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THE GERMLINE STEM CELL NICHE UNIT IN MAMMALIAN TESTES

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Abstract

This review addresses current understanding of the germline stem cell niche unit in mammalian testes. Spermatogenesis is a classic model of tissue-specific stem cell function relying on self-renewal and differentiation of spermatogonial stem cells (SSCs). These fate decisions are influenced by a niche microenvironment composed of a growth factor milieu that is provided by several testis somatic support cell populations. Investigations over the last two decades have identified key determinants of the SSC niche including cytokines that regulate SSC functions and support cells providing these factors, adhesion molecules that influence SSC homing, and developmental heterogeneity of the niche during postnatal aging. Emerging evidence suggests that Sertoli cells are a key support cell population influencing the formation and function of niches by secreting soluble factors and possibly orchestrating contributions of other support cells. Investigations with mice have shown that niche influence on SSC proliferation differs during early postnatal development and adulthood. Moreover, there is mounting evidence of an age-related decline in niche function, which is likely influenced by systemic factors. Defining the attributes of stem cell niches is key to developing methods to utilize these cells for regenerative medicine. The SSC population and associated niche comprise a valuable model system for study that provides fundamental knowledge about the biology of tissue-specific stem cells and their capacity to sustain homeostasis of regenerating tissue lineages. While the stem cell is essential for maintenance of all self-renewing tissues and has received considerable attention, the role of niche cells is at least as important and may prove to be more receptive to modification in regenerative medicine.

I. INTRODUCTION

In mammals, homeostasis relies on stem cells replenishing tissue lineages with differentiating cells that are continually lost due to cytotoxic injury and terminal differentiation. In embryonic and neonatal development, these stem cells are also tasked with establishing tissue lineages while setting aside a self-renewing population that will remain undifferentiated and be sustained throughout life. During steady-state conditions, differentiating cells will continually arise from the stem cell pool to support organ function. All tissue-specific stem cell populations originate from the inner cell mass of the blastocyst during early embryogenesis and upon lineage commitment lose pluripotent potential. In vivo, most tissue-specific stem cells are committed to providing the cell lineage of organs in which they reside and therefore lack plasticity. However, some stem cell populations including the bone marrow-derived hematopoietic stem cells (HSCs) appear to have

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DISCLOSURES

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multipotent potential to derive several different cell lineages when placed in an appropriate environment (33, 53, 70, 105, 112, 146, 151).

To carry out their functions, stem cells possess the capacity for both self-renewal to maintain a pool of stem cells and generation of progenitor cells that are set on a pathway of differentiation. In theory, stem cell division can be symmetrical to generate two new stem cells or two differentiated cells; alternatively, division can be asymmetrical generating one new stem cell and one progenitor cell that will continue to differentiate. Both types of stem cell division may occur simultaneously within a tissue or be regulated in a temporal manner. These fate decisions are tightly regulated by influences from microenvironments referred to as “niches.” In general, stem cell niches are themselves tissue-specific, being composed of a growth factor milieu and architectural support that are dictated by resident support cells. The characteristics of niches may be altered by the contributions of support cells to provide cues that influence symmetric versus asymmetric division depending on the state of tissue function. An example reflecting niche influence on stem cell replenishment is the temporary state of symmetrical division in neural stem cells during embryogenesis that is required for expansion of the stem cell pool, which is followed by transition to asymmetric division to establish the differentiated neural cell lineages and maintain homeostasis (38, 85, 97). Regulation of these different states is likely a result of niche cues received by the stem cells.

The possible immortal nature of stem cells has captured the interest of researchers in the scientific and medical communities as a potential avenue for regenerative medicine to treat a variety of degenerative diseases caused by loss of tissue homeostasis. Achieving this goal relies on the deciphering of molecular mechanisms within stem cells that control fate decisions and defining the components that constitute niche microenvironments. Investigations over the last several decades have led to the identification of tissue-specific stem cell populations in a variety of self-renewing organs including the bone marrow (21, 90, 138, 143), gut (5, 17, 77, 123), hair follicle (27, 88), prostate (73), heart (48), and testis (10, 11). Collectively, studies utilizing these model systems have provided clear evidence that the niche microenvironment has a major influence on stem cell fate decisions. For example, in older animals, stem cells of some tissue systems retain normal function, but overall tissue functional decline is caused by failure of support cells to provide an adequate niche (16, 26, 119). Intriguingly, systemic factors from young individuals were capable of rejuvenating failing niches of aged counterparts to stimulate regeneration of tissue lineages from resident stem cells (26). Thus it is becoming clear that a key element to regenerate stem cell potential will be manipulations of niche microenvironments.

In stem cell biology, the term *niche* describes a microenvironment with anatomical specializations consisting of supporting cells and a growth factor milieu that promotes self-renewal of stem cells. The concept of stem cell niches was first proposed for regulation of HSC function in the bone marrow by Schofield in 1978 (127) and has become a widely accepted concept in stem cell biology over the last 30 years. Decades of research have defined characteristics of niches for a variety of tissue-specific stem cell populations including HSCs (15, 61, 133, 158), intestinal crypt stem cells (5, 84), neural stem cells (31, 106, 129), muscle satellite stem cells (66, 80), and hair follicle bulge stem cells (27). For other systems, understanding the determinants of microenvironments influencing stem cell functions is at the forefront of investigation. Of particular interest is the niche of germline stem cells in mammalian testes, referred to as spermatogonia stem cells (SSCs). Spermatogenesis in the mammalian testis is a classic model of developmental biology that has been studied extensively, but key features of the niche have only recently begun to be described, and these investigations have generated fascinating insights into a robust and complex model of stem cell function.

Spermatogenesis is essential for the continuity of a species, contributes to genetic diversity, and determines sex ratios in most mammalian populations (117, 128). Reduction in or loss of SSC function disrupts spermatogenesis leading to subfertility or infertility in males. It has been estimated that at least 10% of human couples worldwide are infertile, and approximately half of this infertility is male related, much of which is caused by impaired SSC function (79). In addition, because SSCs are the only cells in the body that self-renew and contribute genes to the next generation, they provide an avenue to alter genes within a male's germline. Aside from medical implications in humans, preservation of genetic lines of endangered species and expanded use of gametes from valuable food or companion animals represent a potential application of SSC populations by utilizing their immortal nature and capacity for regeneration of male germlines upon transplantation. Thus, because of the central role of the niche in regulating SSC functions, defining its characteristics is essential to realize potential application of SSCs in regenerative medicine and reproduction. This review focuses on recent findings regarding SSC biology, developmental heterogeneity of their cognate niche, and determinants of normal niche function.

II. SPERMATOGONIAL STEM CELL BIOLOGY

Spermatogenesis is the process by which millions of mature gametes or spermatozoa are generated daily within testes (117, 128). Male fertility relies on continuity of this process, and the foundation is provided by self-renewal and differentiation of SSCs (30, 51, 98, 101). In mammalian testes, development of the SSC population begins shortly after birth in coordination with testicular morphogenesis to establish a stem cell pool and set the stage for spermatogenesis in adulthood (6, 50). During steady-state conditions, the niche provides cues that promote self-renewal to sustain the SSC population, as well as generate progenitor spermatogonia that will continue on a path of differentiation (FIG. 1). Whether asymmetric division to generate one new SSC that remains in the niche and one progenitor germ cell that will initiate differentiation outside of the niche occurs in mammalian testes similar to that of other model organisms including *Drosophila melanogaster* (140) and *Caenorhabditis elegans* (62) has not been determined. Regardless, both self-renewal and differentiation are essential aspects of SSC function, and recent studies indicate that niche factors have a major influence on these activities (83, 102, 104, 119, 131, 132, 156). Knowledge of testicular architecture and the unique characteristics of SSCs are critical to understanding the biology and functions of the germline stem cell niche in maintaining spermatogenesis.

