes Chromosomes Cancer 34(2):168–174

Kratz CP, Han SS, Rosenberg PS, Berndt SI, Burdett L, Yeager M, Korde LA, Mai PL, Pfeiffer R, Greene MH (2011) Variants in or near KITLG, BAK1, DMRT1, and TERT-CLPTM1L predispose to familial testicular germ cell tumour. J Med Genet 48(7):473–476

Krentz AD, Murphy MW, Kim S, Cook MS, Capel B, Zhu R, Matin A, Sarver AL, Parker KL, Griswold MD, Looijenga LH, Bardwell VJ, Zarkower D (2009) The DM domain protein DMRT1 is a dose-sensitive regulator of fetal germ cell proliferation and pluripotency. Proc Natl Acad Sci U S A 106(52):22323–22328

Krentz AD, Murphy MW, Zhang T, Sarver AL, Jain S, Griswold MD, Bardwell VJ, Zarkower D (2013) Interaction between DMRT1 function and genetic background modulates signaling and pluripotency to control tumor susceptibility in the fetal germ line. Dev Biol 377(1):67–78

Kunwar PS, Siekhaus DE, Lehmann R (2006) In vivo migration: a germ cell perspective. Annu Rev Cell Dev Biol 22:237–265

Kurimoto K, Yamaji M, Seki Y, Saitou M (2008) Specification of the germ cell lineage in mice: a process orchestrated by the PR-domain proteins, Blimp1 and Prdm14. Cell Cycle 7(22):3514–3518

Lane N, Dean W, Erhardt S, Hajkova P, Surani A, Walter J, Reik W (2003) Resistance of IAPs to methylation reprogramming may provide a mechanism for epigenetic inheritance in the mouse. Genesis 35(2):88–93

Lange UC, Adams DJ, Lee C, Barton S, Schneider R, Bradley A, Surani MA (2008) Normal germ line establishment in mice carrying a deletion of the Ifitm/Fragilis gene family cluster. Mol Cell Biol 28(15):4688–4696

Lanza DG, Dawson EP, Rao P, Heaney JD (2016) Misexpression of cyclin D1 in embryonic germ cells promotes testicular teratoma initiation. Cell Cycle 15(7):919–930

Lawson KA, Dunn NR, Roelen BA, Zeinstra LM, Davis AM, Wright CV, Korving JP, Hogan BL (1999) Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev 13(4):424–436

Lee J, Inoue K, Ono R, Ogonuki N, Kohda T, Kaneko-Ishino T, Ogura A, Ishino F (2002) Erasing genomic imprinting memory in mouse clone embryos produced from day 11.5 primordial germ cells. Development 129(8):1807–1817

Leitch HG, McEwen KR, Turp A, Encheva V, Carroll T, Grabole N, Mansfield W, Nashun B, Knezovich JG, Smith A, Surani MA, Hajkova P (2013) Naive pluripotency is associated with global DNA hypomethylation. Nat Struct Mol Biol 20(3):311–316

Li E (2002) Chromatin modification and epigenetic reprogramming in mammalian development. Nat Rev Genet 3(9):662–673

Lin Y, Page DC (2005) Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice. Dev Biol 288(2):309–316

Lin YT, Capel B (2015) Cell fate commitment during mammalian sex determination. Curr Opin Genet Dev 32:144–152

Lindelof B, Eklund G (2001) Analysis of hereditary component of cancer by use of a familial index by site. Lancet 358(9294):1696–1698

Litchfield K, Holroyd A, Lloyd A, Broderick P, Nsengimana J, Eeles R, Easton DF, Dudakia D, Bishop DT, Reid A, Huddart RA, Grotmol T, Wiklund F, Shipley J, Houlston RS, Turnbull C (2015a) Identification of four new susceptibility loci for testicular germ cell tumour. Nat Commun 6:8690

Litchfield K, Levy M, Huddart RA, Shipley J, Turnbull C (2016) The genomic landscape of testicular germ cell tumours: from susceptibility to treatment. Nat Rev Urol 13(7):409–419

Litchfield K, Shipley J, Turnbull C (2015b) Common variants identified in genome-wide association studies of testicular germ cell tumour: an update, biological insights and clinical application. Andrology 3(1):34–46

Litchfield K, Summersgill B, Yost S, Sultana R, Labreche K, Dudakia D, Renwick A, Seal S, Al-Saadi R, Broderick P, Turner NC, Houlston RS, Huddart R, Shipley J, Turnbull C (2015c) Whole-exome sequencing reveals the mutational spectrum of testicular germ cell tumours. Nat Commun 6:5973

Litchfield K, Thomsen H, Mitchell JS, Sundquist J, Houlston RS, Hemminki K, Turnbull C (2015d) Quantifying the heritability of testicular germ cell tumour using both population-based and genomic approaches. Sci Rep 5:13889

Looijenga LH, de LH, van OM, van Gurp RJ, Stoop H, Gillis AJ, de Gouveia Brazao CA, Weber RF, Kirkels WJ, van DT, von LM, Valk P, Lajos G, Olah E, Nesland JM, Fossa SD, Oosterhuis JW (2003a) Stem cell factor receptor (c-KIT) codon 816 mutations predict development of bilateral testicular germ-cell tumors. Cancer Res 63(22):7674–7678

Looijenga LH, Gillis AJ, van Gurp RJ, Verkerk AJ, Oosterhuis JW (1997) X inactivation in human testicular tumors. XIST expression and androgen receptor methylation status. Am J Pathol 151(2):581–590

Looijenga LH, Hersmus R, de Leeuw BH, Stoop H, Cools M, Oosterhuis JW, Drop SL, Wolffenbuttel KP (2010) Gonadal tumours and DSD. Best Pract Res Clin Endocrinol Metab 24(2):291–310

Looijenga LH, Hersmus R, Gillis AJ, Pfundt R, Stoop HJ, van Gurp RJ, Veltman J, Beverloo HB, van Drunen E, van Kessel AG, Pera RR, Schneider DT, Summersgill B, Shipley J, McIntyre A, van der Spek P, Schoenmakers E, Oosterhuis JW (2006) Genomic and expression profiling of human spermatocytic seminomas: primary spermatocyte as tumorigenic precursor and DMRT1 as candidate chromosome 9 gene. Cancer Res 66(1):290–302

Looijenga LH, Olie RA, van der Gaag I, van Sluijs FJ, Matoska J, Ploem-Zaaijer J, Knepfle C, Oosterhuis JW (1994) Seminomas of the canine testis. Counterpart of spermatocytic seminoma of men? Lab Investig 71(4):490–496

Looijenga LH, Oosterhuis JW (2002) Pathobiology of testicular germ cell tumors: views and news. Anal Quant Cytol Histol 24(5):263–279

Looijenga LH, Rosenberg C, van Gurp RJ, Geelen E, van Echten-Arends J, de Jong B, Mostert M, Wolter Oosterhuis J (2000) Comparative genomic hybridization of microdissected samples from different stages in the development of a seminoma and a non-seminoma. J Pathol 191(2):187–192

Looijenga LH, Stoop H, de Leeuw HP, de Gouveia Brazao CA, Gillis AJ, van Roozendaal KE, van Zoelen EJ, Weber RF, Wolffenbuttel KP, van DH, Honecker F, Bokemeyer C, Perlman EJ, Schneider DT, Kononen J, Sauter G, Oosterhuis JW (2003b) POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors. Cancer Res 63(9):2244–2250

Looijenga LH, Stoop H, Hersmus R, Gillis AJ, Wolter Oosterhuis J (2007) Genomic and expression profiling of human spermatocytic seminomas: pathogenetic implications. Int J Androl 30(4):328–335; discussion 335–326

Looijenga LH, Verkerk AJ, Dekker MC, van Gurp RJ, Gillis AJ, Oosterhuis JW (1998) Genomic imprinting in testicular germ cell tumours. APMIS 106(1):187–195

Looijenga LH, Zafarana G, Grygalewicz B, Summersgill B, Debiec-Rychter M, Veltman J, Schoenmakers EF, Rodriguez S, Jafer O, Clark J, van Kessel AG, Shipley J, van Gurp RJ, Gillis AJ, Oosterhuis JW (2003c) Role of gain of 12p in germ cell tumour development. APMIS 111(1):161–171; discussion 172–163

MacLean G, Li H, Metzger D, Chambon P, Petkovich M (2007) Apoptotic extinction of germ cells in testes of Cyp26b1 knockout mice. Endocrinology 148(10):4560–4567
Magnusdottir E, Dietmann S, Murakami K, Gunesdogan U, Tang F, Bao S, Diamanti E, Lao K, Gottgens B, Azim Surani M (2013) A tripartite transcription factor network regulates primordial germ cell specification in mice. Nat Cell Biol 15(8):905–915
Mahakali Zama A, Hudson FP 3rd, Bedell MA (2005) Analysis of hypomorphic KitlSl mutants suggests different requirements for KITL in proliferation and migration of mouse primordial germ cells. Biol Reprod 73(4):639–647
Mai PL, Friedlander M, Tucker K, Phillips KA, Hogg D, Jewett MA, Lohynska R, Daugaard G, Richard S, Bonaiti-Pellie C, Heidenreich A, Albers P, Bodrogi I, Geczi L, Olah E, Daly PA, Guilford P, Fossa SD, Heimdal K, Liubchenko L, Tjulandin SA, Stoll H, Weber W, Easton DF, Dudakia D, Huddart R, Stratton MR, Einhorn L, Korde L, Nathanson KL, Bishop DT, Rapley EA, Greene MH (2010) The International Testicular Cancer Linkage Consortium: a clinicopathologic descriptive analysis of 461 familial malignant testicular germ cell tumor kindred. Urol Oncol 28(5):492–499
Manivel JC, Reinberg Y, Niehans GA, Fraley EE (1989) Intratubular germ cell neoplasia in testicular teratomas and epidermoid cysts. Correlation with prognosis and possible biologic significance. Cancer 64(3):715–720
Manivel JC, Simonton S, Wold LE, Dehner LP (1988) Absence of intratubular germ cell neoplasia in testicular yolk sac tumors in children. A histochemical and immunohistochemical study. Arch Pathol Lab Med 112(6):641–645
Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78(12):7634–7638
Matin A, Collin GB, Asada Y, Varnum D, Nadeau JH (1999) Susceptibility to testicular germ-cell tumours in a 129.MOLF-Chr 19 chromosome substitution strain. Nat Genet 23(2):237–240
Matin A, Collin GB, Varnum DS, Nadeau JH (1998) Testicular teratocarcinogenesis in mice—a review. APMIS 106(1):174–182
Matsui Y, Zsebo K, Hogan BL (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70(5):841–847
Matsui Y, Zsebo KM, Hogan BL (1990) Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit. Nature 347(6294):667–669
Mayer F, Honecker F, Looijenga LH, Bokemeyer C (2003) Towards an understanding of the biological basis of response to cisplatin-based chemotherapy in germ-cell tumors. Ann Oncol 14(6):825–832
McIntyre A, Summersgill B, Grygalewicz B, Gillis AJ, Stoop J, van Gurp RJ, Dennis N, Fisher C, Huddart R, Cooper C, Clark J, Oosterhuis JW, Looijenga LH, Shipley J (2005) Amplification and overexpression of the KIT gene is associated with progression in the seminoma subtype of testicular germ cell tumors of adolescents and adults. Cancer Res 65(18):8085–8089
McLaren A (1984) Meiosis and differentiation of mouse germ cells. Symp Soc Exp Biol 38:7–23
McLaren A (1999) Signaling for germ cells. Genes Dev 13(4):373–376
McLaren A (2000) Germ and somatic cell lineages in the developing gonad. Mol Cell Endocrinol 163(1–2):3–9
McLaren A (2003) Primordial germ cells in the mouse. Dev Biol 262(1):1–15
McLaren A, Southee D (1997) Entry of mouse embryonic germ cells into meiosis. Dev Biol 187(1):107–113
Medrano JV, Ramathal C, Nguyen HN, Simon C, Reijo Pera RA (2012) Divergent RNA-binding proteins, DAZL and VASA, induce meiotic progression in human germ cells derived in vitro. Stem Cells 30(3):441–451
Mendis SH, Meachem SJ, Sarraj MA, Loveland KL (2011) Activin A balances Sertoli and germ cell proliferation in the fetal mouse testis. Biol Reprod 84(2):379–391
Menke DB, Koubova J, Page DC (2003) Sexual differentiation of germ cells in XX mouse gonads occurs in an anterior-to-posterior wave. Dev Biol 262(2):303–312
Menke DB, Page DC (2002) Sexually dimorphic gene expression in the developing mouse gonad. Gene Expr Patterns 2(3–4):359–367

Mesnard D, Guzman-Ayala M, Constam DB (2006) Nodal specifies embryonic visceral endoderm and sustains pluripotent cells in the epiblast before overt axial patterning. Development 133(13):2497–2505

Miles DC, van den Bergen JA, Wakeling SI, Anderson RB, Sinclair AH, Western PS (2012) The proto-oncogene Ret is required for male foetal germ cell survival. Dev Biol 365(1):101–109

Miles DC, Wakeling SI, Stringer JM, van den Bergen JA, Wilhelm D, Sinclair AH, Western PS (2013) Signaling through the TGF beta-activin receptors ALK4/5/7 regulates testis formation and male germ cell development. PLoS One 8(1):e54606

Mintz B, Russell ES (1957) Gene-induced embryological modifications of primordial germ cells in the mouse. J Exp Zool 134(2):207–237

Moniot B, Ujjan S, Champagne J, Hirai H, Aritake K, Nagata K, Dubois E, Nidelet S, Nakamura M, Urade Y, Poulat F, Boizet-Bonhoure B (2014) Prostaglandin D2 acts through the Dp2 receptor to influence male germ cell differentiation in the foetal mouse testis. Development 141(18):3561–3571

Moreno SG, Attali M, Allemand I, Messiaen S, Fouchet P, Coffigny H, Romeo PH, Habert R (2010) TGFbeta signaling in male germ cells regulates gonocyte quiescence and fertility in mice. Dev Biol 342(1):74–84

Moshfegh C, Aires L, Kisielow M, Vogel V (2016) A gonogenic stimulated transition of mouse embryonic stem cells with enhanced control of diverse differentiation pathways. Sci Rep 6:25104

Mostert M, Rosenberg C, Stoop H, Schuyer M, Timmer A, Oosterhuis W, Looijenga L (2000) Comparative genomic and in situ hybridization of germ cell tumors of the infantile testis. Lab Investig 80(7):1055–1064

Mostofi FK, Sesterhenn IA, Davis CJ Jr (1988) Developments in histopathology of testicular germ cell tumors. Semin Urol 6(3):171–188

Muller AJ, Teresky AK, Levine AJ (2000) A male germ cell tumor-susceptibility-determining locus, pgct1, identified on murine chromosome 13. Proc Natl Acad Sci U S A 97(15):8421–8426

Muller J, Skakkebaek NE, Ritzen M, Ploen L, Petersen KE (1985) Carcinoma in situ of the testis in children with 45,X/46,XY gonadal dysgenesis. J Pediatr 106(3):431–436

Mummery CL, van Rooijen MA, van den Brink SE, de Laat SW (1987) Cell cycle analysis during retinoic acid induced differentiation of a human embryonal carcinoma-derived cell line. Cell Differ 20(2–3):153–160

Munsterberg A, Lovell-Badge R (1991) Expression of the mouse anti-mullerian hormone gene suggests a role in both male and female sexual differentiation. Development 113(2):613–624

Nathanson KL, Kanetsky PA, Hawes R, Vaughn DJ, Letrero R, Tucker K, Friedlander M, Phillips KA, Hogg D, Jewett MA, Lohynska R, Daugaard G, Richard S, Chompret A, Bonaiti-Pellie C, Heidenreich A, Olah E, Geczi L, Bodrogi I, Ormiston WJ, Daly PA, Oosterhuis JW, Gillis AJ, Looijenga LH, Guilford P, Fossa SD, Heimdal K, Tjulandin SA, Liubchenko L, Stoll H, Weber W, Rudd M, Huddart R, Crockford GP, Forman D, Oliver DT, Einhorn L, Weber BL, Kramer J, McMaster M, Greene MH, Pike M, Cortessis V, Chen C, Schwartz SM, Bishop DT, Easton DF, Stratton MR, Rapley EA (2005) The Y deletion gr/gr and susceptibility to testicular germ cell tumor. Am J Hum Genet 77(6):1034–1043

Nelson VR, Heaney JD, Tesar PJ, Davidson NO, Nadeau JH (2012) Transgenerational epigenetic effects of the Apobec1 cytidine deaminase deficiency on testicular germ cell tumor susceptibility and embryonic viability. Proc Natl Acad Sci U S A 109(41):E2766–E2773

Nicholls PK, Harrison CA, Gilchrist RB, Farnworth PG, Stanton PG (2009) Growth differentiation factor 9 is a germ cell regulator of Sertoli cell function. Endocrinology 150(5):2481–2490

Nitzsche B, Gloesenkamp C, Schrader M, Hoffmann B, Zengerling F, Balabanov S, Honecker F, Hopfner M (2012) Anti-tumour activity of two novel compounds in cisplatin-resistant testicular germ cell cancer. Br J Cancer 107(11):1853–1863

Noel EE, Yeste-Velasco M, Mao X, Perry J, Kudahetti SC, Li NF, Sharp S, Chaplin T, Xue L, McIntyre A, Shan L, Powles T, Oliver RT, Young BD, Shipley J, Berney DM, Joel SP, Lu YJ (2010) The association of CCND1 overexpression and cisplatin resistance in testicular germ cell tumors and other cancers. Am J Pathol 176(6):2607–2615

Noguchi M, Watanabe C, Kobayashi T, Kuwashima M, Sakurai T, Katoh H, Moriwaki K (1996) The ter mutation responsible for germ cell deficiency nor ovarian teratocarcinogenesis in ter/ter congenic mice. Develop Growth Differ 38:59–69

Noguchi T, Stevens LC (1982) Primordial germ cell proliferation in fetal testes in mouse strains with high and low incidences of congenital testicular teratomas. J Natl Cancer Inst 69(4):907–913

Wai-Sum O, Baker TG (1976) Initiation and control of meiosis in hamster gonads in vitro. J Reprod Fertil 48(2):399–401

Ohinata Y, Ohta H, Shigeta M, Yamanaka K, Wakayama T, Saitou M (2009) A signaling principle for the specification of the germ cell lineage in mice. Cell 137(3):571–584

Ohinata Y, Payer B, O'Carroll D, Ancelin K, Ono Y, Sano M, Barton SC, Obukhanych T, Nussenzweig M, Tarakhovsky A, Saitou M, Surani MA (2005) Blimp1 is a critical determinant of the germ cell lineage in mice. Nature 436(7048):207–213

Okashita N, Kumaki Y, Ebi K, Nishi M, Okamoto Y, Nakayama M, Hashimoto S, Nakamura T, Sugasawa K, Kojima N, Takada T, Okano M, Seki Y (2014) PRDM14 promotes active DNA demethylation through the ten-eleven translocation (TET)-mediated base excision repair pathway in embryonic stem cells. Development 141(2):269–280

Oldenburg J, Kraggerud SM, Brydoy M, Cvancarova M, Lothe RA, Fossa SD (2007a) Association between long-term neuro-toxicities in testicular cancer survivors and polymorphisms in glutathione-s-transferase-P1 and -M1, a retrospective cross sectional study. J Transl Med 5:70

Oldenburg J, Kraggerud SM, Cvancarova M, Lothe RA, Fossa SD (2007b) Cisplatin-induced long-term hearing impairment is associated with specific glutathione s-transferase genotypes in testicular cancer survivors. J Clin Oncol 25(6):708–714

Oosterhuis JW, Castedo SM, de JB, Cornelisse CJ, Dam A, Sleijfer DT, Schraffordt KH (1989) Ploidy of primary germ cell tumors of the testis. Pathogenetic and clinical relevance. Lab Investig 60(1):14–21

Oosterhuis JW, Looijenga LH (2005) Testicular germ-cell tumours in a broader perspective. Nat Rev Cancer 5(3):210–222

Oosterhuis JW, Looijenga LH, van EJ, de JB (1997) Chromosomal constitution and developmental potential of human germ cell tumors and teratomas. Cancer Genet Cytogenet 95(1):96–102

Oshimori N, Fuchs E (2012) The harmonies played by TGF-beta in stem cell biology. Cell Stem Cell 11(6):751–764

Ottesen AM, Kirchhoff M, Rajpert De-Meyts E, Maahr J, Gerdes T, Rose H, Lundsteen C, Petersen PM, Philip J, Skakkebaek NE (1997) Detection of chromosomal aberrations in seminomatous germ cell tumours using comparative genomic hybridization. Genes Chromosomes Cancer 20(4):412–418

Ottesen AM, Skakkebaek NE, Lundsteen C, Leffers H, Larsen J, Rajpert-De Meyts E (2003) High-resolution comparative genomic hybridization detects extra chromosome arm 12p material in most cases of carcinoma in situ adjacent to overt germ cell tumors, but not before the invasive tumor development. Genes Chromosomes Cancer 38(2):117–125

Palumbo C, van RK, Gillis AJ, van Gurp RH, de MH, Oosterhuis JW, van Zoelen EJ, Looijenga LH (2002) Expression of the PDGF alpha-receptor 1.5 kb transcript, OCT-4, and c-KIT in human normal and malignant tissues. Implications for the early diagnosis of testicular germ cell tumours and for our understanding of regulatory mechanisms. J Pathol 196(4):467–477

Park SY, Jameson JL (2005) Minireview: transcriptional regulation of gonadal development and differentiation. Endocrinology 146(3):1035–1042

Pauklin S, Vallier L (2015) Activin/Nodal signalling in stem cells. Development 142(4):607–619

Payer B, Saitou M, Barton SC, Thresher R, Dixon JP, Zahn D, Colledge WH, Carlton MB, Nakano T, Surani MA (2003) Stella is a maternal effect gene required for normal early development in mice. Curr Biol 13(23):2110–2117

Pesce M, Scholer HR (2000) Oct-4: control of totipotency and germline determination. Mol Reprod Dev 55(4):452–457

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(1–2):89–98

Peters U, Preisler-Adams S, Hebeisen A, Hahn M, Seifert E, Lanvers C, Heinecke A, Horst J, Jurgens H, Lamprecht-Dinnesen A (2000) Glutathione S-transferase genetic polymorphisms and individual sensitivity to the ototoxic effect of cisplatin. Anti-Cancer Drugs 11(8): 639–643

Pierce GB, Stevens LC, Nakane PK (1967) Ultrastructural analysis of the early development of teratocarcinomas. J Natl Cancer Inst 39(4):755–773

Pleskacova J, Hersmus R, Oosterhuis JW, Setyawati BA, Faradz SM, Cools M, Wolffenbuttel KP, Lebl J, Drop SL, Looijenga LH (2010) Tumor risk in disorders of sex development. Sex Dev 4(4–5):259–269

Popp C, Dean W, Feng S, Cokus SJ, Andrews S, Pellegrini M, Jacobsen SE, Reik W (2010) Genome-wide erasure of DNA methylation in mouse primordial germ cells is affected by AID deficiency. Nature 463(7284):1101–1105

Poynter JN, Hooten AJ, Frazier AL, Ross JA (2012) Associations between variants in KITLG, SPRY4, BAK1, and DMRT1 and pediatric germ cell tumors. Genes Chromosomes Cancer 51(3):266–271

Rajpert-De Meyts E (2006) Developmental model for the pathogenesis of testicular carcinoma in situ: genetic and environmental aspects. Hum Reprod Update 12(3):303–323

Rajpert-De Meyts E, Hanstein R, Jorgensen N, Graem N, Vogt PH, Skakkebaek NE (2004) Developmental expression of POU5F1 (OCT-3/4) in normal and dysgenetic human gonads. Hum Reprod 19(6):1338–1344

Rajpert-de Meyts E, Hoei-Hansen CE (2007) From gonocytes to testicular cancer: the role of impaired gonadal development. Ann N Y Acad Sci 1120:168–180

Rajpert-De Meyts E, Skakkebaek NE (1994) Expression of the c-kit protein product in carcinoma-in-situ and invasive testicular germ cell tumours. Int J Androl 17(2):85–92

Rapley EA, Crockford GP, Teare D, Biggs P, Seal S, Barfoot R, Edwards S, Hamoudi R, Heimdal K, Fossa SD, Tucker K, Donald J, Collins F, Friedlander M, Hogg D, Goss P, Heidenreich A, Ormiston W, Daly PA, Forman D, Oliver TD, Leahy M, Huddart R, Cooper CS, Bodmer JG, Easton DF, Stratton MR, Bishop DT (2000) Localization to Xq27 of a susceptibility gene for testicular germ-cell tumours. Nat Genet 24(2):197–200

Rapley EA, Nathanson KL (2010) Predisposition alleles for testicular germ cell tumour. Curr Opin Genet Dev 20(3):225–230

Rapley EA, Turnbull C, Al Olama AA, Dermitzakis ET, Linger R, Huddart RA, Renwick A, Hughes D, Hines S, Seal S, Morrison J, Nsengimana J, Deloukas P, Rahman N, Bishop DT, Easton DF, Stratton MR (2009) A genome-wide association study of testicular germ cell tumor. Nat Genet 41(7):807–810

Rodriguez E, Houldsworth J, Reuter VE, Meltzer P, Zhang J, Trent JM, Bosl GJ, Chaganti RS (1993) Molecular cytogenetic analysis of i(12p)-negative human male germ cell tumors. Genes Chromosomes Cancer 8(4):230–236

Rodriguez E, Mathew S, Reuter V, Ilson DH, Bosl GJ, Chaganti RS (1992) Cytogenetic analysis of 124 prospectively ascertained male germ cell tumors. Cancer Res 52(8):2285–2291

Rodriguez J, Frigola J, Vendrell E, Risques RA, Fraga MF, Morales C, Moreno V, Esteller M, Capella G, Ribas M, Peinado MA (2006) Chromosomal instability correlates with genome-wide DNA demethylation in human primary colorectal cancers. Cancer Res 66(17):8462–9468

Ross JA, Schmidt PT, Perentesis JP, Davies SM (1999) Genomic imprinting of H19 and insulin-like growth factor-2 in pediatric germ cell tumors. Cancer 85(6):1389–1394

Rossant J, Papaioannou VE (1984) The relationship between embryonic, embryonal carcinoma and embryo-derived stem cells. Cell Differ 15(2–4):155–161

Ruark E, Seal S, McDonald H, Zhang F, Elliot A, Lau K, Perdeaux E, Rapley E, Eeles R, Peto J, Kote-Jarai Z, Muir K, Nsengimana J, Shipley J, Bishop DT, Stratton MR, Easton DF, Huddart RA, Rahman N, Turnbull C (2013) Identification of nine new susceptibility loci for testicular cancer, including variants near DAZL and PRDM14. Nat Genet 45(6):686–689

Saba R, Kato Y, Saga Y (2014a) NANOS2 promotes male germ cell development independent of meiosis suppression. Dev Biol 385(1):32–40

Saba R, Wu Q, Saga Y (2014b) CYP26B1 promotes male germ cell differentiation by suppressing STRA8-dependent meiotic and STRA8-independent mitotic pathways. Dev Biol 389(2):173–181
Saitou M (2009) Germ cell specification in mice. Curr Opin Genet Dev 19(4):386–395
Saitou M, Barton SC, Surani MA (2002) A molecular programme for the specification of germ cell fate in mice. Nature 418(6895):293–300
Saitou M, Kagiwada S, Kurimoto K (2012) Epigenetic reprogramming in mouse pre-implantation development and primordial germ cells. Development 139(1):15–31
Saitou M, Miyauchi H (2016) Gametogenesis from Pluripotent Stem Cells. Cell Stem Cell 18(6):721–735
Saitou M, Payer B, Lange UC, Erhardt S, Barton SC, Surani MA (2003) Specification of germ cell fate in mice. Philos Trans R Soc Lond Ser B Biol Sci 358(1436):1363–1370
Saitou M, Yabuta Y, Kurimoto K (2008) Single-cell cDNA high-density oligonucleotide microarray analysis: detection of individual cell types and properties in complex biological processes. Reprod Biomed Online 16(1):26–40
Saitou M, Yamaji M (2010) Germ cell specification in mice: signaling, transcription regulation, and epigenetic consequences. Reproduction 139(6):931–942
Sasaki H, Matsui Y (2008) Epigenetic events in mammalian germ-cell development: reprogramming and beyond. Nat Rev Genet 9(2):129–140
Schemmer J, Arauzo-Bravo MJ, Haas N, Schafer S, Weber SN, Becker A, Eckert D, Zimmer A, Nettersheim D, Schorle H (2013) Transcription factor TFAP2C regulates major programs required for murine fetal germ cell maintenance and haploinsufficiency predisposes to teratomas in male mice. PLoS One 8(8):e71113
Schmahl J, Capel B (2003) Cell proliferation is necessary for the determination of male fate in the gonad. Dev Biol 258(2):264–276
Schmahl J, Eicher EM, Washburn LL, Capel B (2000) Sry induces cell proliferation in the mouse gonad. Development 127(1):65–73
Schmahl J, Kim Y, Colvin JS, Ornitz DM, Capel B (2004) Fgf9 induces proliferation and nuclear localization of FGFR2 in Sertoli precursors during male sex determination. Development 131(15):3627–3636
Schneider DT, Schuster AE, Fritsch MK, Hu J, Olson T, Lauer S, Gobel U, Perlman EJ (2001) Multipoint imprinting analysis indicates a common precursor cell for gonadal and nongonadal pediatric germ cell tumors. Cancer Res 61(19):7268–7276
Scholer HR (1991) Octamania: the POU factors in murine development. Trends Genet 7(10):323–329
Seki Y, Hayashi K, Itoh K, Mizugaki M, Saitou M, Matsui Y (2005) Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice. Dev Biol 278(2):440–458
Seki Y, Yamaji M, Yabuta Y, Sano M, Shigeta M, Matsui Y, Saga Y, Tachibana M, Shinkai Y, Saitou M (2007) Cellular dynamics associated with the genome-wide epigenetic reprogramming in migrating primordial germ cells in mice. Development 134(14):2627–2638
Sekido R, Lovell-Badge R (2008) Sex determination involves synergistic action of SRY and SF1 on a specific Sox9 enhancer. Nature 453(7197):930–934
Shoji M, Tanaka T, Hosokawa M, Reuter M, Stark A, Kato Y, Kondoh G, Okawa K, Chujo T, Suzuki T, Hata K, Martin SL, Noce T, Kuramochi-Miyagawa S, Nakano T, Sasaki H, Pillai RS, Nakatsuji N, Chuma S (2009) The TDRD9-MIWI2 complex is essential for piRNA-mediated retrotransposon silencing in the mouse male germline. Dev Cell 17(6):775–787
Shovlin TC, Bourc'his D, La Salle S, O'Doherty A, Trasler JM, Bestor TH, Walsh CP (2007) Sex-specific promoters regulate Dnmt3L expression in mouse germ cells. Hum Reprod 22(2):457–467
Silver SA, Wiley JM, Perlman EJ (1994) DNA ploidy analysis of pediatric germ cell tumors. Mod Pathol 7(9):951–956
Singh AM, Dalton S (2009) The cell cycle and Myc intersect with mechanisms that regulate pluripotency and reprogramming. Cell Stem Cell 5(2):141–149

Singhera M, Lees K, Huddart R, Horwich A (2012) Minimizing toxicity in early-stage testicular cancer treatment. Expert Rev Anticancer Ther 12(2):185–193
Skakkebaek NE (1978) Carcinoma in situ of the testis: frequency and relationship to invasive germ cell tumours in infertile men. Histopathology 2(3):157–170
Skakkebaek NE, Rajpert-de ME, Main KM (2001) Testicular dysgenesis syndrome: an increasingly common developmental disorder with environmental aspects. Hum Reprod 16(5):972–978
Skotheim RI, Monni O, Mousses S, Fossa SD, Kallioniemi OP, Lothe RA, Kallioniemi A (2002) New insights into testicular germ cell tumorigenesis from gene expression profiling. Cancer Res 62(8):2359–2364
Smiraglia DJ, Szymanska J, Kraggerud SM, Lothe RA, Peltomaki P, Plass C (2002) Distinct epigenetic phenotypes in seminomatous and nonseminomatous testicular germ cell tumors. Oncogene 21(24):3909–3916
Soosay GN, Bobrow L, Happerfield L, Parkinson MC (1991) Morphology and immunohistochemistry of carcinoma in situ adjacent to testicular germ cell tumours in adults and children: implications for histogenesis. Histopathology 19(6):537–544
Souquet B, Tourpin S, Messiaen S, Moison D, Habert R, Livera G (2012) Nodal signaling regulates the entry into meiosis in fetal germ cells. Endocrinology 153(5):2466–2473
Spiller CM, Bowles J, Koopman P (2013) Nodal/Cripto signaling in fetal male germ cell development: implications for testicular germ cell tumors. Int J Dev Biol 57(2–4):211–219
Spiller CM, Feng CW, Jackson A, Gillis AJ, Rolland AD, Looijenga LH, Koopman P, Bowles J (2012) Endogenous Nodal signaling regulates germ cell potency during mammalian testis development. Development 139(22):4123–4132
Spiller CM, Wilhelm D, Koopman P (2010) Retinoblastoma 1 protein modulates XY germ cell entry into G1/G0 arrest during fetal development in mice. Biol Reprod 82(2):433–443
Stamp IM, Barlebo H, Rix M, Jacobsen GK (1993) Intratubular germ cell neoplasia in an infantile testis with immature teratoma. Histopathology 22(1):69–72
Stamp IM, Jacobsen GK (1995) Infant intratubular germ cell neoplasia. Am J Surg Pathol 19(4):489
Stevens L (1962) Testicular teratomas in fetal mice. J Natl Cancer Inst 28:247–267
Stevens L (1964) Experimental produciton of testicular teratomas in mice. Proc Natl Acad Sci U S A 52:654–661
Stevens L (1966) Development of resistance to teratocarcinogenesis by primordial germ cells in mice. J Natl Cancer Inst 37(6):859–867
Stevens L (1967a) The biology of teratomas. Adv Morphog 6:1–31
Stevens L (1967b) Origin of testicular teratomas from primordial germ cells in mice. J Natl Cancer Inst 38(4):549–552
Stevens L (1973) A new inbred subline of mice (129-terSv) with a high incidence of spontaneous congenital testicular teratomas. J Natl Cancer Inst 50(1):235–242
Stevens L, Bunker M (1964) Karyotpye and sex of primary testicualr teratomas in mice. J Natl Cancer Inst 33:65–78
Stevens L, Hummel K (1957) A description of spontaneous congenital testicular teratomas in strain 129 mice. J Natl Cancer Inst 18(5):719–747
Stevens L, Mackensen JA (1961) Genetic and environmental influences on teratocarcinogenesis in mice. J Natl Cancer Inst 27:443–453
Stevens LC (1958) Studies on transplantable testicular teratomas of strain 129 mice. J Natl Cancer Inst 20(6):1257–1275
Stevens LC (1981) Genetic influences on teratocarcinogenesis and parthenogenesis. Prog Clin Biol Res 45:93–104
Stock C, Ambros IM, Lion T, Haas OA, Zoubek A, Gadner H, Ambros PF (1994) Detection of numerical and structural chromosome abnormalities in pediatric germ cell tumors by means of interphase cytogenetics. Genes Chromosomes Cancer 11(1):40–50
Summersgill B, Goker H, Weber-Hall S, Huddart R, Horwich A, Shipley J (1998) Molecular cytogenetic analysis of adult testicular germ cell tumours and identification of regions of consensus copy number change. Br J Cancer 77(2):305–313

Summersgill B, Osin P, Lu YJ, Huddart R, Shipley J (2001) Chromosomal imbalances associated with carcinoma in situ and associated testicular germ cell tumours of adolescents and adults. Br J Cancer 85(2):213–220

Surani MA (2001) Reprogramming of genome function through epigenetic inheritance. Nature 414(6859):122–128

Surani MA, Hayashi K, Hajkova P (2007) Genetic and epigenetic regulators of pluripotency. Cell 128(4):747–762

Sutton KA (2000) Molecular mechanisms involved in the differentiation of spermatogenic stem cells. Rev Reprod 5(2):93–98

Suzuki A, Saga Y (2008) Nanos2 suppresses meiosis and promotes male germ cell differentiation. Genes Dev 22(4):430–435

Suzuki A, Tsuda M, Saga Y (2007) Functional redundancy among Nanos proteins and a distinct role of Nanos2 during male germ cell development. Development 134(1):77–83

Tam PP, Snow MH (1981) Proliferation and migration of primordial germ cells during compensatory growth in mouse embryos. J Embryol Exp Morphol 64:133–147

Taylor-Weiner A, Zack T, O'Donnell E, Guerriero JL, Bernard B, Reddy A, Han GC, AlDubayan S, Amin-Mansour A, Schumacher SE, Litchfield K, Turnbull C, Gabriel S, Beroukhim R, Getz G, Carter SL, Hirsch MS, Letai A, Sweeney C, Van Allen EM (2016) Genomic evolution and chemoresistance in germ-cell tumours. Nature 540(7631):114–118

Trautmann E, Guerquin MJ, Duquenne C, Lahaye JB, Habert R, Livera G (2008) Retinoic acid prevents germ cell mitotic arrest in mouse fetal testes. Cell Cycle 7(5):656–664

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(5637):1239–1241

Tsuneyoshi N, Sumi T, Onda H, Nojima H, Nakatsuji N, Suemori H (2008) PRDM14 suppresses expression of differentiation marker genes in human embryonic stem cells. Biochem Biophys Res Commun 367(4):899–905

Turnbull C, Rapley EA, Seal S, Pernet D, Renwick A, Hughes D, Ricketts M, Linger R, Nsengimana J, Deloukas P, Huddart RA, Bishop DT, Easton DF, Stratton MR, Rahman N (2010) Variants near DMRT1, TERT and ATF7IP are associated with testicular germ cell cancer. Nat Genet 42(7):604–607

Ulbright TM, Amin MB, Balzer B, Berney DM, Epstein JI, Guo C, Idrees MT, Looijenga L, Paner G, Rajpert De-Meyts E, Skakkebaek NE, Tickoo SK, Yilmaz A, Oosterhuis JW (2016) Tumours of the testis and paratesticular tissue. In: Moch H, Humphrey PA, Ulbright TM, Reuter VE (eds) WHO classification of tumours of the urinary system and male genital organs, vol 8. International Agency for Research on Cancer, pp 185–227

Vallier L, Mendjan S, Brown S, Chng Z, Teo A, Smithers LE, Trotter MW, Cho CH, Martinez A, Rugg-Gunn P, Brons G, Pedersen RA (2009) Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. Development 136(8):1339–1349

van Echten J (1995) Cytogenetic evidence that carcinoma in situ is the precursor lesion for invasive testicular germ cell tumors. Cancer Genet Cytogenet 85(2):133–137

van Gurp RJ, Oosterhuis JW, Kalscheuer V, Mariman EC, Looijenga LH (1994) Biallelic expression of the H19 and IGF2 genes in human testicular germ cell tumors. J Natl Cancer Inst 86(14):1070–1075

Vernet N, Dennefeld C, Rochette-Egly C, Oulad-Abdelghani M, Chambon P, Ghyselinck NB, Mark M (2006) Retinoic acid metabolism and signaling pathways in the adult and developing mouse testis. Endocrinology 147(1):96–110

Vigier B, Watrin F, Magre S, Tran D, Josso N (1987) Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it in vitro. Development 100(1):43–55

Vincent SD, Dunn NR, Sciammas R, Shapiro-Shalef M, Davis MM, Calame K, Bikoff EK, Robertson EJ (2005) The zinc finger transcriptional repressor Blimp1/Prdm1 is dispensable for early axis formation but is required for specification of primordial germ cells in the mouse. Development 132(6):1315–1325

von Eyben FE (2004) Chromosomes, genes, and development of testicular germ cell tumors. Cancer Genet Cytogenet 151(2):93–138
Vos A, Oosterhuis JW, de Jong B, Buist J, Schraffordt KH (1990) Cytogenetics of carcinoma in situ of the testis. Cancer Genet Cytogenet 46(1):75–81
Walt H, Oosterhuis JW, STEVENS LC (1993) Experimental testicular germ cell tumorigenesis in mouse strains with and without spontaneous tumours differs from development of germ cell tumours of the adult human testis. Int J Androl 16(4):267–271
Weber S, Eckert D, Nettersheim D, Gillis AJ, Schafer S, Kuckenberg P, Ehlermann J, Werling U, Biermann K, Looijenga LH, Schorle H (2010) Critical function of AP-2 gamma/TCFAP2C in mouse embryonic germ cell maintenance. Biol Reprod 82(1):214–223
Welter D, MacArthur J, Morales J, Burdett T, Hall P, Junkins H, Klemm A, Flicek P, Manolio T, Hindorff L, Parkinson H (2014) The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. Nucleic Acids Res 42:D1001–D1006
Werling U, Schorle H (2002) Transcription factor gene AP-2 gamma essential for early murine development. Mol Cell Biol 22(9):3149–3156
Western P (2009) Foetal germ cells: striking the balance between pluripotency and differentiation. Int J Dev Biol 53(2–3):393–409
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(2):339–347
Western PS, Ralli RA, Wakeling SI, Lo C, van den Bergen JA, Miles DC, Sinclair AH (2011) Mitotic arrest in teratoma susceptible fetal male germ cells. PLoS One 6(6):e20736
Western PS, van den Bergen JA, Miles DC, Sinclair AH (2010) Male fetal germ cell differentiation involves complex repression of the regulatory network controlling pluripotency. FASEB J 24(8):3026–3035
Wu Q, Fukuda K, Weinstein M, Graff JM, Saga Y (2015) SMAD2 and p38 signaling pathways act in concert to determine XY primordial germ cell fate in mice. Development 142(3):575–586
Wu Q, Kanata K, Saba R, Deng CX, Hamada H, Saga Y (2013) Nodal/activin signaling promotes male germ cell fate and suppresses female programming in somatic cells. Development 140(2):291–300
Yamaguchi S, Kimura H, Tada M, Nakatsuji N, Tada T (2005) Nanog expression in mouse germ cell development. Gene Expr Patterns 5(5):639–646
Yamaji M, Seki Y, Kurimoto K, Yabuta Y, Yuasa M, Shigeta M, Yamanaka K, Ohinata Y, Saitou M (2008) Critical function of Prdm14 for the establishment of the germ cell lineage in mice. Nat Genet 40(8):1016–1022
Yamaji M, Ueda J, Hayashi K, Ohta H, Yabuta Y, Kurimoto K, Nakato R, Yamada Y, Shirahige K, Saitou M (2013) PRDM14 ensures naive pluripotency through dual regulation of signaling and epigenetic pathways in mouse embryonic stem cells. Cell Stem Cell 12(3):368–382
Yan W, Samson M, Jegou B, Toppari J (2000) Bcl-w forms complexes with Bax and Bak, and elevated ratios of Bax/Bcl-w and Bak/Bcl-w correspond to spermatogonial and spermatocyte apoptosis in the testis. Mol Endocrinol 14(5):682–699
Yao HH, DiNapoli L, Capel B (2003) Meiotic germ cells antagonize mesonephric cell migration and testis cord formation in mouse gonads. Development 130(24):5895–5902
Yeom YI, Fuhrmann G, Ovitt CE, Brehm A, Ohbo K, Gross M, Hubner K, Scholer HR (1996) Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells. Development 122(3):881–894
Yi SE, LaPolt PS, Yoon BS, Chen JY, Lu JK, Lyons KM (2001) The type I BMP receptor BmprIB is essential for female reproductive function. Proc Natl Acad Sci U S A 98(14):7994–7999
Youngren KK, Coveney D, Peng X, Bhattacharya C, Schmidt LS, Nickerson ML, Lamb BT, Deng JM, Behringer RR, Capel B, Rubin EM, Nadeau JH, Matin A (2005) The Ter mutation in the dead end gene causes germ cell loss and testicular germ cell tumours. Nature 435(7040):360–364
Yu Z, Ji P, Cao J, Zhu S, Li Y, Zheng L, Chen X, Feng L (2009) Dazl promotes germ cell differentiation from embryonic stem cells. J Mol Cell Biol 1(2):93–103

Zhou X, Zhang Z, Yang X, Chen W, Zhang P (2009) Inhibition of cyclin D1 expression by cyclin D1 shRNAs in human oral squamous cell carcinoma cells is associated with increased cisplatin chemosensitivity. Int J Cancer 124(2):483–489

Zhu R, Heaney J, Nadeau JH, Ali S, Matin A (2010) Deficiency of splicing factor 1 suppresses the occurrence of testicular germ cell tumors. Cancer Res 70(18):7264–7272

Zhu R, Matin A (2014) Tumor loci and their interactions on mouse chromosome 19 that contribute to testicular germ cell tumors. BMC Genet 15:65

Part VI

Tools to Study Spermatogonial Biology

Transplantation and Culture of Spermatogonial Stem Cells

11

Hiroshi Kubota and Ralph L. Brinster

Abstract

Spermatogonial stem cells (SSCs), also called postnatal male germline stem cells, continuously undergo self-renewal and produce daughter spermatogonia that commit to differentiation to spermatozoa, thereby maintaining spermatogenesis and fertility throughout postnatal life. Development of the SSC transplantation technique, in which donor testis cells from a fertile male are microinjected into the seminiferous tubules of an infertile male where SSCs reconstitute donor-derived spermatogenesis and restore fertility, provides a powerful means to unequivocally identify SSCs in a quantitative manner. SSC transplantation is a remarkable breakthrough for SSC research and has established a crucial foundation to study the biology of SSCs. In this chapter, we first describe the transplantation technique that allows characterization of SSCs and their niche, cryopreservation of the germline, and transgenesis. We subsequently describe SSC culture systems that establish a platform for studying SSCs in vitro and enormously enhance their biological value. SSC transplantation, culture, and cryopreservation were originally developed in mice and subsequently in rats, and have since then been extending to other species including domestic animals, endangered or rare species, and primates. Therefore, in the final section, we discuss potential applications of SSCs, for example, the transplantation technique and SSC culture, in human medicine.