A. Testicular Architecture

The architecture of mammalian testes is defined by the seminiferous epithelium and interstitial tissue (FIG. 1). Within the seminiferous epithelium, somatic Sertoli cells associate closely with developing germ cells during embryonic development to form seminiferous cords that are referred to as seminiferous tubules after birth. During the first round of spermatogenesis, Sertoli cells form tight junctions with each other that compartmentalize the seminiferous epithelium into basal and adluminal compartments in the prepubertal male of most mammalian species, and these tight junctions constitute the blood-testis barrier. Within the basal compartment resides the spermatogonial population, which is classified into A, Intermediate, and B subtypes (FIG. 2). The type A spermatogonial population is segregated further into undifferentiated and differentiating subtypes (30, 117). In rodents, the undifferentiated spermatogonial population is further separated into A_{single} or A_{s} (individual spermatogonia), A_{paired} or A_{pr} (cohorts of 2 cells), and A_{aligned} or A_{al} (cohorts of 4, 8, and 16 cells), but in primates, this population is made up of two types of A_{s} spermatogonia referred to as A_{dark} or A_{d} and A_{pale} or A_{p} (FIG. 2). Regardless of the species, the A_{s} spermatogonia are thought to represent or include the SSC population during steady-state conditions, and their close association with the basement membrane of seminiferous

tubules within the basal compartment has led to the theory that the SSC niche is defined by this anatomical localization. Also, because of their intimate association with germ cells, Sertoli cells are widely regarded as the key contributors of the SSC niche. Sertoli cell function is regulated by the gonadotropin follicle stimulating hormone (FSH) to support quantitatively normal spermatogenesis. Thus FSH-regulated secretion of specific growth factors is thought to be a key mechanism of SSC niche functions.

In addition to the highly organized architecture of the seminiferous epithelium and associated Sertoli cell population, the interstitial tissue residing between seminiferous tubules influences the regulation of spermatogenesis and may provide key components of the SSC niche. Composition of interstitial tissue is diverse, consisting of Leydig cells, macrophages, and mesenchymal cells, in addition to networks of capillaries and nerves (FIG. 1). The most widely studied aspect of Leydig cells is their function as an endocrine population for production of testosterone, which is utilized by Sertoli cells to support qualitatively normal spermatogenesis. In addition, the position of SSCs in the basal compartment of the seminiferous epithelium, below Sertoli cell tight junctions that form the blood-testis-barrier, results in their exposure to factors secreted by interstitial cells and those carried in the systemic circulation, but differentiated germ cell types located in the adluminal compartment are sheltered from these. Thus cell populations of the interstitial tissue may provide essential contributions to the SSC niche which do not affect other germ cell types. In support of this notion, recent studies with mice showed that Leydig cells express a key cytokine that regulates self-renewal of SSCs (102). Also, studies by Yoshida et al. (156) in mice indicate that patterning of the vascular network within the interstitial space influences development of undifferentiated spermatogonia, but whether this pertains to SSC functions remains to be determined. Earlier studies also noted a non-random distribution of undifferentiated spermatogonia in mice and rats and revealed preferential positioning in areas of seminiferous tubules that border the interstitium, which contains the vascular network (19, 20). It is important to note that these observations and those of Yoshida et al. (156) are strictly associative, and whether the vascular patterning affects the function of SSCs or other undifferentiated spermatogonia has not been described. In addition to the vasculature, it is likely that contributions from other interstitial cell populations affect SSCs either directly via production of growth factors influencing self-renewal and differentiation or indirectly through regulating niche contributions of other support cells. Over the last decade, emerging evidence indicates that multiple testicular somatic cell populations influence the SSC niche. The contributions of these niche cells likely changes in response to external stimuli to create different environments during embryonic and neonatal development of the germ cell lineage, as well as in adulthood for steady-state spermatogenesis and after cytotoxic injury to the testes. Understanding the origins of the SSC population and their defining characteristics is essential for deciphering how the niche may interact with the SSC during different testicular conditions.

B. Characteristics of Spermatogonial Stem Cells

In embryonic development primordial germ cells (PGCs) arise from the epiblast and migrate to the genital ridge where they associate with somatic cells to form the embryonic gonad (35, 81, 124). During migration and soon after arrival at the genital ridge, PGCs are bipotential, and sexual differentiation of the gonad relies on cues from the somatic cells. In female embryos, PGCs enter meiosis and become arrested in prophase I to form primordial follicles, referred to as oogonia. In comparison, PGCs in male embryos do not enter meiosis and remain proliferative for a short period of time and are referred to as mitotic prospermatogonia (also known as gonocytes) before entering quiescence, a state in which they will remain until after birth. Recent studies with mice revealed that exposure to retinoic acid (RA) during embryonic development between 12.5 and 16.5 days post-conception

induces the onset of meiosis in PGCs of female XX gonads. However, in male XY gonads, somatic Sertoli cell precursors sequester RA via expression of the degrading enzyme Cyp26b1 (65), thereby representing an essential mechanism for formation of prospermatogonia which will transition into the SSC population after birth.

Postnatally, prospermatogonia develop into SSCs during a defined period of neonatal development, which is ~6 days in rodents and several months in livestock species and primates (6, 28, 29, 50, 107). The earliest event in development of the SSC population is migration of prospermatogonia from the center of seminiferous cords where they have resided since sex determination of the embryonic gonad to the basement membrane. In mice, this process occurs in the first 3 days postpartum, and it is not until day 3 that germ cells capable of functioning as SSCs have formed (82). After migration, the morphology of germ cells is distinctly different and they become referred to as the undifferentiated spermatogonial population which consists of SSCs and other non-stem cell progenitors. In the primate, this initial stage is somewhat different, and in the human the spermatogonial population exists primarily as A_d and A_p from ~5 mo of age until spermatogonial differentiation begins at ~10–12 years of age (28, 107). Spermatogenesis can be said to begin when SSCs produce progenitor spermatogonia that give rise to differentiating spermatogonia that subsequently initiate meiosis to become haploid gametes. Prospermatogonia that fail to migrate to the basement membrane undergo apoptosis and are cleared from the seminiferous epithelium. Thus the mechanisms regulating migration of prospermatogonia to the basement membrane are essential for formation of the SSC pool and establishment of niches. Studies with mice indicate that after migration prospermatogonia form two different subtypes. The first develops into the SSC population that contributes progenitor spermatogonia for spermatogenesis during adulthood, whereas the second population transitions directly into differentiating spermatogonia which contribute to the first round of spermatogenesis but do not self-renew (63, 157).

The prevailing model of SSC function in testes of adult rodents during normal conditions states that differentiation of A_s spermatogonia produces A_{pr} progenitor spermatogonia that develop into A_{al} subtypes from which differentiating spermatogonia are generated (FIG. 2). In the human, much less is known about early stages of spermatogenesis. It has been proposed that A_d are very slow cycling and give rise to A_p , which appear to cycle more frequently and produce differentiating B spermatogonia (23–25, 45). However, recent studies with rhesus macaques found that the two populations express similar molecular markers, suggesting that both are actively cycling spermatogonia (46). During steady-state conditions, the number of divisions that occur in A_d and A_p before differentiating B spermatogonia are formed is unclear. However, clones of 2–16 A_p/A_d spermatogonia have been observed during recovery of spermatogenesis following irradiation-induced depletion of the germline in rhesus macaques (1). Maintenance of the SSC pool to provide future cohorts of A_{pr}/A_{al} spermatogonia and differentiating spermatogonia requires self-renewal. While the ability for self-renewal has been used to indicate a stem cell phenotype for many cell populations, this general label is insufficient given that many cell types can proliferate to make “copies” but lack the capacity to sustain a cell lineage for long periods of time. Thus true stem cells must be defined based on a functional capacity for sustaining homeostasis and long-term regeneration of a cell lineage. For germ cells, the capability to support spermatogenesis and reestablish the germline defines SSCs (10–13, 101). The only unequivocal means to assess this capacity is by transplantation of a cell population into the testes of a recipient male in which the endogenous germline has been depleted, ideally using a marked or tagged donor cell population (FIG. 3). Colonies of spermatogenesis arising from the transplanted cells that are maintained for long periods of time are clonally derived from a single SSC (54). Upon transplantation, SSCs in an injected cell suspension must home to open niches for colonization and reestablishment of spermatogenesis (58, 91). Therefore,

manipulation of recipient testes can be used to investigate niche functionality based on the ability of injected SSCs to colonize. Since the first report almost two decades ago (10, 11), the SSC transplantation assay has been used to identify key attributes of the stem cell and its niche in mammalian testes.