Keywords

Spermatogonial stem cell • Germline stem cell • Transplantation • Stem cell culture • Germline modification • Stem cell niche • Fertility • Spermatogonium • Sertoli cell • Spermatogenesis

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11.1 Introduction

Spermatogenesis is the process in which male diploid germ cells undergo meiosis and produce a number of haploid germ cells, spermatozoa, which eventually fertilize female haploid germ cells to generate zygotes. The spermatogenesis process, which takes place in seminiferous tubules in testes, is complex, but well organized, and known as one of the most productive systems in mammalian tissues (Clermont 1972; Potten and Morris 1988; Russell et al. 1990). In the testes, millions (rodents) to hundreds of millions (primates and farm animals) of spermatozoa are produced daily from the onset of spermatogenesis at puberty until death (Amann 1986). In all mammalian species, spermatogenesis consists of three phases, mitotic, meiotic, and maturation phases. In the mitotic phase, diploid spermatogonia on the basal lamina of the seminiferous tubules extensively proliferate to increase cell number before the meiotic phase. There are several types of spermatogonia, which are distinguished by heterochromatin patterns in the nuclei and morphometric analysis with stages of seminiferous epithelium cycles (Russell et al. 1990). Spermatogonia are the initial cell population in spermatogenesis, and the spermatogonial stem cell (SSC) is the foundation cell of spermatogonia and subsequent spermatogenesis. The high productivity of spermatogenesis relies on self-renewal of SSCs and the mitotic phase of spermatogenesis (Clermont and Bustos-Obregon 1968; Huckins 1971; Oakberg 1971).

Although the cell type of the seminiferous epithelium and the process of spermatogenesis are conserved among different species, there exist species differences in the duration of spermatogenesis and subtypes of spermatogonia. The duration from spermatogonia to spermatozoa varies for each mammalian species, and the range in most mammals is approximately 30–75 days (Hess and Renato de Franca 2008; Russell et al. 1990). Murine spermatogonia are the most intensively studied and have become a model for other mammalian species, because spermatogonia of other species including rat, hamster, pig, sheep, and cattle, have many similar characteristics (Russell et al. 1990). On the other hand, primates, including humans, have unique subtypes of spermatogonia (Hermann et al. 2010; Meistrich and van Beek 1993).

During cell divisions associated with differentiation, the daughter spermatogonia do not separate completely, but remain connected by intercellular bridges. Such incomplete cytokinesis is an evolutionally conserved characteristic of germ cells (Greenbaum et al. 2011). This unique cell division is important to synchronize differentiation during spermatogenesis. In the mouse, the most immature spermatogonia that form the initial stages of spermatogenesis are called undifferentiated spermatogonia, which can be further subdivided into type A_{single} (A_s), A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}) based on the number of cohorts connected by intercellular bridges (Huckins 1971; Oakberg 1971). A_s spermatogonia are single cells and represent a very small number of the undifferentiated spermatogonial population (Tegelenbosch and de Rooij 1993). When A_s spermatogonia divide, there are two possible fates of daughter cells. In one case two A_s spermatogonia result, and in the other case two

A_{pr} spermatogonia are connected by an intercellular bridge. The next division of the A_{pr} spermatogonia forms four A_{al} spermatogonia connected by intercellular bridges. Further cell divisions generate 8, 16, and very rarely 32 A_{al} spermatogonia. The last stages of A_{al} spermatogonia differentiate and sequentially form type A1, A2, A3, and A4 spermatogonia, which then differentiate to intermediate (In) spermatogonia, followed by Type B spermatogonia (Russell et al. 1990). The A1 ~ 4, In, and B spermatogonia are called differentiating spermatogonia (de Rooij and Russell 2000). The timing of cell division of differentiating spermatogonia is relatively fixed, whereas that of undifferentiated spermatogonia is not consistent. Type B spermatogonia differentiate into primary spermatocytes that pass through the blood-testis barrier formed by the tight junctions between Sertoli cells and begin meiotic prophase. The tight junctions of Sertoli cells divide the tubular lumen into the basal compartment containing spermatogonia, which is exposed to blood constituents, and the adluminal compartment containing meiotic and maturation germ cell stages, largely separate from blood cells and large molecules (Mruk and Cheng 2015). Meiosis has two sequential cycles of cell division, and one diploid spermatocyte produces four haploid round spermatids that undergo spermiogenesis. During spermiogenesis, round spermatids morphologically and functionally differentiate into spermatozoa.

Classic studies, using histological sections, whole mounts of seminiferous tubules, and H^3-thymidine labeling experiments for cell-kinetics, suggest that some, perhaps many, A_s spermatogonia are SSCs (de Rooij 1973; Huckins 1971; Oakberg 1971). However, these experimental approaches could not assess the biological activity of the A_s spermatogonia; therefore, it was impossible to determine whether they are indeed SSCs. Stem cells are defined by their biological activity, specifically, they are able to self-renew and produce committed cells that eventually differentiate to functional mature cells. Therefore, to unequivocally conclude which cells in the seminiferous tubules are SSCs, an experimental system that could evaluate the biological function of SSCs is required.

11.2 Spermatogonial Stem Cell Transplantation

11.2.1 Development of Spermatogonial Stem Cell Transplantation

A technique for the transplantation of testis cells into seminiferous tubules of recipient males was developed to identify SSCs using mice in 1994 (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). When donor testis cells from a fertile male are microinjected into the lumen of the seminiferous tubules of an infertile recipient male, some donor germ cells reach the basal lamina passing through the blood–testis barrier of Sertoli cells in the opposite direction of normal spermatogenesis. The recipient mice used are prepared by injection of Busulfan, an alkylating agent, to eliminate endogenous germ cells, or are white spotting (*W*) mutant mice that have

congenital deficiencies of germ cell development due to a mutation of the *Kit* gene. After colonization, the donor cells begin proliferating laterally on the basement membrane during the first month, and then gradually differentiate toward the lumen (Nagano et al. 1999). By 1 month following transplantation, donor-derived spermatocytes appear in the adluminal compartment of the seminiferous tubules. By 2 months after transplantation, donor germ cells fill the tubules, and spermatozoa begin to appear. Although 35 days are required for spermatogenesis in mice (Oakberg 1957), nearly twice as long is necessary to produce donor spermatozoa following transplantation. Some of this additional time likely represents a longer mitotic phase of undifferentiated and differentiating spermatogonia from the colonized cells, because extensive lateral expansion of spermatogonia on the basement membrane occurs 1 month after transplantation (Nagano et al. 1999). Other than this, however, many unknown factors would be involved in these different times to obtain mature spermatozoa following transplantation. The spermatozoa differentiated from donor cells are morphologically normal and are able to fertilize eggs, resulting in production of progeny carrying the donor male haplotype, indicating they are functionally normal (Brinster and Avarbock 1994). The reconstituted spermatogenesis continues throughout the remaining life of the recipients. These characteristics collectively prove that some of the transplanted cells have the ability to colonize the basement membrane and have the differentiation capability for continuous production of functional spermatozoa, clearly indicating that the spermatogenesis-reconstituting cells are SSCs (Fig. 11.1A–F).

Three different methods to introduce donor cells into seminiferous tubules of recipient mice were developed (Ogawa et al. 1997). The first method is to inject cells directly into the seminiferous tubule using a micropipette (Fig. 11.1Ca, inset). This method is the most direct way to introduce germ cells into seminiferous tubules. Donor germ cells are forced into the rete testis from the injected tubule and can then enter other seminiferous tubules, because all seminiferous tubules access the rete testis. The cell suspension entering the rete testis can fill many seminiferous tubules. The second method is to insert a micropipette directly into the rete testis and fill the seminiferous tubules (Fig. 11.1Cb, inset). The third method is to insert a micropipette into one of the efferent ducts and thread it into the rete testis (Fig. 11.1Cc, inset). This method is the most accurate in controlling the injection volume because less cell suspension leaks from the insertion site of the micropipette. All three methods have been used for mice and rats and resulted in successful reconstitution of donor spermatogenesis (Brinster and Avarbock 1994; Brinster and Zimmermann 1994; Ogawa et al. 1997, 1999b). For other animals, including pigs, goats, cattle, sheep, monkeys, and humans, the second method, in which donor cells are directly introduced into the rete testis of recipients, has been used (Hermann et al. 2012; Herrid et al. 2006; Honaramooz et al. 2002, 2003a; Izadyar et al. 2003; Kim et al. 2008; Mikkola et al. 2006; Radford 2003; Rodriguez-Sosa et al. 2009; Schlatt et al. 1999), and successful reconstitution of donor-derived spermatogenesis is also reported.

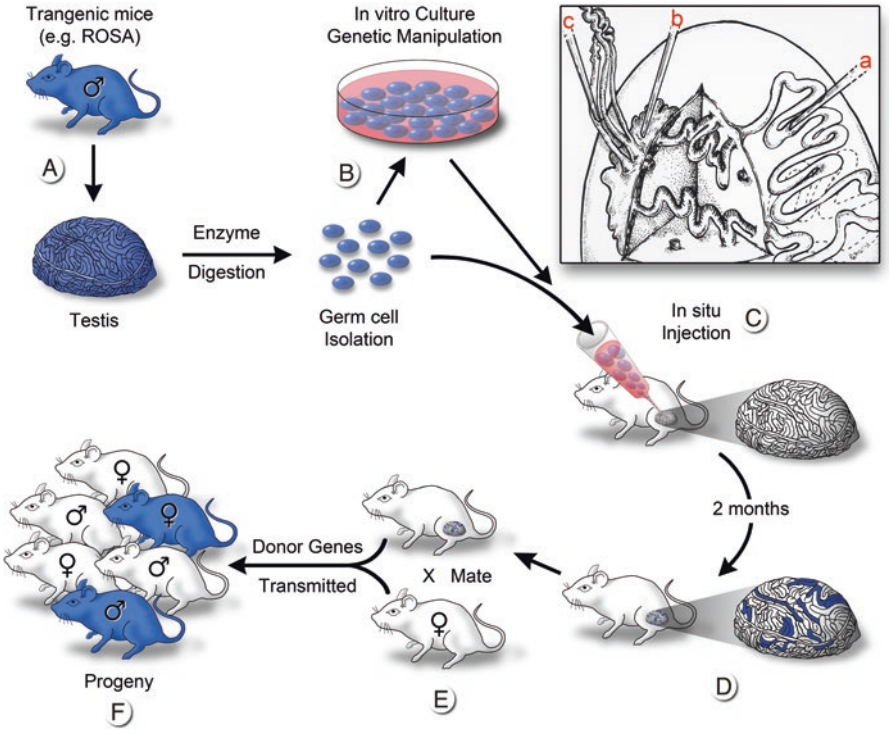

Fig. 11.1 Procedure for testis-cell transplantation as developed in the mouse. (**A**) testis is removed from a fertile male that expresses a reporter transgene, *Escherichia coli lacZ*, and a single-cell suspension is prepared from the testis of the transgenic mouse. (**B**) The testis cells can be cultured with appropriate conditions. Genetic manipulation can be employed during culture. (**C**) Cells are microinjected into the seminiferous tubules of an infertile recipient male. There are three methods for microinjection indicated in the inset: the micropipette can be inserted (*a*) directly into the seminiferous tubules, (*b*) into the rete testis, or (*c*) into an efferent duct. (**D**) Spermatogonial stem cells colonize the basement membrane of the tubules and generate donor-cell-derived spermatogenesis, which can be stained blue using a substrate for the reporter gene product (β-galactosidase). Each blue stretch of cells in the seminiferous tubules of the recipient testis represents a spermatogenic colony derived from a single donor stem cell. (**E**) Mating the recipient male to a wild-type female results in donor-cell-derived spermatozoa fertilizing wild-type oocytes. (**F**) Progeny with the donor haplotype are produced. Modified from Brinster (2002) and Ogawa et al. (1997)

11.2.2 Functional Assay for Spermatogonial Stem Cells

As mentioned above, stem cells are defined by biological function, in which they have the ability to both self-renew and produce large numbers of differentiated functional cells. The transplantation procedure can be used as a quantitative assay for retrospective identification of SSCs. When testis cells from a transgenic mouse that expresses a reporter gene, such as β-galactosidase or green fluorescent protein (GFP), are transplanted into infertile recipient testes, donor-derived spermatogenesis can be unequivocally identified by visualizing the reporter proteins (Brinster

2002; Kubota and Brinster 2008). For example, in recipient testes transplanted with β-galactosidase-expressing germ cells, donor-derived spermatogenesis can be identified as blue colonies after staining with a substrate, X-gal, 2 months after transplantation (Fig. 11.1). These colonies are each derived from a single donor cell (Dobrinski et al. 1999b; Kanatsu-Shinohara et al. 2006c; Zhang et al. 2003), and the colony number does not change from 1 to 4 months, whereas the length of colonies increases (Nagano et al. 1999). Thus, the number of blue colonies represents the number of spermatogenesis-reconstituting cells (SSCs) in donor cell suspensions, which are able to self-renew and continuously produce daughter spermatogonia that eventually differentiate into spermatozoa. Because the colonization efficiency of SSCs into adult recipient testes is 5–12% (Nagano 2003; Ogawa et al. 2003), the actual SSC number in a donor cell suspension is 8- to 20-fold higher than the colony number. The exact reasons of the low colonization efficiency are not clear, but passing through the blood–testis barrier is likely one of the impeding factors.

Although the transplantation technique provides a powerful means to identify SSCs in any donor cell suspension, the technique can be used to identify precursor cells that have the ability to differentiate into SSCs. SSCs are a subpopulation of type A spermatogonia that are derived from gonocytes, or prospermatogonia, a few days after birth in mice (Culty 2013; McCarrey 2013). Gonocytes are mitotically inactive and located in the center of the seminiferous tubules of fetal and newborn testes. When gonocytes from fetal and newborn testes are transplanted into seminiferous tubules of mature testes, donor-derived spermatogenesis occurs, indicating that the donor gonocytes differentiated to SSCs in recipient mature testes (Brinster and Avarbock 1994; Kubota et al. 2004a; Shinohara et al. 2001). Furthermore, when primordial germ cells (PGCs), the precursor of gonocytes, and epiblast cells, the precursor of PGCs, were transplanted into immature testes, but not mature testes, before forming the blood–testis barrier of Sertoli cells, donor-derived spermatogenesis was reconstituted (Chuma et al. 2005; Ohta et al. 2004). Normal fertile progeny were produced by micro-insemination using donor-derived spermatids or spermatozoa, confirming that PGCs and epiblast cells can normally differentiate into SSCs in the postnatal testis (Chuma et al. 2005). This indicates that the transplantation technique can be used for not only a functional assay to identify SSCs, but also for assessing developmental potential of other cell types to produce SSCs.

During the past decade, derivation of spermatozoa from pluripotent stem cells has been attempted in vitro, and several groups reported the successful induction of PGC-like cells from embryonic stem (ES) cells, although no functionally normal haploid germ cells were obtained from the PGC-like cells (Geijsen et al. 2004; Nayernia et al. 2006; Toyooka et al. 2003). However, a recent study reported generation of functionally normal spermatozoa from ES cells and induced pluripotent stem (iPS) cells in the mouse (Hayashi et al. 2011). In this study, initially epiblast-like cells were induced from pluripotent cells, and then further induction to PGC-like cells was carried out in culture. Subsequently, the PGC-like cells were transplanted into the seminiferous tubules of immature infertile mouse testes. Donor-derived spermatogenesis occurred in the recipient testes, and micro-insemination using the spermatozoa from these testes successfully generated fertile offspring without any

defect or abnormality (Hayashi et al. 2011). At present, the transplantation procedure is indispensable for the generation of functional spermatozoa from pluripotent stem cells such as ES cells and iPS cells.

11.2.3 Characterization of Spermatogonial Stem Cells

The availability of a functional assay is essential to identify SSCs based on their biological functions. Because the number of SSCs in the testis is extremely rare, perhaps only 0.03% of all germ cells in mouse testis (Tegelenbosch and de Rooij 1993), determination of the phenotypic characteristics of SSCs is critical to investigate SSCs at the cellular and molecular level, as well as in the context of tissue sections or whole mount analyses. There have been several methods developed that enrich SSCs from postnatal testes, including differential plating, Percoll centrifugation, isolation from cryptorchid testes, and immunoselection (Kubota et al. 2004a; Shinohara et al. 1999, 2000a). Although there are several methods of immunoselection to isolate particular subpopulations from a mixed cell population, fluorescence activated cell sorting (FACS) is the most widely used for identification of stem cell populations from a variety of tissues (Grompe 2012). Originally, an approach using FACS along with a functional transplantation assay was developed for identifying hematopoietic stem cells (HSCs). Although HSCs represent only 0.01% of the cells in bone marrow, the surface characteristics of murine HSCs have been successfully determined by this approach (Osawa et al. 1996; Spangrude et al. 1988). Utilizing a similar experimental approach, the cell surface phenotype of SSCs in rodents was determined. Briefly, testicular cells prepared from testes are stained with fluorochrome-conjugated antibodies against cell surface molecules, followed by flow cytometric analysis to determine cell surface expression of the molecules. When the testicular cell population is divided based on cell surface expression of antigens, each fraction of cells can be isolated by cell sorting and subjected to the transplantation assay. Two months after transplantation of each cell fraction, recipient testes are analyzed to identify donor-derived spermatogenesis. The number of spermatogenic colonies generated by different cell populations indicates the number of SSCs in that population of cells. By repeating this process, surface molecules expressed on SSCs have been determined. Several studies identified the SSC surface phenotype as represented by $ITGA6^{+}$ $ITGB1^{+}$ $THY1^{+}$ $CD9^{+}$ $GFRA1^{+}$ $EPCAM^{+}$ $CD24^{+}$ $MCAM^{+}$ KIT^{-} Major histocompatibility complex class I $(MHC\text{-}I)^{-}$ in mice (Kanatsu-Shinohara et al. 2004b, 2012; Kubota et al. 2003; Shinohara et al. 2000b). At present, no cell surface molecules that are exclusively expressed on SSCs have been identified. However, a combination of surface markers can identify the cell surface phenotype of undifferentiated spermatogonia enriched for SSCs. Although the concentration of SSCs in adult testes is thought to be about 1 in 3000, that of $THY1^{+}$ $ITGA6^{+}$ KIT^{-} $MHC\text{-}I^{-}$ cells in cryptorchid testes or $THY1^{+}$ $ITGA6^{+}$ $ITGAV^{-/lo}$ in normal testes is approximately 1 in 15 ~ 30 (Kubota et al. 2003, 2004a), indicating that FACS in conjunction with the transplantation assay could determine cell surface molecules and facilitate a major enrichment of SSCs. Determination of the antigenic profile of

SSC-enriched cell populations allowed subsequent development of magnetic activated cell sorting (MACS) for the population, which is a simpler and quicker method than FACS (Kubota et al. 2004a). Furthermore, FACS can be used for cell fractionation based on cellular activity, such as efflux pump activity, mitochondrial activity, cell cycle, and intracellular enzymatic activity, which have been used for HSC identification (Ishii et al. 2014; Kanatsu-Shinohara et al. 2013; Kubota et al. 2003; Lo et al. 2005). Using those parameters with cell surface analysis, murine SSCs were further characterized; however, no improvement of SSC enrichment was achieved.

For characterization of SSC-specific genes such as transcription factors, transgenic mice in which a reporter gene, such as GFP, is inserted downstream of the promoter of a putative SSC-specific gene, are useful. Several genes expressed in undifferentiated spermatogonia, including *Pou5f1* (*Oct-3/4*), *Ngn3*, *Nanos2*, and *Id4*, have been investigated (Chan et al. 2014; Ohbo et al. 2003; Ohmura et al. 2004; Sada et al. 2009; Yoshida et al. 2004). Following identification of reporter gene-expressing cells in the testes of the transgenic mice, the cells are isolated by FACS and transplanted into recipient testes. Again, no SSC-specific molecules have been identified, but this approach could allow better SSC enrichment than existing techniques relaying on cell surface markers or physiological characteristics in the future. Furthermore, this approach is important to delineate the ordered expression of gene expression during spermatogonial differentiation from SSCs.

Although A_s spermatogonia have been thought to be SSCs for a long time, recent studies challenge this hypothesis. A live image study of GFP-labeled *Ngn3* spermatogonia indicated a separation or unjoining of A_{al} spermatogonia to A_{pr} or A_s spermatogonia, which then appeared to reinitiate formation of A_{al} spermatogonia from either the A_{pr} or A_s (Hara et al. 2014). Furthermore, a study using FACS and the transplantation assay demonstrated that KIT-expressing differentiating spermatogonia may also possess SSC potential (Barroca et al. 2009). These studies suggest that undifferentiated or differentiating spermatogonia other than A_s spermatogonia could function as stem cells. There is no molecular marker for A_s spermatogonia, and A_s, A_{pr}, and A_{al} spermatogonia can be identified only by whole mount analysis; therefore, identification of each type of undifferentiated spermatogonia by flow cytometry is not feasible. At present, it is not technically easy to determine which undifferentiated spermatogonia possess SSC activity. However, even in a single cohort of A_{al} spermatogonia, it appeared that gene expression patterns in individual spermatogonia might be different (Zheng et al. 2009). If particular spermatogonia within an A_{al} spermatogonia syncytium can be isolated, it would be possible to determine which undifferentiated spermatogonia possess the stem cell activity by the transplantation assay. Assembling all data from whole mount analysis, flow cytometry, and live imaging in conjunction with transplantation assays, we might be able to elucidate cellular and molecular determinants of SSCs, from which our understanding of SSC biology would significantly improve.

11.2.4 Characteristics of the Spermatogonial Stem Cell Niche

The surrounding microenvironment of stem cells is called the stem cell niche, which has been shown to control self-renewal and differentiation of stem cells (Li and Xie 2005; Spradling et al. 2001). The microinjection technique into seminiferous tubules is also useful to investigate the biological function of the SSC niche. When SSCs from adults were transplanted into pups and adult recipient testes, the number of spermatogenic colonies in pup testes was approximately ten times greater than in adult testes (Shinohara et al. 2001). In addition, colonies generated were four times longer in pup testes compared with those in adults (Shinohara et al. 2001). These findings indicated that the SSC niche in pups is more accessible and supportive of transplanted SSCs than that in adults. These transplantation experiments clearly demonstrate that the transplantation assay can be used for evaluating the biological activity of the SSC niche in recipient testes. Components of the stem cell niche comprise neighboring cells, soluble factors, and extracellular matrices. The neighboring cells include Sertoli cells, myoid cells, Leydig cells, potentially other interstitial cells, and spermatogonia. In particular, Sertoli cells play a critical role in establishing the SSC niche, because they produce glial cell line-derived neurotrophic factor (GDNF) and fibroblast growth factor 2 (FGF2), the primary and secondary critical soluble factor for self-renewal and expansion of SSCs (Kubota et al. 2004b; Meng et al. 2000; Mullaney and Skinner 1992). Furthermore, recent studies indicated that the number of Sertoli cells influence the number of niches accessible for colonization of transplanted SSCs in mice (Oatley et al. 2011). In this study, the number of donor-derived spermatogenic colonies significantly increased in the recipient testes with ~50% increase in Sertoli cell numbers following SSC transplantation compared with normal recipient mice. In addition, colony stimulating factor 1 (CSF1), which is produced by Leydig and myoid cells, has been identified as the first specific niche factor that increases the replication and expansion of SSCs (Oatley et al. 2009).

The transplantation of cells into the seminiferous tubules can also be applied to Sertoli cells. In the mouse, expression of the KIT receptor tyrosine kinase by spermatogonia is critical for proliferation and differentiation beyond the undifferentiated spermatogonia stage (Yoshinaga et al. 1991), and the KIT ligand, also known as stem cell factor (SCF), is expressed on and secreted by Sertoli cells in the testes (Ogawa et al. 2000). Steel (*Sl*) mutant mice are infertile due to a congenital defect of the KIT ligand, but transplantation of normal testis cells into seminiferous tubules of *Sl* mice initiates spermatogenesis by transplanted Sertoli cells (Shinohara et al. 2003). Furthermore, infertile *Sl* recipient mice transplanted with Sertoli cells from *W* mice restored endogenous spermatogenesis and fertility, demonstrating the functionality of transplanted Sertoli cells (Kanatsu-Shinohara et al. 2005a). Although the efficiency of Sertoli cell colonization is low (Shinohara et al. 2003; Shinomura et al. 2014), transplantation of Sertoli cells provides an alternative approach to restore fertility in infertile recipients. Moreover, transplantation of xenogeneic Sertoli cells into immunodeficient mice would be able to reconstitute the SSC niche

for foreign species, which provides a foundation for non-rodent SSC studies including human (see below).

11.2.5 Application to Non-mouse Systems

The spermatogonial transplantation technique was initially developed in mice. Following the original mouse system, the technique was extended to other species including rats, monkeys, goats, cattle, pigs, sheep, cats, and dogs (Hermann et al. 2012; Herrid et al. 2006; Honaramooz et al. 2002, 2003a; Izadyar et al. 2003; Jiang and Short 1995; Kim et al. 2006, 2008; Mikkola et al. 2006; Ogawa et al. 1999b; Radford 2003; Rodriguez-Sosa et al. 2009; Schlatt et al. 1999). In rat, the procedure is essentially the same as for mouse. In brief, donor germ cells were collected from transgenic rats that express a β-galactosidase reporter gene and transplanted into infertile recipient rat testes through the efferent duct. Recipient males were prepared by injection of Busulfan to deplete endogenous germ cells (Ogawa et al. 1999b; Ryu et al. 2003). As shown in mice, following transplantation donor SSCs initiate spermatogenesis, and donor-derived spermatogenesis continued during the remaining life of the recipient rats. Donor-derived spermatogenesis was confirmed by X-gal staining. Although the rat system is quite similar to the mouse, there are no rat strains with congenital mutations leading to germ cell deficiency that are suitable for recipient males. Nevertheless, the transplantation system using recipients prepared by Busulfan injection can be used for biological functional assay to identify rat SSCs. Applying the same approach as with mouse SSCs, the surface phenotype of the undifferentiated spermatogonia population enriched for rat SSCs has been determined (Ryu et al. 2004). Furthermore, SSC niche development in postnatal rats was also investigated (Ryu et al. 2003).

In non-rodents, such as farm animals and companion animals, recipient males are prepared by Busulfan injection or local irradiation of testes (Honaramooz et al. 2005; Izadyar et al. 2003; Oatley et al. 2005). Both methods have been shown to be effective for ablation of endogenous germ cells. Although appropriate injection timing and doses for Busulfan must be determined in each species, complete removal of endogenous germ cells is not necessary. Some studies have suggested that residual endogenous spermatogenesis may be helpful to maintain a healthy testicular niche microenvironment to enhance donor spermatogenesis (Ryu et al. 2003). Furthermore, immature pigs and goats without pretreatment can be used as recipients (Honaramooz et al. 2002, 2003a, b). However, recipients with persistence endogenous spermatogenesis can produce both donor-derived and recipient-derived progeny; therefore, genotyping is necessary to determine whether donor or endogenous spermatogenesis produced the progeny. Although there are three methods to inject donor germ cells as described above, donor SSCs in non-rodent system were introduced into the rete testis by ultrasound guidance or surgical dissection (Honaramooz et al. 2002, 2003a; Schlatt et al. 1999). In goat and sheep, progeny with donor SSC-haplotype were successfully generated (Herrid et al. 2009; Honaramooz et al. 2003b, 2008).

11.2.6 Xenotransplantation

Following transplantation of rat SSCs into the seminiferous tubules of infertile immunocompromised mice, rat spermatogenesis was established in recipient testes (Clouthier et al. 1996). The rat spermatozoa in mouse testes are functionally normal, since in a subsequent study normal progeny were generated from the rat spermatozoa developed in the recipient mouse testes (Shinohara et al. 2006). This finding suggests that xenogeneic spermatogenesis could be reconstituted by transplantation of SSCs from variety of species into immunocompromised mice. A striking finding regarding control of timing of germ cell differentiation during spermatogenesis emerged from this rat to mice spermatogonial transplantation experiments (Clouthier et al. 1996). Although the duration time from type A spermatogonia to mature spermatozoa is 35 days in mice, that of rats is 52 days (Russell et al. 1990). It was found that rat spermatogenesis in mouse seminiferous tubules progressed at the rate determined by the transplanted germ cells. Thus, rat spermatogenesis in mouse seminiferous tubules progresses at the slower speed of rat spermatogenesis while supported by mouse Sertoli cells, indicating that the genotype of the germ cell controls this specific timing of spermatogenesis characteristic of different species (França et al. 1998; Russell and Brinster 1996).

In subsequent studies germ cells from various mammalian species, including rabbits, dogs, cats, pigs, cattle, horses, baboon, macaques, and humans, were transplanted into infertile immunocompromised male mice (Dobrinski et al. 1999a, 2000; Hermann et al. 2007; Nagano et al. 2001b, 2002; Oatley et al. 2004). However, complete spermatogenesis from transplanted SSCs was developed only in the combinations between rodents, such as rat to mouse and hamster to mouse (Clouthier et al. 1996; Ogawa et al. 1999a). Although donor-derived spermatogenesis did not occur following transplantation of germ cells from non-rodent mammalian species into the mouse testes, primitive spermatogonia from all mammals examined colonized and proliferated for 1–12 months in the seminiferous tubules of immunocompromised mice (Dobrinski et al. 1999a, 2000; Hermann et al. 2007; Kim et al. 2006; Nagano et al. 2001b, 2002; Oatley et al. 2004). At present, the colony-forming spermatogonia in immunocompromised mice are considered to represent SSCs and perhaps early undifferentiated spermatogonia. The remarkable results of xenogeneic transplantations indicate that factors produced in the mouse SSC niche are able to support proliferation of SSCs from many species. Because the process of spermatogenesis is well conserved among mammalian species, conservation of self-renewing factors for SSCs may not be surprising. On the other hand, species variation in spermatogonial differentiation factors seems to exist among mammalian species. Sertoli cell transplantation from the same species as the donor germ cells may overcome the block in differentiation of xenogeneic donor germ cells into mouse testes. The SSC characteristics in non-rodent mammals including human are largely unknown; however, functional assays to identify these SSCs are under development. Transplantation of Sertoli cells in addition to SSCs from xenogeneic species would reestablish the necessary niche and differentiating factors for foreign species spermatogenesis in immunodeficient mice. If immunocompromised mice with xenogeneic Sertoli cells can

support spermatogenesis from xenogeneic SSCs, such a transplantation system can be used as a functional assay for non-rodent SSCs. It is extremely important to develop functional assays for non-rodent SSCs in which both self-renewal and differentiation can be evaluated.

11.3 Spermatogonial Stem Cell Culture

11.3.1 Mouse

The first demonstration of a long-term culture of murine SSCs was reported in 1998 (Nagano et al. 1998). In this study, testicular cells were cultured on STO (SIM mouse embryo-derived thioguanine and ouabain resistant) feeders in a serum-supplemented medium for approximately 4 months, followed by transplantation into testes of infertile mice in order to examine whether SSCs existed in the culture (Nagano et al. 1998). Donor cell-derived spermatogenesis was generated in the recipient testes, indicating that SSCs existed in the long-term culture. This result clearly demonstrated that SSCs could be maintained in culture for several months, although the number of SSCs was reduced (Nagano et al. 1998). Several cytokines such as FGF2, leukemia inhibitory factor (LIF) and SCF were thought to be candidate mitogens for SSCs. FGF2 and SCF are potent mitogens for PGCs (Matsui et al. 1992; Resnick et al. 1992), while LIF is an essential self-renewing factor for ES cells (Smith et al. 1988; Williams et al. 1988). Using a short-term culture experiment, none of these three cytokines improved survival of SSCs (Nagano et al. 2003). A critical finding came from a study using GDNF-overexpressing and -deficient mice (Meng et al. 2000). Although GDNF was originally discovered as a survival factor for midbrain dopaminergic neurons (Lin et al. 1993), the factor is secreted from Sertoli cells in the testis (Viglietto et al. 2000). In the GDNF-overexpressing mice, dysregulated proliferation of spermatogonia was observed, whereas hemizygous GDNF-targeted mice gradually lost their spermatogonial population, indicating that GDNF regulates spermatogonial proliferation (Meng et al. 2000). Furthermore, gene transfer of a GDNF-expression plasmid into Sertoli cells resulted in an increase of SSC number (Yomogida et al. 2003). In addition to these in vivo studies, an in vitro experiment also indicated a beneficial effect of GDNF on cultured SSCs using serum-supplemented medium in a short-term culture experiment; however, an increase in SSC number was not observed under these culture conditions (Nagano et al. 2003).

In 2003, it was reported that gonocytes from newborn mice of genetic background ICR or BDF1 (C57BL/6 × DBA/2), could form grape-like colonies and proliferate on mouse embryonic fibroblasts (MEF) feeders in a serum-supplemented proprietary StemPro-34® (Life technology)-based medium, which contained the original StemPro-34® supplement plus 16 individual compounds and fetal bovine serum (FBS) with a cytokine mixture of GDNF, FGF2, epidermal growth factor (EGF), and LIF (Kanatsu-Shinohara et al. 2003). Proliferating cells on MEF express several spermatogonial markers such as ITGA6, ITGB1, and EPCAM, and

generated spermatogenic colonies following transplantation into infertile mouse testes, indicating they had SSC potential. In this culture condition, cell proliferation was restricted to the genetic background indicated above, and gonocytes from C57BL/6 or 129/Sv never formed colonies. The grape-like proliferating cells were named GS (germline stem) cells, because they possessed SSC activity, which was equivalent to that of freshly-isolated gonocytes (~12 colonies/10^5 cells transplanted (Kanatsu-Shinohara et al. 2003)). Using the transplantation assay, gonocytes purified by FACS generated ~17 colonies per 10^5 cells transplanted, while purified undifferentiated spermatogonia enriched for SSCs generate 160 ~ 350 colonies per 10^5 cells transplanted (Kubota et al. 2003, 2004a). Under the culture condition, GS cells that arose from gonocytes in culture spontaneously transformed to pluripotent stem cells, which were designated multipotent GS (mGS) cells, with characteristics similar to ES cells (Kanatsu-Shinohara et al. 2004a). Because spontaneous transformation of SSCs in normal mouse testes is extremely rare, this suggests that GS cells might not be identical to normal SSCs. In addition, GS cells could proliferate in an anchorage independent condition (Kanatsu-Shinohara et al. 2006b), which is one criterion of transformed cells. These unique characteristics of GS cells might be acquired during the process of in vitro cultivation. GS-like cell lines, which possess SSC activity with spontaneous dedifferentiation to pluripotent cells, have also been reported to arise from adult testes using similar culture conditions (Guan et al. 2006; Ko et al. 2009; Seandel et al. 2007). Therefore, although SSCs are unipotent stem cells in the testis, the transformation capability of GS cells into pluripotent stem cells makes them a valuable tool to investigate and understand pluripotent characteristics of the germline. Furthermore, a recent study reported that FGF2-dependent GS cells were established in the absence of GDNF (Takashima et al. 2015). Spermatogonial proliferation does not occur in the testes of GDNF-knockout mice (Naughton et al. 2006); therefore, it is not clear whether a population equivalent to FGF2-dependent GS cells exists in normal testes. Because FGF2 is a potent mitogen for PGCs, and the FGF2-dependent GS cells express the KIT receptor, it would be valuable to investigate whether they are capable of transforming into pluripotent stem cells as seen in PGCs or GS cells cultured with GDNF.

To establish long-term culture conditions for SSCs, identification of essential extrinsic factors for self-renewal of SSCs is crucial (Kubota and Brinster 2008). For this purpose, serum-free culture conditions are useful. In addition to considerable batch variation, serum contains complex undefined substances including biologically active compounds, such as hormones and growth factors. In addition, serum contains growth factors that stimulate proliferation of fibroblasts, including FGFs or platelet-derived growth factors (PDGFs); therefore, serum-containing medium supports proliferation of fibroblasts in a selective manner (Sato et al. 1960). Such fibroblast-dominant culture conditions are detrimental for many types of cells, including germ cells. In the 1970s, Gordon Sato's group developed serum-free hormonally defined media, which supported many different types of cells, by adding appropriate hormones or growth factors (Barnes and Sato 1980). Common requirements for all cells in serum-free conditions were insulin, albumin, transferrin, selenium, and fatty acids (Barnes and Sato 1980). Further studies demonstrated that

extracellular matrices that provide anchorage for adherent cells are important components of serum (Enat et al. 1984). Thus, in serum-free conditions, anchorage materials, which can be extracellular matrices or inactive feeder cells, should be added to the culture. For stem cell cultures, feeder cells are preferable, because the initial stem cell number placed in culture is generally low, and feeder cells are beneficial in this situation (Kubota and Brinster 2008). Based on these principles, a culture system for SSCs was developed consisting of a serum-free defined medium containing minimum components and STO feeder cells, which was originally developed for hepatic stem/progenitor cells (Kubota and Reid 2000). The serum-free medium consisted of alpha MEM basal medium containing insulin, transferrin, selenium, putrescine, 2-mercaptoethanol, free fatty acids, HEPES, and antibiotics. This defined culture system was used to screen various growth factors for their effect on SSC proliferation (Kubota et al. 2004a), and it was found that SSCs from DBA background mice continuously proliferated in the presence of GDNF (Kubota et al. 2004b). Although the STO cell line produces LIF and SCF constitutively (Schmitt et al. 1991), these factors did not show any beneficial effect on SSC proliferation (Kubota et al. 2004a). Additionally, EGF did not support proliferation of SSCs (Kubota et al. 2004a). Although GDNF alone supports proliferation of SSCs isolated from DBA/2 background mice, GDNF alone was not sufficient to support continuous proliferation of SSCs from other mouse strains, such as C57BL/6. In the GDNF-containing medium, they initially formed clumps, but ceased proliferation and disappeared within a few weeks (Kubota et al. 2004b). Interestingly, a relatively small amount of FGF2 (~1 ng/mL) was very effective in enhancing proliferation of SSCs from non-DBA/2 mouse strains. Furthermore, addition of soluble GFRA1 enhanced the effect of GDNF on SSCs (Kubota et al. 2004b). The GDNF receptor complex consists of RET receptor tyrosine kinase and GFRA1 that is a glycosil phosphatidylinositol-anchored ligand binding subunit. Soluble GFRA1 has been shown to potentiate RET activation by direct binding with GDNF-GFRA1 complexes (Paratcha et al. 2001). Combining both factors with GDNF, SSCs from all mouse strains examined including 129/Sv, which is one of nonpermissive strains to generate GS cells (Inoue and Ogura 2015), could self-renew and proliferate indefinitely in culture (Kubota et al. 2004b). Under these culture condition, undifferentiated spermatogonia that were freshly isolated and highly enriched for SSCs form morula-like, tightly packed cellular clumps and continuously proliferate (Fig. 11.2A). The proliferating cells were $THY1^{+}$ $ITGA6^{+}$ $ITGAV^{lo}$ and their SSC activity as determined by the functional assay was 300 ~ 400 colonies per 10^{5} cell transplanted, which is essentially equivalent to freshly isolated undifferentiated spermatogonia from postnatal testes (Kubota et al. 2004b).