III. DEVELOPMENTAL HETEROGENEITY OF THE SPERMATOGONIAL STEM CELL NICHE

During the establishment of cell lineages, stem cell actions must be in favor of self-renewal over differentiation to establish a stable pool for long-term support of homeostasis in adulthood. In contrast, during steady-state conditions, stem cell self-renewal is likely a rare event occurring in a temporal manner when new progenitor cells are needed, particularly in systems where large numbers of differentiated cells are produced from multiple divisions, such as hematopoiesis and spermatogenesis. Thus niche cues that regulate stem cell activities are likely different depending on the state of development. Studies of the bone marrow, intestinal epithelium, and hair follicle suggest that resident stem cells are predominately in a quiescent state and periodically give rise to transient amplifying (TA) progenitors that will generate mature cells of the tissue lineage (5, 18, 27, 60, 115, 147, 154). However, TA progenitors are not long-lived and lack the capacity for continual self-renewal, and the population must be periodically replenished from quiescent stem cells that become active and divide asymmetrically to produce new stem cells and TA progenitors. An alternative model suggests that subpopulations of quiescent and active stem cells coexist within tissues at all times (74), implying that the niche provides different signals to stem cells depending on location. In addition, stem cells appear to favor symmetric division during morphogenesis of a tissue to establish a stem cell pool and then switch to asymmetric division to provide homeostasis during steady-state conditions. These schools of thought imply that the niche provides different cues depending on the state of the tissue, that is, the components of niches are different depending on whether stem cells are to be held in quiescence or to be actively cycling in the form of asymmetric or symmetric division. The male germline relies on a period of active SSC self-renewal during neonatal development to establish the stem cell pool, whereas in adulthood SSC renewal may only occur periodically during certain stages of the seminiferous cycle when A_{pr} and A_{al} progenitor spermatogonia form in rodents or in humans when a subpopulation of A_d and A_p spermatogonia transition into differentiating spermatogonia. Therefore, it is likely that niche microenvironments are different in testes during neonatal development and following puberty (see sect. IIIA).

In the mammalian testis, both actual stem cells in the form of SSCs and TA progenitor spermatogonia have been identified (93). However, whether active and quiescent subpopulations of SSCs exist is a matter of debate. Moreover, the propensity of SSCs to undergo asymmetric or symmetric division has not been resolved. Studies by Huckins (49) reported the existence of both quiescent and actively cycling SSCs based on identification of heterogeneity within the A_s spermatogonial population that was found to contain both long-term and short-term label-retaining cells. On the other hand, long-term label-retaining A_s spermatogonia could not be identified in studies by Lok et al. (76), indicating the existence of actively cycling SSCs only. Also, studies of non-human primates indicate that the SSC population is heterogeneous, being composed of A_d and A_p spermatogonia which are akin to the $A_s/A_{pr}/A_{al}$ population in mouse testes (23, 24, 25). It has been suggested that A_p are actively cycling spermatogonia, whereas A_d are the quiescent reserve subpopulation (23, 24, 25). Moreover, it has been suggested that in the nonhuman primate, only a subpopulation of A_d and A_p spermatogonia are functional stem cells (46). Thus it is unknown whether the SSC population in mammalian testes is composed of both quiescent and actively dividing

stem cells. Therefore, makeup of the SSC niche is likely different depending on the stem cell activity required.

A. Remodeling of the Niche During Postnatal Development

In rodents, the first round of spermatogenesis during post-natal development is unique compared with steady-state spermatogenesis in adulthood. During neonatal development, SSC proliferation is required to establish a pool from which future SSCs and progenitor spermatogonia are derived. Studies with mice showed that SSC proliferation in testes of neonatal animals during establishment of the germ cell lineage is greater than in testes of adult mice (132). Collectively, this finding indicates that SSC niches vary at different stages of development. However, it is likely that many niche factors regulating stem cell self-renewal are identical given that SSCs in testes of adults can reestablish spermatogenesis in testes of pups, and vice versa. Perhaps small differences in concentration contribute to differences in SSC functions. Studies of Shinohara et al. (132) found that the number of SSCs expands by greater than 39-fold from birth to adulthood in mice, but the propensity of transplanted SSCs to expand by self-renewal and occupy adjacent niches in testes of adult mutant W mice that lack endogenous germ cells is not different for stem cells from testes of donor neonatal or adult mice. In contrast, colonization of transplanted SSCs in seminiferous tubules of neonatal pups was greater than ninefold more efficient than in adult recipient mice, and the length of regenerated colonies was four times greater (FIG. 4). These findings indicate that niche regulation of SSC function is different during neonatal and adult phases of development. In the neonate when the SSC pool must be established, niche cues likely support a greater rate of self-renewal to expand numbers and fill adjacent niches compared with the microenvironment of niches in adult testes which promote both self-renewal and differentiation of SSCs.

Both in vitro and in vivo studies have shown that glial cell line-derived neurotrophic factor (GDNF) is critical for self-renewal of SSCs in rodents. Meng et al. (83) found that the seminiferous tubules of mice overexpressing GDNF contained overproduction of undifferentiated spermatogonia and lacked later-differentiating stages, whereas germ cell content became depleted during aging in mice deficient for GDNF. In addition, during a state of regeneration following declining tissue function upon aging in adult animals or cytotoxic injury, GDNF expression in the testis is altered (119). For example, with aging, GDNF expression in the testes is at first normal and then decreases as a male becomes infertile (119). In addition, GDNF expression is elevated approximately fivefold in testes of mice treated with chemotoxic drugs to eliminate germ cells. While GDNF is unequivocally a critical factor for self-renewal of mouse SSCs, other factors also play an important role. Thus it has been noted that long-term in vitro expansion of SSCs from testes of adult mice in conditions that support expansion of SSCs from neonatal/prepubertal mice, including supplementation with GDNF, requires an extensive period of in vitro adaption (J. M. Oatley, unpublished observations). Likewise, transplantation studies indicate SSCs change activities. Colonization of mutant W testes by SSCs from adult and pup donor mice seems to be similar (132). However, colonization of wild-type pup recipient testes by adult SSCs from cryptorchid testes is significantly better than when pup donor SSCs are used (131). Thus adult SSCs compete better than pup SSCs for niche space when spermatogenesis is developing in the wild-type pup testis. These in vitro and in vivo analyses from mice strongly support the conclusion that activities of SSCs can be different when exposed to factors and environments that influence their survival and self-renewal capability. Certainly, GDNF is a principal regulator, and SSCs of adult testes may be less responsive in vitro. However, the adult SSCs from cryptorchid testes function more effectively than SSCs from pup testes when competing for available niches in a developing wild-type testis that has endogenous stem cells and spermatogenesis (131). Nonetheless, the role of the niche appears

to exercise the greatest control of SSC function as demonstrated by transplantation into mutant W recipients (FIG. 4), and during aging (see section below) when the niche age appears to be more critical than age of the stem cell. These observations suggest that the response of SSCs to growth factors promoting proliferation may vary in vitro depending on the stage of postnatal development.

B. Age-Related Decline of Niche Support

Tissue-specific stem cells are known to be long-lived and thought to possess the capacity for infinite self-renewal. Thus it is an attractive possibility to consider stem cells as immortal and capable of rejuvenating aged tissues. However, decline in ability of niche support cells to provide a sufficient microenvironment could inhibit these potentials. Investigation of the male germline has provided insights into how reduced supportive properties of the niche contribute to decline of stem cell function in aged animals (119, 159). It is well known that decrease in reproductive function in older males occurs in all mammalian species (34, 69, 116, 126). A characteristic of male reproductive aging is also a decline in spermatogenesis with an associated incidence of seminiferous tubules lacking germ cells (52, 126, 152). Because SSC function is the foundation to sustain the germline, it is possible that these cells are impaired as animals age, and functional analyses in male mice revealed a linear decline in the number of competent SSCs with age (FIG. 5). However, serial transplantation of SSCs into testes of young males prolonged their function for at least 3 years, suggesting that SSC capacity for regenerating the germ cell lineage is sustained at a normal level when supported by adequate niche cues (FIG. 5). These findings indicate that decline of spermatogenesis in older male's results primarily from impaired capacity of the niche to provide an adequate microenvironment that supports SSC functions. Further investigation revealed that Sertoli cell expression of local growth factors regulating SSC self-renewal (i.e., GDNF) is reduced in old animals compared with young counterparts, indicating that one aspect contributing to the decline in capacity of niche cells is failure to provide adequate cues (119).

The functionality of somatic support cell populations in mammalian testes is regulated by systemic factors that likely control contribution to the SSC niche. In fact, the local expression of growth factors regulating proliferation of SSCs by the somatic Sertoli cells in mouse testes was found to be influenced by the gonadotropin FSH (142), which is secreted by the anterior pituitary gland and carried in the systemic circulation. Thus it is likely that deteriorated capacity of somatic support cells to provide an adequate SSC niche microenvironment is caused by reduction in systemic factors that control their local contributions. In support of this idea, recent studies using parabiosis to connect the circulation of young with old animals found that factors in the systemic circulation of young mice rejuvenated niche support of muscle progenitors and HSCs in old individuals (26). Currently, the effects of systemic factors in young males on rejuvenation of SSC niches in old males have not been described. However, it is well known that secretion of gonadotropins and production of testosterone declines in older males (14, 114). Thus it is plausible to suggest that reduction in concentration of these factors that control testicular somatic support cells gradually declines with age, resulting in inadequate function of niche cells and loss of SSC activity. Indeed, a recent study showed that in mice the presence of females prolongs the period of normal fertility in aging male mice (126). This finding indicates that the production of systemic factors regulating testis function can be influenced by extrinsic cues operating through the nervous system to prolong niche support of SSC functions and delay reproductive aging. Identifying the actual niche cells, their contributions, and their regulation by systemic factors is essential for determining how the niche regulates SSC function and the effects of aging on impaired homeostasis.

IV. DETERMINANTS OF THE SPERMATOGONIAL STEM CELL NICHE

Steady-state spermatogenesis during adulthood relies on continual formation of differentiating spermatogonia from undifferentiated progenitors. Self-renewal of SSCs is required to sustain a stem cell pool from which the progenitor spermatogonia will arise. Thus a balance of self-renewal and differentiation must be tightly regulated to ensure continual spermatogenesis at a normal level. Signals emanating from the niche influence all aspects of stem cell function including self-renewal, differentiation, and apoptosis. In general, the defining feature of stem cell niches is a growth factor milieu that is formed by contributions of somatic support cells. Studies with mice have provided valuable insights into the soluble factors regulating SSC division and somatic support cell populations that provide them. In addition, expression of adhesion molecules by SSCs has been proposed as a mechanism that regulates homing to the niche. In the following sections we focus on knowledge that has been gained about the growth factor signals regulating self-renewal and differentiation of SSCs as well as the recent advances in understanding of adhesion molecules that may influence SSC homing.

A. Adhesion Molecules Influencing Spermatogonial Stem Cell Homing

In stem cell biology, the term *homing* refers to migration of stem cells to and retention in cognate niches. This definition includes the migration of stem cells to adjacent or open niches, which is a fundamental aspect for the success of spermatogenesis. Studies with invertebrate models suggest that homing is key to maintenance of a stem cell phenotype and that migration away from the niche drives differentiation (8, 145, 153, 155). This regulation is controlled by cell polarity, which is a defining feature of the germline stem cell population in *Drosophila* (155). However, in gonads of mammals, a role for cell polarity in regulation of stem cells or differentiating germ cells has not been demonstrated. However, evidence over the last several years has suggested that adhesion molecules play important roles in the homing of SSCs to niches following transplantation, which is required to reestablish spermatogenesis.

Seminiferous tubules are enclosed by peritubular tissue that consists of a basement membrane residing between Sertoli cells of the seminiferous epithelium and myoepithelial cells within the interstitial space (FIG. 1). The basement membrane is a modified form of extracellular matrix consisting of fibronectin, collagens, and laminins (40, 72, 108, 111, 148). Within the seminiferous epithelium, Sertoli cells are anchored to the basement membrane and spermatogonia reside in close proximity to it. In cross-sections of seminiferous tubules from rodents, the undifferentiated spermatogonia appear to be flattened along the basement membrane. Thus it has been postulated that expression of cell adhesion molecules by SSCs may be important for homing through an interaction with the basement membrane. The trans-membrane proteins $\alpha 6$ - and $\beta 1$ -integrin are known to bind laminin, and studies of Shinohara et al. (130) revealed that SSCs in mouse testes express both molecules. Moreover, recent studies by Kanatsu-Shinohara et al. (58) found that impaired expression of $\beta 1$ -integrin disrupts the capability of SSCs to reestablish spermatogenesis following transplantation, but the translocation of these cells to the basement membrane was not affected. These findings indicate that adhesion to the basement membrane is important for sustaining SSCs within the basal compartment of the seminiferous epithelium, but their migration to open niches involves other factors.

The binding of adhesion molecules between cells promotes cell-to-cell communication that can affect differentiation, growth, and survival. In mammalian testes, spermatogonia develop as cohorts beginning at the A_{pr} stage that are connected by intercellular bridges. Because of this close association as a group of identical cells during differentiation, it is possible that expression of adhesion molecules is essential for sustaining coordinated

development of the cohort. In addition, SSCs and the A_{pr}/A_{al} progenitor spermatogonia are intimately associated with Sertoli cells, and this interaction involves cell adhesion. In multiple tissue lineages, cell adhesion molecules are known to act as biosensors of the extracellular environment. In this respect, expression of adhesion proteins by stem cells could provide a mechanism for sensing and responding to niche cues. The cadherin family of proteins is a well-studied class of molecules involved in cell-to-cell adhesion. Recent studies of the mouse germline showed that expression of cadherin 1 (CDH1) is localized to undifferentiated spermatogonia in the mouse testis including A_s , A_{pr} , and A_{al} subtypes (94, 144). However, a functional role in regulating the activities of SSCs or the other progenitor spermatogonia has not been described.

Considering that the $\alpha6$ - and $\beta1$ -integrin expressing cell populations are not composed purely of SSCs and contain other germ cell types and Cdh1 is expressed by all subtypes of undifferentiated spermatogonia, it is doubtful that these molecules are specific to SSC homing. Also, most spermatogonia reside in close proximity to the basement membrane; thus adhesion molecules likely play important roles in general maintenance of these cell populations. Indeed, impaired reestablishment of spermatogenesis from $\beta1$ -integrin-deficient SSCs was not observed until after the SSCs had migrated to the basement membrane and initiated germ cell differentiation. Therefore, unlike the critical role of germline stem cell adhesion to somatic support cells in polarized gonads of invertebrates, in the mammalian gonad expression of adhesion molecules may play a general role in regulating the maintenance of undifferentiated spermatogonia and not SSC functions specifically. Alternatively, SSC homing to niches could be influenced by concentration gradients of cytokines that are established by somatic support cells to keep SSCs exposed to growth factor cues that promote self-renewal and prevent differentiation. Removal of the gradient influence could promote generation of progenitor spermatogonia that respond to signals inducing differentiation. Moreover, concentration gradients of specific cytokines could be generated at open niches that have resulted from death of the cognate SSC, which could induce the migration of new SSCs to occupy the niche.