A serum-free culture system containing key extrinsic factors has allowed development of a simple culture condition to support self-renewal and proliferation of SSCs. Once a simple culture condition for ex vivo expansion of SSCs was determined, several minor factors appeared to be important for successful establishment of long-term cultures. One important factor is the catalog number (type) and lot number of bovine serum albumin (BSA). Although the basis is not clear, BSA purified by a heat-shock process rather than the Cohn cold ethanol precipitation process

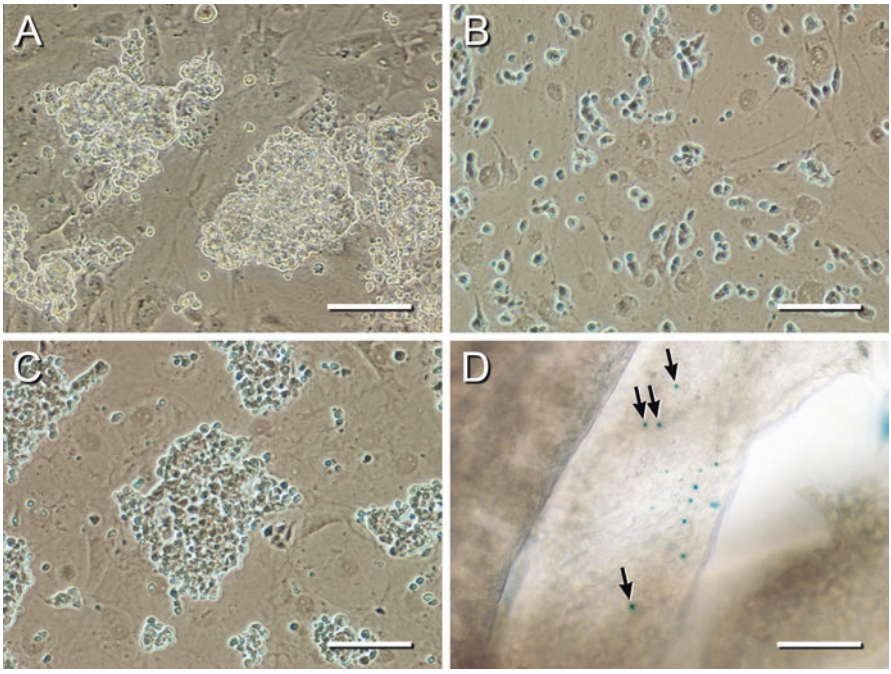

Fig. 11.2 Comparative analysis of mouse SSCs and rabbit SSCs. (**A**–**C**) A phase-culture images of proliferating mouse SSCs clumps on STO feeder cells (**A**), rabbit SSCs cultured on STO feeders (**B**), and rabbit SSCs on C166 mouse endothelial cell feeders (**C**). Although no cellular clumps of rabbit SSCs were formed on STO feeder cells, they formed clumps on C166 feeder layers and continuously proliferated. (**D**) Seminiferous tubules of recipient testes transplanted with the β-galactosidase-expressing rabbit SSCs. Recipient testes were analyzed with X-gal staining at 23 weeks after transplantation. Donor rabbit cells were stained blue (*arrows*). Blue cells and clusters were found throughout the recipient seminiferous tubules. Scale bars = 100 μm

appeared more suitable for SSC culture. The catalog number and lot number of BSA that allows SSC survival and proliferation in culture must be determined empirically. In addition, a culture gas atmosphere of 10% O_2 was significantly better than 21% O_2 (Kubota et al. 2009). In particular, long-term cultures of SSCs from W^v/W^v mice could be established only in a 10% O_2 atmosphere (Kubota et al. 2009). Because the SSC number is extremely low in W^v/W^v testes, it was impossible to enrich SSCs from W^v/W^v mouse testes by antibody-based enrichment such as FACS using markers for SSC. However, even without an enrichment step, a 10% O_2 condition could support proliferation of W^v/W^v SSCs and establish a long-term culture. Furthermore, a 10% O_2 atmosphere made it possible to establish a long-term culture from an individual colony generated from a single SSC colonization event in a seminiferous tubule of a recipient testis (Schmidt et al. 2011). In addition, an increased proliferation rate of SSCs from wild-type C57Bl/6 mice was found in a 10% O_2 atmosphere (Kubota et al. 2009). Significantly, the number of SSCs in a germ cell clump and the cell surface phenotype was not different between 10% O_2 and 21%

O_2 cultures, indicating that O_2 concentration improved the proliferation rate of cultured SSCs.

11.3.2 Rat

Using mouse SSC culture conditions as a foundation, two rat SSC culture systems have been developed (Hamra et al. 2005; Ryu et al. 2005). One of these conditions is essentially the same as the mouse SSC culture system consisting of serum-free medium, STO feeders, and growth factors including, GDNF and FGF2 (Ryu et al. 2005). GFRA1 also has a supportive effect on the GDNF dependent proliferation (Ryu et al. 2005). Under these culture conditions, rat spermatogonia indefinitely proliferated as clumps, and cultured cells generated spermatogenic colonies in infertile recipients following transplantation. When they were transplanted into Busulfan-treated recipient rat testes, offspring were successfully produced, indicating that cultured cells were indeed rat SSCs (Ryu et al. 2005).

However, some modifications of the mouse SSC culture method were beneficial for long-term culture of rat SSCs (Kubota and Brinster 2008). First, although the components (insulin, transferrin, selenium, putrescine, 2-mercaptoethanol, and free fatty acids) of the serum-free medium for rat SSCs are the same as those of the medium for mouse SSCs, the concentration of several components was increased. Second, the medium osmolality was reduced by addition of water to the serum-free medium (Brinster 1965). Third, a reduced concentration of trypsin with a short exposure time during subculture facilitated recovery of cells. Lastly, a low atmospheric concentration of oxygen (5 ~ 10%) was beneficial. In addition, overgrowth of testicular somatic cells was even more of a problem for rat SSC proliferation then previously observed for mouse SSC (Kubota et al. 2004a). Therefore, it is critical that somatic cells be removed from the cultures. Thus, when fibroblasts or other somatic cells were present in the culture, rat germ cell clumps were collected by gentle pipetting of medium across the surface of the feeder layers because regular trypsin-treatment resulted in recovering all somatic cells in the culture. Before placing collected germ cells on fresh STO feeders, they could be digested with a low concentration of trypsin (e.g. 0.01%). All together, these minor modifications are important for establishment of long-term cultures of rat SSCs (Ryu et al. 2005). An important finding arising from rat SSC cultures is that self-renewal of rat SSCs is also dependent on GDNF, suggesting that it may be essential in other species. Furthermore, these studies using defined culture conditions for mouse and rat SSCs have identified the main essential exogenous factors for their unlimited proliferation and made possible generation of large numbers of SSCs in vitro, which is a prerequisite for biochemical and molecular investigation of SSCs (Oatley et al. 2006; Schmidt et al. 2009).

In a second long-term culture system for rat SSCs, a serum-free medium containing proprietary B27 supplement® minus vitamin A (Life technology), with GDNF, FGF2, and MEF feeder cells was used (Hamra et al. 2005). Initially in this system, when serum-supplemented medium was used, the number of rat germ cells

decreased after each subculture (Hamra et al. 2005). Serum was removed from the medium because the decrease in germ cell numbers in the culture seemed to be caused by a combination of serum and testicular somatic cells, which had been demonstrated in mouse germ cell cultures (Kubota et al. 2004a). The basic culture medium was StemPro-34-based GS cell medium, but serum was replaced to the proprietary B27 supplement, a serum-free supplement developed for supporting neural cell cultures (Brewer et al. 1993). Although the original B27 supplement contains vitamin A, the B27 minus vitamin A was used for rat SSC culture because vitamin A is a potent differentiation factor for spermatogonia. When the B27 supplement minus vitamin A replaced serum in the StemPro-34 based GS cell medium, rat SSCs continuously proliferated (Hamra et al. 2005). A subsequent study showed that a medium without StemPro-34 supplement seemed better than medium containing StemPro-34 supplement (Wu et al. 2009b). Interestingly, like rat SSCs, removal of FBS and B27 supplementation in the GS medium improved cell proliferation speed of mouse GS cells, although FBS appeared to be necessary for initial derivation of mouse GS cell lines on primary MEF feeder cells (Kanatsu-Shinohara et al. 2005b). Rat pluripotent stem cell lines have not been developed from cultured rat SSCs in either culture system.

11.3.3 Rabbit

The basic requirements for self-renewal of murine and rat SSCs appear to be identical. Although rodent SSCs have been investigated intensively using the in vitro culture techniques in addition to the functional transplantation assay (Brinster 2002; Kubota and Brinster 2006; Oatley and Brinster 2012), knowledge about non-rodent SSCs is limited. Rabbits diverged phylogenetically from rodents about 60 million years ago (McKenna and Bell 1997), while the time of divergence between mice and rats is considered to be approximately 11 million years ago (Catzeflis et al. 1993). Thus, cultivation of rabbit germ cells could provide an excellent foundation for development of SSC cultures from other species that diverged from the mouse 60 million or more years ago, and this possibility served as one objective for studying rabbit SSCs. When rabbit germ cells were transplanted into immunodeficient mouse testes, putative rabbit SSCs colonized the basement membrane of seminiferous tubules and proliferated for several months; however, no spermatogonial differentiation occurred (Dobrinski et al. 1999a). This indicates that exogenous factors to promote rabbit SSCs exist in mouse seminiferous tubules. GDNF and FGF2 are strong candidates for those factors. However, the rodent SSC culture conditions that consist of STO feeders and serum-free medium containing GDNF and FGF2 could not support proliferation of rabbit germ cells (Kubota et al. 2011). In the serum-free rodent SSC culture system, involvement of mitotically inactive feeder layers for expansion of mouse SSCs is important, because other feeder cells such as Sertoli cell feeders could not support initiation of derivation or maintenance of mouse SSCs (Kubota and Brinster 2008). In particular, testicular fibroblast feeders were detrimental for maintenance of murine SSCs (Kubota et al. 2004a). Similar feeder

effects were observed in the culture system for rat SSCs (Hamra et al. 2005). Besides embryonic fibroblasts, endothelial cells are often used as feeder cells for several types of stem cells (Shen et al. 2004), and we found endothelial cells critical for rabbit SSC culture. Although rabbit spermatogonia could not form clumps and proliferate on STO feeder cells on which rodent SSCs replicate continuously (Fig. 11.2A, B), rabbit germ cells could form clumps on C166 feeders, a yolk sac-derived endothelial cell line, and proliferated continuously in the presence of GDNF (Fig. 11.2C). The proliferation of rabbit germ cell clumps was dependent on GDNF, but FGF2 was not required for continuous proliferation. In addition to expression of DDX4, a definitive germ cell marker, they expressed several SSC marker proteins including ZBTB16, POU5F1, GFRA1, and THY1, which were found in rodent SSCs (Kubota et al. 2011).

To demonstrate that the clump-forming germ cells on C166 feeder cells are rabbit SSCs, functional characterization is necessary. However, an assay to evaluate rabbit SSC activity that includes both self-renewal and differentiation capability to produce functional sperm has not been established. For non-rodent SSCs, colony formation following transplantation into the seminiferous tubules of immunodeficient mouse testes represents the most reliable identification of SSC potential and has been used in several other xenogenetic transplantation assays (Dobrinski et al. 1999a, 2000; Hermann et al. 2007; Nagano et al. 2001b, 2002; Oatley et al. 2004). In the transplantation assay, there are two issues to be carefully addressed. One is identification of donor cells in recipient testes. They should be unequivocally distinguished from recipient cells. The other is characterization of colonized cells. If the phenotype of colonized cells is not undifferentiated spermatogonia, transplanted cells might not be SSCs. In addition, some types of somatic cells could be colonizing (Dobrinski et al. 2000; Shinohara et al. 2003). To avoid any misinterpretation, labeling transplanted cells by introducing a reporter gene, β-galactosidase or GFP, is one of the best techniques. In our experiments, clump-forming rabbit germ cells on C166 feeders were labeled with the β-galactosidase or GFP gene using lentivirus vectors and transplanted into seminiferous tubules of Busulfan-treated nude mice (Kubota et al. 2011). For as long as 23 weeks after transplantation, the β-galactosidase-labeled donor cells were identified by stereomicroscopic analyses (Fig. 11.2D). In addition, flow cytometry readily identified the GFP-labeled donor cells in the testis cell suspension from recipient seminiferous tubules. More importantly, rabbit donor cells in recipient mouse testes retained the undifferentiated spermatogonial phenotype for 6 months, although they did not produce spermatogenesis (Kubota et al. 2011). Thus, it is very likely that the transplanted clump-forming cells cultured on C166 feeders contained rabbit SSCs. To unequivocally demonstrate that the clump-forming germ cells are genuine rabbit SSCs, evaluation by transplantation experiments using allogeneic or ideally syngeneic rabbits as recipients is required, because a transplantation assay is the gold standard of identification of stem cells in any type of tissue. At present there is no report demonstrating a successful long-term cultivation of SSCs derived from non-rodent species. Nonetheless, the rabbit culture system serves as a critical foundation for efforts to culture the SSCs of other non-rodent species, including human SSCs.

11.3.4 Human

Propagation of human SSCs in vitro is of great clinical value. Since the first report of long-term culture of human SSCs in 2009 (Sadri-Ardekani et al. 2009), several culture conditions have been reported (Conrad et al. 2014; Goharbakhsh et al. 2013; Guo et al. 2015; Kokkinaki et al. 2011; Koruji et al. 2012; Lim et al. 2010). Most of these are modified methods of rodent SSC cultures using StemPro34-based media supplemented with GDNF, FGF2, EGF, LIF, and FBS. In these conditions, two types of colonies appeared after about 2–4-weeks in culture. One type consisted of individually visible cells, while the second type consisted of tightly packed colonies, which appeared similar to ES cell colonies (Sadri-Ardekani et al. 2009). The former colonies were designated SSCs, because they expressed several spermatogonial markers, such as ZBTB16, GFRA1, UCHL1, and GPR125 (Sadri-Ardekani et al. 2009, 2011). On the other hand, ES cell-like colonies were considered to be pluripotent stem cells, because they expressed pluripotent markers, such as POU5F1 and NANOG. Culturing human testicular cells in ES cell culture conditions also produced ES-like colonies (Conrad et al. 2008; Golestaneh et al. 2009; Kossack et al. 2009; Mizrak et al. 2010). In order to investigate the biological activity of proliferating cells in cultures, the putative human SSCs were transplanted into infertile immunodeficient mouse testes and the recipient testes analyzed 10 weeks after transplantation. In the recipient testes, a small number of donor-derived cells were identified (Sadri-Ardekani et al. 2009, 2011). Although other research groups also reported culture of human SSCs using similar culture conditions, they were not transplanted (Conrad et al. 2014; Goharbakhsh et al. 2013; Guo et al. 2015; Kokkinaki et al. 2011; Koruji et al. 2012; Lim et al. 2010), and in none of these studies were the transplanted donor human cells recovered from the recipient mouse testes and analyzed for surface markers, as done for the rabbit, or characteristic transcription factors.

The absence of a functional assay system to identify unequivocally human SSCs has resulted in considerable controversy regarding interpretation of results in these and other human SSC culture experiments (Kossack et al. 2013; Langenstroth et al. 2014; Zheng et al. 2014). Although the cellular identity of human SSCs in the cultures was demonstrated by expression of SSC markers previously identified in the SSCs of rodents, recent studies also have clearly demonstrated that many putative markers used for identifying human SSCs are not reliable, due to their expression in non-germ cells of primary and cultured human testis cells (Kossack et al. 2013; Zheng et al. 2014). Importantly, GPR125, ZBTB16, UCHL1, ITGA6, and GFRA1, which were used to identify human SSCs in previous studies, were expressed in testicular somatic cells. Furthermore, in the transplantation experiments, donor germ cells cannot differentiate in the xenotransplantation system, and non-germ cells can colonize recipient seminiferous tubules as well; therefore, characterization of colonized cells would require demonstrating that the colonized cells were human undifferentiated spermatogonia. Information describing molecular signatures and transcriptome analysis of human undifferentiated spermatogonia in recent studies will be useful to identify donor cells in recipient testes (Valli et al. 2014; Wu et al.

2009a). Possible approaches to avoid the problem would be elimination of non-germ cells prior to transplantation or definitive characterization of colonized cells as donor-derived germ cells, both of which represent formidable challenges.

Collectively, while several reports proposed that human SSCs could be continuously cultured over months using StemPro-34-based culture medium containing a growth factor cocktail with FBS, these published reports of long-term human SSC culture are not universally accepted (Medrano et al. 2016). Development of a functional assay to evaluate the self-renewal and differentiation capability for human SSCs and confirm their identity will be essential to the widespread acceptance of any published techniques.

11.4 Potential Applications of Spermatogonial Stem Cells and Future Directions

The transplantation technique has made it possible to generate functional spermatozoa from SSCs isolated from a donor cell population introduced into the seminiferous tubules of a recipient animal. Furthermore, it has been shown that SSCs can be cryopreserved for at least 14 years and will regenerate spermatogenesis and functional spermatozoa after thawing and transplantation (Avarbock et al. 1996; Wu et al. 2012). Thus, cryopreservation of SSCs or SSC containing cell populations provides an effective mechanism by which to preserve the germline of individual males for long periods providing a potentially immortal lifespan for male germlines. Although semen cryopreservation is commonly used to preserve the germline of certain economically, biologically, or scientifically valuable males, including livestock breeds or endangered animal species, semen cryopreservation methods must be developed for each species. Cryopreservation of SSCs is more suitable for this purpose, because simple cryopreservation procedures for somatic cells are applicable to SSCs (Avarbock et al. 1996). Although semen preservation techniques have only been developed for a limited number of species (Barbas and Mascarenhas 2009), SSCs of many species are readily cryopreserved by regular procedures for somatic cells (Avarbock et al. 1996; Dobrinski et al. 1999a, 2000; Hermann et al. 2007; Nagano et al. 2001b, 2002; Oatley et al. 2004). In addition, the potential genetic recombination possibilities of a germline are only partially conserved with spermatozoa since variability is limited by the number of spermatozoa present in the sample, whereas, the stem cell preserves all the potential recombination possibilities of any germline. Moreover, SSCs have the potential to be expanded in culture. Although long-term culture techniques are available for a few species at present, techniques for many of the valuable species, including human, livestock, or endangered animals are certain to be worked out in the future (Kubota and Brinster 2006).

Functional spermatozoa can be obtained by transplantation of SSCs from immature males before puberty or even from fetuses. Thus, an important and potential clinical application for human SSCs is in prepubertal boys undergoing chemotherapy or radiation treatment (Brinster 2007; Kubota and Brinster 2006) (Fig. 11.3). Germ cells including SSCs are extremely sensitive to chemotherapeutic agents and

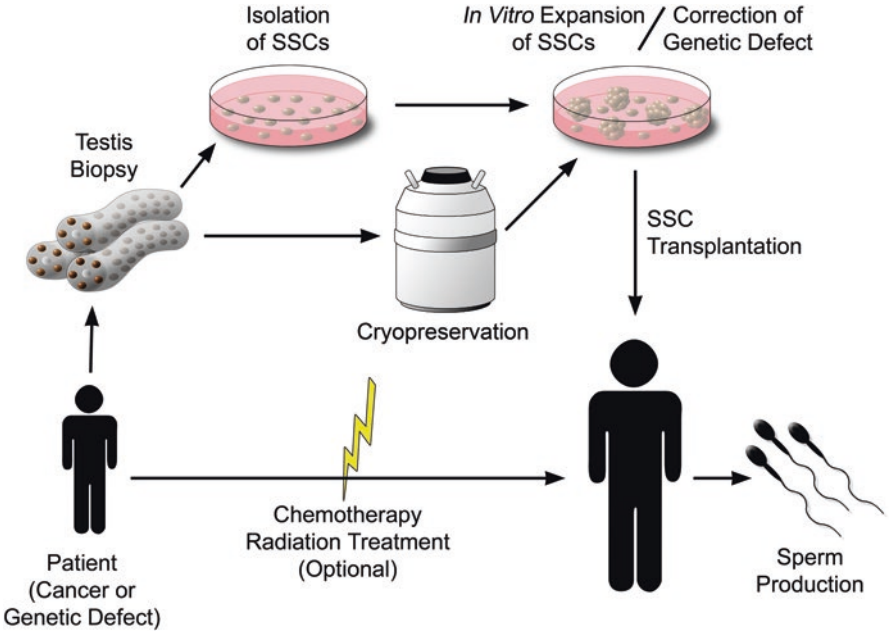

Fig. 11.3 A proposed clinical application of human SSCs. Before treatment for cancer by chemotherapy or irradiation, a prepubertal patient could undergo a testicular biopsy to recover SSCs. The SSCs could be cryopreserved and/or cultured to expand in vitro. After treatment, the SSCs would be transplanted to the patient's testes for the production of spermatozoa. A possible step for genetic correction to rescue a genetic disorder is indicated prior to transplantation. Modified from Kubota and Brinster (2006)

radiation, and it is estimated that approximately 1 in 5000 male cancer survivors of reproductive age are infertile or extremely sub-fertile as a result of treatments for childhood cancer (Ginsberg et al. 2010). While adults can cryopreserve semen before germ cell destroying therapies for future use in artificial insemination or in vitro fertilization, this option is not available for prepubertal boys, because complete spermatogenesis has not been established. For prepubertal boys, cryopreservation of a testicular biopsy can be used for future autologous transplantation into the seminiferous tubules following successful cancer treatments (Ginsberg et al. 2014). The biopsy contains SSCs, which have the potential to colonize and restore spermatogenesis following transplantation. In addition, when efficient culture methods to allow ex vivo expansion of human SSCs become available, the number of SSCs can be greatly increase before cryopreservation or transplantation, which maximizes recovery of spermatogenesis in recipient testes (Fig. 11.3).

An important concern is potential contamination of malignant cells in donor cell suspensions. To avoid this, it is important to determine the unique surface phenotype of human SSCs, which will allow both enrichment of human SSCs and elimination of cancer cells before transplantation. The antigenic profile of mouse SSCs is highly conserved in putative human SSCs. In particular, THY1 is a useful cell

surface marker to enrich putative SSCs in human, and the characteristic of MHC-I negative is also valuable to eliminate malignant cells, because MHC-I is strongly expressed on almost all somatic cells, including tumorigenic cells but not on SSCs (Hermann et al. 2011). Recent studies suggest that contamination of tumorigenic cells in donor cell suspension is avoidable by FACS with combinations of several surface markers (Dovey et al. 2013).

An enormously valuable application of SSCs is for germline modification (Brinster 2002). The first transgenic animal using SSCs was created by transduction of a retrovirus vector containing the β-galactosidase gene into mouse SSCs (Nagano et al. 2001a). Although retroviral transduction was used in the initial approach, subsequent development of a long-term culture system now allows a variety of techniques to select successful modifications, resulting in generation of not only knock-out mice by homologous recombination (Kanatsu-Shinohara et al. 2006a), but also gene-editing mice using the TALEN or, in particular, the CRISPR/Cas9 system (Sato et al. 2015; Wu et al. 2015). In rats, similar to the mouse system, the first SSC-based transgenic rats were generated using a lentiviral vector (Hamra et al. 2002), and the CRISPR/Cas9 system in rat SSCs has been developed (Chapman et al. 2015; Sato et al. 2015; Wu et al. 2015). In addition, recent studies demonstrated the possibility of germline gene therapy using the CRISPR/Cas9 system in mice (Wu et al. 2015). These gene-editing approaches will eventually be applicable to a variety of animals, for instance, valuable breeds of companion animals and farm animals (Tan et al. 2013).

A number of genetic mutations causing human disease have been identified, and at some future date germline gene-editing may be considered for therapeutic correction. However, a recent report of gene-editing in human preimplantation embryos using CRISPR/Cas9 is highly controversial (Kang et al. 2016; Liang et al. 2015), because it raises serious ethical concerns (Bosley et al. 2015). The scientific community has agreed that this technology is not sufficiently developed and should not be used for human clinical reproductive purpose (Baltimore et al. 2015). In 2016, only a few countries have approved the use of gene-editing techniques on human embryos for research purposes. Once cultivation and differentiation techniques of human SSCs become available, controversy will inevitably arise regarding the ethics of research designed to generate spermatozoa from gene-edited human SSCs.

It can be seen from the above discussion that SSC transplantation, culture and cryopreservation have revolutionized the study of the male germline of not only research species, but of all mammals, including companion animals, farm animals, primates, and endangered species. The possibility to increase knowledge about SSCs and spermatogenesis regarding biological regulation and for practical purposes is now limited only by future development and understanding of male germline stem cells and their differentiated daughters, which is a rapidly emerging field as seen in the contents of other chapters in this volume.

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References

Amann RP (1986) Detection of alterations in testicular and epididymal function in laboratory animals. Environ Health Perspect 70:149–158

Avarbock MR, Brinster CJ, Brinster RL (1996) Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nat Med 2:693–696

Baltimore D, Berg P, Botchan M, Carroll D, Charo RA, Church G, Corn JE, Daley GQ, Doudna JA, Fenner M et al (2015) A prudent path forward for genomic engineering and germline gene modification. Science 348:36–38

Barbas JP, Mascarenhas RD (2009) Cryopreservation of domestic animal sperm cells. Cell Tissue Bank 10:49–62

Barnes D, Sato G (1980) Serum-free cell culture: a unifying approach. Cell 22:649–655

Barroca V, Lassalle B, Coureuil M, Louis JP, Le Page F, Testart J, Allemand I, Riou L, Fouchet P (2009) Mouse differentiating spermatogonia can generate germinal stem cells in vivo. Nat Cell Biol 11:190–196

Bosley KS, Botchan M, Bredenoord AL, Carroll D, Charo RA, Charpentier E, Cohen R, Corn J, Doudna J, Feng G et al (2015) CRISPR germline engineering[mdash]the community speaks. Nat Biotech 33:478–486

Brewer GJ, Torricelli JR, Evege EK, Price PJ (1993) Optimized survival of hippocampal neurons in B27-supplemented Neurobasal, a new serum-free medium combination. J Neurosci Res 35:567–576

Brinster RL (1965) Studies on the development of mouse embryos in vitro. I. The effect of osmolarity and hydrogen ion concentration. J Exp Zool 158:49–57

Brinster RL (2002) Germline stem cell transplantation and transgenesis. Science 296:2174–2176

Brinster RL (2007) Male germline stem cells: from mice to men. Science 316:404–405

Brinster RL, Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 91:11303–11307

Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 91:11298–11302

Catzeflis FM, Dickman DA, Michaux J, Kirsch JAW (1993) DNA hybridization and rodent phylogeny, vol 2. Springer, New York

Chan F, Oatley MJ, Kaucher AV, Yang QE, Bieberich CJ, Shashikant CS, Oatley JM (2014) Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev 28:1351–1362

Chapman KM, Medrano GA, Jaichander P, Chaudhary J, Waits AE, Nobrega MA, Hotaling JM, Ober C, Hamra FK (2015) Targeted germline modifications in rats using CRISPR/Cas9 and spermatogonial stem cells. Cell Rep 10:1828–1835

Chuma S, Kanatsu-Shinohara M, Inoue K, Ogonuki N, Miki H, Toyokuni S, Hosokawa M, Nakatsuji N, Ogura A, Shinohara T (2005) Spermatogenesis from epiblast and primordial germ cells following transplantation into postnatal mouse testis. Development 132:117–122

Clermont Y (1972) Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 52:198–236

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

Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL (1996) Rat spermatogenesis in mouse testis. Nature 381:418–421

Conrad S, Azizi H, Hatami M, Kubista M, Bonin M, Hennenlotter J, Renninger M, Skutella T (2014) Differential gene expression profiling of enriched human spermatogonia after short- and long-term culture. Biomed Res Int 2014:138350

Conrad S, Renninger M, Hennenlotter J, Wiesner T, Just L, Bonin M, Aicher W, Buhring HJ, Mattheus U, Mack A et al (2008) Generation of pluripotent stem cells from adult human testis. Nature 456:344–349

Culty M (2013) Gonocytes, from the fifties to the present: is there a reason to change the name? Biol Reprod 89(46):41–46

de Rooij DG (1973) Spermatogonial stem cell renewal in the mouse. I. Normal situation. Cell Tissue Kinet 6:281–287

de Rooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21:776–798

Dobrinski I, Avarbock MR, Brinster RL (1999a) Transplantation of germ cells from rabbits and dogs into mouse testes. Biol Reprod 61:1331–1339

Dobrinski I, Avarbock MR, Brinster RL (2000) Germ cell transplantation from large domestic animals into mouse testes. Mol Reprod Dev 57:270–279

Dobrinski I, Ogawa T, Avarbock MR, Brinster RL (1999b) Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice. Mol Reprod Dev 53:142–148

Dovey SL, Valli H, Hermann BP, Sukhwani M, Donohue J, Castro CA, Chu T, Sanfilippo JS, Orwig KE (2013) Eliminating malignant contamination from therapeutic human spermatogonial stem cells. J Clin Invest 123:1833–1843

Enat R, Jefferson DM, Ruiz-Opazo N, Gatmaitan Z, Leinwand LA, Reid LM (1984) Hepatocyte proliferation in vitro: its dependence on the use of serum-free hormonally defined medium and substrata of extracellular matrix. Proc Natl Acad Sci U S A 81:1411–1415

França LR, Ogawa T, Avarbock MR, Brinster RL, Russell LD (1998) Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol Reprod 59:1371–1377

Geijsen N, Horoschak M, Kim K, Gribnau J, Eggan K, Daley GQ (2004) Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 427:148–154

Ginsberg JP, Carlson CA, Lin K, Hobbie WL, Wigo E, Wu X, Brinster RL, Kolon TF (2010) An experimental protocol for fertility preservation in prepubertal boys recently diagnosed with cancer: a report of acceptability and safety. Hum Reprod 25:37–41

Ginsberg JP, Li Y, Carlson CA, Gracia CR, Hobbie WL, Miller VA, Mulhall J, Shnorhavorian M, Brinster RL, Kolon TF (2014) Testicular tissue cryopreservation in prepubertal male children: an analysis of parental decision-making. Pediatr Blood Cancer 61:1673–1678

Goharbakhsh L, Mohazzab A, Salehkhou S, Heidari M, Zarnani AH, Parivar K, Akhondi MM (2013) Isolation and culture of human spermatogonial stem cells derived from testis biopsy. Avicenna J Med Biotechnol 5:54–61

Golestaneh N, Kokkinaki M, Pant D, Jiang J, DeStefano D, Fernandez-Bueno C, Rone JD, Haddad BR, Gallicano GI, Dym M (2009) Pluripotent stem cells derived from adult human testes. Stem Cells Dev 18:1115–1126

Greenbaum MP, Iwamori T, Buchold GM, Matzuk MM (2011) Germ cell intercellular bridges. Cold Spring Harb Perspect Biol 3:a005850

Grompe M (2012) Tissue stem cells: new tools and functional diversity. Cell Stem Cell 10:685–689

Guan K, Nayernia K, Maier LS, Wagner S, Dressel R, Lee JH, Nolte J, Wolf F, Li M, Engel W et al (2006) Pluripotency of spermatogonial stem cells from adult mouse testis. Nature 440:1199–1203

Guo Y, Liu L, Sun M, Hai Y, Li Z, He Z (2015) Expansion and long-term culture of human spermatogonial stem cells via the activation of SMAD3 and AKT pathways. Exp Biol Med (Maywood, NJ) 240:1112–1122

Hamra FK, Chapman KM, Nguyen DM, Williams-Stephens AA, Hammer RE, Garbers DL (2005) Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. Proc Natl Acad Sci U S A 102:17430–17435

Hamra FK, Gatlin J, Chapman KM, Grellhesl DM, Garcia JV, Hammer RE, Garbers DL (2002) Production of transgenic rats by lentiviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A 99:14931–14936

Hara K, Nakagawa T, Enomoto H, Suzuki M, Yamamoto M, Simons BD, Yoshida S (2014) Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. Cell Stem Cell 14:658–672

Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M (2011) Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell 146:519–532

Hermann BP, Sukhwani M, Hansel MC, Orwig KE (2010) Spermatogonial stem cells in higher primates: are there differences from those in rodents? Reproduction 139:479–493

Hermann BP, Sukhwani M, Lin CC, Sheng Y, Tomko J, Rodriguez M, Shuttleworth JJ, McFarland D, Hobbs RM, Pandolfi PP et al (2007) Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult rhesus macaques. Stem Cells 25:2330–2338

Hermann BP, Sukhwani M, Salati J, Sheng Y, Chu T, Orwig KE (2011) Separating spermatogonia from cancer cells in contaminated prepubertal primate testis cell suspensions. Hum Reprod (Oxford, England) 26:3222–3231

Hermann BP, Sukhwani M, Winkler F, Pascarella JN, Peters KA, Sheng Y, Valli H, Rodriguez M, Ezzelarab M, Dargo G et al (2012) Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell 11:715–726

Herrid M, Olejnik J, Jackson M, Suchowerska N, Stockwell S, Davey R, Hutton K, Hope S, Hill JR (2009) Irradiation enhances the efficiency of testicular germ cell transplantation in sheep. Biol Reprod 81:898–905

Herrid M, Vignarajan S, Davey R, Dobrinski I, Hill JR (2006) Successful transplantation of bovine testicular cells to heterologous recipients. Reproduction 132:617–624

Hess RA, Renato de Franca L (2008) Spermatogenesis and cycle of the seminiferous epithelium. Adv Exp Med Biol 636:1–15

Honaramooz A, Behboodi E, Blash S, Megee SO, Dobrinski I (2003a) Germ cell transplantation in goats. Mol Reprod Dev 64:422–428

Honaramooz A, Behboodi E, Hausler CL, Blash S, Ayres S, Azuma C, Echelard Y, Dobrinski I (2005) Depletion of endogenous germ cells in male pigs and goats in preparation for germ cell transplantation. J Androl 26:698–705

Honaramooz A, Behboodi E, Megee SO, Overton SA, Galantino-Homer H, Echelard Y, Dobrinski I (2003b) Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol Reprod 69:1260–1264

Honaramooz A, Megee S, Zeng W, Destrempes MM, Overton SA, Luo J, Galantino-Homer H, Modelski M, Chen F, Blash S et al (2008) Adeno-associated virus (AAV)-mediated transduction of male germ line stem cells results in transgene transmission after germ cell transplantation. FASEB J 22:374–382

Honaramooz A, Megee SO, Dobrinski I (2002) Germ cell transplantation in pigs. Biol Reprod 66:21–28

Huckins C (1971) The spermatogonial stem cell population in adult rats. I. Their morphology, proliferation and maturation. Anat Rec 169:533–557

Inoue K, Ogura A (2015) In quest of genomic treasure. J Reprod Dev 61:489–493

Ishii K, Kanatsu-Shinohara M, Shinohara T (2014) Cell-cycle-dependent colonization of mouse spermatogonial stem cells after transplantation into seminiferous tubules. J Reprod Dev 60:37–46

Izadyar F, DenOuden K, Stout T, Stout J, Coret J, Lankveld DP, Spoormakers TJ, Colenbrander B, Oldenbroek JK, VanderPloeg KD et al (2003) Autologous and homologous transplantation of bovine spermatogonial stem cells. Reproduction 126:765–774

Jiang FX, Short RV (1995) Male germ cell transplantation in rats: apparent synchronization of spermatogenesis between host and donor seminiferous epithelia. Int J Androl 18:326–330

Kanatsu-Shinohara M, Ikawa M, Takehashi M, Ogonuki N, Miki H, Inoue K, Kazuki Y, Lee J, Toyokuni S, Oshimura M et al (2006a) Production of knockout mice by random or targeted mutagenesis in spermatogonial stem cells. Proc Natl Acad Sci U S A 103:8018–8023

Kanatsu-Shinohara M, Inoue K, Lee J, Miki H, Ogonuki N, Toyokuni S, Ogura A, Shinohara T (2006b) Anchorage-independent growth of mouse male germline stem cells in vitro. Biol Reprod 74:522–529

Kanatsu-Shinohara M, Inoue K, Lee J, Yoshimoto M, Ogonuki N, Miki H, Baba S, Kato T, Kazuki Y, Toyokuni S et al (2004a) Generation of pluripotent stem cells from neonatal mouse testis. Cell 119:1001–1012

Kanatsu-Shinohara M, Inoue K, Miki H, Ogonuki N, Takehashi M, Morimoto T, Ogura A, Shinohara T (2006c) Clonal origin of germ cell colonies after spermatogonial transplantation in mice. Biol Reprod 75:68–74

Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T (2005a) Germline niche transplantation restores fertility in infertile mice. Hum Reprod 20:2376–2382
Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T (2005b) Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod 72:985–991
Kanatsu-Shinohara M, Mori Y, Shinohara T (2013) Enrichment of mouse spermatogonial stem cells based on aldehyde dehydrogenase activity. Biol Reprod 89:140
Kanatsu-Shinohara M, Morimoto H, Shinohara T (2012) Enrichment of mouse spermatogonial stem cells by melanoma cell adhesion molecule expression. Biol Reprod 87:139
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
Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2004b) CD9 is a surface marker on mouse and rat male germline stem cells. Biol Reprod 70:70–75
Kang X, He W, Huang Y, Yu Q, Chen Y, Gao X, Sun X, Fan Y (2016) Introducing precise genetic modifications into human 3PN embryos by CRISPR/Cas-mediated genome editing. J Assist Reprod Genet 33:581–588
Kim Y, Selvaraj V, Dobrinski I, Lee H, McEntee MC, Travis AJ (2006) Recipient preparation and mixed germ cell isolation for spermatogonial stem cell transplantation in domestic cats. J Androl 27:248–256
Kim Y, Turner D, Nelson J, Dobrinski I, McEntee M, Travis AJ (2008) Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. Reproduction 136:823–831
Ko K, Tapia N, Wu G, Kim JB, Bravo MJ, Sasse P, Glaser T, Ruau D, Han DW, Greber B et al (2009) Induction of pluripotency in adult unipotent germline stem cells. Cell Stem Cell 5:87–96
Kokkinaki M, Djourabtchi A, Golestaneh N (2011) Long-term culture of human SSEA-4 positive spermatogonial stem cells (SSCs). J Stem Cell Res Ther 2
Koruji M, Shahverdi A, Janan A, Piryaei A, Lakpour MR, Gilani Sedighi MA (2012) Proliferation of small number of human spermatogonial stem cells obtained from azoospermic patients. J Assist Reprod Genet 29:957–967
Kossack N, Meneses J, Shefi S, Nguyen HN, Chavez S, Nicholas C, Gromoll J, Turek PJ, Reijo-Pera RA (2009) Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells. Stem Cells 27:138–149
Kossack N, Terwort N, Wistuba J, Ehmcke J, Schlatt S, Schöler H, Kliesch S, Gromoll J (2013) A combined approach facilitates the reliable detection of human spermatogonia in vitro. Hum Reprod 28:3012–3025
Kubota H, Avarbock MR, Brinster RL (2003) Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. Proc Natl Acad Sci U S A 100:6487–6492
Kubota H, Avarbock MR, Brinster RL (2004a) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71:722–731
Kubota H, Avarbock MR, Brinster RL (2004b) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 101:16489–16494
Kubota H, Avarbock MR, Schmidt JA, Brinster RL (2009) Spermatogonial stem cells derived from infertile Wv/Wv mice self-renew in vitro and generate progeny following transplantation. Biol Reprod 81:293–301
Kubota H, Brinster RL (2006) Technology insight: in vitro culture of spermatogonial stem cells and their potential therapeutic uses. Nat Clin Pract Endocrinol Metab 2:99–108
Kubota H, Brinster RL (2008) Culture of rodent spermatogonial stem cells, male germline stem cells of the postnatal animal. Methods Cell Biol 86:59–84
Kubota H, Reid LM (2000) Clonogenic hepatoblasts, common precursors for hepatocytic and biliary lineages, are lacking classical major histocompatibility complex class I antigen. Proc Natl Acad Sci U S A 97:12132–12137

Kubota H, Wu X, Goodyear SM, Avarbock MR, Brinster RL (2011) Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. FASEB J 25:2604–2614

Langenstroth D, Kossack N, Westernstroer B, Wistuba J, Behr R, Gromoll J, Schlatt S (2014) Separation of somatic and germ cells is required to establish primate spermatogonial cultures. Hum Reprod (Oxford, England) 29:2018–2031

Li L, Xie T (2005) Stem cell niche: structure and function. Annu Rev Cell Dev Biol 21:605–631

Liang P, Xu Y, Zhang X, Ding C, Huang R, Zhang Z, Lv J, Xie X, Chen Y, Li Y et al (2015) CRISPR/Cas9-mediated gene editing in human tripronuclear zygotes. Protein Cell 6:363–372

Lim JJ, Sung SY, Kim HJ, Song SH, Hong JY, Yoon TK, Kim JK, Kim KS, Lee DR (2010) Long-term proliferation and characterization of human spermatogonial stem cells obtained from obstructive and non-obstructive azoospermia under exogenous feeder-free culture conditions. Cell Prolif 43:405–417

Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260:1130–1132

Lo KC, Brugh VM 3rd, Parker M, Lamb DJ (2005) Isolation and enrichment of murine spermatogonial stem cells using rhodamine 123 mitochondrial dye. Biol Reprod 72:767–771

Matsui Y, Zsebo K, Hogan BL (1992) Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70:841–847

McCarrey JR (2013) Toward a more precise and informative nomenclature describing fetal and neonatal male germ cells in rodents. Biol Reprod 89:1–9

McKenna M, Bell S (1997) Classification of mammals: above the species level. Columbia University Press, New York

Medrano JV, Rombaut C, Simon C, Pellicer A, Goossens E (2016) Human spermatogonial stem cells display limited proliferation in vitro under mouse spermatogonial stem cell culture conditions. Fertil Steril 106:1539–1549

Meistrich ML, van Beek MEAB (1993) Spermatogonial stem cells. In: Desjardins C, Ewing LL (eds) Cell and molecular biology of the testis. Oxford University Press, New York, pp 266–295

Meng X, Lindahl M, Hyvonen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M et al (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287:1489–1493

Mikkola M, Sironen A, Kopp C, Taponen J, Sukura A, Vilkki J, Katila T, Andersson M (2006) Transplantation of normal boar testicular cells resulted in complete focal spermatogenesis in a boar affected by the immotile short-tail sperm defect. Reprod Domest Anim 41:124–128

Mizrak SC, Chikhovskaya JV, Sadri-Ardekani H, van Daalen S, Korver CM, Hovingh SE, Roepers-Gajadien HL, Raya A, Fluiter K, de Reijke TM et al (2010) Embryonic stem cell-like cells derived from adult human testis. Hum Reprod 25:158–167

Mruk DD, Cheng CY (2015) The mammalian blood-testis barrier: its biology and regulation. Endocr Rev 36:564–591

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

Nagano M, Avarbock MR, Brinster RL (1999) Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. Biol Reprod 60:1429–1436

Nagano M, Avarbock MR, Leonida EB, Brinster CJ, Brinster RL (1998) Culture of mouse spermatogonial stem cells. Tissue Cell 30:389–397

Nagano M, Brinster CJ, Orwig KE, Ryu BY, Avarbock MR, Brinster RL (2001a) Transgenic mice produced by retroviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A 98:13090–13095

Nagano M, McCarrey JR, Brinster RL (2001b) Primate spermatogonial stem cells colonize mouse testes. Biol Reprod 64:1409–1416

Nagano M, Patrizio P, Brinster RL (2002) Long-term survival of human spermatogonial stem cells in mouse testes. Fertil Steril 78:1225–1233