To date, the existence of concentration gradients for specific cytokines that influence SSC behavior has not been described. However, it is well established that PGC migration from the hindgut to the genital ridge during embryonic morphogenesis of the gonad requires the establishment of cytokine concentration gradients (3, 36, 37, 78, 87). In particular, stromal derived factor 1 (SDF-1), which is expressed by somatic cells of the genital ridge, promotes PGC migration (3, 87). Postnatally, recent studies showed that prospermatogonia derived from PGCs fail to migrate from the center of seminiferous tubules to the basement membrane in mice lacking expression of the transcription factor Sin3a by Sertoli cells (109). Further investigation found that expression of SDF-1 is impaired in Sertoli cells from Sin3a-deficient mice, indicating a role in migration of spermatogonia after birth (109). Moreover, responsiveness to SDF-1 signaling is important for the homing of hematopoietic stem cells to bone marrow niches. Thus it is plausible to theorize that somatic support cells establish concentration gradients of key cytokines to regulate the homing of stem cells, and there may be extensive conservation among multiple tissue-specific stem cell populations. Strong evidence for the existence of cytokine gradients in mammalian testes is the affinity of transplanted SSCs to translocate from the tubular lumen through several layers of germ cells undergoing differentiation and reach the basement membrane (10, 11, 91). This opposite direction of migration includes passage through the Sertoli cell tight junctions that compartmentalize the tubule.

B. Growth Factor Signals Influencing Spermatogonial Stem Cell Self-Renewal

Use of transgenic mouse models and derivation of culture methods for maintenance of rodent undifferentiated spermatogonia have provided platforms from which seminal discoveries of growth factor actions on SSC activity have been made. With the use of these tools, a multitude of studies have identified key growth factors that influence SSC self-renewal including GDNF, fibroblast growth factor 2 (FGF2), and colony stimulating factor 1 (CSF-1). Importantly, these factors are also known to influence the self-renewal of stem cells in other tissue lineages, suggesting that at least some components of niches are common among different stem cell-dependent tissues in mammals.

Studies over the last decade have provided definitive evidence that GDNF is an essential regulator of SSC self-renewal. However, the importance of GDNF in promoting the capacity for undifferentiated cells to sustain homeostasis was initially described based on the discoveries of a critical role in morphogenesis of the kidney (113, 122, 125, 149) and maintenance of the neural progenitor populations (4, 44, 75, 95, 125). In 2000, Meng et al. (83) reported that mutant mice with GDNF haploinsufficiency have severe fertility defects and disrupted spermatogenesis. Examination of testes from adult GDNF-deficient mice revealed that a majority of seminiferous tubules lacked germ cells and contained Sertoli cells only, indicating an impaired ability of undifferentiated spermatogonia to sustain the germline. In addition, testes of transgenic mice harboring ubiquitous overexpression of GDNF were found to contain an accumulation of undifferentiated spermatogonia resulting in formation of germ cell tumors (83). Collectively, these findings indicated that GDNF plays an essential role in maintenance of the undifferentiated spermatogonial population, but effects on SSC proliferation were not determined. The essential role of GDNF in regulating SSC self-renewal was revealed through studies that identified conditions for long-term maintenance of undifferentiated spermatogonia as a proliferative population *in vitro*. In short-term cultures of mouse germ cells, Nagano et al. (92) found that supplementation with recombinant GDNF enhanced the maintenance of cells capable of reestablishing spermatogenesis after transplantation, indicating effects on SSC survival. While many culture media for SSCs contain GDNF among a cocktail of growth factors, definitive evidence of the absolute requirement for GDNF in SSC self-renewal came from studies of Kubota et al. (67) that showed addition of recombinant GDNF to serum-free chemically defined medium promoted long-term expansion of mouse SSCs for periods greater than 6 mo. Studies with both rat and rabbit SSCs in culture also show that GDNF is essential for SSC maintenance and self-renewal in these species, suggesting that GDNF regulation may be widespread and perhaps ubiquitous for many mammalian SSCs (42, 68, 118). While these studies clearly show that GDNF is a key regulator of SSC self-renewal, the receptor complex for GDNF that consists of c-RET and GDNF family receptor α 1 (GFR α 1) is not localized specifically to A_s spermatogonia in the mouse germline but is also expressed by A_{pr} and A_{al} spermatogonial subtypes (39, 96, 141). Also, recent studies revealed that testis cell fractions expressing c-RET and GFR α 1 have varying concentrations of SSCs depending on the stage of testis development. Studies by Ebata et al. (32) found that the GFR α 1 expressing fraction in testes of mice at the early developmental stage of 6 days postpartum is slightly enriched for SSCs. However, further studies by Ebata et al. (32) and those of Grisanti et al. (39) found that the GFR α 1 expressing cell population in testes of adult mice is depleted of SSCs. Collectively, these findings indicate that GDNF plays a general role in regulating the proliferation and survival of undifferentiated spermatogonia, and other factors likely play a greater role in influencing the decision of self-renewal or differentiation during SSC division.

Culture of undifferentiated spermatogonia with GDNF as the sole growth factor supplement promotes long-term self-renewal of SSCs from neonatal mouse pups of a DBA/2J genetic

background (57, 67). However, for mice of other genetic backgrounds, a second growth factor stimulus is required for long-term support of SSC self-renewal. Studies of Kubota et al. (67) revealed that cosupplementation with FGF2 and GDNF promoted the growth of SSCs from mice of a C57BL/6 and other genetic backgrounds. In accordance, Kanatsu-Shinohara et al. (56) found that addition of either FGF2 or epidermal growth factor (EGF) to serum-free medium along with GDNF supports the long-term expansion of SSCs from mice of DBA background. After binding to its receptor, GDNF signaling activates Src family kinase (SFK) and AKT intercellular cascades to promote self-renewal and survival of SSCs (9, 99). Also, Lee et al. (71) found that genetically modified undifferentiated spermatogonia constitutively expressing an activated form of AKT could be maintained for long periods of time without exposure to GDNF as long as FGF2 or EGF was added to the culture media. These findings indicate that FGF2 and EGF signaling play essential roles in regulating SSC self-renewal. However, although the number of undifferentiated spermatogonia increased in cultures expressing activated AKT, the concentration of SSCs declined, indicating that self-renewal was compromised and growth of the other non-stem cell spermatogonia was promoted (71). Thus, while FGF2 and EGF influence proliferation of SSCs in combination with GDNF, they are not specific for self-renewal. Moreover, GDNF and FGF2 supplementation promotes both expansion of SSC numbers and production of non-stem cell progenitor spermatogonia in vitro, indicating that other growth factors must be involved in the specific process of self-renewal.

In search of additional extrinsic factors that influence proliferation of SSCs, several studies have examined the gene expression profiles of undifferentiated spermatogonia isolated from mouse testes (64, 100, 102). These investigations revealed enriched expression of the colony stimulating factor 1 receptor (Csf1r) gene, implicating the corresponding ligand CSF-1 as a potential regulator of SSC functions (64, 102). Addition of recombinant CSF-1 to cultures of mouse undifferentiated spermatogonia also supplemented with GDNF and FGF2 did not significantly affect germ cell proliferation over a period of greater than 2 mo (FIG. 6; Ref. 102). However, the germ cell transplantation assay demonstrated a significant increase in SSC content after 21 days of culture, which became greater with time in culture during which exposure to CSF-1 continued (FIG. 6; Ref. 102). Because spermatogonial number was not affected by exposure to CSF-1, the increase in SSC content must have resulted from an alteration in the balance of self-renewal and generation of progenitor spermatogonia that lack stem cell capacity. These findings indicated that CSF-1 is a specific regulator of mouse SSC self-renewal that does not also affect progenitor spermatogonia. Therefore, CSF-1 can be considered the first identified component of the SSC niche in mice that specifically alters self-renewal, but whether it influences the activity of SSCs from other mammalian species has not been determined.

In addition to GDNF, FGF2, and CSF-1, studies with mouse spermatogonia suggest that leukemia inhibitory factor (LIF) and insulin-like growth factor I (IGF-I) may also influence the survival and self-renewal of SSCs. Kubota et al. (67) found that SSC content in cultures of undifferentiated spermatogonia exposed to IGF-I along with GDNF and FGF2 was increased by almost threefold after 6 wk compared with cultures supplemented with GDNF and FGF2 only. This finding indicates a positive effect of IGF-I on self-renewal of SSCs in the presence of other growth factors that promote proliferation of undifferentiated spermatogonia. However, it remains to be defined whether the influence of IGF-I is specific to SSCs or more general for promoting the proliferation of multiple spermatogonial subtypes. LIF is a major extrinsic cue for maintenance of pluripotency in mouse embryonic stem (ES) cells (137), and studies of Kanatsu-Shinohara et al. (55) found that addition of LIF to mouse newborn testis cell cultures facilitated establishment of germ cell colonies but did not affect self-renewal of SSCs. These findings suggest that LIF is involved in the

maturation of gonocytes into spermatogonia but is not likely a specific regulator of SSC function.

Collectively, a multitude of in vitro studies have implicated GDNF, FGF2, CSF-1, IGF-I, and LIF as extrinsic signals that influence SSC functions. While the studies have clearly shown that SSC self-renewal requires GDNF signaling and is enhanced by exposure to FGF2, these factors affect the proliferation of other undifferentiated spermatogonia as well and are therefore not specific factors of the SSC niche. Also, IGF-I appears to enhance SSC self-renewal, but whether this results from increased survival and proliferation of all undifferentiated spermatogonia or has a specific effect on SSCs remains to be defined. The effects of LIF signaling appear to be restricted to general spermatogonial survival and not self-renewal of SSCs. In contrast to these other factors, CSF-1 influences SSC self-renewal specifically without affecting progenitor spermatogonia, making it the only niche factor in mammalian testes identified by functional transplantation assays as a specific regulator of SSC activity.

C. Growth Factor Signals Regulating Spermatogonial Stem Cell Differentiation

Spermatogenesis initiates with the formation of A_{pr} progenitor spermatogonia from SSCs. Thus the mechanisms driving this differentiation are crucial for sustaining the germ cell lineage, but deciphering these pathways is a daunting task due to the absence of known markers for distinguishing SSCs and A_{pr} progenitors. This limitation has impaired the ability to study factors promoting SSC differentiation in vivo. Therefore, investigations over the last decade have relied on in vitro approaches in combination with transplantation assays to measure a loss of stem cell potential in cultures of undifferentiated spermatogonia. Studies of Nagano et al. (92) demonstrated that exposure to Activin A or bone morphogenic protein 4 (BMP4) resulted in a decline of SSC numbers in cultures of undifferentiated spermatogonia, suggesting an influence on differentiation. Further investigations by Pellegrini et al. (110) found that receptors to BMP4 are expressed by spermatogonia in mouse testes, although the specific subtypes were not determined. Also, addition of BMP4 to cultures of spermatogonia promoted the expression of c-Kit, which is localized to differentiating spermatogonia and a hallmark of germ cell differentiation (110). In contrast, studies of germ cells from prepubertal mice with reduced levels of bioactive Activin A revealed increased expression of markers for differentiated germ cells including c-Kit, indicating an important role in regulating an undifferentiated state (86). Collectively, these findings suggest that BMP4 is a regulator of SSC differentiation in the mouse germline, although a direct link to formation of A_{pr} progenitor spermatogonia has not been shown. Furthermore, it is suggested that Activin A stimulates a loss of stem cell function but is needed for maintenance of progenitor spermatogonia which make up the other component of the undifferentiated spermatogonial population.

Studies with rat undifferentiated spermatogonia found that an immortalized mouse embryonic fibroblast cell line termed STO-SNL promoted the formation of chained cells when utilized as feeder cell monolayers, suggesting the secretion of factors promoting differentiation of SSCs and likely A_{pr} spermatogonia (41). In contrast, culture of undifferentiated spermatogonia in identical conditions but with the immortalized Sertoli cell line MSC-1 as feeders did not promote formation of chained spermatogonia. Characterization of soluble factors secreted from the different feeder cell lines led to the identification of Neuregulin 1 as a STO-SNL factor (41). Supplementation of undifferentiated spermatogonial cultures maintained on MSC-1 feeders with recombinant Neuregulin 1 promoted formation of spermatogonial chains, confirming the activity of this factor as an inducer of differentiation. While it appears to be a potent stimulator of undifferentiated spermatogonia to form chains in vitro, the effects of Neuregulin 1 on spermatogonial differentiation in vivo have not been determined. Also, whether Neuregulin

1 influences differentiation of spermatogonia from other mammalian species in addition to rats has not been reported.

If factors influencing SSC differentiation such as BMP4, Activin A, and Neuregulin 1 act on SSCs directly, their expression must be regulated in a temporal manner when new progenitor spermatogonia are needed, otherwise exhaustion of the SSC pool could occur. Thus somatic support cells must regulate the balance between secretion of niche factors influencing self-renewal and differentiation to maintain the stem cell pool while also promoting the formation and further development of progenitor spermatogonia. It is likely that these factors influence the differentiation of most, if not all, undifferentiated spermatogonia including SSCs, and the maintenance of a stem cell phenotype by SSCs requires suppressing the response to these differentiating stimuli through inhibiting expression of receptors or preventing the intracellular responses elicited by their binding. Clearly, the fate of SSCs is dependent on the contributions of somatic cells to provide a niche microenvironment that will both maintain stem cell phenotype in a subgroup of cells, while stimulating formation of differentiating cells and adjust for changes to maintain tissue function.

D. Somatic Support Cells of the Spermatogonial Stem Cell Niche

The principal somatic cell populations of mammalian testes include Sertoli, Leydig, and peritubular myoid cells, and recent studies have implicated all of these as contributors to the SSC niche. Sertoli cells are the only somatic cells within seminiferous tubules, and they intimately associate with developing germ cells. For these reasons, they have been regarded as the most likely key somatic cell population contributing to the SSC niche. Indeed, expression of GDNF and FGF2 has been localized to Sertoli cells in mouse testes (89, 142). However, examinations of human testes suggest that GDNF is also produced by peritubular myoid cells (139). Additional studies with mice revealed that CSF-1 expression is localized to clusters of Leydig cells in the interstitial space between seminiferous tubules and select peritubular myoid cells but not Sertoli cells (102). Together, these findings implicate Sertoli, Leydig, and myoid cell populations as contributors of the growth factor milieu that constitutes the SSC niche. The finding that interstitial cell populations may influence SSC functions was surprising given that they do not directly contact the germ cells. However, previous studies have established the existence of cross-communication between Sertoli and Leydig cells that influence spermatogenesis (7, 22, 121, 135). Thus it is possible that Leydig and myoid cell contributions to the SSC niche are influenced by communication from Sertoli cells.

Investigation of testis histological cross-sections from adult mice and rats showed that undifferentiated spermatogonia are in a higher concentration in areas of the seminiferous tubules where the basement membrane is in close association with interstitial tissue (19, 20). While not specific for SSCs, this finding suggests that the stem cell niche of mouse testes is regulated by somatic cell populations that makeup the interstitial tissue. In addition to Leydig cells, the interstitial tissue contains a vascular network that delivers factors of the systemic circulation to the seminiferous epithelium. Using live imaging of seminiferous tubules from a transgenic mouse line in which spermatogonia are marked by expression of a reporter transgene, Yoshida et al. (156) tracked the migration patterns of undifferentiated spermatogonia. Those investigations suggested that upon differentiation the undifferentiated spermatogonia migrate away from areas of seminiferous tubules that are associated with the interstitial vasculature. This finding led the authors to conclude that SSC niches are influenced by the testicular vascular network. However, these findings are associative, and functional evidence that factors from the vasculature influence SSC functions directly or indirectly through somatic cells has yet to be demonstrated.