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
Nagano MC (2003) Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. Biol Reprod 69:701–707
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
Nayernia K, Nolte J, Michelmann HW, Lee JH, Rathsack K, Drusenheimer N, Dev A, Wulf G, Ehrmann IE, Elliott DJ et al (2006) In vitro-differentiated embryonic stem cells give rise to male gametes that can generate offspring mice. Dev Cell 11:125–132
Oakberg EF (1957) Duration of spermatogenesis in the mouse. Nature 180:1137–1138
Oakberg EF (1971) Spermatogonial stem-cell renewal in the mouse. Anat Rec 169:515–531
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 U S A 103:9524–9529
Oatley JM, Brinster RL (2012) The germline stem cell niche unit in mammalian testes. Physiol Rev 92:577–595
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
Oatley JM, Reeves JJ, McLean DJ (2004) Biological activity of cryopreserved bovine spermatogonial stem cells during in vitro culture. Biol Reprod 71:942–947
Oatley JM, Tibary A, de Avila DM, Wheaton JE, McLean DJ, Reeves JJ (2005) Changes in spermatogenesis and endocrine function in the ram testis due to irradiation and active immunization against luteinizing hormone-releasing hormone. J Anim Sci 83:604–612
Oatley MJ, Racicot KE, Oatley JM (2011) Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. Biol Reprod 84:639–645
Ogawa T, Arechaga JM, Avarbock MR, Brinster RL (1997) Transplantation of testis germinal cells into mouse seminiferous tubules. Int J Dev Biol 41:111–122
Ogawa T, Dobrinski I, Avarbock MR, Brinster RL (1999a) Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. Biol Reprod 60:515–521
Ogawa T, Dobrinski I, Avarbock MR, Brinster RL (2000) Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med 6:29–34
Ogawa T, Dobrinski I, Brinster RL (1999b) Recipient preparation is critical for spermatogonial transplantation in the rat. Tissue Cell 31:461–472
Ogawa T, Ohmura M, Yumura Y, Sawada H, Kubota Y (2003) Expansion of murine spermatogonial stem cells through serial transplantation. Biol Reprod 68:316–322
Ohbo K, Yoshida S, Ohmura M, Ohneda O, Ogawa T, Tsuchiya H, Kuwana T, Kehler J, Abe K, Scholer HR et al (2003) Identification and characterization of stem cells in prepubertal spermatogenesis in mice. Dev Biol 258:209–225
Ohmura M, Yoshida S, Ide Y, Nagamatsu G, Suda T, Ohbo K (2004) Spatial analysis of germ stem cell development in Oct-4/EGFP transgenic mice. Arch Histol Cytol 67:285–296
Ohta H, Wakayama T, Nishimune Y (2004) Commitment of fetal male germ cells to spermatogonial stem cells during mouse embryonic development. Biol Reprod 70:1286–1291
Osawa M, Hanada K, Hamada H, Nakauchi H (1996) Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 273:242–245
Paratcha G, Ledda F, Baars L, Coulpier M, Besset V, Anders J, Scott R, Ibanez CF (2001) Released GFRalpha1 potentiates downstream signaling, neuronal survival, and differentiation via a novel mechanism of recruitment of c-Ret to lipid rafts. Neuron 29:171–184
Potten CS, Morris RJ (1988) Epithelial stem cells in vivo. J Cell Sci Suppl 10(45–62):45–62
Radford J (2003) Restoration of fertility after treatment for cancer. Horm Res 59(Suppl 1):21–23
Resnick JL, Bixler LS, Cheng L, Donovan PJ (1992) Long-term proliferation of mouse primordial germ cells in culture. Nature 359:550–551
Rodriguez-Sosa JR, Silvertown JD, Foster RA, Medin JA, Hahnel A (2009) Transduction and transplantation of spermatogonia into the testis of ram lambs through the extra-testicular rete. Reprod Domest Anim 44:612–620

Russell LD, Brinster RL (1996) Ultrastructural observations of spermatogenesis following transplantation of rat testis cells into mouse seminiferous tubules. J Androl 17:615–627
Russell LD, Ettlin RA, Sinha Hikim AP, Clegg ED (1990) Histological and histopathological evaluation of the testis. Cache River Press, Clearwater, FL
Ryu BY, Kubota H, Avarbock MR, Brinster RL (2005) Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc Natl Acad Sci U S A 102:14302–14307
Ryu BY, Orwig KE, Avarbock MR, Brinster RL (2003) Stem cell and niche development in the postnatal rat testis. Dev Biol 263:253–263
Ryu BY, Orwig KE, Kubota H, Avarbock MR, Brinster RL (2004) Phenotypic and functional characteristics of spermatogonial stem cells in rats. Dev Biol 274:158–170
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
Sadri-Ardekani H, Akhondi MA, van der Veen F, Repping S, van Pelt AM (2011) In vitro propagation of human prepubertal spermatogonial stem cells. JAMA 305:2416–2418
Sadri-Ardekani H, Mizrak SC, van Daalen SK, Korver CM, Roepers-Gajadien HL, Koruji M, Hovingh S, de Reijke TM, de la Rosette JJ, van der Veen F et al (2009) Propagation of human spermatogonial stem cells in vitro. JAMA 302:2127–2134
Sato G, Zaroff L, Mills SE (1960) Tissue culture populations and their relation to the tissue of origin. Proc Natl Acad Sci U S A 46:963–972
Sato T, Sakuma T, Yokonishi T, Katagiri K, Kamimura S, Ogonuki N, Ogura A, Yamamoto T, Ogawa T (2015) Genome editing in mouse spermatogonial stem cell lines using TALEN and double-nicking CRISPR/Cas9. Stem Cell Rep 5:75–82
Schlatt S, Rosiepen G, Weinbauer GF, Rolf C, Brook PF, Nieschlag E (1999) Germ cell transfer into rat, bovine, monkey and human testes. Hum Reprod 14:144–150
Schmidt JA, Abramowitz LK, Kubota H, Wu X, Niu Z, Avarbock MR, Tobias JW, Bartolomei MS, Brinster RL (2011) In vivo and in vitro aging is detrimental to mouse spermatogonial stem cell function. Biol Reprod 84:698–706
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
Schmitt RM, Bruyns E, Snodgrass HR (1991) Hematopoietic development of embryonic stem cells in vitro: cytokine and receptor gene expression. Genes Dev 5:728–740
Seandel M, James D, Shmelkov SV, Falciatori I, Kim J, Chavala S, Scherr DS, Zhang F, Torres R, Gale NW et al (2007) Generation of functional multipotent adult stem cells from GPR125+ germline progenitors. Nature 449:346–350
Shen Q, Goderie SK, Jin L, Karanth N, Sun Y, Abramova N, Vincent P, Pumiglia K, Temple S (2004) Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science 304:1338–1340
Shinohara T, Avarbock MR, Brinster RL (1999) beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 96:5504–5509
Shinohara T, Avarbock MR, Brinster RL (2000a) Functional analysis of spermatogonial stem cells in Steel and cryptorchid infertile mouse models. Dev Biol 220:401–411
Shinohara T, Kato M, Takehashi M, Lee J, Chuma S, Nakatsuji N, Kanatsu-Shinohara M, Hirabayashi M (2006) Rats produced by interspecies spermatogonial transplantation in mice and in vitro microinsemination. Proc Natl Acad Sci U S A 103:13624–13628
Shinohara T, Orwig KE, Avarbock MR, Brinster RL (2000b) Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci U S A 97:8346–8351
Shinohara T, Orwig KE, Avarbock MR, Brinster RL (2001) Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc Natl Acad Sci U S A 98:6186–6191
Shinohara T, Orwig KE, Avarbock MR, Brinster RL (2003) Restoration of spermatogenesis in infertile mice by Sertoli cell transplantation. Biol Reprod 68:1064–1071
Shinomura M, Kishi K, Tomita A, Kawasumi M, Kanezashi H, Kuroda Y, Tsunekawa N, Ozawa A, Aiyama Y, Yoneda A et al (2014) A novel Amh-Treck transgenic mouse line allows toxin-dependent loss of supporting cells in gonads. Reproduction 148:H1–H9

Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336:688–690

Spangrude GJ, Heimfeld S, Weissman IL (1988) Purification and characterization of mouse hematopoietic stem cells. Science 241:58–62

Spradling A, Drummond-Barbosa D, Kai T (2001) Stem cells find their niche. Nature 414:98–104

Takashima S, Kanatsu-Shinohara M, Tanaka T, Morimoto H, Inoue K, Ogonuki N, Jijiwa M, Takahashi M, Ogura A, Shinohara T (2015) Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. Stem Cell Rep 4:489–502

Tan W, Carlson DF, Lancto CA, Garbe JR, Webster DA, Hackett PB, Fahrenkrug SC (2013) Efficient nonmeiotic allele introgression in livestock using custom endonucleases. Proc Natl Acad Sci U S A 110:16526–16531

Tegelenbosch RA, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 290:193–200

Toyooka Y, Tsunekawa N, Akasu R, Noce T (2003) Embryonic stem cells can form germ cells in vitro. Proc Natl Acad Sci U S A 100:11457–11462

Valli H, Sukhwani M, Dovey SL, Peters KA, Donohue J, Castro CA, Chu T, Marshall GR, Orwig KE (2014) Fluorescence- and magnetic-activated cell sorting strategies to isolate and enrich human spermatogonial stem cells. Fertil Steril 102:566–580.e567

Viglietto G, Dolci S, Bruni P, Baldassarre G, Chiariotti L, Melillo RM, Salvatore G, Chiappetta G, Sferratore F, Fusco A et al (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

Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM (1988) Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature 336:684–687

Wu X, Goodyear SM, Abramowitz LK, Bartolomei MS, Tobias JW, Avarbock MR, Brinster RL (2012) Fertile offspring derived from mouse spermatogonial stem cells cryopreserved for more than 14 years. Hum Reprod 27:1249–1259

Wu X, Schmidt JA, Avarbock MR, Tobias JW, Carlson CA, Kolon TF, Ginsberg JP, Brinster RL (2009b) Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. Proc Natl Acad Sci U S A 106:21672–21677

Wu Y, Zhou H, Fan X, Zhang Y, Zhang M, Wang Y, Xie Z, Bai M, Yin Q, Liang D et al (2015) Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. Cell Res 25:67–79

Wu Z, Falciatori I, Molyneux LA, Richardson TE, Chapman KM, Hamra FK (2009a) Spermatogonial culture medium: an effective and efficient nutrient mixture for culturing rat spermatogonial stem cells. Biol Reprod 81:77–86

Yomogida K, Yagura Y, Tadokoro Y, Nishimune Y (2003) Dramatic expansion of germinal stem cells by ectopically expressed human glial cell line-derived neurotrophic factor in mouse Sertoli cells. Biol Reprod 69:1303–1307

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

Yoshinaga K, Nishikawa S, Ogawa M, Hayashi S, Kunisada T, Fujimoto T (1991) Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function. Development 113:689–699

Zhang X, Ebata KT, Nagano MC (2003) Genetic analysis of the clonal origin of regenerating mouse spermatogenesis following transplantation. Biol Reprod 69:1872–1878

Zheng K, Wu X, Kaestner KH, Wang PJ (2009) The pluripotency factor LIN28 marks undifferentiated spermatogonia in mouse. BMC Dev Biol 9:38

Zheng Y, Thomas A, Schmidt CM, Dann CT (2014) Quantitative detection of human spermatogonia for optimization of spermatogonial stem cell culture. Hum Reprod 29:2497–2511

In Vitro Differentiation of Spermatogonia

12

Takehiko Ogawa

Abstract

Presence of functional spermatogonial population secures continuing spermatogenesis, by repeated commencement of spermatogenic cycles from them. In vitro manipulation of spermatogonia, thus, can be divided into two aspects: proliferation of spermatogonia for their maintenance and initiation of their differentiation toward sperm formation. In this chapter, experiments on in vitro differentiation of spermatogonia using an organ culture technique are described. Principle of the method and results, using testis tissues of pup and adult mice as well as an infertile mutant mouse, are introduced. Spermatogenesis from isolated or cultured spermatogonia is also described. Finally, future challenges and prospects are stated.

Keywords

Organ culture method • In vitro • Spermatogenesis • Spermatogonia

12.1 Introduction

Spermatogenesis is an extremely complicated process of cellular differentiation. The details of cellular and molecular mechanism underlying the process remain to be elucidated. In order to study such mechanism, an in vitro system which can recapitulate the whole process is desirable or even mandatory. Such in vitro system should be also useful for studying the pathogenic mechanism of spermatogenic failure of infertile patients and could be applied as therapeutic modalities.

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In order to make sperm in vitro from spermatogonia, or particularly spermatogonial stem cells, several methods could be possible. Broadly, they are classified into four strategies; organ culture, cell culture, 3-D culture, and direct induction with transfection. Among these, the organ culture method alone successfully recapitulates complete spermatogenesis at present. In this chapter, organ culture experiments for spermatogenesis along with other in vitro methods are discussed.

12.2 Organ Culture

12.2.1 Gas–Liquid Interphase Method

Spermatogenesis cannot be completed by spermatogenic germ cells alone. It needs various supports from the surrounding somatic cells, especially Sertoli cells, peritubular myoid cells, and interstitial Leydig cells. Thus, it would be practical to culture the testis tissue as a whole rather than culturing germ cells separately. In fact, the history of culture experiments started with organ culture. The study of in vitro spermatogenesis which started in the early twentieth century also used an organ culture method, principle of which is called the gas–liquid interphase method. In the 1960s, Anne and Emil Steinberger extensively worked on in vitro spermatogenesis and obtained many important findings. They actually succeeded in inducing meiosis from primitive spermatogonia up to pachytene stage using neonatal rat testis tissues (Steinberger et al. 1964). However, even with this method, the in vitro spermatogenesis did not advance beyond their achievement. As a result, pachytene stage was considered a barrier for in vitro spermatogenesis when using the organ culture method. However, when considering the in vivo conditions where spermatogenic germ cells are located, it appears that the architecture of the seminiferous tubule may have an important influence on spermatogenesis. Firstly, Sertoli cells form the so-called blood–testis barrier (BTB) by tight junction between each other which separates seminiferous epithelial space into two compartments; basal and adluminal. The spermatogonia reside in the basal compartment, while spermatocytes undergoing meiosis move up to adluminal compartment. Sertoli cells, in addition, are adaptable and flexible in shape holding each germ cell by extending their cytoplasmic projections. These features of Sertoli cells can make microenvironments specific to each stage of spermatogenic cells. In particular, the two compartments, basal and adluminal, should be sharply different in condition; the former supports mitosis of spermatogonia, while the latter supports meiosis of spermatocytes. Secondly, germ cells maintain intercellular cytoplasmic bridges after cell divisions which function to synchronize the cell kinetics of the comradery of cells. This syncytia formation is vital for them as its disruption ends up in spermatogenic failure (Greenbaum et al. 2011). These facts emphasize the importance of architecture of the seminiferous tubules for proper spermatogenesis, which supports the strategy using an organ culture method for in vitro spermatogenesis rather than cell culture methods. Figure 12.1 shows our organ culture method using agarose gel block as a stand to which testis tissue pieces are placed.

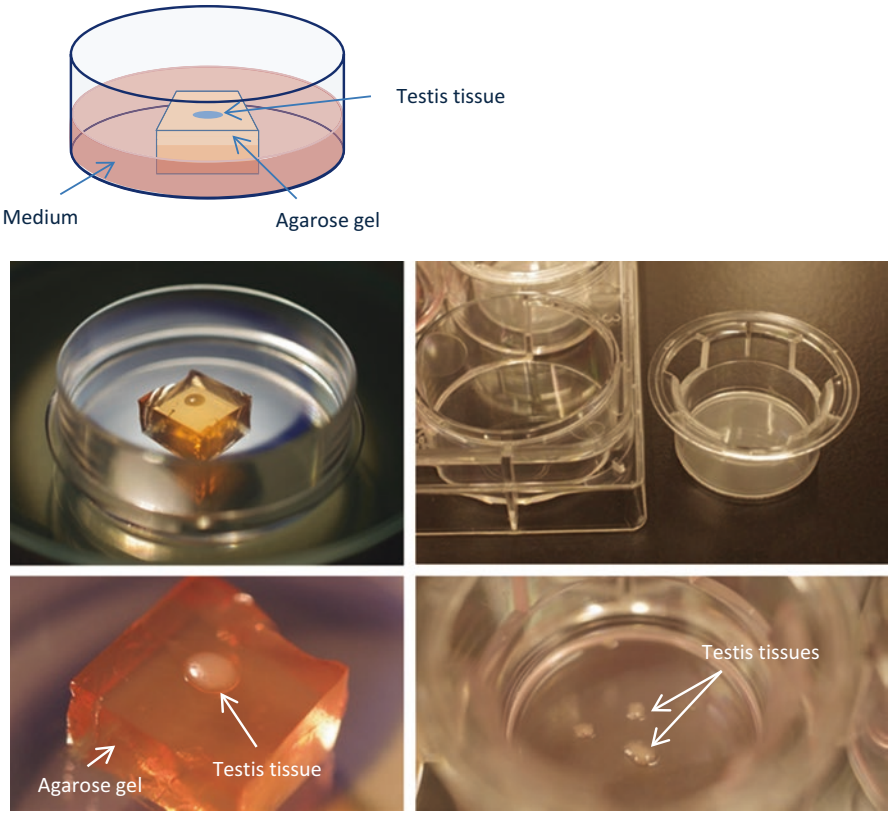

Fig. 12.1 A scheme of organ culture method using agarose gel as the platform of the testis tissue. Photos show testis tissues spreading on the agarose gel. The well-insert is also available for the organ culture

An important technical point for the study of in vitro spermatogenesis is how to monitor the progression of spermatogenesis. The traditional approach depended on histological evaluations. This is reasonable and most reliable because spermatogenesis accompanies dynamic morphological changes of germ cells themselves. Nonetheless, easier and more objective methods have been developed by introduced transgenic mice whose germ cells express GFP at particular stage of spermatogenesis; *Gsg2*-GFP and *Acr*-GFP transgenic mouse lines (Sato et al. 2011a). In the *Gsg2*-GFP mouse, the germ cells express GFP in the cytoplasm beginning at the end-meiotic stage of maturation. In the *Acr*-GFP mouse, GFP is expressed in the mid-meiotic stage of spermatocyte maturation and GFP accumulates into the acrosome structure when the cell becomes spermatids. The GFP-positive acrosome changes its shape from original dot to cap-like as the spermatid proceeds from round to elongating forms. These morphological changes bestow additional advantages to monitor the progression of spermatogenesis by observing the shape of a GFP-positive acrosome. Using these transgenic mice, it becomes possible to monitor the

progression of spermatogenesis by observing the culture tissue under a stereomicroscope equipped for GFP-excitation light throughout the culture experiment.

In many culture experiments, fetal bovine serum (FBS) is an effective supplement in the culture medium and in many cases required for cell survival and growth. However, FBS has proved to not be effective for induction of mouse spermatogenesis in vitro (Sato et al. 2011a). Instead, a serum replacement, Knockout Serum Replacement (KSR) or a purified albumin product, AlbuMAX, when added in basal medium, are effective for the induction of spermatogenesis. When FBS was supplemented to cultures of mouse testicular tissue, spermatogenesis arrested at the pachytene stage of meiosis. On the other hand, with KSR or AlbuMAX, complete spermatogenesis to sperm formation was supported (Sato et al. 2011a; Yokonishi et al. 2013a). AlbuMAX is a purified but lipid-rich albumin derived from bovine serum by column chromatography. KSR is reported to contain AlbuMAX at 83 mg/mL. Thus, it is likely that the effective components for spermatogenic induction in KSR is AlbuMAX (Price et al. 1998).

The time frame for progression of spermatogenesis in cultured testicular tissue almost parallels the in vivo situation. For instance, *Acr*-GFP expression appears around 15 dpp in mouse pups which is also the case or 1–2 days delay in the organ culture experiments (Sato et al. 2011a; Kojima et al. 2016). In the case of *Gsg2*-GFP mouse, the GFP appears around 20 dpp in meiotic phase germ cells in vivo, which also occurs in cultured testis tissues. Interestingly, the most effective temperature for in vitro mouse spermatogenesis is 34 °C. The standard 37 °C temperature used for many cells lines does not support spermatogenesis and although culture at 32 °C is permissive, the spermatogenic process appears to be delayed (Gohbara et al. 2010).

Because the organ culture method maintains the tissue unit as a whole, namely seminiferous tubules and interstitial components together, authentic maintenance of the microenvironments of the in vivo situation are maintained. There are, however, several critical differences. For example, sperm produced in testis tissue in vivo transit into the epididymis where final maturation for fertilization competence occurs; however, this has yet to be replicated in culture systems. The cultured seminiferous tubules do not seem to generate a flow of fluid, thus expelling of sperm is impaired. Lack of an outlet for sperm could be a serious defect in the organ culture method for normal and complete spermatogenesis to occur. Indeed, finding elongated spermatids are produced in limited cases using currently developed organ culture methodologies (Nakamura et al. 2017). However, the haploid cells including round and elongating spermatids that are produced have been shown to be viable for generation of normal offspring using micro-insemination procedures.

12.2.2 Adult Testis Tissue Culture

Previous studies have demonstrated that complete spermatogenesis originating from spermatogonial stem cells (SSCs) occurs in cultured testis tissue from neonatal or pup mice. When it comes to adult mouse testis tissue, however, in vitro spermatogenesis is less efficient. In contrast to neonatal and pup testis tissue, seminiferous

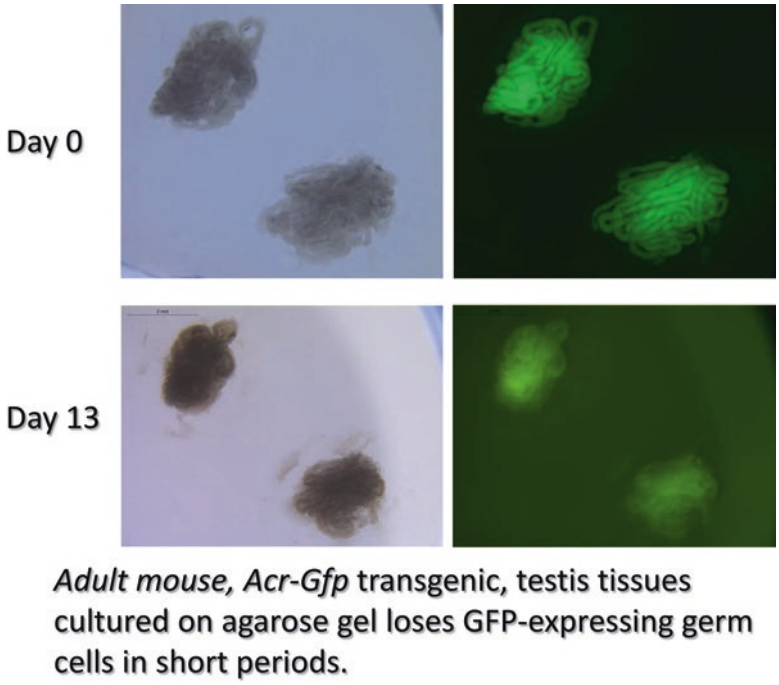

Fig. 12.2 Adult testis culture is difficult. A piece of testis tissue taken from an adult Acr-Gfp transgenic mouse was cultured on agarose gel. The GFP expression dramatically decreased in a week and almost disappeared in 2 weeks. The tissue architecture also destructed

tubules of adult testes contain all stages of male germ cells from SSCs to sperm. In addition, the size of seminiferous tubules in adults is much larger at about 200 μm in diameter compared to tubules in neonatal and pup testes. They express full extent of GFP in case of *Acr*-GFP Tg mouse of adults. When this GFP-expressing testis was cultured, the GFP disappear very rapidly because the germ cells in the seminiferous tubules do not survive (Sato et al. 2015a). These findings suggest that the in vitro conditions that support testis tissue from neonates and pups cannot support the continuation of spermatogenesis which is proceeding at very high efficiency in adult tissue. The high level of spermatogenesis occurring in adult tissue demands high nutrition and oxygen supply from the capillaries in the body, which is not met in the culture condition. Unfortunately, GFP expression disappears almost completely by 2 weeks of culture for adult tissue, demonstrating loss of all advanced germ cells (Fig. 12.2). However, in some cases GFP expression is observable in small foci of tissue for extended periods of time in which complete spermatogenesis persists. In fact, when testis tissue from adult animals treated with vitamin A-deficient diet that contain undifferentiated sperm, atogonia only were cultured, GFP expression appeared gradually over time signifying maturation of germ cells. Thus, in vitro spermatogenesis with adult testis tissue is certainly possible (Sato et al. 2015a), but the efficiency of spermatogenesis that has been achieved with neonatal and pup tissue has not been achieved.

12.2.3 Organ Culture of *Sl/Sl*^{d} Mouse Testis

The role of Sertoli cells on spermatogenesis cannot be over emphasized. Sertoli and germ cells have intimate cell–cell contact and form attachment complexes that are critical for spermatogenesis. At present, the molecules functioning at cell surface between Sertoli and germ cells are undefined. One such prime example is c-Kit on germ cells and its ligand KitL on Sertoli cells. Sertoli cells produce two forms of KitL; membrane-bound and secreted. The membrane-bound KitL contains transmembrane sequence by which it is anchored on the surface of Sertoli cells. The extracellular domain of the KitL can bind to its receptor c-Kit on differentiating spermatogonia. The KitL signal to the differentiating spermatogonia is necessary for their proliferation and differentiation (Vincent et al. 1998; Ohta et al. 2000). On the other hand, the secreted form of KitL is a truncated, being devoid of transmembrane sequence. This secreted KitL can also bind to c-Kit and transmit a signal but is not sufficient for maintaining the differentiating spermatogonia. Several lines of mutant mice possessing genetic mutations of the gene of KitL, named Steel mice, have been derived. Inactivation of the KitL gene in a homozygous manner leads to embryonic lethal. However, a mutant line named Steel dicke (*Sl*^{d}) cannot produce membrane-bound type KitL but can produce the secreted form. The *Sl/Sl*^{d} mouse is viable but their testis contains only a few primitive spermatogonia and spermatogenesis is ablated. Although the *Sl/Sl*^{d} mouse is sterile, the exact cause of ablated spermatogenesis is clear. Therefore, several strategies have been proposed to treat the infertility of this mouse since 2000, which include germ cell transplantation, Sertoli cell transplantation, and gene-therapy with viral vectors (Ogawa et al. 2000; Ikawa et al. 2002; Kanatsu-Shinohara et al. 2002, 2005). However, these modalities proved to be invasive to the infertile mouse and not efficient in regenerating spermatogenesis. To address this, a testis organ culture method was devised as a new therapeutic scheme by simply supplementing KitL in the culture medium (Sato et al. 2012). Although this scheme does not seem logical because Sertoli cells in the *Sl/Sl*^{d} mouse produce the secreted form of KitL even though they lack spermatogenesis. Nonetheless, the addition of recombinant KitL to culture medium led to the induction of spermatogenesis in the cultured testis tissue from *Sl/Sl*^{d} mice (Sato et al. 2012). Importantly, germ cell maturation beyond the meiotic phase up to haploid cell formation was supported and micro-insemination with the round spermatids succeeded in producing offspring that harbored the KitL mutation. This example demonstrated a possible utility of organ culture method for treating spermatogenic impairment by supplementing factors lacking in the tissue.

12.2.4 Spermatogenesis from Isolated Spermatogonia

The organ culture method can produce sperm from spermatogonia which innately reside in the cultured tissue. On the other hand, primary cultures of spermatogonia from mice can be maintained for long periods of time. With recent advancement in genome editing technologies, such as CRISPR/Cas9, modifying the genome of

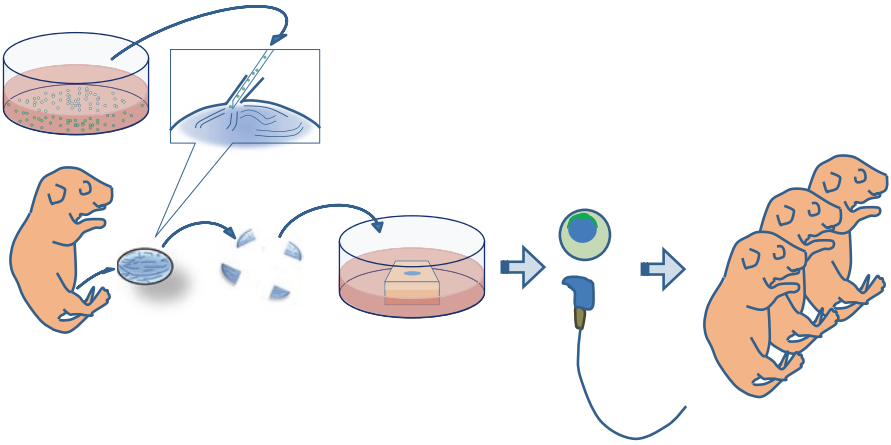

Fig. 12.3 In vitro germ cell transplantation. Cultured spermatogonia injected into the seminiferous tubules of a testis taken out from a pup mouse. The spermatogonia colonized in the tubules and formed spermatogenic colonies under culture condition. Produced haploid cells could be used for microinsemination to generate offspring

germ cells has become more efficient (Sato et al. 2015b). The ability to produce sperm from primary cultures of spermatogonia would broaden technological potential of the germline cell lineage and the organ culture method has such potential. Indeed, recent studies have begun to demonstrate feasibility of this approach. Injection of cultured spermatogonia into seminiferous tubules of pup testes in an organ culture format led to regeneration of spermatogenesis (Sato et al. 2011b) (Fig. 12.3). Interestingly, the injected cultured spermatogonia initially floated in the organ cultured seminiferous tubules but then migrate toward periphery and settled at the basement membrane beside Sertoli cells where they proliferated and then generated colonies of spermatogenesis, similar to what occurs with spermatogonial transplantation in vivo (Nagano et al. 1999). Within the regenerated colonies sperm were produced, although the efficiency was low and offspring were generated by microinsemination of haploid cells (Sato et al. 2011b, 2013). At present, use of the organ culture platform is the only method for production of sperm in vitro from isolated spermatogonia.

12.2.5 In Vitro Reconstruction of Testis Tissue

Previous studies have demonstrated that after disassociation fetal or neonatal testes can reorganize the histological structure at various places in a body such as the subrenal or subdermal spaces (Kita et al. 2007; Honaramooz et al. 2007; Matoba and Ogura 2011). In these reconstituted testis tissues, spermatogenesis is able to proceeds to sperm formation. This reconstitution of testis tissue architecture has also been observed in vitro and spermatogenesis can proceed to a certain extent (Yokonishi et al. 2013b). Single cell suspensions from disassociated mouse pup

testes can re-aggregate when cultured in a floating small well for 2 days and be subsequently moved to an agarose gel for "organ culture." Within a 2 week period, Sertoli cells in the aggregate attach together to form tubular structures and spermatogonia become incorporated. The spermatogonia left outside the tubule seemed to disappear relatively soon. On the contrary, between the tubules, Leydig cells were observed along with undetermined types of cells. The spermatogonia in the tubule can multiply and differentiate to commence spermatogenesis. However, the efficiency is not high and sperm formation has not been observed. Using this phenomenon, we mixed cultured spermatogonia cells with dissociated pup testis cells to reconstitute chimeric testis tissue (Fig. 12.4). When the cultured spermatogonia cells were incorporated in the newly formed tubules, they executed spermatogenesis up to the mid-meiotic phase.

This so-called in vitro reconstruction of testis tissue method is not efficient in inducing spermatogenesis and rarely results in haploid cell formation. Nonetheless, there is advantage to using isolated testis cells versus testis tissue. Using testis tissue always needs animals, mice or whatever. When it comes to human, in particular, it is not practically possible to use testis tissue as experimental material. In this regard, it is really desirable if the testis tissue were produced from cells available with ease. Thus, making testis tissue from cells, iPS for instance, is an attractive challenge, which leads to the establishment of new in vitro system for spermatogenesis.

12.3 Cultured Spermatogonia and Beyond

In this chapter, I have described the recent progress of research on in vitro spermatogenesis using the organ culture method which, at present, is the most efficient and reliable for inducing spermatogenesis and producing sperm. However, over the past few years several reports have emerged about limited success with inducing spermatogenesis from cultured spermatogonia only and three-dimensional culture platforms. Now, it is possible to use primary cultures of spermatogonia as starting material for in vitro spermatogenesis. Up until now, to our best knowledge, there are no reports to induce meiosis and produce haploid cells from cultured spermatogonia only. Indeed, when aggregated with Sertoli cells, cultured spermatogonia do not progress into meiosis (Kanatsu-Shinohara et al. 2012). Retinoic acid is a pivotal factor for germ cells in inducing spermatogonia into meiosis. However, simply adding RA in the culture medium does not seem to be sufficient for induction of meiosis in cultured spermatogonia (Travers et al. 2013).

Beyond mammals, research with several fish species has demonstrated that induction of complete spermatogenesis is possible even when using standard cell culture conditions. Use of Sertoli cell lines as feeders appears to be sufficient for supporting germ cell maturation to sperm in minimal culture conditions (Kurita and Sakai 2004). Interestingly, different feeder cell lines appear to have different capacities to support germ cell activities. While one line supports proliferation of spermatogonia, another line induced differentiation. This findings might suggest that two basic role of the Sertoli cell, one for self-renewing proliferation of

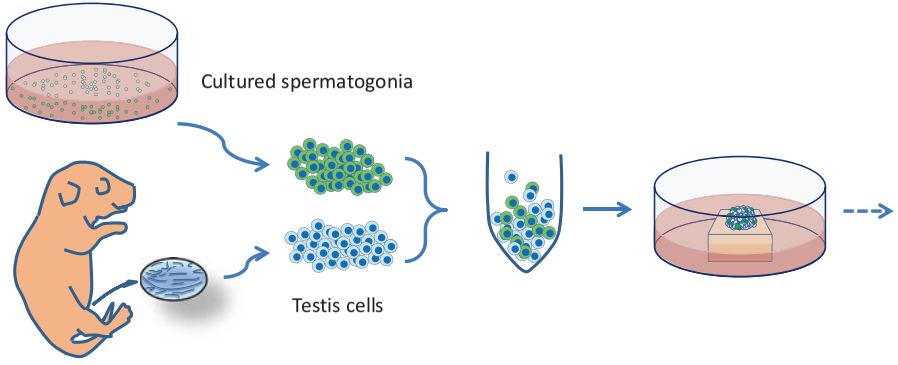

Fig. 12.4 In vitro reconstruction. Cultured spermatogonia were mixed with testicular somatic cells for making chimeric aggregation. The aggregation reconstructs itself into the tubular architecture and could support spermatogenesis in them

spermatogonial stem cells and the other for promoting their differentiation to sperm can be separated in each cell line of Sertoli cell. In fact, two functions were performed in a different location in the seminiferous tubule; basal- and adluminal-compartments, respectively. In the regular culture condition, such spatical distinction, particularly when it comes to vertical segregation, would be really difficult to produce. Therefore, production of these zebrafish Sertoli cell-lines was a unique example and suggests that facets of Sertoli cell function when properly modulated in culture conditions would suffice to support differentiation of germ cells up to sperm formation. It is not, however, clear if this would hold true for mammalian spermatogenesis.

12.4 Challenge to Produce Sperm from Spermatogonia by Reprograming Method

Several studies have reported the ability to make sperm from spermatogonia by transfecting genes to immortalize them in culture (Hofmann et al. 1994; Feng et al. 2002). Such an idea that spermatogenic cells might have spontaneous tendency to proceed along spermatogenesis when placed under simple undisturbed conditions seems unrealistic in light of recent development. Spermatogenic cells certainly need, delicate environmental to support their differentiation into meiosis and spermiogenesis which is at present seems to only be provided by intact seminiferous tubules along with contributions from the interstitial compartment. However, we are now in age of being able to produce different types of cells from cells of distinct origin by introducing a set of transcription factors. This idea could be applicable to making sperm from spermatogonia or even from cells of other sources in a culture dish. In fact, cells with primordial germ cell like properties have been engineered from ES/iPS cells not only by tuning culture conditions (Hayashi et al. 2011) but also introducing specific transcription factors (Nakaki et al. 2013). With the availability of sophisticated tools for manipulating the extrinsic and intrinsic environments of cells, it will be interesting to see what the future holds in deriving complete spermatogenesis in vitro and the impacts this will have on treating various causes of male infertility.

References

Feng LX, Chen Y, Dettin L, Pera RA, Herr JC, Goldberg E, Dym M (2002) Generation and in vitro differentiation of a spermatogonial cell line. Science 297(5580):392–395

Gohbara A, Katagiri K, Sato T, Kubota Y, Kagechika H, Araki Y, Araki Y, Ogawa T (2010) In vitro murine spermatogenesis in an organ culture system. Biol Reprod 83(2):261–267. https://doi.org/10.1095/biolreprod.110.083899.

Greenbaum MP, Iwamori T, Buchold GM, Matzuk MM (2011) Germ cell intercellular bridges. Cold Spring Harb Perspect Biol 3(8):a005850. https://doi.org/10.1101/cshperspect.a005850

Hayashi K, Ohta H, Kurimoto K, Aramaki S, Saitou M (2011) Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell 146(4):519–532

Hofmann MC, Hess RA, Goldberg E, Millán JL (1994) Immortalized germ cells undergo meiosis in vitro. Proc Natl Acad Sci U S A 91(12):5533–5537

Honaramooz A, Megee SO, Rathi R, Dobrinski I (2007) Building a testis: formation of functional testis tissue after transplantation of isolated porcine (Sus scrofa) testis cells. Biol Reprod 76(1):43–47

Ikawa M et al (2002) Restoration of spermatogenesis by lentiviral gene transfer: offspring from infertile mice. Proc Natl Acad Sci U S A 99:7524–7529

Kanatsu-Shinohara M et al (2002) Adenovirus-mediated gene delivery and in vitro microinsemination produce offspring from infertile male mice. Proc Natl Acad Sci U S A 99:1383–1388

Kanatsu-Shinohara M et al (2005) Germline niche transplantation restores fertility in infertile mice. Hum Reprod 20:2376–2382

Kanatsu-Shinohara M, Inoue K, Takashima S, Takehashi M, Ogonuki N, Morimoto H, Nagasawa T, Ogura A, Shinohara T (2012) Reconstitution of mouse spermatogonial stem cell niches in culture. Cell Stem Cell 11(4):567–578

Kita K, Watanabe T, Ohsaka K, Hayashi H, Kubota Y, Nagashima Y, Aoki I, Taniguchi H, Noce T, Inoue K, Miki H, Ogonuki N, Tanaka H, Ogura A, Ogawa T (2007) Production of functional spermatids from mouse germline stem cells in ectopically reconstituted seminiferous tubules. Biol Reprod 76(2):211–217

Kojima K, Sato T, Naruse Y, Ogawa T (2016) Spermatogenesis in explanted fetal mouse testis tissues. Biol Reprod 95(3):63

Kurita K, Sakai N (2004) Functionally distinctive testicular cell lines of zebrafish to support male germ cell development. Mol Reprod Dev 67(4):430–438

Matoba S, Ogura A (2011) Generation of functional oocytes and spermatids from fetal primordial germ cells after ectopic transplantation in adult mice. Biol Reprod 84(4):631–638

Nagano M, Avarbock MR, Brinster RL (1999) Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. Biol Reprod 60(6):1429–1436

Nakaki F, Hayashi K, Ohta H, Kurimoto K, Yabuta Y, Saitou M (2013) Induction of mouse germ-cell fate by transcription factors in vitro. Nature 501(7466):222–226

Nakamura N, Merry GE, Inselman AL, Sloper DT, Del Valle PL, Sato T, Ogawa T, Hansen DK (2017) Evaluation of culture time and media in an in vitro testis organ culture system. Birth Defects Res B Dev Reprod Toxicol

Ogawa T, Dobrinski I, Avarbock MR, Brinster RL (2000) Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med 6:29–34

Ohta H, Yomogida K, Dohmae K, Nishimune Y (2000) Regulation of proliferation and differentiation in spermatogonial stem cells: the role of c-kit and its ligand SCF. Development 127:2125–2131

Price PJ, Goldsborough MD, Tilkins ML (1998) Embryonic stem cell serum replacement. Patent: WO 1998030679 A1. http://www.google.com/patents/WO1998030679A1?cl=en

Sato T et al (2011a) In vitro production of functional sperm in cultured neonatal mouse testes. Nature 471:504–507

Sato T, Katagiri K, Yokonishi T, Kubota Y, Inoue K, Ogonuki N, Matoba S, Ogura A, Ogawa T (2011b) In vitro production of fertile sperm from murine spermatogonial stem cell lines. Nat Commun 2:472. https://doi.org/10.1038/ncomms1478

Sato T, Yokonishi T, Komeya M, Katagiri K, Kubota Y, Matoba S, Ogonuki N, Ogura A, Yoshida S, Ogawa T (2012) Testis tissue explantation cures spermatogenic failure in c-Kit ligand mutant mice. Proc Natl Acad Sci U S A 109(42):16934–16938

Sato T, Katagiri K, Kubota Y, Ogawa T (2013) In vitro sperm production from mouse spermatogonial stem cell lines using an organ culture method. Nat Protoc 8(11):2098–2104. https://doi.org/10.1038/nprot.2013.138

Sato T, Katagiri K, Kojima K, Komeya M, Yao M, Ogawa T (2015a) In vitro spermatogenesis in explanted adult mouse testis tissues. PLoS One 10(6):e0130171. https://doi.org/10.1371/journal.pone.0130171

Sato T, Sakuma T, Yokonishi T, Katagiri K, Kamimura S, Ogonuki N, Ogura A, Yamamoto T, Ogawa T (2015b) Genome editing in mouse spermatogonial stem cell lines using TALEN and double-nicking CRISPR/Cas9. Stem Cell Rep 5(1):75–82

Steinberger A et al (1964) Mammalian testes in organ culture. Exp Cell Res 36:19–27
Travers A, Arkoun B, Safsaf A, Milazzo JP, Absyte A, Bironneau A, Perdrix A, Sibert L, Macé B, Cauliez B, Rives N (2013) Effects of vitamin A on in vitro maturation of pre-pubertal mouse spermatogonial stem cells. PLoS One 8(12):e82819
Vincent S et al (1998) Stage-specific expression of the Kit receptor and its ligand (KL) during male gametogenesis in the mouse: a Kit-KL interaction critical for meiosis. Development 125:4585–4593
Yokonishi T, Sato T, Katagiri K, Ogawa T (2013a) In vitro spermatogenesis using an organ culture technique. Methods Mol Biol 927:479–488
Yokonishi T, Sato T, Katagiri K, Komeya M, Kubota Y, Ogawa T (2013b) In vitro reconstruction of mouse seminiferous tubules supporting germ cell differentiation. Biol Reprod 89(1):15, 1–6

Part VII

Therapeutic Potentials and Applications of Spermatogonia

Fertility Preservation in Cancer Patients

13

Sherin David and Kyle E. Orwig

Abstract

Chemotherapy and radiation treatments for cancer or other conditions can cause permanent infertility. This condition not only affects the ability to bear children after cure, but may also have a lasting impact on psychosocial well-being, relationships and overall health. Adolescent and adult patients may have the options to preserve eggs, sperm, or embryos prior to treatment to preserve their future fertility. These options are not available to prepubertal patients who are not producing mature eggs or sperm. This is a critical human health concern because most children will survive their cancer and still have their entire reproductive life in front of them. This review focuses on stem cell-based methods that may provide new fertility-sparing options for boys receiving gonadotoxic therapies for cancer or other conditions.

Keywords

Spermatogonial stem cells • Fertility preservation • Testis • Testis tissue cryopreservation • SSC transplantation • SSC culture • Organ culture • Xenografting • De novo testicular morphogenesis • In vitro derivation of germ cells

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13.1 Introduction

Radiation and chemotherapy have been shown to cause temporary or permanent fertility loss in cancer survivors (Wallace et al. 2005; Meistrich 2009). In a recent study, germ cell dysfunction was observed in 66.4% of adults previously treated for pediatric cancer (Hudson et al. 2013). Given the survival rate of individuals diagnosed with cancer between the ages 0–19 has risen to about 87% in recent decades, it has become increasingly important to address factors that affect patient quality of life after cure (Ward et al. 2014). A number of surveys have indicated that psychosocial distress due to iatrogenic loss of fertility is common among cancer survivors (Hammond et al. 2007; Wenzel et al. 2005; Zebrack et al. 2004). However, many patients undergo gonadotoxic treatments without receiving information about their risk for infertility or about the fertility preservation options that may be available to them (Osterberg et al. 2014; Quinn et al. 2009). To improve the number of patients that can benefit from fertility preservation methods, the American Society of Clinical Oncology and the American Society for Reproductive Medicine have recommended counseling all patients on the reproductive risks and available fertility preservation options, ideally before the initiation of gonadotoxic therapies (Lee et al. 2006; Loren et al. 2013; Ethics Committee of the American Society for Reproductive Medicine 2005).