For many tissue-specific stem cells, the niche is orchestrated by a single support cell population that provides extrinsic cues and directs the contributions of other resident support cells. Examples include the neural stem cell population which receives cues from endothelial cells lining blood vessels (129) and hematopoietic stem cells whose functions are regulated by contributions from bone marrow stromal cells (15, 158). Because of their central role as “nurse” cells in spermatogenesis, Sertoli cells have been regarded as the likely orchestrators of SSC niches. Indeed, recent studies found that experimentally increasing the number of Sertoli cells in testes of mice increases the number of niches accessible for colonization by SSCs following transplantation (104). Testes from mice with ~50% increase in Sertoli cell numbers were found to contain greater than threefold more niches accessible for colonization by transplanted SSCs compared with normal recipient male mice (FIG. 7). Also, the association of seminiferous tubules with the vasculature or interstitial tissue was not altered in testes with an increased number of accessible niches. Moreover, the size of testes containing greater niche content was not increased compared with normal mice, indicating that increased colonization of SSCs was not a result of greater total area available for colonization by individual SSCs. Collectively, these findings indicate that an increase in Sertoli cell number alone was the determining factor for greater SSC niche accessibility and demonstrate that Sertoli cells are a critical support cell population that regulates stem cell niches in mouse testes. Further studies are needed to determine how Sertoli cells regulate SSC niches, but it is possible they orchestrate the contributions of other support cells, such as myoid and Leydig cells, in response to cues from SSCs and other spermatogonia. In fact, cross-communication of Sertoli cells with interstitial cell populations including Leydig cells has been established (47, 134, 136, 150), but whether this mechanism influences SSC niches remains to be determined.

V. CONCLUSIONS

Defining the components of SSC niches in mammalian testes is important for understanding the foundation of continual spermatogenesis. This knowledge could aid in developing approaches for treating instances of male infertility caused by impaired functions of SSCs and refining in vitro culture methods for maintaining and expanding SSCs. The capability to maintain SSCs in vitro provides a valuable model to study the molecular mechanisms controlling SSC fate decisions and provides a means to preserve and expand male germlines. Moreover, SSCs are a valuable model for deciphering conserved mechanisms regulating the functions of stem cells in other renewing tissue lineages. In fact, SSCs are the only adult tissue-specific stem cell population that can be maintained in long-term culture as a self-renewing population and for which an in vivo transplantation functional assay is available for studying their biology. These capabilities allow for robust investigation of the extrinsic niche cues influencing SSC function and the molecular mechanisms activated or suppressed within SSCs that ultimately control the balance of self-renewal and differentiation.

Investigations over the last two decades with rodent models have begun to identify components of niches that regulate SSC functions including the growth factor milieu provided by somatic support cell populations (FIG. 8). The cytokines GDNF, FGF2, LIF, and IGF-I have been shown to promote the proliferation and survival of SSCs and progenitor spermatogonia, whereas CSF-1 specifically influences the process of SSC self-renewal in vitro without affecting proliferation of progenitor spermatogonia. In contrast, exposure to Neuregulin 1 and BMP4 in vitro appears to promote the differentiation of SSCs. Expression of most of these factors has been linked to one or more somatic support cell populations in vivo including Sertoli, Leydig, and myoid cells. However, recent evidence indicates that Sertoli cells play a major role in establishing the SSC niche in mouse testes, and they may achieve this through orchestrating the contributions of other somatic cell populations.

Several fundamental questions need to be addressed in the future to further define niche microenvironments in mammalian testes. Currently, only studies on GDNF have provided direct *in vivo* evidence of a role in regulating spermatogonia and likely SSC functions. Knowledge of the other factors has come from investigating their effects on SSC maintenance and proliferation *in vitro*. Thus, whether FGF2, LIF, IGF-I, CSF-1, Neuregulin 1, and BMP4 also influence the self-renewal and differentiation of SSCs *in vivo* will need to be addressed. Another unanswered question is whether the niche cues influencing SSC function in testes of rodents are conserved in other mammalian species. A recent report demonstrated that SSCs of rabbits require GDNF for continual expansion *in vitro* (68), but long-term proliferation of SSCs from other higher order species has been difficult to confirm. Several studies have shown that exposure to GDNF affects the proliferation of bovine undifferentiated spermatogonia *in vitro*, but long-term maintenance of SSC self-renewal has not been described (2, 103). Also, recent studies have indicated that human undifferentiated spermatogonia can be maintained *in vitro* for long periods of time in medium supplemented with GDNF (43, 120). However, evidence that SSCs are present in these cultures or that exposure to GDNF is essential for their survival and proliferation has not been provided. Investigations of mice at different developmental stages found that expansion of SSCs is greater during the early postnatal period compared with in adulthood (132). This finding indicates that the niche microenvironment provides different cues depending on the state of testes function. It is possible that during early postnatal development when expansion of SSC numbers is required to establish the stem cell pool that makeup of the niche is designed to promote a high rate of self-renewal without differentiation, whereas in adulthood during steady-state spermatogenesis an appropriate balance of self-renewal and differentiation is supported by different signals emanating from the niche. An important future step will be defining the differences between niches promoting a high frequency of SSC self-renewal and niches maintaining less self-renewal and more differentiation. Knowledge of these influences would aid in refining culture conditions to promote greater expansion of SSC numbers for rodents and development of culture conditions to expand SSCs from other species. Moreover, the potential to rejuvenate failing niches and rescue male fertility would be enhanced greatly by understanding of the cues altering SSC behavior *in vivo*. This potential is especially applicable to defining the effects of aging on niche function and how failing niches contribute to loss of homeostasis in the mammalian testis which leads to impaired spermatogenesis. Recent evidence indicates that systemic factors influence niche functions to support stem cell activities (26, 119). Thus defining these factors and how they affect support cell contributions to the niche may be key to realizing the regenerative potentials of tissue-specific stem cells for treating degenerative diseases. Aside from the information gained about their role in sustaining spermatogenesis, the SSC population provides a valuable model for study that could yield seminal discoveries about niche function in a multitude of stem cell-dependent tissue lineages.

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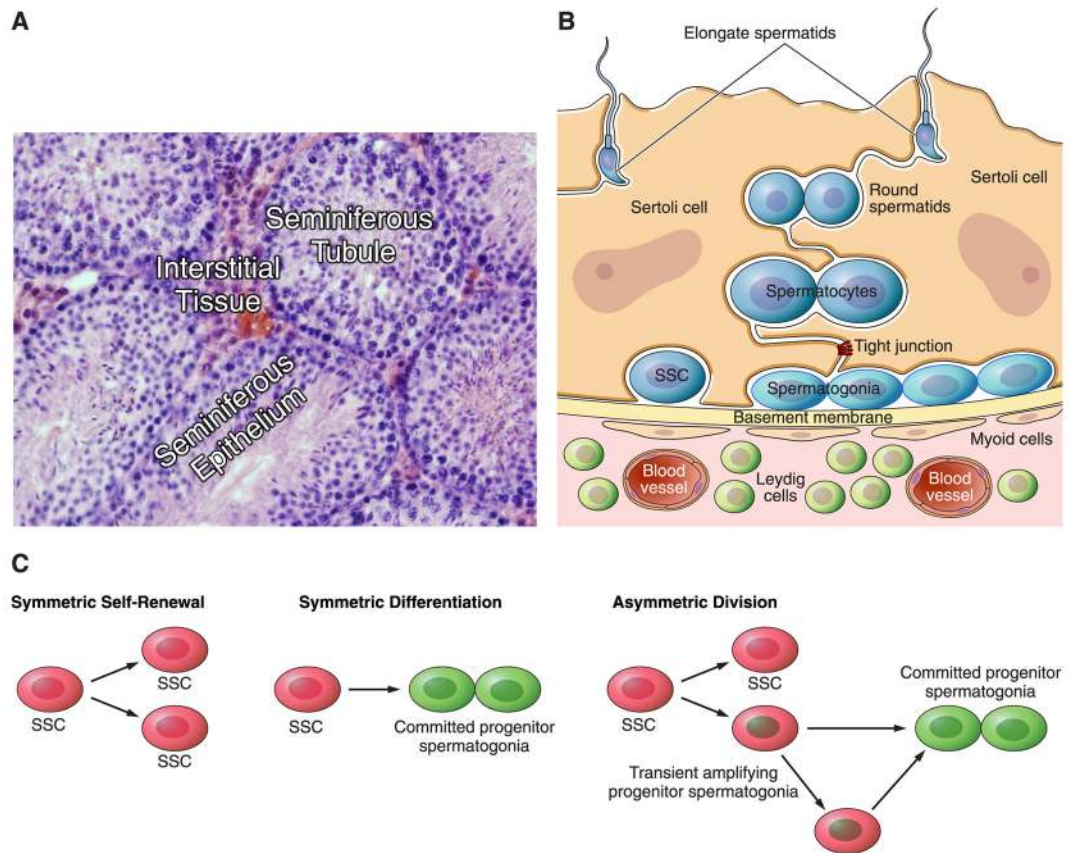
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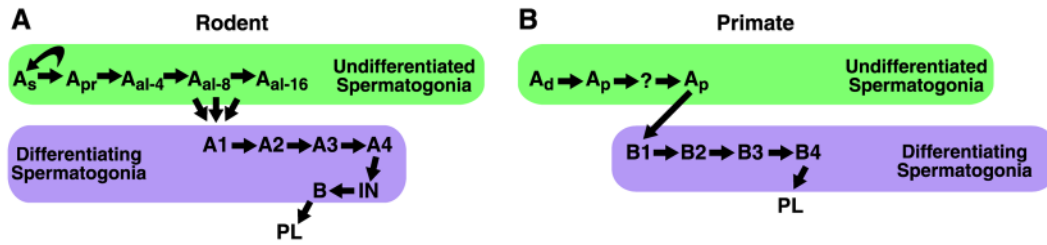
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**FIGURE 1.**