The extent of chemotherapy-associated azoospermia depends on the type and dosage of the chemotherapeutic agent used. Alkylating agents such as Busulfan, cyclophosphamide, chlorambucil, melphalan, and procarbazine have been associated with significant risk of azoospermia (reviewed in Meistrich (2009)). The degree of azoospermia resulting from radiation therapy is determined by factors such as total dose, number of fractions, and duration of exposure to radiation. Doses greater than 2 Gy have been reported to cause permanent infertility (Shalet 1993). Certain forms of cancer, including testicular cancer and Hodgkin's disease have been associated with an increased risk for gonadal dysfunction even before the onset of treatment (Petersen et al. 1999; Chapman et al. 1981; Vigersky et al. 1982). Therefore, the extent and permanence of azoospermia depends on a combination of several factors, including the disease itself, the stress resulting from the disease and the therapeutic regimen used to treat the disease (reviewed in Agarwal et al. (2014)). Although about 85% of cancer patients recover normal levels of spermatogenesis within 5 years post treatment, the algorithms for predicting which patients will recover fertility are imperfect due to the biological heterogeneity among human subjects and the constantly evolving treatment regimens (Achille et al. 2006; Howell and Shalet 2005).

For adult males, sperm cryopreservation is a well-established method of fertility preservation prior to cancer therapy (Saito et al. 2003; Bahadur et al. 2002; Kelleher et al. 2001; Lass et al. 1998; Naysmith et al. 1998). Cryopreserved sperm can be thawed at a later date and used to achieve pregnancy through various procedures such as intrauterine insemination (IUI), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI) (Palermo et al. 1992; Sanger et al. 1992; Steptoe and Edwards 1978). Intrauterine insemination, also known as artificial insemination,

involves the direct introduction of sperm into the uterus thereby increasing the concentration at the site of fertilization (reviewed in Bensdorp et al. (2007)). In conventional IVF, oocytes are retrieved using transvaginal ultrasound (TVUS)-guided needle aspiration. Each oocyte is then incubated with about 50,000 sperm and fertilization is allowed to occur naturally (Stephens et al. 2013). IUI and IVF require a large number of motile sperm for successful fertilization to occur and hence are less effective when semen parameters such as concentration, motility, and morphology are below reference values (Wang and Sauer 2006). In ICSI, one sperm is selected and injected directly into each oocyte (Palermo et al. 1992). Semen samples cryopreserved for as long as 40 years have been used with assisted reproduction to achieve pregnancy and live births (Feldschuh et al. 2005; Szell et al. 2013; Schmidt et al. 2004). Men who fail to preserve sperm samples prior to being treated for cancer may still be able to achieve pregnancy by undergoing testicular sperm extraction (TESE) (Hsiao et al. 2011). In this technique testicular spermatozoa are directly retrieved from focal areas of spermatogenesis produced by rare SSCs that survived gonadotoxic treatment. Sperm retrieval rate by TESE from infertile male cancer survivors is 37% with a 57% fertilization rate by ICSI and 50% pregnancy rate (Hsiao et al. 2011). Currently, there are no alternative options to treat infertile survivors with no sperm after TESE.

All fertility preservation techniques currently available in the clinic rely on the isolation of mature sperm from male patients. These methods are available to pubertal and adult males, but not prepubertal patients who are not yet producing sperm. This problem affects a significant proportion of the population; about 10,380 individuals under the age of 15 will be diagnosed with cancer in the United States in 2016 and hence may be exposed to chemotherapy or radiation (American Cancer Society). Retrospective data from the Childhood Cancer Survivor Study (CCSS) indicates that adult survivors of childhood cancers are significantly less likely to sire offspring than their siblings without cancer (Green et al. 2010). In addition, prepubertal males may undergo hematopoietic stem cell transplantation for a variety of nonmalignant disorders including severe aplastic anemia, Fanconi's anemia, B-Thalassemia major, congenital immunodeficiency disorders, and inherited metabolic disorders (Sevilla et al. 2005; Storb et al. 2001; Mahmoud et al. 2015). Myeloablative conditioning prior to stem cell transplantation employs the use of radiotherapy and/or gonadotoxic drugs such as busulfan and cyclophosphamide, leading to a significant risk of irreversible azoospermia (Borgmann-Staudt et al. 2012).

While pre-pubertal males do not produce sperm, they do have spermatogonial stem cells (SSCs) in their testes that are poised to initiate spermatogenesis at puberty. Several centers around the world, including our own, are freezing testicular tissue for prepubertal boys with anticipation that the tissue (containing SSCs) can be used in the future to achieve natural or assisted pregnancy (Clark et al. 2011; Goossens et al. 2013; Wyns et al. 2011; Keros et al. 2007; Ginsberg et al. 2010; Picton et al. 2015; Orwig et al. n.d.). Testicular tissue is typically obtained via biopsy before the initiation of gonadotoxic therapies. Therefore, it is incumbent on the medical and research communities to responsibly develop technologies that will

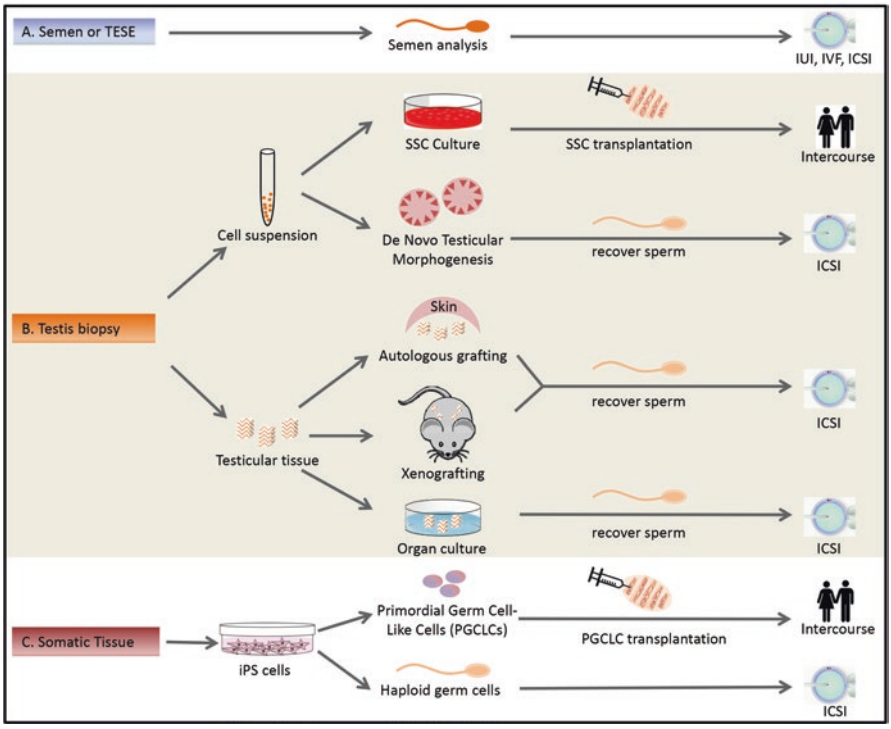

Fig. 13.1 Standard and experimental options to treat male infertility. (**a**) Sperm obtained from ejaculated semen, or by testicular sperm extraction (TESE) of infertile men, can be used to achieve pregnancy by intrauterine insemination (IUI), in vitro fertilization (IVF), or IVF with intracytoplasmic sperm injection (ICSI). (**b**) When it is not possible to obtain sperm, testicular tissue containing spermatogonial stem cells (SSCs) can be obtained by biopsy. Testicular tissue can be digested with enzymes to produce a cell suspension from which SSCs can be expanded in culture and/or transplanted into the testes of the patient. This method has the potential to regenerate spermatogenesis and possibly natural fertility. Heterogeneous testicular cell suspensions also have the potential undergo de novo testicular morphogenesis with seminiferous tubules and a polarized epithelium surrounded by a basement membrane with germ cells inside and interstitial cells outside the tubules. Sperm generated in the "rebuilt" testes can be used to fertilize eggs by ICSI. Intact testicular tissues from prepubertal animals can be grafted or xenografted under the skin or in the scrotum and produce mature sperm that can be used to fertilize eggs by ICSI. Sperm can also be generated when immature testicular tissues are maintained in organ culture and used to fertilize eggs by ICSI. (**c**) Patient-specific induced pluripotent stem (iPS) cells can be derived from patient somatic tissues (e.g., skin or blood) and differentiated into germline stem cells (GSCs) to be transplanted into patient testes. This method may have the potential to regenerate spermatogenesis and natural fertility. It may also be possible to differentiate iPS cells into sperm that can be used to fertilize eggs by ICSI. This figure and legend are reproduced with permission and with minor modification from Gassei and Orwig, *Fertility & Sterility* 2016 (Gassei and Orwig 2016)

allow patients to use their cryopreserved tissues for reproductive purposes in the future. SSC transplantation, de novo testicular morphogenesis, testicular tissue grafting/xenografting, testicular organ culture, and in vitro derivation of germ cells are methods that are currently in the research pipeline and have produced sperm and live offspring in one or more animal models (Fig. 13.1) (Gassei and Orwig 2016). The following sections of this chapter review stem cell-based methods that are being developed for potential application to preserve and restore fertility.

13.2 Spermatogonial Stem Cell Transplantation

Spermatogonial stem cells, like other adult tissue stem cells, have the potential generate or regenerate the dependent lineage: spermatogenesis. Ralph Brinster and colleagues pioneered the technique for spermatogonial stem cell transplantation in mice in 1994, demonstrating that donor SSCs could engraft the seminiferous tubules of chemotherapy-treated recipient mice and regenerate spermatogenesis leading to the production of sperm and viable offspring through normal breeding (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). SSC transplantation has now been reported in mice, rats, pigs, goats, bulls, sheep, dogs, and monkeys, including the production of donor-derived embryos or offspring in mice, rats, goats, sheep, and monkeys (Ogawa et al. 2000; Shinohara et al. 2001; Nagano et al. 2001; Brinster et al. 2003; Honaramooz et al. 2003; Izadyar et al. 2003a; Mikkola et al. 2006; Kim et al. 2008; Herrid et al. 2009; Hermann et al. 2012; Hamra et al. 2002). SSCs from donors of all ages, newborn to adult, can regenerate spermatogenesis (Shinohara et al. 2001; Ryu et al. 2003; Jahnukainen et al. 2011) and SSCs can be cryopreserved and retain spermatogenic function upon thawing and transplantation (Kanatsu-Shinohara et al. 2003a; Nagano and Brinster 1998; Dobrinski et al. 1999, 2000). Therefore, a prepubertal boy should be able to cryopreserve SSCs prior to treatment and have these cells reintroduced into his testes at a later date. Wu and colleagues reported that frozen and thawed SSCs were competent to regenerate spermatogenesis after more than 14 years of cryostorage (Wu et al. 2012).

Radford and colleagues from Manchester, United Kingdom pioneered testicular tissue cryopreservation for human cancer patients in in 1999 (Radford 2003; Brook et al. 2001). Testicular biopsies from 12 non-Hodgkin's lymphoma patients were cryopreserved as cell suspensions prior to the initiation of chemotherapy. After treatment, seven patients returned to the clinic to have their cryopreserved samples injected back into their testes through the rete testis. To our knowledge, there have been no follow-up reports on the fertility status of those patients (Radford et al. 1999; Valli et al. 2014a). Nonetheless, this study established that fertility is important enough to cancer survivors that they were willing to undergo an experimental stem cell therapy with no guaranteed outcome and, as far as we know, there were no adverse outcomes.

13.3 Cryopreservation of Testicular Tissue and Cell Suspension

Fertility preservation strategies for cancer survivors rely on effective methods for cryopreservation and long-term storage. As described above, sperm cryopreservation is a well-established method to preserve the fertility of post-pubertal men (Tournaye et al. 2014; Anderson et al. 2015). For prepubertal patients who are not producing sperm, many centers have experimental protocols to freeze testicular tissues with anticipation that SSCs in those tissues can eventually be used to produce sperm via one of a myriad of cell-based and tissue-based methods that are reviewed in Fig. 13.1. The efficacy of these techniques depends on the viability and function or cryopreserved tissues or cells after thawing.

Most centers are freezing intact pieces of testicular tissue rather than testicular cell suspensions for patients because this preserves the option for both tissue-based and cell-based therapies in the future (Goossens et al. 2013; Wyns et al. 2011; Keros et al. 2007; Ginsberg et al. 2010; Picton et al. 2015; Valli et al. 2015). Tissues are typically cut into small pieces (1–9 mm^3); suspended in a DMSO-based freezing medium and frozen at a controlled slow rate using a programmable freezing machine or device (Wyns et al. 2007, 2011; Keros et al. 2007; Ginsberg et al. 2010; Picton et al. 2015; Orwig et al. n.d.; Valli et al. 2015). Some centers have reported using ethylene glycol-based freezing medium instead of DMSO (Brook et al. 2001; Kvist et al. 2006; Unni et al. 2012). However, a study in Rhesus macaque suggests that prepubertal testicular biopsies cryopreserved with 1.4M DMSO had a higher rate of survival and spermatogenic development after xenografting into immunodeficient mice compared with biopsies frozen in 0.7M DMSO or ethylene glycol (Jahnukainen et al. 2007). Some centers have reported that viability of vitrified testicular tissue is similar to tissue frozen at a controlled slow rate (Baert et al. 2013; Curaba et al. 2011; Poels et al. 2013; Sa et al. 2012) and this may improve access to testicular tissue freezing technology in centers that do not have programmable freezing machines. Systematic studies on prepubertal human testicular tissues with evaluation of both cell-based and tissue-based endpoints are needed. It is possible that the optimal freezing condition depends on the intended use of the tissue or cells.

13.4 Spermatogonial Stem Cell Culture

For the successful application of stem cell transplant therapies, methods are needed to isolate and expand the small population of SSCs present in patient testis biopsies. Currently, there is limited information on the true identity of human SSCs or the cellular mechanisms that regulate proliferation and self-renewal of these cells. In contrast, progress characterizing the role of testicular somatic cells and their secreted factors in the survival, expansion, or differentiation of spermatogonia have been instructive for the development of mouse SSC cultures.

In the testis, SSCs reside on the basement membrane of seminiferous tubules in association with Sertoli cells within the tubules and the adjacent interstitial compartment comprised of Leydig cells, peritubular myoid cells, endothelial cells and others (de Rooij 2009). Glial cell-line derived neurotropic factor (GDNF), secreted by Sertoli cells, is required for SSC self-renewal by the activation of AKT and Src family kinase (SFK) signaling (Meng et al. 2000; Oatley et al. 2007). Fibroblast growth factor 2 (FGF2 or bFGF), also secreted by Sertoli cells, has been shown to promote self-renewal by the upregulation of *Bcl6b* and *Etv5* via mitogen-activated protein kinase (MAPK) activation (Ishii et al. 2012; Oatley and Brinster 2008; Takashima et al. 2015). Sertoli cells also secrete factors such as Bone morphogenetic protein 4 (BMP4) and Activin A that promote SSC differentiation (de Rooij 2009; Nagano et al. 2003; Pellegrini et al. 2003). Peritubular myoid cells secrete factors including leukemia inhibitory factor (LIF), GDNF, and monocyte chemotactic protein 1 (MCP1) that contribute to the maintenance of spermatogenesis in the seminiferous tubules (Chen et al. 2014; Dorval-Coiffec et al. 2005; Mayerhofer 2013). Leydig cells affect the activity of SSCs both directly, through factors such as the colony stimulating factor 1 (CSF1), which promotes SSC self-renewal, and indirectly through Sertoli cells, which are the only cells within the seminiferous tubules that express the androgen receptor (AR) for testosterone (Kokkinaki et al. 2009; Oatley et al. 2009; Walker and Cheng 2005). In fact, it has been observed in rodent testicular cross-sections that a higher number of SSCs are present in the areas of seminiferous tubules directly in contact with large patches of interstitial tissue, indicating the role played by interstitial cells on SSC maintenance (Chiarini-Garcia et al. 2001, 2003; Yoshida et al. 2007).

In addition to growth factors, Sertoli cells and peritubular myoid cells secrete collagen α1(IV), α2(IV) and α3(IV) chains, which along with laminin, heparin sulfate proteoglycan and entactin form the basement membrane of the seminiferous tubule (Hadley and Dym 1987; Lian et al. 1992; Siu and Cheng 2008; Skinner et al. 1985). These ECM components of the basement membrane are important for stem cell regulation and abnormalities in the structure and components of the basement membrane are associated with infertility (Hager et al. 2005; Salomon and Hedinger 1982; Volkmann et al. 2011). In addition to providing structural support, the ECM harbors proteins, growth factors, and cytokines (Dym 1994).

In rodents, SSCs can be maintained in long-term culture with exponential expansion in numbers. Cultured SSCs remain competent to produce spermatogenesis and restore fertility upon transplantation (Hamra et al. 2005; Kanatsu-Shinohara et al. 2003b, 2008; Kubota et al. 2004a; Richardson et al. 2009; Ryu et al. 2005). Several factors were critical to the establishment of long-term SSC cultures. First, methods were needed (e.g., FACS or MACS and/or differential attachment and replating) to enrich the SSCs and remove somatic cells that can overwhelm the culture. Second, development of serum-free, defined medium facilitated the discovery of essential growth factors, such as GDNF, to maintain and expand rodent SSCs in culture (Nagano et al. 2003; Kanatsu-Shinohara et al. 2003b, 2008; Kubota et al. 2004a, b). The effects of GDNF in mice and rats is enhanced by the addition of fibroblast growth factor 2 (FGF2) (Kubota et al. 2004a; Ryu et al. 2005). Third, STO (SIM

mouse embryo-derived thioguanine and ouabain resistant) fibroblast or MEF (mouse embryonic fibroblast) feeder cells are often needed for the survival of rodent SSCs (Kanatsu-Shinohara et al. 2003b; Kubota et al. 2004b; Nagano et al. 1998); although it is now possible to maintain mouse SSCs in feeder-free conditions (Kanatsu-Shinohara et al. 2005, 2014). SSC cultures are often established from mouse pup testes (5–12 days postpartum) because SSCs are enriched at this stage of development due to the absence of differentiating germ cells and because SSCs are predisposed to active cell cycle (Nagano et al. 2002). However, SSC cultures can be established from all ages (from neonates to adults) (Kanatsu-Shinohara et al. 2003b; Kubota et al. 2004a).

Mouse SSC culture is a *robust technology that has been replicated with slight modification by numerous research groups worldwide* and also extended to rats (Hamra et al. 2005; Ryu et al. 2005), hamsters (Kanatsu-Shinohara et al. 2008) and rabbits (Kubota et al. 2011). Progress in developing SSC cultures in mice and other species provides a valuable foundation, but it is likely that human SSC cultures will present unique challenges for maintenance and expansion that are related to the species-specific biology. For example, mouse SSCs can be maintained in StemPro medium, but hamster SSCs cannot (Kanatsu-Shinohara et al. 2008). Rabbit SSCs can be maintained on C166 endothelial cells, but not on STO fibroblast feeder cells that are often used for mouse SSC cultures (Kubota et al. 2011). Medrano and colleagues reported that human SSCs display limited proliferation under mouse SSC culture conditions. Therefore, *it is essential to discover the unique characteristics of SSCs and the testicular environment in human testes* because this will inform the development of human SSC culture methods. Initial methods for culturing human SSCs were derived from rodent methods, with the rationale that there would be conservation between species. Wu et al. compared prepubertal human spermatogonia and mouse gonocytes/prospermatogonia to show a significant level of conservation between the two species. This finding prompted them to use mouse feeder cell lines, STO and C166 with medium supplemented with GDNF and GFRA1 to culture prepubertal human SSCs (Wu et al. 2009). Those conditions did not support long-term maintenance of human SSCs, but suggested that the role of GDNF in stimulating self-renewal is conserved from rodents to humans. Subsequent studies employed the use of feeder-based conditions with feeder cells such as human Sertoli cells, human embryonic stem cells-derived fibroblasts and THY1+ testicular somatic cells, and feeder-free methods such as human laminin-coated plates (Chen et al. 2009; Liu et al. 2011; Sadri-Ardekani et al. 2009, 2011).

Indeed, several groups have reported extending SSC culture to large animal species (Eildermann et al. 2012; Izadyar et al. 2003b; Kala et al. 2012; Kuijk et al. 2009; Langenstroth et al. 2014; Luo et al. 2006; Oatley et al. 2016) and humans (Wu et al. 2009; Chen et al. 2009; Liu et al. 2011; Sadri-Ardekani et al. 2009, 2011; He et al. 2010; Mirzapour et al. 2012; Lim et al. 2010; Goharbakhsh et al. 2013; Akhondi et al. 2013; Smith et al. 2014; Guo et al. 2015; Abdul Wahab et al. 2016; Medrano et al. 2016; Baert et al. 2015; Kokkinaki et al. 2011; Nowroozi et al. 2011; Piravar et al. 2013; Zheng et al. 2014), including two from the testes of prepubertal patients (Wu et al. 2009; Sadri-Ardekani et al. 2011) (Table 13.1). Each laboratory

Table 13.1 Reports on human SSC culture

Authors	Year	Tissue	SSC enrichment	Growth factors	Feeders/ substrate	Endpoint	Ref.
Sadri-Ardekani et al.	2009	Adult	Differential plating	EGF, GDNF, LIF	Laminin	Xenotransplants; ICC—ZBTB16; RT-PCR—ZBTB6, ITGA6, ITGB1	Sadri-Ardekani et al. (2009)
Wu et al.	2009	Prepubertal	Isolated by micromanipulator	GDNF, GFRα1 and FGF	C166	ICC—UCHL1	Wu et al. (2009)
Chen et al.	2009	Fetal	MACS for ITGA	FGF, GDNF, LIF	hESCdFs	ICC—OCT4, SSEA1, ITGA6;m RT-PCR—OCT4, STRA8, DAZL, NOTCH1, NGN3, SOX3, KIT	Chen et al. (2009)
Lim et al.	2010	Adult	Differential plating followed by MACS—CD9	GDNF, bFGF, EGF, LIF	Laminin	RT-PCR—OCT4, ITGA6, ITGB1, cKIT, TH2B, SYCP3, TP-1; MTT assay; TUNEL assay; ICC—GFRA1, CD-9, ITGA6; Alkaline phosphatase staining	Lim et al. (2010)
He et al.	2010	Adult	Differential plating and MACS for GFR125	GDNF, GFRA1-Fc, NUDT6, LIF, EGF, TGFB, Nodal	0.1% gelatin	ICC—GPR125, ITGA6, GFRA1, THY1	He et al. (2010)
Kokkinaki et al.	2011	Adult	MACS for SSEA4	EGF, FGF, GDNF and LIF	Matrigel	RT-PCR—EPCAM, GPR125, SSEA4, ITGA6	Kokkinaki et al. (2011)

(continued)

Table 13.1 (continued)

Authors	Year	Tissue	SSC enrichment	Growth factors	Feeders/ substrate	Endpoint	Ref.
Sadri-Ardekani et al.	2011	Prepubertal	Differential plating	EGF, GDNF, LIF	Laminin	Xenotransplants and RT-PCR—ZBTB16, ITGA6, ITGB1, CD9, GFRA1, GPR125, UCHL1	Sadri-Ardekani et al. (2011)
Nowroozi et al.	2011	Adult	Differential plating	None	Sertoli cells	ICC—OCT4, Vimentin; Alkaline phosphatase staining	Nowroozi et al. (2011)
Liu et al.	2011	Fetal	Percoll separation and differential plating	None	Sertoli cells	ICC and Fc—OCT4, SSEA4	Liu et al. (2011)
Mirzapour et al.	2012	Adult	Differential plating	FGF and LIF	Sertoli cells	Xenotransplants; Alkaline phosphatase staining; ICC—OCT4, Vimentin; RT-PCR—OCT4, NANOG, STRA8, PIWIl2, VASA	Mirzapour et al. (2012)
Goharbakhsh et al.	2013	Adult	Differential plating	GDNF, bFGF, EGF, LIF	Laminin	ICC—GPR125	Goharbakhsh et al. (2013)
Piravar et al.	2013	Adult	Differential plating	GDNF, EGF, LIF, bFGF	Laminin	RT-PCR—UCHL1	Piravar et al. (2013)
Akhondi et al.	2013	Adult	Differential plating	GDNF, EGF, LIF		ICC—OCT4, qPCR—PLZF	Akhondi et al. (2013)
Smith et al.	2014	Adult	FACS—CD45neg, THY1 neg, SSEA4 pos	EGF, GDNF, LIF	Human THY1+ testicular somatic cells	ICC—SSEA4, VASA	Smith et al. (2014)

Authors	Year	Tissue	SSC enrichment	Growth factors	Feeders/ substrate	Endpoint	Ref.
Zheng et al.	2014	Adult	Differential plating	GDNF, LIF, bFGF, EGF	None listed	Flow cytometry—SSEA4; RT-PCR—UTF1, FGFR3, SALL4, ZBTB16, DAZL, VIM, ACTA2, GATA4	Zheng et al. (2014)
Baert et al.	2015	Adult	Differential plating	None listed	None listed	ICC and RT-PCR—VASA, UCHL1	Baert et al. (2015)
Guo et al.	2015	Adult	Differential plating followed by MACS—GPR125	GDNF, bFGF, EGF, LIF	Stem easy hydrogel	ICC—UCHL1, GPR125, THY1, PLZF; qPCR—GPR123, GFRa1, RET, PLZF, UCHL1, MAGEA4, SYCP3, PRM1 and TNP1	Guo et al. (2015)
Wahab et al.	2016	Adult	No enrichment	bFGF	Plastic	IF—ITGA6, ITGB1, CD9, GFRA1	Abdul Wahab et al. (2016)
Medrano et al.	2016	Adult	FACS—HLA-/ EPCAM+	EGF, LIF, bFGF, GDNF	Y-irradiated testicular somatic cells	ICC—Ki67; TUNEL; RT-PCR—UTF1, DAZL, VASA, PLZF, FGFR3, UCHL1; Elecsys Testosterone II competitive immunoassay; ELISA—Inhibin B; ICC—VASA, UTF1, UCHL1	Medrano et al. (2016)

Abbreviations: *ICC* immunocytochemistry, *RT-PCR* real-time (quantitative) PCR, *IF* immunofluorescence, *TUNEL* terminal deoxynucleotidyl transferase dUTP nick end labeling

has used a different approach to culture human SSCs and different methods to assess outcomes (reviewed in Table 13.1) and to date; *no human SSC culture method has been independently replicated by another laboratory*. To move the field forward, human SSC culture methods need to be independently replicated in other laboratories; evaluated systematically and quantitatively using validated markers of human spermatogonia (e.g., UTF1, UCHL1, SSEA4, PLZF, SALL4, ENO2) and/or by functional assay, such as xenotransplantation to nude mice (Dovey et al. 2013; Valli et al. 2014b).

13.5 Sorting Methods for the Enrichment of Spermatogonia and Elimination of Malignant Contaminants

Several studies have reported enrichment of putative human SSCs by fluorescence activated cell sorting (FACS) and magnetic activated cell sorting (MACS) based on cell surface markers including GPR125, EpCAM, SSEA4, ITGA6, and CD9 (He et al. 2010; Dovey et al. 2013; Izadyar et al. 2011; Zohni et al. 2012). Isolation and enrichment of SSCs facilitates (1) fundamental investigations; (2) establishment of SSC culture by removing testicular somatic cells and (3) regeneration of spermatogenesis after transplantation of the enriched cell population. In the context of the cancer survivor, sorting might also be used to remove malignant contamination in the stored samples, especially those from patients with hematopoietic or lymphoid malignancies. This is an important concern as Jahnukainen and colleagues reported that transplantation of rat testicular cells with as few as 20 contaminating leukemia cells consistently transmitted the disease to recipients (Jahnukainen et al. 2001). To address this aspect of safety, Fujita and colleagues established a sorting method based on cancer cell-specific surface antigens, MHC class I and CD45 to negatively select germ cells. Mice transplanted with the germ cell-enriched fraction (MHC class I-/CD45-) did not develop cancer whereas all mice transplanted with unsorted cells showed signs of terminal leukemia within 40 days (Fujita et al. 2005). This approach is not universally applicable, as the same group later used the MHC class I and CD45-based sorting method to eliminate malignant contamination from human testis cells, demonstrating that 7 of the 8 malignant cell lines could be eliminated from the germ-cell enriched fraction (Fujita et al. 2006). Geens et al. used FACS to distinguish leukemic cells from testicular cell suspensions based on HLA class I expression but were unable to remove malignant cells (Geens et al. 2007). Similarly, CD49f MACS to enrich spermatogonia followed by selective adhesion using collagen I and laminin failed to eliminate malignant contamination (Geens et al. 2011). These initial studies show that negative selection based on leukemic markers is not sufficient to eliminate cancer cells from a human testicular cell suspension and that a multiparametric sort may provide more stringent selection. Our group showed in monkeys and humans, that positive/negative selection using cell surface markers THY1 (CD90, expressed by spermatogonia) and CD45 (expressed by MOLT-4 leukemia cells) followed by singlet discrimination effectively removed leukemic cells from the spermatogonial fraction of primate testicular cell

suspensions (Hermann et al. 2011). A similar strategy was employed to eliminate MOLT-4 leukemia cells (EpCAM−/HLA-ABC+/CD49e+) from the spermatogonial fraction (EpCAM+/HLA-ABC−/CD49e-) of human testicular cell suspensions (Dovey et al. 2013). These studies show that by using a combination of spermatogonial and malignant cell markers, it may be possible to remove malignant contamination from human testis cell suspensions. However, considering that (1) different criteria may have to be optimized for each cancer cell type, (2) there is substantial stem cell loss associated with most sorting protocols and (3) there is a possibility of failure; we would not recommend autologous transplantation of testicular tissues or cells into patients with hematopoietic or lymphoid cancers.

13.6 Testicular Tissue Grafting

One way to circumvent the risk of malignant contamination is to use an animal host to mature testicular tissues or cells. Testicular tissue grafting was initially developed as a tool to study the somatic compartment of the testis and steroidogenesis (Kuopio et al. 1989; Arregui and Dobrinski 2014; Johnson et al. 1996). Xenotransplantation of SSCs into the seminiferous tubules of recipient mice from donors of increased phylogenetic distances causes donor spermatogonia to arrest before undergoing differentiation. Honaramooz and colleagues recognized that this phenomenon is caused by SSC niche incompatibility and used testis tissue xenografting as a way of providing donor SSCs with their homologous niche (Honaramooz et al. 2002). In this collaboration between the Dobrinski and Schlatt labs, testis tissue fragments from neonatal mice, pigs and goats were transplanted under the skin of immune deficient recipient mice and gave rise to complete spermatogenesis from all donor species. A subsequent study reported that testicular tissue pieces from sexually immature (13 month old) Rhesus monkeys could be grafted into immune deficient mice, giving rise to complete spermatogenesis in 4% of all tubules within 7 months post-grafting (Honaramooz et al. 2004). Mature spermatozoa were isolated and used to fertilize eggs via ICSI and produce pre-implantation embryos, in vitro (Honaramooz et al. 2002, 2004). This technique has been replicated using testicular grafts from several species including dog, hamster, ferret, rabbit, domestic cattle, cat, etc. (Oatley et al. 2005a; Shirazi et al. 2014; Snedaker et al. 2004; Schlatt et al. 2002, 2010; Shinohara et al. 2002; Abrishami et al. 2010; Gourdon and Travis 2011; Kim et al. 2007).

Effective grafting is established by the formation of a vascular network between small capillaries made by the graft itself and larger subcutaneous blood vessels formed around the graft by the host (Schlatt et al. 2010). Treatment of recipient tissue with vascular endothelial growth factor (VEGF) has been shown to improve grafting efficiency (Schmidt et al. 2006). Vascularization is essential for the survival of the graft and for the establishment of a feedback loop between the donor endocrine cells and the murine hypothalamic-pituitary axis (Arregui and Dobrinski 2014). Leydig cells and Sertoli cells present in the graft can respond to murine luteinizing hormone and follicle stimulating hormone, respectively. Androgens and

inhibins secreted by the donor somatic compartment in response to murine gonadotropins, in turn, can provide feedback to the murine hypothalamic-pituitary axis. A significant amount of variability has been observed in the spermatogenic potential of grafts between species. Grafts of porcine and ovine origin xenografted into mice have been reported to have complete spermatogenesis in over 50% of seminiferous tubules (Zeng et al. 2006). In contrast, less than 10% of seminiferous tubules have been observed to have elongated spermatids in grafts from bull, equine, and nonhuman primate testes xenografted into mice (Oatley et al. 2004; Rathi et al. 2006, 2008). These differences could be due to dissimilarities in the structure of gonadotropins between species leading to differences in the efficiency of interaction between donor gonadotropin receptors and murine gonadotropins (Arregui and Dobrinski 2014). In some studies using the primate model, supplementing recipient mice with primate gonadotropins improved graft size and the extent of spermatogenesis (Schlatt et al. 2002; Rathi et al. 2008; Ehmcke et al. 2011).

The donor age at the time of xenografting plays an important role in the spermatogenic potential of the graft. Most studies using adult donor testicular grafts have reported graft degeneration, incomplete spermatogenesis, and arrest at the spermatocyte stage or the presence of Sertoli cell only phenotype (Abrishami et al. 2010; Kim et al. 2007; Oatley et al. 2005b; Arregui et al. 2008a). The mechanism behind the differential spermatogenic potential and grafting efficiency with age is not completely understood, however, some hypotheses have been proposed. Immature testis tissue may have an increased resistance to transient hypoxia induced by the grafting process and may have a higher angiogenic capacity compared with adult tissue (Arregui et al. 2008a, 2012). Arregui and colleagues hypothesized that the degree of sperm production in donor tissue at the time of grafting negatively affects grafting efficiency due to high metabolic demands of cell division and differentiation, thereby making the tissue more susceptible to hypoxia. They showed that suppression of spermatogenesis in adult donor mice dramatically improved grafting efficiency with the complete recovery of spermatogenesis, while control grafts from mice with ongoing spermatogenesis degenerated and no spermatogenesis was observed (Arregui et al. 2012). Sexually immature testes at different donor ages also have been shown to have different grafting efficiencies, with prepubertal tissue having a higher spermatogenic potential compared to neonatal tissue (Oatley et al. 2005a; Kim et al. 2007). This occurrence could be due to the immaturity of the developing somatic compartment and their inability to respond to circulating gonadotropins (Plant et al. 2005).

Testicular tissue xenografting using human tissue has been performed in several studies. However, these studies failed to achieve complete spermatogenesis with production of haploid germ cells (Yu et al. 2006; Sato et al. 2010; Geens et al. 2006). Unlike immature testicular tissue grafting from other species where complete spermatogenesis was observed, grafts using human fetal or infant testis tissue resulted only in the maintenance of spermatogonia for extended periods of time (Yu et al. 2006; Sato et al. 2010). Orthotopic xenografts of prepubertal human tissue into the scrotum of immunodeficient mice also led to the maintenance of spermatogonia (Van Saen et al. 2011) and some studies have reported maturation of germ cells up

to the spermatocyte stage in grafts from infant, prepubertal, and postpubertal donors (Sato et al. 2010; Van Saen et al. 2011; Wyns et al. 2008). Adult human donor-derived xenografts have been shown to regress over time with the presence of few spermatogonia in the tubules (Geens et al. 2006; Schlatt et al. 2006). These results from human to mouse xenografts are less promising than results from other species, indicating the further development is needed. Some approaches that appear to enhance xenograft results from other species, such as pretreatment of recipient tissue with VEGF or homologous gonadotropin supplementation, may be tested in context of human xenografting. Perhaps species other than mice would be better hosts for human testicular tissue xenografts. While human to animal xenografting might circumvent safety issues associated with malignant contamination of the tissue, zoonosis issues will have to be addressed on the road to clinical application.

If malignant contamination is not a concern, autologous grafting of testicular tissues back into the patient may be an option and provide the ideal host for the grafted tissue. Wistuba and colleagues have performed autologous testicular grafting in two studies in marmoset monkeys (Luetjens et al. 2008; Wistuba et al. 2006) and reported that complete spermatogenesis can be obtained in orthotopic (in the scrotum), but not ectopic (under the back skin) grafts. The same group also grafted frozen and thawed tissue, but these were only transplanted ectopically and did not produce spermatogenesis (Luetjens et al. 2008). These results raise the following questions: (1) can frozen and thawed prepubertal primate testis tissue grafts produce haploid gametes; (2) what are the optimal freezing conditions; (3) If haploid gametes are produced, are they competent to fertilize monkey oocytes and give rise to healthy offspring? The latter question is one that can only be answered using animal models and continued progress in the nonhuman primate model should have important implications for the clinic. Some of these questions were answered in a later study from Jahnukainen and colleagues, who demonstrated that prepubertal/pubertal testicular tissue could be frozen, thawed, and grafted autologously to produce complete spermatogenesis. Similar to the study from Wistuba and colleagues, it appears that sperm were produced in orthotopic grafts (in the scrotum), but not ectopic grafts (Jahnukainen et al. 2012).

13.7 De Novo Testicular Morphogenesis

Sertoli cells and peritubular myoid cells isolated from immature testes have the potential to reorganize themselves into testicular cords in vitro or when ectopically grafted into recipient mice (Dufour et al. 2002; Gassei et al. 2006, 2010). Honaramooz and colleagues showed that ectopic grafting of immature porcine testicular cells (single cell suspension) under the dorsal skin of immunocompromised mice led to the formation of testicular cords within a week post-grafting. The organization of the somatic compartment developed further to form seminiferous tubules with the migration of germ cells to the basement membrane by 10 weeks and proliferation of germ cells was observed by week 25. Grafts recovered after 30 weeks exhibited complete spermatogenesis with elongated spermatids in about 11% of the

tubules (Honaramooz et al. 2007). Subsequent studies have reported similar morphogenetic potential of cells obtained from rodent, zebrafish, ovine and bovine testes (Kita et al. 2007; Zhang et al. 2008; Arregui et al. 2008b; Kawasaki et al. 2010). Yokonoshi and coworkers used the same principle to culture aggregates of cells isolated from neonatal mouse testes on the surface of agarose gel platforms, using the gas–liquid interface method (described in the next section). These cellular aggregates formed tubules in vitro to generate complete spermatogenesis (Yokonishi et al. 2013). De novo testicular morphogenesis could potentially be used as a fertility preservation method for prepubertal patients who elect to preserve testicular tissue biopsies, and like xenografting, could circumvent issues of malignant contamination.

13.8 In Vitro Spermatogenesis Using Organ Culture

In the 1950s–1960s a series of studies were performed on various culture parameters such as pH, temperature, energy substrate, and oxygen tension to develop an appropriate organ culture method for testis tissue (Trowell 1959; Steinberger and Steinberger 1965; Steinberger et al. 1964; Staub 2001). In vitro germ cell differentiation was first demonstrated in a culture system developed by Steinberger and coworkers in 1966 in which neonatal rat testis fragments were placed on an agar platform at the medium–gas interface and incubated at 31 °C and 5% CO_2 in a defined culture medium supplemented with pyruvate, vitamins A, C and E and glutamine. Pachytene spermatocytes were observed within 3 weeks of culture (Steinberger and Steinberger 1966). Sato and colleagues from the Ogawa laboratory used the same method to culture neonatal (2.5–3.5 days post-partum) mouse testis fragments and found that by replacing fetal bovine serum (FBS) with knockout serum replacement (KSR), cultured tissue differentiated to produce spermatids and spermatozoa. Spermatids and sperm were isolated from the cultured tissues after 23–42 days and used to fertilize oocytes by round spermatid injection (ROSI) and ICSI that resulted in the production of live and fertile offspring (Sato et al. 2011a). The same group then performed another set of experiments where donor SSCs expanded in culture were injected into recipient mouse testes either before or after castration. The recipient testis fragments were then cultured in vitro. Donor SSCs successfully colonized the basement membrane of the recipient seminiferous tubules and underwent complete spermatogenesis in organ culture to produce round spermatids and sperm which were used to fertilize oocytes and generate healthy offspring (Sato et al. 2011b). The same result was subsequently reported from frozen and thawed testicular tissue (Sato et al. 2011a; Yokonishi et al. 2014), suggesting that this approach could be used to preserve the fertility of prepubertal cancer patients and allow them to have biological children in the future. Adult testis tissues do not perform as well in organ culture (Sato et al. 2015).

13.9 In Vitro Spermatogenesis from ES Cells and iPSCs

The elucidation of signals and transcription factors involved in the formation of primordial germ cells (PGCs) during embryonic development has established the blueprint for derivation of PGC-like cells (PGC-LCs) from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC), in vitro. During embryonic development in mice, the blastocyst differentiates to form three layers: epiblast, trophectoderm, and primitive endoderm. While the trophectoderm and the primitive endoderm form extraembryonic tissues, the epiblast gives rise to the embryo proper. In mice, on embryonic day (E) 6.25 after implantation, PGC precursors arise in the proximal epiblast in response to bone morphogenetic protein 4 (BMP 4) and BMP8b from the extraembryonic tissue (Lawson et al. 1999; Saitou 2009). PGC specification continues as the cells migrate into the extraembryonic allantois mesoderm. PGC specification occurs via the suppression of the somatic differentiation program through the action of transcription regulators, BLIMP1 and AP2γ (Ohinata et al. 2005; Weber et al. 2010). PRDM14-driven reacquisition of pluripotency through the activation of SOX2 and NANOG expression is also essential for specification of PGC-fate (Yamaji et al. 2008; Yabuta et al. 2006). Between E8–E10 PGCs migrate back into the embryo proper, through the hindgut and the dorsal mesentery and colonize the gonadal ridge. PGC proliferation takes place through the period of migration and ceases upon entry into gonads. In the male embryo, PGCs undergo mitotic arrest at E13.5 and are referred to as gonocytes or prospermatogonia (Ge et al. 2015). Prospermatogonia migrate to the basement membrane of the seminiferous tubules after birth in rodents and give rise to the first round of spermatogenesis and SSCs that maintain continuous sperm production in the adult (Manku and Culty 2015; Culty 2009).

Hayashi and colleagues demonstrated that ESCs derived from E3.5 mouse blastocysts could be induced to form epiblast-like cells (EpiLCs) upon exposure to Activin A, bFGF and 1% knockout serum replacement (KSR). Furthermore, Day 2 EpiLCs, on stimulation with 15%KSR, BMP4, BMP8b, LIF, Epidermal growth factor (EGF), and Stem cell factor (SCF), formed PGCLCs that could generate complete spermatogenesis and give rise to offspring when transplanted into the seminiferous tubules of an infertile recipient (Hayashi et al. 2011). A similar approach has been used to derive PGCLCs from human ESCs (Irie et al. 2015), but of course it is impossible to test the spermatogenic potential of those cells by transplantation or production of offspring. Murine PGCLCs can also be directly induced from ESCs and EpiLCs by co-expression of BLIMP1, AP2γ, and PRDM14 (Magnúsdóttir et al. 2013; Nakaki et al. 2013). While these findings provide a great tool for studying the mechanisms behind the embryonic development of germ cells, clinical translation of these methods must be approached with caution due to the risk that occult pluripotent cells or early germ cells can produce benign tumors called teratomas. This problem can potentially be circumvented by complete differentiation from pluripotent cells to haploid germ cells in vitro, which may then be used to fertilize oocytes through ICSI.

Previous studies have reported that ES cells can be differentiated in vitro to form embryoid bodies (EBs) that support the formation of PGCs and cells of the somatic compartment of the testis (Geijsen et al. 2004; Toyooka et al. 2003). Geijsen and coworkers further demonstrated that PGCs generated in EBs differentiated into haploid germ cells that expressed Acrosin and FE-J1, both markers of male germ cell maturation. Haploid Fe-J1$^+$ cells were microinjected into oocytes to give rise to blastocysts. The generation of offspring was not evaluated in this study (Geijsen et al. 2004). More recently, Zhou and colleagues co-cultured PGCLCs derived from mouse ESCs with neonatal mouse testicular somatic cells and observed initiation of meiosis upon exposure to activin A, BMP-2/4/7 and retinoic acid (RA). Completion of meiosis to generate haploid cells was subsequently achieved by withdrawing the 3 morphogens and introducing FSH, testosterone and bovine pituitary extract into culture. Haploid spermatid-like cells (SLCs) generated using this method were competent to fertilize oocytes by ICSI and produced offspring (Zhou et al. 2016). Translating this technique to human iPSCs could provide a powerful tool for treating male infertility.

These approaches need to be replicated by other investigators and in other animal models to establish feasibility, safety and reproductive competence of the in vitro germ cells. However, if the potential is fully realized in humans, it will provide a valuable tool for studying germ lineage development in a species that is not amenable to genetic manipulation or transplantation approaches. There are also important implications for the cancer patient. If germ cells can be produced from skin or other somatic tissues after cure, it will obviate the need for "fertility preservation" at the time of cancer diagnosis and treatment. Rather, the survivors can make fertility decisions when they are ready to start their families.

13.10 Concluding Remarks

Assisted reproductive technologies that enabled an infertile couple in Great Britain to have the world's first "test tube" baby (Louise Brown, born July 25th, 1978), have now produced millions of children worldwide. However, there are still no fertility treatment options for men or women who cannot produce eggs or sperm due to medical treatment, injury, genetics, or other circumstance. It is amazing that every method reviewed in this chapter and summarized in Fig. 13.1 has produced sperm and live offspring in at least one species. SSC transplantation, testicular tissue grafting and de novo spermatogenesis have been replicated in numerous species over the past 10–20 years and might be considered mature technologies that merit consideration for translation to the human clinic. Studies in nonhuman primates and with human tissues are critical last steps on the road to the clinic. Nonhuman primate studies have the advantage that experimental germ cells can be tested functionally by transplantation, fertilization and production of offspring, but are hampered by expense and the need for specialized infrastructure. Human tissue studies are most relevant to the target patients, but hampered by the inability to test function of experimental germ cells. This necessarily lowers the bar for burden of proof in the

most important human focused studies. Creative approaches to test the function of human experimental germ cells within the parameters of funding or legislative restrictions will have an immeasurable impact on the quality and pace of research toward to next generation of assisted reproductive technologies.

References

Abdul Wahab AY, Md Isa ML, Ramli R (2016) Spermatogonial stem cells protein identification in in vitro culture from non-obstructive Azoospermia patient. Malays J Med Sci 23(3):40–48

Abrishami M, Abbasi S, Honaramooz A (2010) The effect of donor age on progression of spermatogenesis in canine testicular tissue after xenografting into immunodeficient mice. Theriogenology 73(4):512–522

Achille MA et al (2006) Facilitators and obstacles to sperm banking in young men receiving gonadotoxic chemotherapy for cancer: the perspective of survivors and health care professionals. Hum Reprod 21(12):3206–3216

Agarwal A, Ong C, Durairajanayagam D (2014) Contemporary and future insights into fertility preservation in male cancer patients. Transl Androl Urol 3(1):27–40

Akhondi MM et al (2013) Propagation of human germ stem cells in long-term culture. Iran J Reprod Med 11(7):551–558

Anderson RA et al (2015) Cancer treatment and gonadal function: experimental and established strategies for fertility preservation in children and young adults. Lancet Diabetes Endocrinol 3(7):556–567

Arregui L, Dobrinski I (2014) Xenografting of testicular tissue pieces: twelve years of an in vivo spermatogenesis system. Reproduction (Cambridge, England) 148(5):R71–R84

Arregui L et al (2008a) Xenografting of adult mammalian testis tissue. Anim Reprod Sci 106(1–2):65–76

Arregui L et al (2008b) Xenografting of sheep testis tissue and isolated cells as a model for preservation of genetic material from endangered ungulates. Reproduction 136(1):85–93

Arregui L et al (2012) Suppression of spermatogenesis before grafting increases survival and supports resurgence of spermatogenesis in adult mouse testis. Fertil Steril 97(6):1422–1429

Baert Y et al (2013) What is the best cryopreservation protocol for human testicular tissue banking? Hum Reprod 28(7):1816–1826

Baert Y et al (2015) Cryopreservation of testicular tissue before long-term testicular cell culture does not alter in vitro cell dynamics. Fertil Steril 104(5):1244–1252.e4

Bahadur G et al (2002) Semen quality and cryopreservation in adolescent cancer patients. Hum Reprod 17(12):3157–3161

Bensdorp A et al (2007) Intra-uterine insemination for male subfertility. Cochrane Database Syst Rev 4

Borgmann-Staudt A et al (2012) Fertility after allogeneic haematopoietic stem cell transplantation in childhood and adolescence. Bone Marrow Transplant 47(2):271–276

Brinster RL, Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 91(24):11303–11307

Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 91(24):11298–11302

Brinster CJ et al (2003) Restoration of fertility by germ cell transplantation requires effective recipient preparation. Biol Reprod 69(2):412–420

Brook PF et al (2001) Isolation of germ cells from human testicular tissue for low temperature storage and autotransplantation. Fertil Steril 75(2):269–274

Chapman RM, Sutcliffe SB, Malpas JS (1981) Male gonadal dysfunction in Hodgkin's disease. A prospective study. JAMA 245(13):1323–1328

Chen B et al (2009) Xeno-free culture of human spermatogonial stem cells supported by human embryonic stem cell-derived fibroblast-like cells. Asian J Androl 11(5):557–565
Chen LY et al (2014) Peritubular myoid cells participate in male mouse spermatogonial stem cell maintenance. Endocrinology 155(12):4964–4974
Chiarini-Garcia H et al (2001) Distribution of type A spermatogonia in the mouse is not random. Biol Reprod 65(4):1179–1185
Chiarini-Garcia H, Raymer AM, Russell LD (2003) Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. Reproduction 126(5):669–680
Clark AT, Phillips BT, Orwig KE (2011) Fruitful progress to fertility: male fertility in the test tube. Nat Med 17(12):1564–1565
Culty M (2009) Gonocytes, the forgotten cells of the germ cell lineage. Birth Defects Res C Embryo Today 87(1):1–26
Curaba M et al (2011) Can prepubertal human testicular tissue be cryopreserved by vitrification? Fertil Steril 95(6):2123. e9–12
de Rooij DG (2009) The spermatogonial stem cell niche. Microsc Res Tech 72(8):580–585
Dobrinski I, Avarbock MR, Brinster RL (1999) Transplantation of germ cells from rabbits and dogs into mouse testes. Biol Reprod 61(5):1331–1339
Dobrinski I, Avarbock MR, Brinster RL (2000) Germ cell transplantation from large domestic animals into mouse testes. Mol Reprod Dev 57(3):270–279
Dorval-Coiffec I et al (2005) Identification of the leukemia inhibitory factor cell targets within the rat testis. Biol Reprod 72(3):602–611
Dovey SL et al (2013) Eliminating malignant contamination from therapeutic human spermatogonial stem cells. J Clin Invest 123(4):1833–1843
Dufour JM, Rajotte RV, Korbutt GS (2002) Development of an in vivo model to study testicular morphogenesis. J Androl 23(5):635–644
Dym M (1994) Basement membrane regulation of Sertoli cells. Endocr Rev 15(1):102–115
Ehmcke J et al (2011) Immature rhesus monkey (Macaca mulatta) testis xenografts show increased growth, but not enhanced seminiferous differentiation, under human chorionic gonadotropin treatment of nude mouse recipients. Int J Androl 34(5pt2):e459–e467
Eildermann K, Gromoll J, Behr R (2012) Misleading and reliable markers to differentiate between primate testis-derived multipotent stromal cells and spermatogonia in culture. Hum Reprod 27(6):1754–1767
Ethics Committee of the American Society for Reproductive Medicine (2005) Fertility preservation and reproduction in cancer patients. Fertil Steril 83(6):1622–1628
Feldschuh J et al (2005) Successful sperm storage for 28 years. Fertil Steril 84(4):1017
Fujita K et al (2005) Transplantation of spermatogonial stem cells isolated from leukemic mice restores fertility without inducing leukemia. J Clin Invest 115(7):1855–1861
Fujita K et al (2006) Isolation of germ cells from leukemia and lymphoma cells in a human in vitro model: potential clinical application for restoring human fertility after anticancer therapy. Cancer Res 66(23):11166–11171
Gassei K, Orwig KE (2016) Experimental methods to preserve male fertility and treat male factor infertility. Fertil Steril 105(2):256–266
Gassei K, Schlatt S, Ehmcke J (2006) De novo morphogenesis of seminiferous tubules from dissociated immature rat testicular cells in xenografts. J Androl 27(4):611–618
Gassei K et al (2010) Immature rat seminiferous tubules reconstructed in vitro express markers of Sertoli cell maturation after xenografting into nude mouse hosts. Mol Hum Reprod 16(2):97–110
Ge W et al (2015) In vitro differentiation of germ cells from stem cells: a comparison between primordial germ cells and in vitro derived primordial germ cell-like cells. Cell Death Dis 6:e1906
Geens M et al (2006) Spermatogonial survival after grafting human testicular tissue to immunodeficient mice. Hum Reprod 21(2):390–396
Geens M et al (2007) The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients. Hum Reprod 22(3):733–742

Geens M, Goossens E, Tournaye H (2011) Cell selection by selective matrix adhesion is not sufficiently efficient for complete malignant cell depletion from contaminated human testicular cell suspensions. Fertil Steril 95(2):787–791
Geijsen N et al (2004) Derivation of embryonic germ cells and male gametes from embryonic stem cells. Nature 427(6970):148–154
Ginsberg JP et al (2010) An experimental protocol for fertility preservation in prepubertal boys recently diagnosed with cancer: a report of acceptability and safety. Hum Reprod 25(1):37–41
Goharbakhsh L et al (2013) Isolation and culture of human spermatogonial stem cells derived from testis biopsy. Avicenna J Med Biotechnol 5(1):54–61
Goossens E, Van Saen D, Tournaye H (2013) Spermatogonial stem cell preservation and transplantation: from research to clinic. Hum Reprod 28(4):897–907
Gourdon JC, Travis AJ (2011) Spermatogenesis in ferret testis xenografts: a new model. Comp Med 61(2):145–149
Green DM et al (2010) Fertility of male survivors of childhood cancer: a report from the childhood cancer survivor study. J Clin Oncol 28(2):332–339
Guo Y et al (2015) Expansion and long-term culture of human spermatogonial stem cells via the activation of SMAD3 and AKT pathways. Exp Biol Med (Maywood) 240(8):1112–1122
Hadley MA, Dym M (1987) Immunocytochemistry of extracellular matrix in the lamina propria of the rat testis: electron microscopic localization. Biol Reprod 37(5):1283–1289
Hager M et al (2005) Laminin {alpha}1 chain corrects male infertility caused by absence of laminin {alpha}2 chain. Am J Pathol 167(3):823–833
Hammond C, Abrams JR, Syrjala KL (2007) Fertility and risk factors for elevated infertility concern in 10-year hematopoietic cell transplant survivors and case-matched controls. J Clin Oncol 25(23):3511–3517
Hamra FK et al (2002) Production of transgenic rats by lentiviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A 99(23):14931–14936
Hamra FK et al (2005) Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. Proc Natl Acad Sci U S A 102(48):17430–17435
Hayashi K et al (2011) Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells. Cell 146(4):519–532
He Z et al (2010) Isolation, characterization, and culture of human spermatogonia. Biol Reprod 82(2):363–372
Hermann BP et al (2011) Separating spermatogonia from cancer cells in contaminated prepubertal primate testis cell suspensions. Hum Reprod 26(12):3222–3231
Hermann BP et al (2012) Spermatogonial stem cell transplantation into rhesus testes regenerates spermatogenesis producing functional sperm. Cell Stem Cell 11(5):715–726
Herrid M et al (2009) Irradiation enhances the efficiency of testicular germ cell transplantation in sheep. Biol Reprod 81(5):898–905
Honaramooz A et al (2002) Sperm from neonatal mammalian testes grafted in mice. Nature 418(6899):778–781
Honaramooz A et al (2003) Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol Reprod 69(4):1260–1264
Honaramooz A et al (2004) Accelerated maturation of primate testis by xenografting into mice. Biol Reprod 70(5):1500–1503
Honaramooz A et al (2007) Building a testis: formation of functional testis tissue after transplantation of isolated porcine (Sus scrofa) testis cells. Biol Reprod 76(1):43–47
Howell SJ, Shalet SM (2005) Spermatogenesis after cancer treatment: damage and recovery. J Natl Cancer Inst Monogr (34):12–17
Hsiao W et al (2011) Successful treatment of postchemotherapy Azoospermia with microsurgical testicular sperm extraction: the weill cornell experience. J Clin Oncol 29(12):1607–1611
Hudson MM et al (2013) Clinical ascertainment of health outcomes among adults treated for childhood cancer. JAMA 309(22):2371–2381
Irie N et al (2015) SOX17 is a critical specifier of human primordial germ cell fate. Cell 160(1–2):253–268

Ishii K et al (2012) FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation. Development 139(10):1734–1743
Izadyar F et al (2003a) Autologous and homologous transplantation of bovine spermatogonial stem cells. Reproduction 126(6):765–774
Izadyar F et al (2003b) Proliferation and differentiation of bovine type A spermatogonia during long-term culture. Biol Reprod 68(1):272–281
Izadyar F et al (2011) Identification and characterization of repopulating spermatogonial stem cells from the adult human testis. Hum Reprod 26(6):1296–1306
Jahnukainen K et al (2001) Intratesticular transplantation of testicular cells from leukemic rats causes transmission of leukemia. Cancer Res 61(2):706–710
Jahnukainen K et al (2007) Effect of cold storage and cryopreservation of immature non-human primate testicular tissue on spermatogonial stem cell potential in xenografts. Hum Reprod 22(4):1060–1067
Jahnukainen K et al (2011) Testicular recovery after irradiation differs in prepubertal and pubertal non-human primates, and can be enhanced by autologous germ cell transplantation. Hum Reprod 26(8):1945–1954
Jahnukainen K et al (2012) Autologous ectopic grafting of cryopreserved testicular tissue preserves the fertility of prepubescent monkeys that receive sterilizing cytotoxic therapy. Cancer Res 72(20):5174–5178
Johnson L et al (1996) Effect of developmental age or time after transplantation on Sertoli cell number and testicular size in inbred Fischer rats. Biol Reprod 54(5):948–959
Kala S et al (2012) In vitro culture and morphological characterization of prepubertal buffalo (Bubalus bubalis) putative spermatogonial stem cell. J Assist Reprod Genet 29(12):1335–1342
Kanatsu-Shinohara M et al (2003a) Restoration of fertility in infertile mice by transplantation of cryopreserved male germline stem cells. Hum Reprod 18(12):2660–2667
Kanatsu-Shinohara M et al (2003b) Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 69(2):612–616
Kanatsu-Shinohara M et al (2005) Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod 72(4):985–991
Kanatsu-Shinohara M et al (2008) Long-term culture of male germline stem cells from hamster testes. Biol Reprod 78(4):611–617
Kanatsu-Shinohara M et al (2014) Improved serum- and feeder-free culture of mouse germline stem cells. Biol Reprod 91(4):88
Kawasaki T et al (2010) Regeneration of spermatogenesis and production of functional sperm by grafting of testicular cell aggregates in Zebrafish (Danio rerio). Biol Reprod 83(4):533–539
Kelleher S et al (2001) Long-term outcomes of elective human sperm cryostorage. Hum Reprod 16(12):2632–2639
Keros V et al (2007) Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. Hum Reprod 22(5):1384–1395
Kim Y et al (2007) Effect of donor age on success of spermatogenesis in feline testis xenografts. Reprod Fertil Dev 19(7):869–876
Kim Y et al (2008) Production of donor-derived sperm after spermatogonial stem cell transplantation in the dog. Reproduction 136(6):823–831
Kita K et al (2007) Production of functional spermatids from mouse germline stem cells in ectopically reconstituted seminiferous tubules. Biol Reprod 76(2):211–217
Kokkinaki M et al (2009) The molecular signature of spermatogonial stem/progenitor cells in the 6-day-old mouse testis. Biol Reprod 80(4):707–717
Kokkinaki M, Djourabtchi A, Golestaneh N (2011) Long-term culture of human SSEA-4 positive spermatogonial stem cells (SSCs). J Stem Cell Res Ther 2(2)
Kubota H, Avarbock MR, Brinster RL (2004a) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 101(47):16489–16494
Kubota H, Avarbock MR, Brinster RL (2004b) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71(3):722–731

Kubota H et al (2011) Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. FASEB J 25(8):2604–2614
Kuijk EW, Colenbrander B, Roelen BA (2009) The effects of growth factors on in vitro-cultured porcine testicular cells. Reproduction 138(4):721–731
Kuopio T et al (1989) Transplantation of newborn rat testis under the kidney capsule of adult host as a model to study the structure and function of Leydig cells. J Androl 10(5):335–345
Kvist K et al (2006) Cryopreservation of intact testicular tissue from boys with cryptorchidism. Hum Reprod 21(2):484–491
Langenstroth D et al (2014) Separation of somatic and germ cells is required to establish primate spermatogonial cultures. Hum Reprod 29(9):2018–2031
Lass A et al (1998) A programme of semen cryopreservation for patients with malignant disease in a tertiary infertility centre: lessons from 8 years' experience. Hum Reprod 13(11):3256–3261
Lawson KA et al (1999) Bmp4 is required for the generation of primordial germ cells in the mouse embryo. Genes Dev 13(4):424–436
Lee SJ et al (2006) American Society of Clinical Oncology recommendations on fertility preservation in cancer patients. J Clin Oncol 24(18):2917–2931
Lian G, Miller KA, Enders GC (1992) Localization and synthesis of entactin in seminiferous tubules of the mouse. Biol Reprod 47(3):316–325
Lim JJ et al (2010) Long-term proliferation and characterization of human spermatogonial stem cells obtained from obstructive and non-obstructive azoospermia under exogenous feeder-free culture conditions. Cell Prolif 43(4):405–417
Liu S et al (2011) Isolation and characterization of human spermatogonial stem cells. Reprod Biol Endocrinol 9(1):1–9
Loren AW et al (2013) Fertility preservation for patients with cancer: American Society of Clinical Oncology clinical practice guideline update. J Clin Oncol 31(19):2500–2510
Luetjens CM et al (2008) Complete spermatogenesis in orthotopic but not in ectopic transplants of autologously grafted marmoset testicular tissue. Endocrinology 149(4):1736–1747
Luo J et al (2006) Protein gene product 9.5 is a spermatogonia-specific marker in the pig testis: application to enrichment and culture of porcine spermatogonia. Mol Reprod Dev 73(12):1531–1540
Magnúsdóttir E et al (2013) A tripartite transcription factor network regulates primordial germ cell specification in mice. Nat Cell Biol 15(8):905–915
Mahmoud HK et al (2015) Allogeneic hematopoietic stem cell transplantation for non-malignant hematological disorders. J Adv Res 6(3):449–458
Manku G, Culty M (2015) Mammalian gonocyte and spermatogonia differentiation: recent advances and remaining challenges. Reproduction 149(3):R139–R157
Mayerhofer A (2013) Human testicular peritubular cells: more than meets the eye. Reproduction 145(5):R107–R116
Medrano JV et al (2016) Human spermatogonial stem cells display limited proliferation in vitro under mouse spermatogonial stem cell culture conditions. Fertil Steril 106(6):1539–1549.e8
Meistrich ML (2009) Male gonadal toxicity. Pediatr Blood Cancer 53(2):261–266
Meng X et al (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287(5457):1489–1493
Mikkola M et al (2006) Transplantation of normal boar testicular cells resulted in complete focal spermatogenesis in a boar affected by the immotile short-tail sperm defect. Reprod Domest Anim 41(2):124–128
Mirzapour T et al (2012) Effects of basic fibroblast growth factor and leukaemia inhibitory factor on proliferation and short-term culture of human spermatogonial stem cells. Andrologia 44(Suppl 1):41–55
Nagano M, Brinster RL (1998) Spermatogonial transplantation and reconstitution of donor cell spermatogenesis in recipient mice. APMIS 106(1):47–55; discussion 56–7
Nagano M et al (1998) Culture of mouse spermatogonial stem cells. Tissue Cell 30(4):389–397

Nagano M et al (2001) Transgenic mice produced by retroviral transduction of male germ-line stem cells. Proc Natl Acad Sci U S A 98(23):13090–13095
Nagano M et al (2002) Lentiviral vector transduction of male germ line stem cells in mice. FEBS Lett 524(1–3):111–115
Nagano M et al (2003) Maintenance of mouse male germ line stem cells in vitro. Biol Reprod 68(6):2207–2214
Nakaki F et al (2013) Induction of mouse germ-cell fate by transcription factors in vitro. Nature 501(7466):222–226
Naysmith TE et al (1998) Do men undergoing sterilizing cancer treatments have a fertile future? Hum Reprod 13(11):3250–3255
Nowroozi MR et al (2011) In vitro colonization of human spermatogonia stem cells: effect of patient's clinical characteristics and testicular histologic findings. Urology 78(5):1075–1081
Oatley JM, Brinster RL (2008) Regulation of spermatogonial stem cell self-renewal in mammals. Annu Rev Cell Dev Biol 24:263–286
Oatley JM et al (2004) Spermatogenesis and germ cell transgene expression in xenografted bovine testicular tissue. Biol Reprod 71(2):494–501
Oatley JM, Reeves JJ, McLean DJ (2005a) Establishment of spermatogenesis in neonatal bovine testicular tissue following ectopic xenografting varies with donor age. Biol Reprod 72(2):358–364
Oatley JM, Reeves JJ, McLean DJ (2005b) Establishment of spermatogenesis in neonatal bovine testicular tissue following ectopic xenografting varies with donor age. Biol Reprod 72(2):358–364
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(35):25842–25851
Oatley JM et al (2009) Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development 136(7):1191–1199
Oatley MJ et al (2016) Conditions for long-term culture of cattle undifferentiated spermatogonia. Biol Reprod 95(1):14
Ogawa T et al (2000) Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med 6(1):29–34
Ohinata Y et al (2005) Blimp1 is a critical determinant of the germ cell lineage in mice. Nature 436(7048):207–213
Orwig KE, Valli H, Peters KA, Freihling E, Sanfilippo JS, Cannon GM, Orwig JJ Fertility preservation program of Magee-Womens Hospital in Pittsburgh. http://fertilitypreservationpittsburgh.org
Osterberg EC et al (2014) Current practices in fertility preservation in male cancer patients. Urology Annals 6(1):13–17
Palermo G et al (1992) Pregnancies after intracytoplasmic injection of single spermatozoon into an oocyte. Lancet 340(8810):17–18
Pellegrini M et al (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(Pt 16):3363–3372
Petersen PM et al (1999) Semen quality and reproductive hormones before orchiectomy in men with testicular cancer. J Clin Oncol 17(3):941–947
Picton HM et al (2015) A European perspective on testicular tissue cryopreservation for fertility preservation in prepubertal and adolescent boys. Hum Reprod 30(11):2463–2475
Piravar Z et al (2013) In vitro culture of human testicular stem cells on feeder-free condition. J Reprod Infertility 14(1):17–22
Plant TM et al (2005) Postnatal and pubertal development of the rhesus monkey (Macaca mulatta) testis. Ann N Y Acad Sci 1061:149–162
Poels J et al (2013) Vitrification preserves proliferation capacity in human spermatogonia. Hum Reprod 28(3):578–589

Quinn GP et al (2009) Physician referral for fertility preservation in oncology patients: a national study of practice behaviors. J Clin Oncol 27(35):5952–5957
Radford J (2003) Restoration of fertility after treatment for cancer. Horm Res 59(Suppl 1):21–23
Radford J, Shalet S, Lieberman B (1999) Fertility after treatment for cancer. Questions remain over ways of preserving ovarian and testicular tissue. BMJ 319(7215):935–936
Rathi R et al (2006) Germ cell development in equine testis tissue xenografted into mice. Reproduction 131(6):1091–1098
Rathi R et al (2008) Maturation of testicular tissue from infant monkeys after xenografting into mice. Endocrinology 149(10):5288–5296
Richardson TE et al (2009) Sterile testis complementation with spermatogonial lines restores fertility to DAZL-deficient rats and maximizes donor germline transmission. PLoS One 4(7):e6308
Ryu BY et al (2003) Stem cell and niche development in the postnatal rat testis. Dev Biol 263(2):253–263
Ryu BY et al (2005) Conservation of spermatogonial stem cell self-renewal signaling between mouse and rat. Proc Natl Acad Sci U S A 102(40):14302–14307
Sa R et al (2012) Cryopreservation of human testicular diploid germ cell suspensions. Andrologia 44(6):366–372
Sadri-Ardekani H et al (2009) Propagation of human spermatogonial stem cells in vitro. JAMA 302(19):2127–2134
Sadri-Ardekani H et al (2011) In vitro propagation of human prepubertal spermatogonial stem cells. JAMA 305(23):2416–2418
Saito K et al (2003) Semen cryopreservation for patients with malignant or non-malignant disease: our experience for 10 years. Nihon Hinyokika Gakkai Zasshi 94(4):513–520
Saitou M (2009) Specification of the germ cell lineage in mice. Front Biosci (Landmark Ed) (14):1068–1087
Salomon F, Hedinger CE (1982) Abnormal basement membrane structures of seminiferous tubules in infertile men. Lab Investig 47(6):543–554
Sanger WG, Olson JH, Sherman JK (1992) Semen cryobanking for men with cancer—criteria change. Fertil Steril 58(5):1024–1027
Sato Y et al (2010) Xenografting of testicular tissue from an infant human donor results in accelerated testicular maturation. Hum Reprod 25(5):1113–1122
Sato T et al (2011a) In vitro production of functional sperm in cultured neonatal mouse testes. Nature 471(7339):504–507
Sato T et al (2011b) In vitro production of fertile sperm from murine spermatogonial stem cell lines. Nat Commun 2:472
Sato T et al (2015) In vitro spermatogenesis in explanted adult mouse testis tissues. PLoS One 10(6):e0130171
Schlatt S, Kim SS, Gosden R (2002) Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts. Reproduction 124(3):339–346
Schlatt S et al (2006) Limited survival of adult human testicular tissue as ectopic xenograft. Hum Reprod 21(2):384–389
Schlatt S et al (2010) Donor-host involvement in immature rat testis xenografting into nude mouse hosts. Biol Reprod 82(5):888–895
Schmidt KLT et al (2004) Assisted reproduction in male cancer survivors: fertility treatment and outcome in 67 couples. Hum Reprod 19(12):2806–2810
Schmidt JA, de Avila JM, McLean DJ (2006) Effect of vascular endothelial growth factor and testis tissue culture on spermatogenesis in bovine ectopic testis tissue xenografts. Biol Reprod 75(2):167–175
Sevilla J et al (2005) Hematopoietic transplantation for bone marrow failure syndromes and thalassemia. Bone Marrow Transplant 35(S1):S17–S21
Shalet SM (1993) Effect of irradiation treatment on gonadal function in men treated for germ cell cancer. Eur Urol 23(1):148–151; discussion 152

Shinohara T et al (2001) Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc Natl Acad Sci U S A 98(11):6186–6191
Shinohara T et al (2002) Birth of offspring following transplantation of cryopreserved immature testicular pieces and in-vitro microinsemination. Hum Reprod 17(12):3039–3045
Shirazi A, Khadivi A, Shams-Esfandabadi N (2014) Male pronuclear formation using dog sperm derived from ectopic testicular xenografts, testis, and epididymis. Avicenna J Med Biotechnol 6(3):140–146
Siu MK, Cheng CY (2008) Extracellular matrix and its role in spermatogenesis. Adv Exp Med Biol 636:74–91
Skinner MK, Tung PS, Fritz IB (1985) Cooperativity between Sertoli cells and testicular peritubular cells in the production and deposition of extracellular matrix components. J Cell Biol 100(6):1941–1947
Smith JF et al (2014) Testicular niche required for human spermatogonial stem cell expansion. Stem Cells Transl Med 3(9):1043–1054
Snedaker AK, Honaramooz A, Dobrinski I (2004) A game of cat and mouse: xenografting of testis tissue from domestic kittens results in complete cat spermatogenesis in a mouse host. J Androl 25(6):926–930
Staub C (2001) A century of research on mammalian male germ cell meiotic differentiation in vitro. J Androl 22(6):911–926
Steinberger A, Steinberger E (1965) Differentiation of rat seminiferous epithelium in organ culture. J Reprod Fertil 9:243–248
Steinberger A, Steinberger E (1966) Stimulatory effect of vitamins and glutamine on the differentiation of germ cells in rat testes organ culture grown in chemically defined media. Exp Cell Res 44(2):429–435
STEINBERGER E, STEINBERGER A, PERLOFF WH (1964) Initiation of spermatogenesis in vitro. Endocrinology 74(5):788–792
Stephens SM, Arnett DM, Meacham RB (2013) The use of in vitro fertilization in the management of male infertility: what the urologist needs to know. Rev Urology 15(4):154–160
Steptoe PC, Edwards RG (1978) Birth after the reimplantation of a human embryo. Lancet 2(8085):366
Storb R et al (2001) Cyclophosphamide and antithymocyte globulin to condition patients with aplastic anemia for allogeneic marrow transplantations: the experience in centers. Biol Blood Marrow Transplant 7(1):39–45
Szell AZ et al (2013) Live births from frozen human semen stored for 40 years. J Assist Reprod Genet 30(6):743–744
Takashima S et al (2015) Functional differences between GDNF-dependent and FGF2-dependent mouse spermatogonial stem cell self-renewal. Stem Cell Rep 4(3):489–502
Tournaye H, Dohle GR, Barratt CL (2014) Fertility preservation in men with cancer. Lancet 384(9950):1295–1301
Toyooka Y et al (2003) Embryonic stem cells can form germ cells in vitro. Proc Natl Acad Sci 100(20):11457–11462
Trowell OA (1959) The culture of mature organs in a synthetic medium. Exp Cell Res 16(1):118–147
Unni S et al (2012) Efficient cryopreservation of testicular tissue: effect of age, sample state, and concentration of cryoprotectant. Fertil Steril 97(1):200–208. e1
Valli H et al (2014a) Germline stem cells: toward the regeneration of spermatogenesis. Fertil Steril 101(1):3–13
Valli H et al (2014b) Fluorescence- and magnetic-activated cell sorting strategies to isolate and enrich human spermatogonial stem cells. Fertil Steril 102(2):566–580. e7
Valli H, Gassei K, Orwig KE (2015) Stem cell therapies for male infertility: where are we now and where are we going? In: Carrell DT et al (eds) Biennial review of infertility, vol 4. Springer, Cham, pp 17–39
Van Saen D et al (2011) Meiotic activity in orthotopic xenografts derived from human postpubertal testicular tissue. Hum Reprod 26(2):282–293

Vigersky RA et al (1982) Testicular dysfunction in untreated Hodgkin's disease. Am J Med 73(4):482–486
Volkmann J et al (2011) Disturbed spermatogenesis associated with thickened lamina propria of seminiferous tubules is not caused by dedifferentiation of myofibroblasts. Hum Reprod 26(6):1450–1461
Walker WH, Cheng J (2005) FSH and testosterone signaling in Sertoli cells. Reproduction 130(1):15–28
Wallace WH, Anderson RA, Irvine DS (2005) Fertility preservation for young patients with cancer: who is at risk and what can be offered? Lancet Oncol 6(4):209–218
Wang J, Sauer MV (2006) In vitro fertilization (IVF): a review of 3 decades of clinical innovation and technological advancement. Ther Clin Risk Manag 2(4):355–364
Ward E et al (2014) Childhood and adolescent cancer statistics, 2014. CA Cancer J Clin 64(2):83–103
Weber S et al (2010) Critical function of AP-2gamma/TCFAP2C in mouse embryonic germ cell maintenance. Biol Reprod 82(1):214–223
Wenzel L et al (2005) Defining and measuring reproductive concerns of female cancer survivors. J Natl Cancer Inst Monogr (34):94–98
Wistuba J et al (2006) Meiosis in autologous ectopic transplants of immature testicular tissue grafted to Callithrix jacchus. Biol Reprod 74(4):706–713
Wu X et al (2009) Prepubertal human spermatogonia and mouse gonocytes share conserved gene expression of germline stem cell regulatory molecules. Proc Natl Acad Sci 106(51):21672–21677
Wu X et al (2012) Fertile offspring derived from mouse spermatogonial stem cells cryopreserved for more than 14 years. Hum Reprod 27(5):1249–1259
Wyns C et al (2007) Spermatogonial survival after cryopreservation and short-term orthotopic immature human cryptorchid testicular tissue grafting to immunodeficient mice. Hum Reprod 22(6):1603–1611
Wyns C et al (2008) Long-term spermatogonial survival in cryopreserved and xenografted immature human testicular tissue. Hum Reprod 23(11):2402–2414
Wyns C et al (2011) Management of fertility preservation in prepubertal patients: 5 years' experience at the Catholic University of Louvain. Hum Reprod 26(4):737–747
Yabuta Y et al (2006) Gene expression dynamics during germline specification in mice identified by quantitative single-cell gene expression profiling. Biol Reprod 75(5):705–716
Yamaji M et al (2008) Critical function of Prdm14 for the establishment of the germ cell lineage in mice. Nat Genet 40(8):1016–1022
Yokonishi T et al (2013) Vitro reconstruction of mouse seminiferous tubules supporting germ cell differentiation. Biol Reprod 89(1):15
Yokonishi T et al (2014) Offspring production with sperm grown in vitro from cryopreserved testis tissues. Nat Commun 5:4320
Yoshida S, Sukeno M, Nabeshima Y (2007) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317(5845):1722–1726
Yu J et al (2006) Development of neonatal mouse and fetal human testicular tissue as ectopic grafts in immunodeficient mice. Asian J Androl 8(4):393–403
Zebrack BJ et al (2004) Fertility issues for young adult survivors of childhood cancer. Psychooncology 13(10):689–699
Zeng W et al (2006) The length of the spermatogenic cycle is conserved in porcine and ovine testis xenografts. J Androl 27(4):527–533
Zhang Z et al (2008) Bovine sertoli cells colonize and form tubules in murine hosts following transplantation and grafting procedures. J Androl 29(4):418–430
Zheng Y et al (2014) Quantitative detection of human spermatogonia for optimization of spermatogonial stem cell culture. Hum Reprod 29(11):2497–2511
Zhou Q et al (2016) Complete meiosis from embryonic stem cell-derived germ cells in vitro. Cell Stem Cell 18(3):330–340
Zohni K et al (2012) CD9 is expressed on human male germ cells that have a long-term repopulation potential after transplantation into mouse testes. Biol Reprod 87(2):27

Application of Spermatogonial Transplantation in Agricultural Animals

14

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Abstract

Spermatogenesis is a precisely orchestrated and efficient process that relies on the presence of spermatogonial stem cells (SSCs) in the testes to maintain the continuous production of spermatozoa. In addition to their ability to reestablish male fertility upon transplantation, the study of SSCs has been recently stimulated by their potential to transmit genetic modifications to the offspring. Introduction of genetic changes through male germline modifications could overcome problems associated with the prevailing methods of generating genetically modified large animals. Male germ cell transplantation, a technique pioneered in the mouse, has been successfully performed in several domestic species. However, there are several limitations to its widespread application. Establishment of standard protocols for recipient preparation is required for each species in order to increase the efficiency of donor SSC colonization and the representation of the donor haplotype in offspring. Characterization of spermatogonia from large animal species showed a variable degree of conservation in the expression patterns of several genes that are known to play important roles in rodent germ cells. The functional significance of those genes in spermatogenesis of large animal species remains to be determined. Despite ongoing efforts, a long-term culture system that supports, expands and maintains SSCs from agricultural animals has not yet been developed. Future efforts to increase utility of

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germ cell transplantation in agricultural animals would likely be directed to the identification of factors produced in the SSC niche that are essential to SSC maintenance and the establishment of a reliable and robust culture system for SSCs from agricultural animals.

Keywords
Germ cell transplantation • Gonocytes • Large animal models • Livestock • Spermatogonia • Spermatogonial stem cells • Transgenesis

14.1 Introduction

Spermatogenesis is the process by which germ cells develop from diploid spermatogonia to haploid spermatozoa in the seminiferous tubules of the parenchyma of the testis. This process is constantly supplying large numbers of spermatozoa, from puberty throughout the reproductive life of a male due to the existence of a stem cell pool, the spermatogonial stem cells (SSCs) (De Jonge and Barratt 2006; Kerr et al. 2006). The SSCs, in a tightly regulated testicular environment, will either self-renew to produce more stem cells that will maintain the lineage or will commit to differentiation to give rise to the male gamete.

The highly organized process of spermatogenesis encompasses different cell associations or stages of the seminiferous epithelium. The changes that occur in a specific area of the seminiferous epithelium between two appearances of the same developmental stage constitute the cycle of the seminiferous epithelium (Leblond and Clermont 1952). The length of the cycle of the seminiferous epithelium is constant and under the control of the germ cell phenotype (Franca et al. 1998). The cycle length is species-specific and determines the duration of spermatogenesis, lasting from 30 to 75 days in mammals (Franca et al. 2005). Determining the cycle of the seminiferous epithelium is essential to understand spermatogenesis and quantify spermatogenic efficiency, i.e. the number of spermatozoa produced per gram of testicular parenchyma (Johnson et al. 2000) and to enable comparisons among species (Johnson et al. 2000). Spermatogenesis is a very efficient process. Based on the number of divisions, a spermatogonium is capable to produce 2048–4096 of spermatozoa (de Rooij and Russell 2000; Russell et al. 1990). However, only 2–3 spermatozoa, out of a possible of 10, are generated form each type A1 spermatogonium in most mammals (Franca et al. 2005) due to the germ cell loss that occurs during spermatogenesis. Comparative data regarding the length of the seminiferous cycle, the duration and efficiency of spermatogenesis in farm and domestic species are represented in Table 14.1.

In farm and companion animals, the spermatogonial subtypes and the number of differentiated spermatogonial generations is similar to those in mouse and rat, whereas in primates, including man, the number of spermatogonial generations and subtypes differ (Franca et al. 2005). Historically, spermatogonia were classified by their chromatin characteristics in type A (do not display heterochromatin),

Table 14.1 Comparative data on the number of differentiated spermatogonial generations, duration of the cycle of the seminiferous epithelium, duration of spermatogenesis and efficiency of spermatogenesis in several mammalian species

Species	Spermatogonial generations	Seminiferous epithelium cycle length (days)	Duration of spermatogenesis (days)	Spermatogenic efficiency[a]
Boar	6 (A1–A4, In, B)	8.6–9.0	38.7–40.5	23
Bull	6 (A1 to A3, In, B1–B2)	13.5	60.8	12
Ram and buck	6 (A1–A3, In, B1–B2)	10.6	47.7	21 (ram); 30 (buck)
Cat	6 (A1–A4, In, B)	10.4	46.8	16
Dog	6 (A1–A4, In, B)	13.6	61.2	17
Stallion	5 (A1–A3, B1–B2)	12.2	54.9	16–19
Donkey (mule)	5 (A1–A3, B1–B2)	10.5 (10.1)	47.2 (45.5)	42
Mouse	6 (A1–A4, In, B)	8.6	38.7	4
Rat	6 (A1–A4, In, B)	12.9	58.0	20–24

Type A spermatogonia (A); intermediate spermatogonia (In) and type B spermatogonia (B). (Franca et al. 1999, 2005; Franca and Godinho 2003; Neves et al. 2002, 2014; Blanco-Rodriguez 2002; Soares et al. 2009; Auharek et al. 2011; Johnson et al. 2000; Klein 2012; Leal et al. 2004)

[a]Number of spermatozoa produced per gram of testicular parenchyma ($\times 10^6$)

intermediate (In, intermediate amount of chromatin), and type B spermatogonia (heterochromatin present in the nucleus), with type B being at a more differentiated stage than the type A spermatogonia. Within the type A spermatogonia, the most primitive cell is the A-single (As), morphologically characterized by the absence of intercellular bridges. These As spermatogonia divide by mitosis giving rise to pairs (Apr) and then, chains of 4–16 aligned (Aal) spermatogonia. Aligned spermatogonia will give rise to a set of "differentiated" generations of spermatogonia: A1–A4; In; and B1–B2 subtypes with some variations according to the species (Table 14.1). The As spermatogonia are generally considered as SSCs, but stem cell activity is defined based on behavioral criteria, rather than on morphological features. The Aal spermatogonia and the majority of Apr will give rise to differentiated generations of spermatogonia.

Three major schemes of spermatogonial multiplication and stem cell renewal have been proposed (de Rooij and Griswold 2012), but there is controversy in the field as to which one is representative of the in vivo situation. All the data have been generated from studies in the mouse and in the case of non-rodent species, very little is known about the kinetics of spermatogonia in the testis.

Based on the unique properties of SSCs, they have great potential for preserving and restoring male fertility, but also have created interest in the generation of genetically modified animals. Since genetic modifications introduced in the SSCs genome will be transmitted to the offspring and maintained across generations, the transplantation of male germ cells arose as a powerful technique in the process of generating genetically modified animals for improving agricultural traits or as models of human diseases.

Although poultry and aquaculture species are also considered as agricultural animals, the information included in this book chapter describes current knowledge on the male germline in agricultural mammals, including the molecular characterization of gonocytes (also known as prospermatogonia) and spermatogonia; described efforts to culture these cells and the advancements achieved with the use of spermatogonia transplantation. The SSC transplantation technique was adapted in the last decade to fish species, and it is quickly evolving and opening new avenues in the application of reproductive technologies in these species with promising results. The relative simplicity of germ cell transplantation in fish species, the plasticity of SSCs to generate both spermatogonia and oogonia, and the capability of SSCs to colonize and generate gametes in xenogenic host fish species with good adaptability to captivity and relatively simple life cycles make SSCs transplantation a transferable technology for the recovery of endangered populations of fish and has applications for aquaculture of commercial fish species. For more information on SSC physiology, germ cell transplantation and its application in fish species the reader is referred to the published literature in the field (Lacerda et al. 2012; Yoshizaki et al. 2011, 2012). In avian species, germ cell transplantation has immediate applications for avoiding genetic erosion and the generation of genetically modified birds, especially those technologies involving the use of primordial germ cells (PGCs). Unlike in other species, avian PGCs use the bloodstream for their transport to the future gonad during embryonic development (reviewed in Nakamura et al. (2013)), which greatly facilitates their transplantation into recipients. The advantages of using PGCs in avian species is that both male and female fertility can be restored and that a long-term culture for PGCs has been established, allowing their propagation and manipulation (Lee et al. 2015; Nakamura et al. 2013).

14.2 Germ Cell Transplantation in Large Animals

In 1994, the germ cell transplantation (GCT) technique developed by Brinster and colleagues revolutionized the field of study on the male germline in mammals. Studies demonstrated that germ cells from a donor mouse testis, when transplanted into the seminiferous tubules of an infertile recipient testis, were able to colonize the recipient testis and reestablished long-term donor-derived spermatogenesis (Brinster and Avarbock 1994; Brinster and Zimmermann 1994). It is generally accepted that only SSCs in the donor cell suspension are able to colonize the stem cell niche in the seminiferous epithelium of recipient mice and initiate spermatogenesis. Pioneered in rodents, GCT has later been adapted and extended to large animal species such as sheep, goats, cattle, and pigs (Izadyar et al. 2003b; Herrid et al. 2006; Honaramooz et al. 2002, 2003a; Rodriguez-Sosa et al. 2006) (Table 14.2).

To date, germ cell transplantation is the only unequivocal functional assay to determine SSC activity in a cell population. It contributed significantly to our understanding of various aspects of SSC biology such as self-renewal and differentiation, homing and colonization, as well as Sertoli cell-SSC interaction (Nagano et al. 1999; Nagano 2003; Oatley et al. 2006, 2011b). The GCT technique also provides

Table 14.2 Germ cell transplantation in agricultural animals

Species	Recipient preparation	Donor SSCs	Donor-derived sperm	Transmission of donor haplotype	References
Pig	Busulfan	Homologous; wild-type	Yes	ND	Mikkola et al. (2006)
	None	Homologous; transgenic	Yes	Yes	Zeng et al. (2013)
	In utero busulfan	Homologous; transgenic	Yes	Yes	Zeng et al. (2013)
Goat	None	Homologous; transgenic	Yes	Yes	Honaramooz et al. (2003b)
	Irradiation	Homologous; transgenic	Yes	Yes	Honaramooz et al. (2008)
	Irradiation	Homologous; transgenic	Yes	Yes	Zeng et al. (2012)
Sheep	Irradiation	Homologous; microsatellite[a]	Yes	Yes	Herrid et al. (2009)
	None	Homologous; transgenic	ND[b]	ND	Rodriguez-Sosa et al. (2009)
Cattle	Irradiation	Autologous and homologous	ND[c]	ND	Izadyar et al. (2003b)
	No	Heterologous (*Bos taurus* to *Bos indicus*); microsatellite	Yes	ND	Stockwell et al. (2009)

ND not determined

[a]Donor-derived sperm were identified based on microsatellite analysis

[b]Recipient testes were analyzed 2 months after transplantation by immunohistochemistry. Donor-derived spermatogenesis was not investigated

[c]Spermatozoa were present in the tubules of transplanted recipients based on histology. Donor-derived spermatogenesis could not be distinguished from the endogenous spermatogenesis due to the lack of a genetic marker

an exciting alternative for generating large transgenic animals as any genetic modifications engineered in SSCs can be passed on to next generations (Zeng et al. 2012, 2013; Honaramooz et al. 2003b, 2008).

14.2.1 Principle

In general, the GCT technique involves three coordinated steps that together contribute to the outcome of GCT. Firstly, recipient animals need to be properly prepared in advance if necessary. Secondly, a cell suspension containing germ cells is prepared from either fresh tissue or culture prior to transplantation. Thirdly, the cell suspension needs to be injected to the lumen of the seminiferous tubules.

In rodents, germ cells can be injected into the seminiferous tubules through three routes: directly into the exposed seminiferous tubules, indirectly into the tubules

through the efferent ducts or through the rete testis (Ogawa et al. 1997). The injection through the efferent ducts is the most widely used approach due to its simplicity and easy accessibility. In large animals, the ultrasound-guided injection into the rete testis is the most efficient and widely practiced approach for GCT due to the differences in testis anatomy (Schlatt et al. 1999).

There are two approaches that can be taken to improve the outcome of germ cell transplantation: (1) prepare recipient testis in a way that endogenous germ cells are depleted to create empty niches for transplanted SSCs; (2) increase the number of SSCs in the donor cell suspension so that more SSCs can colonize the niches and contribute to donor-derived spermatogenesis.

14.2.2 Recipient Preparation

In mice, recipients need to be immunologically tolerant to donor testicular cells. This means that the recipient and the donor are either genetically matched or the recipient is immune-deficient. In the case of large animals such as pigs, goats, and cattle, homologous germ cell transplantation (into unrelated same-species animals) is feasible without any apparent immune reaction (Izadyar et al. 2003b; Honaramooz et al. 2002, 2003a). This bears significant importance in the practical application of GCT in large animals for which matching animals genetically would be a daunting and expensive task.

In rodents, the colonization efficiency of transplanted SSCs can be significantly improved in recipient testis where endogenous SSCs have been depleted (Ogawa et al. 1999). As endogenous SSCs occupy the SSC niche for which transplanted SSCs have to compete, it is desirable to eliminate endogenous SSCs. Alternatively, males which are naturally devoid of spermatogenesis (e.g. *W/Wv* mice) can be used as recipients for germ cell transplantation (Brinster and Zimmermann 1994; Ogawa et al. 2000). The immature mouse pup testis (day 6) has also been used as recipient as it provides a more accessible and favorable microenvironment to transplanted SSCs (Shinohara et al. 2001).

Since there are no naturally infertile recipients readily available for germ cell transplantation in large animals, germ cell depletion by irradiation or busulfan has been performed to prepare recipients (see Table 14.2). Regardless of the methods implemented, the key to a successful recipient preparation is a balance act between the effectiveness of the depletion of endogenous spermatogonia and the maintenance of functional integrity of the somatic cell microenvironment.

Busulfan, a DNA-alkylating agent that targets proliferating cells, is widely used for conditioning adult rodent testis for transplantation. However, its application in large animals has been very limited. So far, it has only been used in pigs to prepare recipients (Honaramooz et al. 2005; Mikkola et al. 2006). The administration of busulfan to pregnant sows at late gestation proved to be more successful in generating male recipients than post-natal treatment of piglets (Honaramooz et al. 2005). The prepubertal piglets that received busulfan at effective doses succumbed to systematic toxicity, resulting in unacceptable high mortality rate (Honaramooz et al.

2005). Busulfan was also given orally for 4 consecutive days to adult recipients 5 weeks prior to transplantation to suppress endogenous spermatogenesis (Mikkola et al. 2006). However, it is not possible to evaluate the effectiveness and applicability of this treatment scheme due to the very small number ($n = 2$) of experimental animals and the very specific genotype of recipients (homozygous mutant with an immotile short-tail sperm defect).

Irradiation appears to be the preferred method for germ cell depletion in large animals (Table 14.2). It offers local treatment of recipient testes, sparing systemic effects seen with chemotherapeutic drugs. However, the irradiation equipment is expensive and the procedure requires anesthesia to perform the treatment efficiently. Irradiation dosage, frequency, and the age of animal at the time of treatment are variable between species (Izadyar et al. 2003b; Honaramooz et al. 2005; Herrid et al. 2009). Therefore, practical protocols need to be established and optimized for each species to achieve desirable results.

The recipient treatment scheme that uses either irradiation or cytotoxic drugs has presented a challenge for the widespread application and commercialization of GCT in agricultural animals due to the cost and the lack of predictable and consistent responses in recipients. This obstacle was alleviated by research showing that depletion of endogenous SSCs is not required for germ cell transplantation into immature testis of large animals such as goats, pigs, sheep, and cattle (Zeng et al. 2013; Honaramooz et al. 2003b; Herrid et al. 2006; Rodriguez-Sosa et al. 2009). This finding suggests that there may be vacant niches easily accessible in the prepubertal testis for transplanted cells to colonize. Additionally, the structure of the seminiferous epithelium in immature testis likely provides a more hospitable environment for colonization and expansion of transplanted SSCs as demonstrated in mice (Shinohara et al. 2001). Although depletion of endogenous spermatogonia is not required, it may still be advantageous when using adult recipients. In one research report using adult sheep as recipients, preparation of recipients by irradiation was shown to enhance the success rate of GCT and increase the proportion of donor-derived spermatozoa (Herrid et al. 2009). How applicable this finding is to other large animal species remains to be determined.

Unless an infertile recipient is used, donor-derived spermatogenesis eventually faces competition from endogenous spermatogenesis in the recipient testis regardless of whether an immature or depleted testis is used. Although it is possible to detect the presence of the donor haplotype in ejaculates with the aid of molecular techniques, transmission of the donor haplotype to the next generations by breeding or IVF is a daunting and expensive task for large animals. An ideal recipient would be one with a functional testicular environment but devoid of endogenous spermatogenesis, such as *W* mutant mice. A recent report on the generation of DAZL knockout pigs by engineered nucleases presents an exciting possibility of using those mutants as infertile recipients (Tan et al. 2013). Mice that are deficient for DAZL are infertile and their testis can support full spermatogenesis of wild-type donor SSCs (Schrans-Stassen et al. 2001; Rilianawati et al. 2003). The fertility status of DAZL knockout pigs is currently under investigation and its applicability as recipients remains to be determined.

14.2.3 Donor Cell Preparation

The efficiency of germ cell transplantation is also dependent on the relative abundance of SSCs in the donor cell preparation. SSCs represent a rare cell population with an estimated number of 35,000 per testis, constituting ~0.03% of all germ cells in the adult mouse testis (Tegelenbosch and de Rooij 1993). In rodents, several strategies have been implemented to increase the proportion of undifferentiated spermatogonia (including SSCs) in donor cell preparations by either excluding/eliminating differentiating germ cells or separating germ cells from somatic cells. Neonatal and prepubertal testes have been used as the preferred source for donor cells as gonocytes/spermatogonia are the only type of germ cells present in the seminiferous tubules during those developmental stages (Bellve et al. 1977). Surgical induction of cryptorchidism in murine donors effectively eliminated differentiating germ cells and resulted in ~25 fold of enrichment of SSCs in a testicular cell preparation (Shinohara et al. 2000a). Vitamin A deficiency and hyperthermical treatment of donor testis have also been shown to provide an enriched in vivo source of mouse SSCs (McLean et al. 2002).

In vitro enrichment of SSCs can be achieved by using cell separation procedures that take advantage of differences in density and size of various cell populations (such as Percoll gradient centrifugation) or that relies on differential adhesion properties of somatic cells and germ cells to a substratum in culture (such as differential plating) (Bellve et al. 1977; van Dissel-Emiliani et al. 1989; Morena et al. 1996; Luo et al. 2006; Bahadorani et al. 2012). With the identification of surface markers present on a subset(s) of spermatogonia, magnetic-activated cell sorting (MACS) and fluorescence-activated cell sorting (FACS) have been used to facilitate SSC enrichment by using antibodies against surface markers such as Thy1, GFR-α1, c-kit, CD9, Integrin-α6, and Integrin-β1 (Kanatsu-Shinohara et al. 2004; Shinohara et al. 2000b; Kubota et al. 2004; Ebata et al. 2005). Efficiency of SSC enrichment is dependent on several factors such as species, the age of donor testis, protocols and reagents used. Although most of those approaches generally yield enrichment of SSCs (several fold change), a combination of approaches can result in a higher degree of SSC enrichment. For example, a combination of in vivo and in vitro enrichment procedures resulted in a 166-fold enrichment of mouse SSCs (Shinohara et al. 2000b).

Unlike in rodents where surface markers have been routinely and reliably used for enriching SSCs, the prevailing methods for enriching SSCs from agricultural animals are differential plating, Percoll density gradient centrifugation, or a combination of both (summarized in Table 14.4). A highly enriched cell population with ~50–90% gonocytes/spermatogonia can be obtained by using those enrichment methods and neonatal/prepubertal donors (Luo et al. 2006; Izadyar et al. 2003a; Aponte et al. 2006; Fujihara et al. 2011; Oatley et al. 2016). Due to big variations among the breed and age of donor animals and experimental procedures that different research groups used, it is difficult to identify a surface marker that can be reliably and consistently used for enriching SSCs in agricultural animals (reviewed in González and Dobrinski 2015). So far, among all the surface markers reported for rodents, only Thy1 seemed to be a

Table 14.3 Summary of current approaches to generate genetically modified large animals

Technique	Methodology	Advantages	Disadvantages	Species/application/genetically modified animal	References
PN injection	Microinjection of foreign DNA into one of the pronuclei (usually the male pronucleus) of a zygote	– One of the few available techniques in large animals for generating genetically modified animals	– Random integration of the transgene and silencing – Requires specialized equipment and advanced technical skills – Low efficiency – Variable expression of the transgene	– Ovine model of Huntington's disease – Transgenic cattle, goats, sheep, and pigs	Jacobsen et al. (2010) Reviewed in Robl et al. (2007)
ESC-based	In vitro modifications of ES cells and injection into an embryo where the ES cells will contribute to the germline	– Precise gene alterations through HR	– Only available in mice – No ESCs with germline competency available in agricultural animals	– Not achieved in large animals	NA

(continued)

Table 14.3 (continued)

Technique	Methodology	Advantages	Disadvantages	Species/application/genetically modified animal	References
Somatic cell nuclear transfer (SCNT)	Injection/fusion of the modified donor cell into enucleated MII oocytes. This is followed by the activation of the reconstructed oocyte and embryo culture	– Precise gene alterations through HR – Ratio of genetically modified offspring per offspring born is high	– Gene targeting is done in somatic cells which have low frequency of HR and limited lifespan in culture – Requires specialized equipment and advanced technical skills – Low efficiency – SCNT-generated embryos had low developmental competence	– Several genetically modified pigs as biomedical models and other domestic species, including cattle, goat, sheep, dog, and cat	Cho et al. (2009), Luo et al. (2012), Rogers et al. (2008), Gomez et al. (2009), Hong et al. (2009), Jeong et al. (2012), Kim et al. (2011), McCreath et al. (2000), Schnieke et al. (1997), Wang et al. (2008), Yin et al. (2008), and Zhang et al. (2014)
Hand-made cloning (HMC)	ZP is digested and the nucleus of the oocyte is removed with blades, followed by fusion of the ooplast with the modified somatic cell and subsequent oocyte activation	– Simplified methodology of SCNT – Allows the fusion of several cytoplasts to increase the developmental competence of the reconstructed embryo	– Gene targeting is done in somatic cells which have low frequency of HR and limited lifespan in culture – Low efficiency	– Pig (Alzheimer's disease)	Kragh et al. (2009)
				– Pig (BRCA1 KO, breast cancer model)	Luo et al. (2011)
				– Sheep (rich in omega-3 fatty acids)	Zhang et al. (2013)

Sperm-mediated gene transfer (SMGT)	Use of the spermatozoon as a natural carrier for transferring genetic material into an oocyte, through DNA binding to the head of the male gamete	– Low cost and simplicity – Theoretically feasible in multiple species due to the easy access to sperm collection	– Low efficiency – Random integration and mosaicism	– Pigs expressing the hDAF transgene	Reviewed in Luo et al. (2012)
Intracytoplasmic sperm injection-mediated gene transfer (ICSI-SMGT)	Extension of SMGT, but by injecting the spermatozoon into the cytoplasm of an oocyte	– Introduction of large fragments of DNA	– Requires micromanipulation, advanced technical skills and specialized equipment	– Transgenic pig for type 3 diabetes	Umeyama et al. (2009)
SSC-based	In vitro genetic modifications of SSCs and intratesticular transplantation into recipients where the donor cells will resume spermatogenesis and result in transgenic sperm	– Already committed to the germ cell lineage – Circumvents problems associated with embryo manipulation – Requirement for equipment and technical skills less demanding than for micromanipulations – Shortens the time required to generate modified animals	– SSCs proliferate slowly – No efficient culture systems in species other than rodents which makes SSC manipulation difficult in livestock species	– EGFP transgene was transmitted through modifications in the male germline in pigs (transgene detected in sperm and IVF-generated embryos)	Honaramooz et al. (2008) and Zeng et al. (2013)
				– Nonviral gene delivery into goat male germline generated transgenic sperm	Zeng et al. (2012)
				– Genetic manipulations of male germline resulted in transgenic sperm in dogs	Harkey et al. (2013)

EGFP enhanced green fluorescent protein, *ESC* embryonic stem cell, *hDAF* human decay-accelerating factor, *HR* homologous recombination, *IVF* in vitro fertilization, *KI* knock-in, *KO* knock-out, *MII* metaphase of the meiosis II, *NA* not applicable, *PN* pronuclear, *SSC* spermatogonial stem cell, *ZP* zona pellucida

Table 14.4 Summary of in vitro culture conditions for male germ cells in agricultural animals

Species	Age of donor	Starting cell population	Enrichment method	Best culture conditions presented[a]	Culture length	Outcome[b]	References
Pig	2 and 10 weeks	Enriched gonocytes (9.3% UCH-L1^{+ve}) Enriched spermatogonia (41–70% UCH-L1^{+ve})	Differential plating (2 weeks) StaPut velocity sedimentation + differential plating (10 weeks)	DMEM + 5% FBS at 37 °C	2–3 weeks	– Colonies formed contained cells with different morphology (round and elongated) – Colonies contained UCH-L1^{+ve} cells	Luo et al. (2006)
	2 days to 3 weeks	Enriched gonocytes (69.6% DBA^{+ve})	Discontinuous Percoll gradient centrifugation	DMEM/F12 + 10% FBS at 37 °C	7 days	– Flat focal colonies that contained pairs and chains of DBA^{+ve} cells started to form within 2–3 days – Colonies grew three dimensional, resembling murine ESCs by day 7	Goel et al. (2007)
	3–4 days	Enriched gonocytes (no information on gonocyte percentage)	Differential plating	SSCM + LIF (1000 U/mL) + GDNF (10 ng/mL) + EGF (20 ng/mL) + bFGF (10 ng/mL) on laminin-coated plates at 37 °C	Up to 9 passages (passage every 4–6 days)	– Three types of colonies observed after 8–14 days. One type resembled mouse SSC lines – SSC-like colonies cultured on laminin-coated plates, grew as a monolayer and could be cultured up to 9 passages	Kuijk et al. (2009)

Pig	1 week	Enriched gonocytes (16% UCH-L1^{+ve})	Differential plating	DMEM + 1% FBS + bFGF (5 ng/mL) and EGF (10 ng/mL) at 37 °C	20–30 days	– Colonies of various sizes were observed in culture – Xenotransplantation of cultured cells into mouse testes showed the presence of labeled pig cells after 2 months	Zheng et al. (2013)
	5 days	Non-enriched testicular cell suspension	NA	SSCM + mEGF (20 ng/mL) + bFGF (10 ng/mL) + GDNF (10 ng/mL) + LIF (1000 U/mL) at 31 °C	Up to 8 passages (passage every 7–8 days)	– 31 °C supported gonocyte culture better than 34 and 37 °C – Round and compact colonies appeared after 3 days of culture and remained compact along culture – Colonies were maintained in culture over 8 passages at 31 °C – Labeled cultured cells colonized mouse testis	Lee et al. (2013)
Goat	1 month	Non-enriched testicular cell suspension	NA	DMEM + 10% FCS + GDNF (40 ng/mL), LIF (100 ng/mL), bFGF (10 ng/mL), EGF (20 ng/mL) at 38 °C	2 weeks	– Somatic cells became confluent within a few days – Some colonies of mixed germ cells and somatic cells formed in 2 weeks of culture	Heidari et al. (2012)

(continued)

Table 14.4 (continued)

Species	Age of donor	Starting cell population	Enrichment method	Best culture conditions presented[a]	Culture length	Outcome[b]	References
	20–40 days (neonatal)	Enriched gonocytes (9.2% VASA^{+ve})	Differential plating on DSA	Rat SSC medium + GDNF (40 ng/mL), bFGF (20 ng/mL), EGF (20 ng/mL) + 1% FBS at 37 °C	7 days	– Mixed colonies of somatic and germ cells formed at day 4 of culture – High serum concentrations (>5%) resulted in over proliferation of somatic cells	Bahadorani et al. (2012)
Goat	New born to adult	Enriched gonocytes/ spermatogonia (~75% Integrin-α6^{+ve})	Differential plating on fibronectin, laminin, gelatin, or Matrigel	DMEM +20% KSR + bFGF (5 ng/mL) + 2.5 uM BIO at 37 °C	7 days and longer	– Colonies started to form at day 4 of culture – Cell culture from younger animals (<2 month) was maintained longer than culture from older animals by a few more passages	Zhu et al. (2013)
	1–2 weeks	Enriched gonocytes (no information on germ cell percentage)	Differential plating on DSA	BM + GDNF (100 ng/mL) + IGF1 (10 ng/mL) + bFGF (10 ng/mL) at 37 °C	7 days	– Mixed colonies of somatic and germ cells appeared at day 4 of culture – Labeled cultured cells persisted in recipient mouse testis after 4–5 weeks post-transplantation	Bahadorani et al. (2015)

Cattle	5 months	Enriched type A spermatogonia (65–87%)	Differential plating + discontinuous Percoll gradient centrifugation	MEM + 2.5% FCS at 37 °C	2–4 weeks (short term) Up to 150 days (long term)	– Type A spermatogonial colonies with intercellular bridges were observed within 2–4 weeks – Most spermatogonia underwent differentiation and ultimately cells with molecular and morphological characteristics of spermatocytes and spermatids were formed	Izadyar et al. (2003a)
Cattle	4–6 months	Enriched type A spermatogonia (~72%)	Differential plating + discontinuous Percoll gradient centrifugation	MEM + 2.5% FCS + GDNF (100 ng/mL) at 37 °C	4–25 days	– Small clones with single, pairs and chains of type A spermatogonia were present within a week – GDNF enhanced spermatogonial survival and ratio of Asingle/Apaired-Aaligned – Germ cell transplantation into mouse testis revealed two fold increase in the stem cell activity after exposure to GDNF	Aponte et al. (2006)

(continued)

Table 14.4 (continued)

Species	Age of donor	Starting cell population	Enrichment method	Best culture conditions presented[a]	Culture length	Outcome[b]	References
	4–6 months	Enriched type A spermatogonia (50–80%)	Differential plating + discontinuous Percoll gradient centrifugation	SSCM + 1% FCS + LIF (100 ng/mL), EGF (20 ng/mL), bFGF (10 ng/mL), GDNF (40 ng/mL) at 37 °C	15 days (short-term) 26–30 days (long-term)	– Compact somatic-germ cell colonies developed during short-term culture (15 days) – Sections of colonies revealed organized round structures that contained spermatogonia and Sertoli cells, resembling the seminiferous tubule structure – Somatic cell numbers increased during culture – No colonies formed during subculture although the number of type A spermatogonia increased (26–30 days) – Germ cell transplantation into mouse testis revealed increased SSC number during 30 days of culture (claimed ~10,000 fold)	Aponte et al. (2008)

Cattle	1–5 week (neonatal) and 2–5 years (adult)	Enriched gonocytes from neonatal (70–90%) Enriched spermatogonia from adult (20–40%)	Discontinuous Percoll gradient centrifugation	DMEM/F12 + 10% FBS at 37 °C	7 days (spermatogonia) 1.5 months (gonocytes)	– Gonocytes: formed colonies within a week, containing DBA^{+ve} cells; the number of colonies gradually decreased in each passage (6 passages, 1.5 month) – Spermatogonia: formed colonies within a week that were lost after passaging	Fujihara et al. (2011)
	4–5 months	Enriched spermatogonia (~74%)	Discontinuous Percoll gradient centrifugation + differential plating on gelatin	BFF feeders at 35 °C under 10% O_2 and feeder-free on laminin-coated plates. MEMα + GDNF (20 ng/mL) + bFGF (2 ng/mL) + LIF (100 ng/mL)	2 month (BFF) or 1 month (feeder-free)	– Germ cell colonies (clumps) appeared within a week, containing ZBTB16 and $LIN28^{+ve}$ cells; passaged every 7–10 days. Proliferated, but declined with time	Oatley et al. (2016)
Buffalo	4–6 months (prepubertal)	Enriched spermatogonia (55% $UCH\text{-}L1^{+ve}$)	Differential plating + discontinuous Percoll gradient centrifugation	DMEM/F12 + 10% FBS at 37 °C	7 days	– Colonies containing proliferative somatic and germ cells appeared after 4–5 days	Goel et al. (2010)
	3–7 months	Enriched spermatogonia (no information on germ cell content)	Differential plating on DSA + discontinuous Percoll gradient centrifugation	HEPES modified DMEM + 10% FBS + GDNF (50 ng/mL) with inactivated Sertoli cell feeder layer	4 weeks	– Colonies started to form at day 5–7 – The number of colonies declined during culture	Kala et al. (2012)

(continued)

Table 14.4 (continued)

Species	Age of donor	Starting cell population	Enrichment method	Best culture conditions presented[a]	Culture length	Outcome[b]	References
Buffalo	3–6 months	Enriched spermatogonia (no information on germ cell content)	Differential plating on lectin + discontinuous Percoll gradient centrifugation	DMEM + 10% FBS + GDNF (40 ng/mL) + EGF (10 ng/mL) + bFGF (10 ng/mL) with inactivated buffalo Sertoli cell feeder layer	33–36 days	– Colonies containing undifferentiated type A spermatogonia formed in 15–18 days – No information on colony numbers	Kadam et al. (2013)

Summary of culture conditions published by various research groups to culture spermatogonia from agricultural animals. [a]In publications where authors compared several culture options for in vitro culture, only the condition that yielded the best results is presented in the table. [b]The culture outcome only presents a simplified overview of cell/colony behavior from the best conditions described. For information on molecular characterization of cultures, please refer to the cited references

BFF bovine fetal fibroblasts; *bFGF* basic fibroblast growth factor; *BIO* 6-bromoindirubin-3′-oxime, a glycogen synthase kinase-3 inhibitor; *BM* base medium; *DBA Dolichos biflorus* agglutinin; *DMEM* Dulbecco's Modified Eagle's medium; *DSA Datura stramonium* agglutinin; *EGF* epidermal growth factor; *FBS* Fetal bovine serum; *GDNF* glial cell line-derived neurotropic factor; *KSR* knock-out serum replacement; *LIN28* Lin-28 Homolog; *LIF* leukemia inhibitory factor; *MEF* mouse embryonic fibroblast feeder; *MEM* minimal essential medium; *Rat SSC medium* rat spermatogonial stem cell medium used by (Wu et al. 2009); *SSCM* spermatogonial stem cell medium used by (Kanatsu-Shinohara et al. 2005); *UCH-L1* ubiquitin C-terminal hydrolase-L1; *ZBTB16* Zinc Finger and BTB Domain Containing 16

conserved surface marker that can be used for enriching undifferentiated spermatogonia in prepubertal goats, pigs and cattle (Abbasi et al. 2013; Reding et al. 2010; Zheng et al. 2014). Cell sorting facilitated by surface markers can yield a highly enriched cell population, which can be very valuable for various research applications such as molecular profiling, genetic modification and establishment of a culture system. However, the relatively low yield of sorting by MACS or FACS limits its application in germ cell transplantation in large animals as a large number of donor cells are needed for efficient colonization in recipient testes.

14.3 Applications of Male Germ Cell Transplantation in Agricultural Animals

The main applications of male germ cell transplantation in agricultural animals are (1) preservation of fertility for the propagation of genetically valuable animals; (2) the generation of genetically modified animals for improving agricultural traits or for the generation of large animal biomedical models; and (3) the study of spermatogenesis.

14.3.1 Fertility Preservation and Propagation of Genetically Valuable Animals

Harvesting and cryopreserving spermatogonia from animals with great genetic value has important applications for fertility preservation of desirable males or for the maintenance of certain alleles that can be propagated between animal populations separated both spatially and temporarily. Spermatogonia are theoretically a continuous source of self-renewing cells which have not undergone meiosis and therefore, contain the full set of genetic information of the individual. Spermatogonia can be collected from animals that have not reached reproductive maturity and from adult animals; allowing the rescue of reproductive material even from animals that died before being able to reproduce, from seasonal breeders that are outside of the breeding season or, from animals affected by some diseases or environmental factors that negatively impact sperm production.

Male germ cell transplantation could be useful for the propagation of the genetic traits of an already proven sire (Hausler and Russell 1999). Theoretically, the transplantation of spermatogonia in bulls would also shorten the time needed for testing a potential dairy sire if spermatogonia of an immature male are transplanted into a mature recipient bull (Hausler and Russell 1999). Male germ cell transplantation could be also used for the selection of animals based on carcass value. If spermatogonia were harvested from pig testes after orchiectomy and cryopreserved at the time of slaughter, animals with outstanding carcass traits could retrospectively be used for germ cell transplantation and transmit the genes for carcass merit to the offspring (Hausler and Russell 1999). The same principle can be applied to other domestic species and nontraditional farming species such as deer, camelids, and buffalo. The deer farming industry has quickly expanded in the last decades in some countries, mirrored by an increasing interest in the application and development of

reproductive technology in these species (Asher et al. 2000; Garde et al. 2006). Harvesting reproductive material from trophy males is also of great interest in those species that are hunted (Asher et al. 2000; Garde et al. 2006).

Livestock production demands have changed in the last decades and the modern farming systems have contributed to the reduction in the diversity of domestic breeds, even to the extent that many domestic breeds have become extinct, endangered or at risk of extinction (www.fao.org, accessed on May 21, 2015). Traditional breeds are generally more resilient to diseases and resistant to temperature changes and therefore, livestock biodiversity is essential for livestock production and livelihood. The FAO has recognized the important role of genome resource banks (GRBs) for maintaining the current animal genetic resources. Banking the genomic resources in GRBs is essential to safeguard for the future, biomaterials from local livestock breeds, wildlife species or other economically and ecologically important genotypes. Many countries worldwide have invested substantial resources in the generation and maintenance of GRBs. Various tissues and cell types, as well as other biomaterials can be cryobanked, making valuable contributions to the maintenance of animal genetic resources and advancement of scientific research. More detailed information regarding the importance, responsibilities, the applications of GRBs and the available GRBs worldwide has been reviewed elsewhere (Agca 2012; Blackburn 2004). Thus, in addition to the preservation of semen, the inclusion of spermatogonia (i.e. SSCs) in GRBs is essential to preserve the genetic information of a male, especially when semen collection is not feasible. More effort is needed in order to develop successful protocols for the preservation of spermatogonia obtained from large animals. The cryopreservation and in vitro expansion of male germ cells are two essential steps for practical applications of germ cell transplantation in livestock animals.

14.3.2 Generation of Genetically Modified Animals

Natural selection and continuous selection of desired animal traits by pastoralists, farmers and animal breeders have contributed to the establishment of current livestock and other domestic species. Several tools have been developed for the generation of genetically modified animals with the purpose to shorten the selection process of traits by crossbreeding (Table 14.3). So far there are no authentic embryonic stem (ES) cell lines established from livestock species. Even though the establishment of induced pluripotent stem (iPS) cells has been reported in non-rodent species, their germline competence has not been described (Nowak-Imialek et al. 2011). The generation of genetically modified animals by means of modifying SSCs has sparked interest. Basically, the procedure involves introducing desired genetic modifications into male germ cells, which upon transplantation into male recipients, will resume spermatogenesis and produce transgenic sperm. The genetic wmodifications can be transmitted to the offspring by natural breeding of the recipients or performing in vitro fertilization using transgenic sperm. The advantages of using SSCs for the generation of genetically modified animals are that they are

already committed to the germ cell lineage; germ cell transplantation circumvents problems associated with manipulations of oocytes and embryos; and, the procedure requires lower technical skills and less sophisticated equipment than the techniques where micromanipulations are involved. Another important advantage for the generation of transgenic large animals via SSCs is that it shortens the time required for generating modified animals, reducing the high costs of obtaining founders with the desired modifications. The main drawback so far is the lack of long-term culture systems to maintain and expand the male germ cells from domestic and livestock species in vitro. The features of the approaches, including modifications of the male germ cells, to generate genetically modified large animals have been summarized in Table 14.3. The biotechnology available for the generation of genetically modified animals has found applications in the agricultural and biomedical fields. Public acceptance to use of lower organisms for the production of biopharmaceutical substances is more generalized than acceptance to generate genetically modified organisms for agricultural or medical purposes (Einsiedel 2005; Pardo et al. 2009). The two main applications that can benefit from the use of male germ cell transplantation in livestock species is the generation of genetically modified animals for improving agricultural traits or for the generation of large animal biomedical models.

14.3.2.1 Generation of Genetically Modified Animals for Improving Agricultural Traits, Resistance to Diseases, and Better Adaptation of Breeds to Different Environments

The agricultural applications for the generation of genetically modified livestock have been described in detail (Wheeler 2007), which include:

- Modification of milk: either changes in the milk composition or the production of new proteins in milk. This would be useful for improving the growth and survival of offspring, facilitate the manufacturing process and deliver better products to the market.
- Modification of growth and carcass composition: possibilities include the manipulation of carcass composition altering the fat content or the introduction of beneficial fatty acids as well as the manipulation of growth factors. The introduction of enzymes, such as phytase into the pig's gut could be beneficial for the reduction in environmental phosphorus pollution (Golovan et al. 2001).
- Modification of hair, wool, and fiber: for fabric and yarn production modifying the elasticity, fiber strength, and shrinkage.
- Modification of reproductive performance and prolificacy targeting genes involved in the regulation of these traits, such as the Boroola fecundity (Davis et al. 1982), Inverdale (Braw-Tal et al. 1993) and estrogen receptor genes.
- Modification of disease resistance and adaptation to different environmental conditions (Donovan et al. 2005).

- Modification of traits with repercussions in animal welfare, such as the generation of dairy breeds without horns (carrying the *POLLED* allele) without reducing the genetic merit of the generated animals (Tan et al. 2013). This will avoid the painful procedure of dehorning and reduce the risks of injury to operators and animals.

14.3.2.2 Generation of Genetically Modified Animals as Biomedical Models

Current attrition rates for pharmaceutical substances in Phase II clinical trials are approximately 80% (Arrowsmith and Miller 2013) and thus, developing better translational animal models is gaining interest. In spite of the knowledge acquired with the study of non-rodent species, the value of livestock has been usually underestimated in translational research. Recent advancements in molecular biology and whole genome sequencing projects are contributing to our knowledge of livestock genomes, which will increase the value of agricultural species in translational research.

The pig has become an important biomedical model and substantial effort has been invested into the generation of genetically modified pigs. Genetically modified pigs serve as models for human diseases such as cystic fibrosis, diabetes, osteoporosis, retinitis pigmentosa, cardiovascular (atherosclerosis and myocardial infarction), and neurodegerative diseases (Alzheimer's and Huntington's disease), among others (Aigner et al. 2010; Luo et al. 2012). Pigs are also a potential source of organs for transplantation and therefore, there is great interest in the generation of genetically modified pigs as donors for xenotransplantation. The main constraints for the use of pig organs for xenotransplantation are the host immunological reactions and safety issues due to the presence of endogenous retroviruses in the porcine genome that could infect humans (reviewed in Luo et al. (2012)). Efforts to generate multi-transgenic pig models addressing these issues are under way (Luo et al. 2012). Bovine models are not widely used in the biomedical field, although there are some examples where they could be useful (Casal and Haskins 2006). Other non-rodent species including the dog and the cat are of particular interest for the study of human genetic diseases and neurological disorders as well (Casal and Haskins 2006; O'Brien et al. 2002). The list of large animal models for human genetic disorders is increasing. The Faculty of Veterinary Science in Sydney has created a comprehensive online database for genetic disorders and genetic traits in species other than human, rat and mouse where potential models for human diseases can be consulted (Online Mendelian Inheritance in Animals, OMIA. Faculty of Veterinary Science, University of Sydney, (Accessed on August 20, 2015). www.http://omia.angis.org.au/).

14.3.3 Study of Spermatogenesis

Besides providing a functional assay to evaluate the presence of stem cell activity in a testicular cell population, germ cell transplantation offers an approach for studying the process of spermatogenesis, helping to elucidate mechanisms of male

fertility. Very little is known about the kinetics of SSCs in large animals. Most of the knowledge on stemness and differentiation properties of spermatogonia has been generated in mice. The lack of a culture system to maintain male germ cells from species other than rodents limits the study of spermatogenesis in domestic animals and livestock. Germ cell transplantation is a useful tool to evaluate modifications of the male germ cells at the SSC level and study certain features of spermatogonia, such as homing to the niche, the ability to reestablish spermatogenesis and maintain the production of spermatozoa after exposure to certain factors. Thus, germ cell transplantation will provide some insights into our knowledge of spermatogenesis in large animals, providing benefit in our understanding of male reproduction. This new generated knowledge will be helpful for controlling male reproduction in livestock, to extrapolate protocols/reproductive techniques to their wildlife counterparts and provide a platform for translational research to ultimately apply to humans.

14.4 Current State of Male Germ Cell Culture in Agricultural Animals

14.4.1 Characterization of Gonocytes/Spermatogonia

For over 10 years, great efforts have been put in to characterize germ cells from various non-rodent species (reviewed in González and Dobrinski 2015). Molecular markers that have been well characterized in mice and known to function in SSC/spermatogonia have been investigated in non-rodent species. An overview of the expression of some germ cell markers in a few agricultural animals is presented in Fig. 14.1.

Promyelocytic leukemia zinc finger protein (PLZF) is a transcriptional repressor expressed in undifferentiated spermatogonia in mice and is essential for SSC maintenance (Costoya et al. 2004). Its expression is conserved in agricultural animals, being reported in gonocytes and undifferentiated spermatogonia from pigs, goats, sheep, and cattle (Reding et al. 2010; Bahadorani et al. 2011; Luo et al. 2006). Whether PLZF is also essential for SSC maintenance in those species remains to be determined. UCH-L1, a ubiquitin c-terminal hydrolase with unknown function in germ cells, is also found to be a highly conserved spermatogonial marker in non-rodent species (Luo et al. 2006; Fujihara et al. 2011; Rodriguez-Sosa et al. 2006; Valli et al. 2014; Abbasi et al. 2013; Goel et al. 2010). DEAD-box polypeptide 4 (DDX4, also known as VASA) and deleted in azoospermia-like (DAZL) are RNA-binding proteins that are essential for germ cell development in mice as demonstrated by infertility phenotype in null mice (Tanaka et al. 2000; Schrans-Stassen et al. 2001). Similar to the expression pattern in mice, VASA and DAZL were also present in various stages of germ cell development from gonocytes to spermatocytes in pigs, goats, cattle, sheep, buffalo, and horses (Fujihara et al. 2011; Bahadorani et al. 2011; Luo et al. 2009; Goel et al. 2010). Although homeobox transcription factors Nanog and POU5F1 (also known as Oct-4) are expressed in gonocytes/spermatogonia in the neonatal/prepubertal testis of pigs and cattle, their expression is not restricted to undifferentiated germ cells in adult animals with proteins being

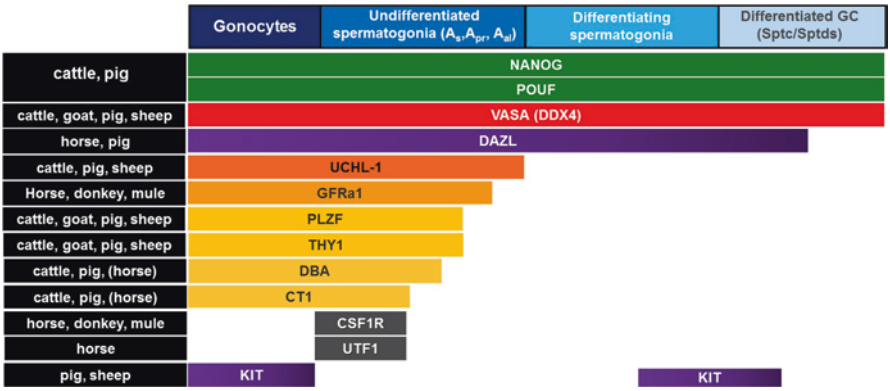

Fig. 14.1 Schematic representation of germ cell markers in agricultural species. The characterization of male germ cells in large animals from the neonatal to the adult stage remains mostly unaddressed. This diagram represents a schematic summary of germ cell markers in domestic species. The information included in the figure was compiled from the literature and based on the information reviewed in (González and Dobrinski 2015). The information gathered is based on different studies done in domestic species where immunocytochemistry was performed on cross-sections of testicular tissue or testicular cells. The figure is meant to reflect the overall knowledge on the biochemical characterization of germ cells in non-rodent animals. Expression of markers is largely unknown and it is expected that it might differ among species. Abbreviations: *CSF1R* colony stimulating factor 1; *CT1* a monoclonal antibody specific for cytotoxic T (CT) cells that recognizes the Sda/GM-2 glycan; *DAZL* deleted in azoospermia-like; *DBA Dolichos biflorus* agglutinin; *DDX4* DEAD-box polypeptide 4 (also known as VASA); *GFRα1* glial cell line-derived neurothrophic factor family receptor alpha 1; *KIT* tyrosine protein kinase kit (CD117); *NANOG* homeobox transcription factor Nanog; *PLZF* promyelocytic leukemia zinc finger protein; *POU5F1 (Oct3/4)* POU domain, class 5, transcription factor 1; *THY1* thymocyte differentiating antigen 1; *UCH-L1* ubiquitin C-terminal hydrolase-L1; *UTF1* undifferentiated embryonic cell transcription factor 1

detected in differentiated germ cells (Goel et al. 2008; Fujihara et al. 2011). The inhibitor of DNA binding 4 (ID4) is considered a marker for undifferentiated spermatogonia and SSCs in the mouse, where it is expressed by a subpopulation of As spermatogonia and plays a role in self-renewal of SSCs (Oatley et al. 2011a; Chan et al. 2014). ID4 was also found to be expressed by cultured bovine germ cell clumps (spermatogonia) (Oatley et al. 2016). Expression of the paired box 7 (PAX7) transcription factor has been recently identified in a subpopulation of As spermatogonia in mice and has been implicated in maintenance of spermatogenesis in the steady state and recovery of spermatogenesis after germ cell ablation. Surprisingly, Pax7 was not functionally required in spermatogenesis in mice since Pax7 inactivation did not caused male infertility (Aloisio et al. 2014). PAX7 expression in spermatogonia is conserved across mammalian species, including domestic animals (Aloisio et al. 2014). Functional studies to determine the function of $PAX7^{+ve}$ cells in non-rodent species have not been performed yet.

Identification of surface markers that can be used for characterization and enrichment of subsets of spermatogonia has also been an active part of germ cell research in agricultural animals. The thymocyte differentiating antigen 1 (THY-1) has been found on a subset of gonocytes/undifferentiated spermatogonia in neonatal/

prepubertal pig, goat and cattle testes (Zheng et al. 2014; Reding et al. 2010). The lectin *Dolichos biflorus* agglutinin (DBA) has been used to mark spermatogonia in different species; however, its affinity for germ cells is not consistent across species. In pigs, DBA's binding to germ cells is progressively lost with age with strong binding in gonocytes and no binding in spermatogonia (Goel et al. 2007). In cattle, DBA binding can be found in type A spermatogonia that include both c-kit positive and c-kit negative populations (Izadyar et al. 2002). The monoclonal antibody specific for cytotoxic T (CT) cells (CT1) that recognizes the Sda/GM2-glycan has also been used to mark gonocytes and a subset of spermatogonia in species such as cattle, pigs, and horses (Klisch et al. 2011). The stage-specific embryonic antigen-1 (SSEA-1), a marker for pluripotent murine embryonic cells, has also been found in undifferentiated spermatogonia from pigs (Kim et al. 2013). Some of those surface markers have been used in MACS or FACS to sort a subset of gonocytes/spermatogonia in various species. However, it is difficult to evaluate the efficiency of enrichment by those markers as functional data was either missing or inconclusive.

14.4.2 Gonocytes/Spermatogonia Culture

SSCs represent a very rare cell population in the testis. An in vitro culture system that supports maintenance and expansion of SSCs as well as maintains their stem cell potency is essential for characterization and manipulation of SSCs. This will not only contribute to the knowledge of stem cell biology and spermatogenesis, but also help researchers to harness their power for therapeutic applications. Long-term culture systems have been established for mouse and rat SSCs for over 10 years (Kanatsu-Shinohara et al. 2003; Kubota et al. 2004; Hamra et al. 2005). The culture of rodent SSCs is usually carried out on feeder cells, which might lead to certain complications for downstream applications, such as flow cytometry or germ cell transplantation. Recent development in the field allows serum-free and feeder-free culture of mouse SSCs for extended periods of time with normal karyotype, androgenetic DNA methylation patterns and functionality (Kanatsu-Shinohara et al. 2014).

Despite ongoing efforts to culture enriched spermatogonia from agricultural animals, the conditions that can maintain SSCs are largely unknown. Published culture conditions and reported outcomes are summarized in Table 14.4. The effects of feeder cells, the substrate, the culture temperatures, the medium composition, the concentration of serum, and the presence of growth factors have been investigated (see Table 14.4 for details). Enriched gonocytes or spermatogonia from neonatal or prepubertal animals were usually used as a starting population to seed on either a monolayer of feeder cells or a substratum. Germ cells and contaminating somatic cells (mostly Sertoli cells) were able to form mixed colonies of various morphological characteristics within a week or two as long as the culture medium contained at least 1% serum (Zheng et al. 2013; Aponte et al. 2008; Table 14.4). Higher serum concentration (such as 10 and 15%) resulted in over proliferation of somatic cells and had adverse effect on germ cell culture (Goel et al. 2007; Fujihara et al. 2011;

Heidari et al. 2012). The StemPro-based SSC medium used for mouse SSC culture failed to maintain SSCs from pigs and cattle for long term; however, it seemed to be superior to DMEM or DMEM/F12 for short-term culture of pig and cattle male germ cells (30–60 days) (Aponte et al. 2008; Lee et al. 2013). Although supplementation of a cocktail of growth factors that have been used for rodent SSC culture such as GDNF, bFGF, EGF, LIF proved to be somehow beneficial, neither a single factor nor a combination of those factors supported long-term SSC culture from agricultural animals investigated so far (see Table 14.4 for details). This suggests that essential niche factors that support SSCs in large animals are yet to be discovered. Lower culture temperature (31 °C) appeared to be better compared to routinely used 37 °C in maintaining porcine gonocytes and resulted in a higher percentage of proliferating germ cells (Lee et al. 2013). In vitro maintenance of undifferentiated spermatogonia was influenced by low oxygen and reduced temperature (35 °C) in cattle as well (Oatley et al. 2016).

In most cases, male germ cell cultures may be maintained for 1–4 weeks and eventually succumb to over-proliferation of somatic cells and ceased proliferation and apoptosis of germ cells. Most studies based their evaluation of culture conditions on the morphological criteria of the heterogeneous colonies (such as the number and size of colonies) and the qualitative expression of some marker genes (such as UCH-L1, NANOG, OCT4, VASA, among others) in culture containing mixed populations of cells (see Table 14.4 for details). How reliable those assessments are for evaluation of culture conditions remains to be tested by other researchers. It is important to mention that the presence of colonies, especially in primary cultures obtained from testicular cell suspensions are not indicators of spermatogonia proliferation. Both male germ cells and testicular somatic cells form colonies, usually with different morphology (Aponte et al. 2008; Oatley et al. 2016). Spermatogonia from rodents form clusters or clumps of cells in culture, giving a grape-like appearance to the generated colonies (Kanatsu-Shinohara and Shinohara 2010). Similar cell clumps to those in bone fide primary cultures of rodent spermatogonia have been observed in primary cultures of bovine spermatogonia (Oatley et al. 2016). Moreover, immunostaining of those cattle male germ cell clumps confirmed the expression of markers of undifferentiated spermatogonia (Oatley et al. 2016). Therefore, this morphological feature of clumping might be conserved among rodents and livestock putative spermatogonia (Oatley et al. 2016). However, the morphology of the colonies should not be considered as a sole indicator of spermatogonia proliferation. In the mouse, the morphology of colonies formed by SSCs changed depending on the substrate and the availability of GDNF (Kanatsu-Shinohara et al. 2005).

Only a few studies used xenotransplantation of large animal-derived cells into the testis of immunodeficient mice to assess their culture conditions (Aponte et al. 2006, 2008; Zheng et al. 2013). The retention of transplanted labeled donor cells in recipient mouse testis was observed and either quantitative or qualitative assessment was performed. However, xenotransplantation of cultured cells into mouse testes does not represent a full functional assay as transplanted germ cells cannot undergo full spermatogenesis due to phylogenetic distance between donor and

recipient animals (reviewed in Dobrinski and Hill 2007). Moreover, the definition of SSC colonies and their quantitation in the recipient testis is very subjective and prone to large variations.

In summary, several issues present big challenges to the establishment of long-term culture conditions for non-rodent animals. Firstly, there are no genetic animal models readily available to investigate intrinsic and extrinsic factors essential for self-renewal and differentiation of SSCs. The knowledge we have gained from rodent models may not directly translate to other species. Secondly, different research groups have access to different breeds and ages of donor animals. In addition, different research groups adopt different experimental protocols for tissue digestion and cell enrichment. All those factors result in variations in the starting cell population, which make it difficult to develop a culture system that is readily reproducible by other research groups. Thirdly, unlike rodents, it is logistically-challenging and cost-prohibitive to use germ cell transplantation to evaluate culture conditions in agricultural animals. The large amount of cells needed for transplantation also requires a very robust culture system. Xenotransplantation of cultured cells into immuno-deficient mouse testis may serve as an alternative functional assay; however, its inherent limitation (no spermatogenesis) likely results in inconclusive data.

14.5 Conclusions

Even though the transplantation of spermatogonia has experienced an increased interest in livestock animals in the last decades, it is still not widely performed in these species due to several limitations. In order to increase its usefulness and spread its use, several steps need to be optimized. First, increasing the efficiency of the technique in terms of spermatogonial colonization and transmission of the donor haplotype is mandatory. This might be achieved by increasing the percentage of undifferentiated spermatogonia present in the transplanted population of cells and/or increasing the number of available niches in the recipient's testes by establishing efficient and safe protocols for ablating endogenous spermatogenesis in the recipient male. Second, a comprehensive biochemical characterization of spermatogonia in large animals will facilitate the use of sorting techniques for the isolation of spermatogonia from other testicular cells. Third, an efficient culture system for spermatogonia from large animals is necessary. This would allow to expand and manipulate the spermatogonia of these species for future research and downstream applications. The generation of genetically modified animals by means of male germline modifications could overcome the problems associated with the prevailing methods of generating genetically modified large animals. Notwithstanding current limitations, there have been advances in the field and "proof of principle" studies have reported that modifications of SSCs can be transmitted through the male germline in agricultural animal models with the generation of spermatozoa or embryos carrying the genetic modification. Therefore, as the technology improves to overcome current limitations, it is expected that genetically modified livestock animals will be generated by targeting the male germline.

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References

Abbasi H, Tahmoorespur M, Hosseini SM, Nasiri Z, Bahadorani M, Hajian M, Nasiri MR, Nasr-Esfahani MH (2013) THY1 as a reliable marker for enrichment of undifferentiated spermatogonia in the goat. Theriogenology 80:923–932. https://doi.org/10.1016/j.theriogenology.2013.07.020

Agca Y (2012) Genome resource banking of biomedically important laboratory animals. Theriogenology 78:1653–1665. https://doi.org/10.1016/j.theriogenology.2012.08.012

Aigner B, Renner S, Kessler B, Klymiuk N, Kurome M, Wunsch A, Wolf E (2010) Transgenic pigs as models for translational biomedical research. J Mol Med 88:653–664. https://doi.org/10.1007/s00109-010-0610-9

Aloisio GM, Nakada Y, Saatcioglu HD, Pena CG, Baker MD, Tarnawa ED, Mukherjee J, Manjunath H, Bugde A, Sengupta AL, Amatruda JF, Cuevas I, Hamra FK, Castrillon DH (2014) PAX7 expression defines germline stem cells in the adult testis. J Clin Invest 124:3929–3944. https://doi.org/10.1172/jci75943

Aponte PM, Soda T, Teerds KJ, Mizrak SC, van de Kant HJ, de Rooij DG (2008) Propagation of bovine spermatogonial stem cells in vitro. Reproduction 136:543–557. https://doi.org/10.1530/rep-07-0419

Aponte PM, Soda T, van de Kant HJ, de Rooij DG (2006) Basic features of bovine spermatogonial culture and effects of glial cell line-derived neurotrophic factor. Theriogenology 65:1828–1847. https://doi.org/10.1016/j.theriogenology.2005.10.020

Arrowsmith J, Miller P (2013) Trial watch: phase II and phase III attrition rates 2011–2012. Nat Rev Drug Discov 12:569. https://doi.org/10.1038/nrd4090

Asher GW, Berg DK, Evans G (2000) Storage of semen and artificial insemination in deer. Anim Reprod Sci 62(1–3):195–211

Auharek SA, Avelar GF, Lara NLM, Sharpe RM, Franca LR (2011) Sertoli cell numbers and spermatogenic efficiency are increased in inducible nitric oxide synthase mutant mice. Int J Androl 34:e621–e629

Bahadorani M, Hosseini SM, Abedi P, Abbasi H, Nasr-Esfahani MH (2015) Glial cell line-derived neurotrophic factor in combination with insulin-like growth factor 1 and basic fibroblast growth factor promote in vitro culture of goat spermatogonial stem cells. Growth Factors:1–11. https://doi.org/10.3109/08977194.2015.1062758

Bahadorani M, Hosseini SM, Abedi P, Hajian M, Afrough M, Azhdari-Tafti Z, Azizi H, Hosseini SE, Vahdati A, Baharvand H, Nasr-Esfahani MH (2011) Comparative immnunochemical analysis of VASA, PLZF and THY in goats and sheep suggests that these markers are also conserved in these species. J Cytol Histol 2:126. https://doi.org/10.4172/2157-7099.1000126

Bahadorani M, Hosseini SM, Abedi P, Hajian M, Hosseini SE, Vahdati A, Baharvand H, Nasr-Esfahani MH (2012) Short-term in-vitro culture of goat enriched spermatogonial stem cells using different serum concentrations. J Assist Reprod Genet 29(1):39–46. https://doi.org/10.1007/s10815-011-9687-5

Bellve AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M (1977) Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. J Cell Biol 74:68–85. https://doi.org/10.1083/jcb.74.1.68

Blackburn HD (2004) Development of national animal genetic resource programs. Reprod Fertil Dev 16(1–2):27–32. 10.10371/rd03075

Blanco-Rodriguez J (2002) DNA replication and germ cell apoptosis during spermatogenesis in the cat. J Androl 23(4):484–490. https://doi.org/10.1002/j.1939-4640.2002.tb02269.x

Braw-Tal R, McNatty KP, Smith P, Heath DA, Hudson NL, Phillips DJ, McLeod BJ, Davis GH (1993) Ovaries of ewes homozygous for the X-linked Inverdale gene (FecXI) are devoid of sec-

ondary and tertiary follicles but contain many abnormal structures. Biol Reprod 49:895–907. https://doi.org/10.1095/biolreprod49.5.895
Brinster RL, Avarbock MR (1994) Germline transmission of donor haplotype following spermatogonial transplantation. Proc Natl Acad Sci U S A 91:11303–11307
Brinster RL, Zimmermann JW (1994) Spermatogenesis following male germ-cell transplantation. Proc Natl Acad Sci U S A 91:11298–11302
Casal M, Haskins M (2006) Large animal models and gene therapy. Eur J Med Genet 14:266–272. https://doi.org/10.1038/sj.ejhg.5201535
Chan F, Oatley MJ, Kaucher AV, Yang QE, Bieberich CJ, Shashikant CS, Oatley JM (2014) Functional and molecular features of the Id4+ germline stem cell population in mouse testes. Genes Dev 28:1351–1362
Cho SK, Hwang KC, Choi YJ, Bui HT, Nguyen VT, Park C, Kim JH, Kim JH (2009) Production of transgenic pigs harboring the human erythropoietin (hEPO) gene using somatic cell nuclear transfer. J Reprod Dev 55:128–136. https://doi.org/10.1262/jrd.20102
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(6):653–659. https://doi.org/10.1038/ng1367
Davis GH, Montgomery GW, Allison AJ, Kelly RW, Bray AR (1982) Segregation of a major gene influencing fecundity in progeny of Booroola sheep. New Zeal J Agr Res 25:525–529. https://doi.org/10.1080/00288233.1982.10425216
De Jonge CJ, Barratt LR (eds) (2006) The sperm cell: production, maturation, fertilization, regeneration. Cambridge University Press, Cambridge, UK
de Rooij DG, Griswold MD (2012) Questions about spermatogonia posed and answered since 2000. J Androl 33(6):1085–1095. https://doi.org/10.2164/jandrol.112.016832
de Rooij DG, Russell LD (2000) All you wanted to know about spermatogonia but were afraid to ask. J Androl 21(6):776–798. https://doi.org/10.1002/j.1939-4640.2000.tb03408.x
Dobrinski I, Hill JR (2007) Germ cell transplantation—a novel approach to 'designer' animals? In: Swain D, Charmley E, Steel J, Coffey S (eds) Redesigning animal agriculture: the challenge of the 21st century. CAB International, Oxford, UK; Cambridge, MA, pp 81–93
Donovan DM, Kerr DE, Wall RJ (2005) Engineering disease resistant cattle. Transgenic Res 14:563–567. https://doi.org/10.1007/s11248-005-0670-8
Ebata KT, Zhang X, Nagano MC (2005) Expression patterns of cell-surface molecules on male germ line stem cells during postnatal mouse development. Mol Reprod Dev 72:171–181. https://doi.org/10.1002/mrd.20324
Einsiedel EF (2005) Public perceptions of transgenic animals. Rev Sci Tech 24:149–157
Franca LR, Avelar GF, Almeida FF (2005) Spermatogenesis and sperm transit through the epididymis in mammals with emphasis on pigs. Theriogenology 63:300–318. https://doi.org/10.1016/j.theriogenology.2004.09.014
Franca LR, Becker-Silva SC, Chiarini-Garcia H (1999) The length of the cycle of seminiferous epithelium in goats (*Capra hircus*). Tissue Cell 31:274–280. https://doi.org/10.1054/tice.1999.0044
Franca LR, Godinho CL (2003) Testis morphometry, seminiferous epithelium cycle length, and daily sperm production in domestic cats (*Felis catus*). Biol Reprod 68:1554–1561. https://doi.org/10.1095/biolreprod.102.010652
Franca LR, Ogawa T, Avarbock MR, Brinster RL, Russell LD (1998) Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol Reprod 59:1371–1377. https://doi.org/10.1095/biolreprod59.6.1371
Fujihara M, Kim SM, Minami N, Yamada M, Imai H (2011) Characterization and in vitro culture of male germ cells from developing bovine testis. J Reprod Dev 57:355–364. https://doi.org/10.1262/jrd.10-185M
Garde JJ, Martinez-Pastor F, Gomendio M, Malo AF, Soler AJ, Fernandez-Santos MR, Esteso MC, Garcia AJ, Anel L, Roldan ER (2006) The application of reproductive technologies to natural populations of red deer. Reprod Dom Anim 41(Suppl 2):93–102. https://doi.org/10.1111/j.1439-0531.2006.00773.x

Goel S, Fujihara M, Minami N, Yamada M, Imai H (2008) Expression of NANOG, but not POU5F1, points to the stem cell potential of primitive germ cells in neonatal pig testis. Reproduction 135:785–795. https://doi.org/10.1530/rep-07-0476

Goel S, Reddy N, Mandal S, Fujihara M, Kim SM, Imai H (2010) Spermatogonia-specific proteins expressed in prepubertal buffalo (*Bubalus bubalis*) testis and their utilization for isolation and *in vitro* cultivation of spermatogonia. Theriogenology 74:1221–1232. https://doi.org/10.1016/j.theriogenology.2010.05.025

Goel S, Sugimoto M, Minami N, Yamada M, Kume S, Imai H (2007) Identification, isolation, and in vitro culture of porcine gonocytes. Biol Reprod 77(1):127–137. https://doi.org/10.1095/biolreprod.106.056879

Golovan SP, Meidinger RG, Ajakaiye A, Cottrill M, Wiederkehr MZ, Barney DJ, Plante C, Pollard JW, Fan MZ, Hayes MA, Laursen J, Hjorth JP, Hacker RR, Phillips JP, Forsberg CW (2001) Pigs expressing salivary phytase produce low-phosphorus manure. Nat Biotechnol 19:741–745. https://doi.org/10.1038/90788

Gomez MC, Pope CE, Kutner RH, Ricks DM, Lyons LA, Ruhe MT, Dumas C, Lyons J, Dresser BL, Reiser J (2009) Generation of domestic transgenic cloned kittens using lentivirus vectors. Cloning Stem Cells 11:167–176. https://doi.org/10.1089/clo.2008.0054

González R, Dobrinski I (2015) Beyond the mouse monopoly: studying the male germ line in domestic animal models. ILAR J 56:83–98. https://doi.org/10.1093/ilar/ilv004

Hamra FK, Chapman KM, Nguyen DM, Williams-Stephens AA, Hammer RE, Garbers DL (2005) Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture. Proc Natl Acad Sci U S A (48):17430–17435. https://doi.org/10.1073/pnas.0508780102

Harkey MA, Asano A, Zoulas ME, Torok-Storb B, Nagashima J, Travis A (2013) Isolation, genetic manipulation, and transplantation of canine spermatogonial stem cells: progress toward transgenesis through the male germ-line. Reproduction 146:75–90. https://doi.org/10.1530/rep-13-0086

Hausler CL, Russell LD (1999) Prospects for spermatogonial transplantation in livestock and endangered species. In: Gagnon C (ed) The male gamete. From basic science to clinical applications. Cache River Press, Vienna, IL, pp 37–45

Heidari B, Rahmati-Ahmadabadi M, Akhondi MM, Zarnani AH, Jeddi-Tehrani M, Shirazi A, Naderi MM, Behzadi B (2012) Isolation, identification, and culture of goat spermatogonial stem cells using c-kit and PGP9.5 markers. J Assist Reprod Genet 29:1029–1038. https://doi.org/10.1007/s10815-012-9828-5

Herrid M, Olejnik J, Jackson M, Suchowerska N, Stockwell S, Davey R, Hutton K, Hope S, Hill JR (2009) Irradiation enhances the efficiency of testicular germ cell transplantation in sheep. Biol Reprod 81:898–905. https://doi.org/10.1095/biolreprod.109.078279

Herrid M, Vignarajan S, Davey R, Dobrinski I, Hill JR (2006) Successful transplantation of bovine testicular cells to heterologous recipients. Reproduction 132:617–624. https://doi.org/10.1530/rep.1.01125

Honaramooz A, Behboodi E, Blash S, Megee SO, Dobrinski I (2003a) Germ cell transplantation in goats. Mol Reprod Dev 64:422–428. https://doi.org/10.1002/mrd.10205 [doi]

Honaramooz A, Behboodi E, Hausler CL, Blash S, Ayres S, Azuma C, Echelard Y, Dobrinski I (2005) Depletion of endogenous germ cells in male pigs and goats in preparation for germ cell transplantation. J Androl 26:698–705. https://doi.org/10.2164/jandrol.05032

Honaramooz A, Behboodi E, Megee SO, Overton SA, Galantino-Homer H, Echelard Y, Dobrinski I (2003b) Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. Biol Reprod 69:1260–1264. https://doi.org/10.1095/biolreprod.103.018788

Honaramooz A, Megee S, Zeng W, Destrempes MM, Overton SA, Luo J, Galantino-Homer H, Modelski M, Chen F, Blash S, Melican DT, Gavin WG, Ayres S, Yang F, Wang PJ, Echelard Y, Dobrinski I (2008) Adeno-associated virus (AAV)-mediated transduction of male germ line stem cells results in transgene transmission after germ cell transplantation. FASEB J 22:374–382. https://doi.org/10.1096/fj.07-8935com

Honaramooz A, Megee SO, Dobrinski I (2002) Germ cell transplantation in pigs. Biol Reprod 66:21–28. https://doi.org/10.1095/biolreprod66.1.21

Hong SG, Kim MK, Jang G, HJ O, Park JE, Kang JT, Koo OJ, Kim T, Kwon MS, Koo BC, Ra JC, Kim DY, Ko C, Lee BC (2009) Generation of red fluorescent protein transgenic dogs. Genesis 47:314–322. https://doi.org/10.1002/dvg.20504

Izadyar F, Den Ouden K, Creemers LB, Posthuma G, Parvinen M, De Rooij DG (2003a) Proliferation and differentiation of bovine type A spermatogonia during long-term culture. Biol Reprod 68:272–281. https://doi.org/10.1095/biolreprod.102.004986

Izadyar F, Den Ouden K, Stout TA, Stout J, Coret J, Lankveld DP, Spoormakers TJ, Colenbrander B, Oldenbroek JK, Van der Ploeg KD, Woelders H, Kal HB, De Rooij DG (2003b) Autologous and homologous transplantation of bovine spermatogonial stem cells. Reproduction 126:765–774. https://doi.org/10.1530/rep.0.1260765

Izadyar F, Spierenberg GT, Creemers LB, den Ouden K, de Rooij DG (2002) Isolation and purification of type A spermatogonia from the bovine testis. Reproduction 124:85–94. https://doi.org/10.1530/rep.0.1240085

Jacobsen JC, Bawden CS, Rudiger SR, McLaughlan CJ, Reid SJ, Waldvogel HJ, MacDonald ME, Gusella JF, Walker SK, Kelly JM, Webb GC, Faull RL, Rees MI, Snell RG (2010) An ovine transgenic Huntington's disease model. Human Mol Genet 19:1873–1882. https://doi.org/10.1093/hmg/ddq063

Jeong YW, Lee GS, Kim JJ, Park SW, Ko KH, Kang M, Kim YK, Jung EM, Hyun SH, Shin T, Jeung EB, Hwang WS (2012) Establishment of a canine model of human type 2 diabetes mellitus by overexpressing phosphoenolypyruvate carboxykinase. Int J Mol Cell Med 30:321–329. https://doi.org/10.3892/ijmm.2012.993

Johnson L, Varner DD, Roberts ME, Smith TL, Keillor GE, Scrutchfield WL (2000) Efficiency of spermatogenesis: a comparative approach. Anim Reprod Sci 60-61:471–480. https://doi.org/10.1016/S0378-4320(00)00108-1

Kadam PH, Kala S, Agrawal H, Singh KP, Singh MK, Chauhan MS, Palta P, Singla SK, Manik RS (2013) Effects of glial cell line-derived neurotrophic factor, fibroblast growth factor 2 and epidermal growth factor on proliferation and the expression of some genes in buffalo (*Bubalus bubalis*) spermatogonial cells. Reprod Fertil Dev 25:1149–1157. https://doi.org/10.1071/RD12330

Kala S, Kaushik R, Singh KP, Kadam PH, Singh MK, Manik RS, Singla SK, Palta P, Chauhan MS (2012) In vitro culture and morphological characterization of prepubertal buffalo (*Bubalus bubalis*) putative spermatogonial stem cell. J Assist Reprod Genet 29:1335–1342. https://doi.org/10.1007/s10815-012-9883-y

Kanatsu-Shinohara M, Miki H, Inoue K, Ogonuki N, Toyokuni S, Ogura A, Shinohara T (2005) Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod 72:985–991. https://doi.org/10.1095/biolreprod.104.036400

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. https://doi.org/10.1095/biolreprod.103.017012

Kanatsu-Shinohara M, Ogonuki N, Matoba S, Morimoto H, Ogura A, Shinohara T (2014) Improved serum- and feeder-free culture of mouse germline stem cells. Biol Reprod 91:88. https://doi.org/10.1095/biolreprod.114.122317

Kanatsu-Shinohara M, Shinohara T (2010) Germline modification using spermatogonial stem cells. Methods Enzymol 477:17–36. https://doi.org/10.1016/S0076-6879(10)77002-6

Kanatsu-Shinohara M, Toyokuni S, Shinohara T (2004) CD9 is a surface marker on mouse and rat male germline stem cells. Biol Reprod 70(1):70–75. https://doi.org/10.1095/biolreprod.103.020867

Kerr JB, Loveland KL, O'Bryan MK, Kretser DM (2006) Cytology of the testis and intrinsic control mechanisms. In: Neill JD, Pfaff DW, Plant TM, Richards JS, Wassarman PM (eds) Knobil and Neill's physiology of reproduction, 3rd edn. Academic Press, Oxford, UK, pp 827–947

Kim MJ, Oh HJ, Park JE, Kim GA, Hong SG, Jang G, Kwon MS, Koo BC, Kim T, Kang SK, Ra JC, Ko C, Lee BC (2011) Generation of transgenic dogs that conditionally express green fluorescent protein. Genesis 49:472–478. https://doi.org/10.1002/dvg.20737

Kim YH, Kim BJ, Kim BG, Lee YA, Kim KJ, Chung HJ, Hwang S, Woo JS, Park JK, Schmidt JA, Pang MG, Ryu BY (2013) Stage-specific embryonic antigen-1 expression by undifferentiated spermatogonia in the prepubertal boar testis. J Anim Sci 91:3143–3154. https://doi.org/10.2527/jas.2012-6139

Klein BG (2012) Reproductive physiology of the male. In: Klein BG (ed) Cunningham's textbook of veterinary physiology, 5th edn. Elsevier Saunders, St. Louis MO, p 624

Klisch K, Contreras DA, Sun X, Brehm R, Bergmann M, Alberio R (2011) The Sda/GM2-glycan is a carbohydrate marker of porcine primordial germ cells and of a subpopulation of spermatogonia in cattle, pigs, horses and llama. Reproduction 142:667–674. https://doi.org/10.1530/rep-11-0007

Kragh PM, Nielsen AL, Li J, Du Y, Lin L, Schmidt M, Bogh IB, Holm IE, Jakobsen JE, Johansen MG, Purup S, Bolund L, Vajta G, Jorgensen AL (2009) Hemizygous minipigs produced by random gene insertion and handmade cloning express the Alzheimer's disease-causing dominant mutation APPsw. Transgenic Res 18:545–558. https://doi.org/10.1007/s11248-009-9245-4

Kubota H, Avarbock MR, Brinster RL (2004) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71(3):722–731. https://doi.org/10.1095/biolreprod.104.029207

Kuijk EW, Colenbrander B, Roelen BA (2009) The effects of growth factors on in vitro-cultured porcine testicular cells. Reproduction 138:721–731. https://doi.org/10.1530/rep-09-0138

Lacerda SMSN, Aponte PM, Costa GMJ, Campos-Junior PHA, Segatelli TM, Silva MA, Franca LR (2012) An overview of spermatogonial stem cell physiology, niche and transplantation in fish. Anim Reprod:798–808

Leal MC, Becker-Silva SC, Chiarini-Garcia H, Franca LR (2004) Sertoli cell efficiency and daily sperm production in goats (*Capra hircus*). Anim Reprod 1:122–128

Leblond CP, Clermont Y (1952) Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N Y Acad Sci 55:548–573. https://doi.org/10.1111/j.1749-6632.1952.tb26576.x

Lee HJ, Lee HC, Han JY (2015) Germline modification and engineering in avian species. Mol Cells 38:743–749. 10.14348/molcells.2015.0225

Lee WY, Park HJ, Lee R, Lee KH, Kim YH, Ryu BY, Kim NH, Kim JH, Kim JH, Moon SH, Park JK, Chung HJ, Kim DH, Song H (2013) Establishment and in vitro culture of porcine spermatogonial germ cells in low temperature culture conditions. Stem Cell Res 11:1234–1249. https://doi.org/10.1016/j.scr.2013.08.008

Luo J, Megee S, Dobrinski I (2009) Asymmetric distribution of UCH-L1 in spermatogonia is associated with maintenance and differentiation of spermatogonial stem cells. J Cell Physiol 220:460–468. https://doi.org/10.1002/jcp.21789

Luo J, Megee S, Rathi R, Dobrinski I (2006) Protein gene product 9.5 is a spermatogonia-specific marker in the pig testis: application to enrichment and culture of porcine spermatogonia. Mol Reprod Dev 73:1531–1540. https://doi.org/10.1002/mrd.20529

Luo Y, Li J, Liu Y, Lin L, Du Y, Li S, Yang H, Vajta G, Callesen H, Bolund L, Sorensen CB (2011) High efficiency of BRCA1 knockout using rAAV-mediated gene targeting: developing a pig model for breast cancer. Transgenic Res 20:975–988. https://doi.org/10.1007/s11248-010-9472-8

Luo Y, Lin L, Bolund L, Jensen TG, Sorensen CB (2012) Genetically modified pigs for biomedical research. J Inherit Metab Dis 35:695–713. https://doi.org/10.1007/s10545-012-9475-0

McCreath KJ, Howcroft J, Campbell KH, Colman A, Schnieke AE, Kind AJ (2000) Production of gene-targeted sheep by nuclear transfer from cultured somatic cells. Nature 405:1066–1069. https://doi.org/10.1038/35016604

McLean DJ, Russell LD, Griswold MD (2002) Biological activity and enrichment of spermatogonial stem cells in vitamin A-deficient and hyperthermia-exposed testes from mice based on colonization following germ cell transplantation. Biol Reprod 66:1374–1379. https://doi.org/10.1095/biolreprod66.5.1374

Mikkola M, Sironen A, Kopp C, Taponen J, Sukura A, Vilkki J, Katila T, Andersson M (2006) Transplantation of normal boar testicular cells resulted in complete focal spermatogenesis in a boar affected by the immotile short-tail sperm defect. Reprod Domest Anim 41:124–128. https://doi.org/10.1111/j.1439-0531.2006.00651.x

Morena AR, Boitani C, Pesce M, De Felici M, Stefanini M (1996) Isolation of highly purified type A spermatogonia from prepubertal rat testis. J Androl 17:708–717. https://doi.org/10.1002/j.1939-4640.1996.tb01856.x

Nagano M, Avarbock MR, Brinster RL (1999) Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes. Biol Reprod 60:1429–1436. https://doi.org/10.1095/biolreprod60.6.1429

Nagano MC (2003) Homing efficiency and proliferation kinetics of male germ line stem cells following transplantation in mice. Biol Reprod 69:701–707. https://doi.org/10.1095/biolreprod.103.016352

Nakamura Y, Kagami H, Tagami T (2013) Development, differentiation and manipulation of chicken germ cells. Develop Growth Differ 55:20–40. https://doi.org/10.1111/dgd.12026

Neves EM, Costa GMJ, França LR (2014) Sertoli cell and spermatogenic efficiencies in Pêga Donkey (*Equus asinus*). Anim Reprod 11:517–525

Neves ES, Chiarini-Garcia H, Franca LR (2002) Comparative testis morphometry and seminiferous epithelium cycle length in donkeys and mules. Biol Reprod 67:247–255. https://doi.org/10.1095/biolreprod67.1.247

Nowak-Imialek M, Kues W, Carnwath JW, Niemann H (2011) Pluripotent stem cells and reprogrammed cells in farm animals. Microsc Microanal 17:474–497. https://doi.org/10.1017/s1431927611000080

O'Brien SJ, Menotti-Raymond M, Murphy WJ, Yuhki N (2002) The feline genome project. Annu Rev Genet 36:657–686. https://doi.org/10.1146/annurev.genet.36.060602.145553

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 U S A 103:9524–9529. https://doi.org/10.1073/pnas.0603332103

Oatley MJ, Kaucher AV, Racicot KE, Oatley JM (2011a) 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

Oatley MJ, Racicot KE, Oatley JM (2011b) Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. Biol Reprod 84:639–645. https://doi.org/10.1095/biolreprod.110.087320

Oatley MJ, Kaucher AV, Yang Q, Waqas MS, Oatley JM (2016) Conditions for long-term culture of cattle undifferentiated spermatogonia. Biol Reprod 95(14):1–10. https://doi.org/10.1095/biolreprod.116.139832

Ogawa T, Arechaga JM, Avarbock MR, Brinster RL (1997) Transplantation of testis germinal cells into mouse seminiferous tubules. Int J Dev Biol 41:111–122

Ogawa T, Dobrinski I, Avarbock MR, Brinster RL (2000) Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med 6:29–34. https://doi.org/10.1038/71496

Ogawa T, Dobrinski I, Brinster RL (1999) Recipient preparation is critical for spermatogonial transplantation in the rat. Tissue Cell 31:461–472. https://doi.org/10.1054/tice.1999.0060

Pardo R, Engelhard M, Hagen K, Jorgensen RB, Rehbinder E, Schnieke A, Szmulewicz M, Thiele F (2009) The role of means and goals in technology acceptance. A differentiated landscape of public perceptions of pharming. EMBO Rep 10:1069–1075. https://doi.org/10.1038/embor.2009.208

Reding SC, Stepnoski AL, Cloninger EW, Oatley JM (2010) THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. Reproduction 139:893–903. https://doi.org/10.1530/rep-09-0513

Rilianawati SR, Taggart M, Cooke HJ (2003) Spermatogenesis in testes of Dazl null mice after transplantation of wild-type germ cells. Reproduction 126:599–604. https://doi.org/10.1530/rep.0.1260599

Robl JM, Wang Z, Kasinathan P, Kuroiwa Y (2007) Transgenic animal production and animal biotechnology. Theriogenology 67:127–133. https://doi.org/10.1016/j.theriogenology.2006.09.034

Rodriguez-Sosa JR, Dobson H, Hahnel A (2006) Isolation and transplantation of spermatogonia in sheep. Theriogenology 66:2091–2103. https://doi.org/10.1016/j.theriogenology.2006.03.039

Rodriguez-Sosa JR, Silvertown JD, Foster RA, Medin JA, Hahnel A (2009) Transduction and transplantation of spermatogonia into the testis of ram lambs through the extra-testicular rete. Reprod Domest Anim 44:612–620. https://doi.org/10.1111/j.1439-0531.2007.01030.x

Rogers CS, Stoltz DA, Meyerholz DK, Ostedgaard LS, Rokhlina T, Taft PJ, Rogan MP, Pezzulo AA, Karp PH, Itani OA, Kabel AC, Wohlford-Lenane CL, Davis GJ, Hanfland RA, Smith TL, Samuel M, Wax D, Murphy CN, Rieke A, Whitworth K, Uc A, Starner TD, Brogden KA, Shilyansky J, PB MC Jr, Zabner J, Prather RS, Welsh MJ (2008) Disruption of the CFTR gene produces a model of cystic fibrosis in newborn pigs. Science 321:1837–1841. https://doi.org/10.1126/science.1163600

Russell LD, Ettlin RA, Sinha Hikim AP, Clegg ED (1990) Histological and histopathological evaluation of the testis. Cache River Press, Clearwater, FL

Schlatt S, Rosiepen G, Weinbauer GF, Rolf C, Brook PF, Nieschlag E (1999) Germ cell transfer into rat, bovine, monkey and human testes. Hum Reprod 14:144–150. https://doi.org/10.1093/humrep/14.1.144

Schnieke AE, Kind AJ, Ritchie WA, Mycock K, Scott AR, Ritchie M, Wilmut I, Colman A, Campbell KH (1997) Human factor IX transgenic sheep produced by transfer of nuclei from transfected fetal fibroblasts. Science 278:2130–2133. https://doi.org/10.1126/science.278.5346.2130

Schrans-Stassen BH, Saunders PT, Cooke HJ, de Rooij DG (2001) Nature of the spermatogenic arrest in Dazl −/− mice. Biol Reprod 65:771–776. https://doi.org/10.1095/biolreprod65.3.771

Shinohara T, Avarbock MR, Brinster RL (2000a) Functional analysis of spermatogonial stem cells in Steel and cryptorchid infertile mouse models. Dev Biol 220:401–411. https://doi.org/10.1006/dbio.2000.9655

Shinohara T, Orwig KE, Avarbock MR, Brinster RL (2000b) Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. Proc Natl Acad Sci U S A 97:8346–8351. https://doi.org/10.1073/pnas.97.15.8346

Shinohara T, Orwig KE, Avarbock MR, Brinster RL (2001) Remodeling of the postnatal mouse testis is accompanied by dramatic changes in stem cell number and niche accessibility. Proc Natl Acad Sci U S A 98:6186–6191. https://doi.org/10.1073/pnas.111158198

Soares JM, Avelar GF, Franca LR (2009) The seminiferous epithelium cycle and its duration in different breeds of dog (*Canis familiaris*). J Anat 215:462–471. https://doi.org/10.1111/j.1469-7580.2009.01122.x

Stockwell S, Herrid M, Davey R, Brownlee A, Hutton K, Hill JR (2009) Microsatellite detection of donor-derived sperm DNA following germ cell transplantation in cattle. Reprod Fertil Dev 21:462–468. dx.doi.org. https://doi.org/10.1071/RD08130

Tan W, Carlson DF, Lancto CA, Garbe JR, Webster DA, Hackett PB, Fahrenkrug SC (2013) Efficient nonmeiotic allele introgression in livestock using custom endonucleases. Proc Natl Acad Sci U S A 110:16526–16531. https://doi.org/10.1073/pnas.1310478110

Tanaka SS, Toyooka Y, Akasu R, Katoh-Fukui Y, Nakahara Y, Suzuki R, Yokoyama M, Noce T (2000) The mouse homolog of Drosophila Vasa is required for the development of male germ cells. Genes Dev 14:841–853. https://doi.org/10.1101/gad.14.7.841

Tegelenbosch RA, de Rooij DG (1993) A quantitative study of spermatogonial multiplication and stem cell renewal in the C3H/101 F1 hybrid mouse. Mutat Res 290:193–200

Umeyama K, Watanabe M, Saito H, Kurome M, Tohi S, Matsunari H, Miki K, Nagashima H (2009) Dominant-negative mutant hepatocyte nuclear factor 1alpha induces diabetes in transgenic-cloned pigs. Transgenic Res 18:697–706. https://doi.org/10.1007/s11248-009-9262-3

Valli H, Sukhwani M, Dovey SL, Peters KA, Donohue J, Castro CA, Chu T, Marshall GR, Orwig KE (2014) Fluorescence- and magnetic-activated cell sorting strategies to isolate and enrich human spermatogonial stem cells. Fertil Steril 102(566–580):e567. https://doi.org/10.1016/j.fertnstert.2014.04.036

van Dissel-Emiliani FM, de Rooij DG, Meistrich ML (1989) Isolation of rat gonocytes by velocity sedimentation at unit gravity. J Reprod Fertil 86(2):759–766. https://doi.org/10.1530/jrf.0.0860759

Wang J, Yang P, Tang B, Sun X, Zhang R, Guo C, Gong G, Liu Y, Li R, Zhang L, Dai Y, Li N (2008) Expression and characterization of bioactive recombinant human alpha-lactalbumin in the milk of transgenic cloned cows. J Dairy Sci 91:4466–4476. https://doi.org/10.3168/jds.2008-1189

Wheeler MB (2007) Agricultural applications for transgenic livestock. Trends Biotechnol 25:204–210. https://doi.org/10.1016/j.tibtech.2007.03.006

Wu Z, Falciatori I, Molyneux LA, Richardson TE, Chapman KM, Hamra FK (2009) Spermatogonial culture medium: an effective and efficient nutrient mixture for culturing rat spermatogonial stem cells. Biol Reprod 81:77–86. https://doi.org/10.1095/biolreprod.108.072645

Yin XJ, Lee HS, XF Y, Choi E, Koo BC, Kwon MS, Lee YS, Cho SJ, Jin GZ, Kim LH, Shin HD, Kim T, Kim NH, Kong IK (2008) Generation of cloned transgenic cats expressing red fluorescence protein. Biol Reprod 78:425–431. https://doi.org/10.1095/biolreprod.107.065185

Yoshizaki G, Fujinuma K, Iwasaki Y, Okutsu T, Shikina S, Yazawa R, Takeuchi Y (2011) Spermatogonial transplantation in fish: a novel method for the preservation of genetic resources. Comp Biochem Physiol Part D Genomics Proteomics 6:55–61. https://doi.org/10.1016/j.cbd.2010.05.003

Yoshizaki G, Okutsu T, Morita T, Terasawa M, Yazawa R, Takeuchi Y (2012) Biological characteristics of fish germ cells and their application to developmental biotechnology. Reprod Domest Anim 47(Suppl 4):187–192. https://doi.org/10.1111/j.1439-0531.2012.02074.x

Zeng W, Tang L, Bondareva A, Honaramooz A, Tanco V, Dores C, Megee S, Modelski M, Rodriguez-Sosa JR, Paczkowski M, Silva E, Wheeler M, Krisher RL, Dobrinski I (2013) Viral transduction of male germline stem cells results in transgene transmission after germ cell transplantation in pigs. Biol Reprod 88:27. https://doi.org/10.1095/biolreprod.112.104422

Zeng W, Tang L, Bondareva A, Luo J, Megee SO, Modelski M, Blash S, Melican DT, Destrempes MM, Overton SA, Gavin WG, Ayres S, Echelard Y, Dobrinski I (2012) Non-viral transfection of goat germline stem cells by nucleofection results in production of transgenic sperm after germ cell transplantation. Mol Reprod Dev 79:255–261. https://doi.org/10.1002/mrd.22014

Zhang P, Liu P, Dou H, Chen L, Chen L, Lin L, Tan P, Vajta G, Gao J, Du Y, Ma RZ (2013) Handmade cloned transgenic sheep rich in omega-3 Fatty acids. PLoS One 8:e55941. https://doi.org/10.1371/journal.pone.0055941

Zhang Q, Chen JQ, Lin J, QH Y, HQ Y, XJ X, Liu GH, Yang Q (2014) Production GH transgenic goat improving mammogenesis by somatic cell nuclear transfer. Mol Biol Rep 41:4759–4768. https://doi.org/10.1007/s11033-014-3347-7

Zheng Y, He Y, An J, Qin J, Wang Y, Zhang Y, Tian X, Zeng W (2014) THY1 is a surface marker of porcine gonocytes. Reprod Fertil Dev 26:533–539. https://doi.org/10.1071/RD13075

Zheng Y, Tian X, Zhang Y, Qin J, An J, Zeng W (2013) In vitro propagation of male germline stem cells from piglets. J Assist Reprod Genet 30:945–952. https://doi.org/10.1007/s10815-013-0031-0

Zhu H, Liu C, Li M, Sun J, Song W, Hua J (2013) Optimization of the conditions of isolation and culture of dairy goat male germline stem cells (mGSC). Anim Reprod Sci 137:45–52. https://doi.org/10.1016/j.anireprosci.2012.12.005

Index

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