Spermatogenesis in mammalian testes. Testicular parenchyma consists of seminiferous tubules and interstitial tissue. *A*: cross-section of testicular parenchyma from an adult mouse stained with hematoxylin and eosin. *B*: schematic recreation of the seminiferous epithelium and interstitial tissue. Germ cell maturation from undifferentiated spermatogonia to elongate spermatids occurs within seminiferous tubules that are bound by a basement membrane. Within seminiferous tubules, the developing germ cells associate with somatic Sertoli cells to make up the seminiferous epithelium. Tight junctions between adjacent Sertoli cells separate the seminiferous epithelium into a basal compartment that houses the spermatogonia and an adluminal compartment, which contains maturing meiotic spermatocytes, haploid spermatids, and spermatozoa. Rigidity of the seminiferous tubules is provided by myoepithelial cells or myoid cells, which line the outside of the basement membrane. Interstitial tissue is located between seminiferous tubules and consists primarily of clusters of Leydig cells that secrete testosterone, the vascular network, and various immune cell populations. *C*: potential outcomes of SSC division in mammalian testes. During neonatal development of the germline and regeneration of steady-state spermatogenesis after cytotoxic insult, symmetrical self-renewal may predominate to establish a stem cell pool. During steady-state spermatogenesis, a balance of symmetrical self-renewal and differentiation may occur at defined frequencies to maintain a stem cell pool and provide the next cohort of progenitor spermatogonia that are committed to further differentiation. Recent evidence suggests that during steady-state conditions in rodent testes true SSCs and transient amplifying progenitor spermatogonia that have limited proliferative potential before giving rise to committed progenitor are present. This model suggests that asymmetric division of an SSC produces one new SSC and one transient amplifying progenitor.

**FIGURE 2.**

Spermatogonial hierarchy in rodents (A) and primates (B). In rodents, the undifferentiated spermatogonial population consists of A_{single} (A_s), A_{paired} (A_{pr}), and $A_{aligned}$ (A_{al}) spermatogonia. Most A_{al} undifferentiated spermatogonia are capable of giving rise to differentiating spermatogonia at the A1 spermatogonial stage, which develop further into A2–4 spermatogonia followed by intermediate (IN) and B spermatogonia, which initiate meiotic prophase to become preleptotene spermatocytes (PL). The A_s spermatogonia are widely considered to represent the true SSC population that self-renew and differentiate to produce A_{pr} spermatogonia, which marks the beginning of spermatogenesis. In primates, the undifferentiated spermatogonial population consists of A_{dark} (A_d) and A_{pale} (A_p) spermatogonia. These germ cells have been considered the resting (A_d) and active (A_p) SSC populations. After a set number of divisions that is currently undefined, the A_p spermatogonia give rise to differentiating spermatogonia beginning with B1 spermatogonia followed by B2–4 spermatogonia from which preleptotene spermatocytes (PL) are derived.

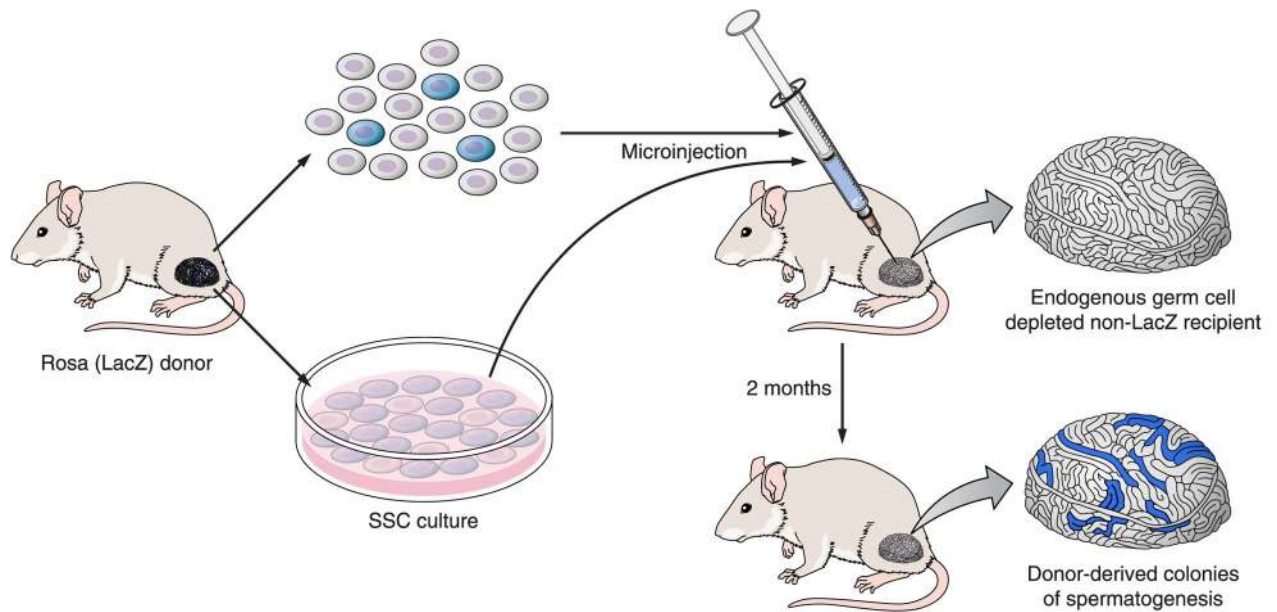


FIGURE 3.

The functional SSC transplantation assay in mice. Stem cells are defined by the functional ability to maintain and reestablish a tissue lineage. For spermatogenesis, SSCs are defined by an ability to reform spermatogenesis following colonization in a recipient's testes. For this methodology, a testis cell population can be collected fresh from a donor testis or following a culture period and microinjected into the testes of recipient males that have been depleted of germ cells by treatment with chemotoxic compounds or by using sterile mutant males that lack germ cells (e.g., W/W^v mice). Colonies of donor-derived spermatogenesis can be detected several months later in the recipient testes if SSCs were present in the injected cell suspension. Colonies of donor-derived spermatogenesis can easily be detected and quantified if transgenic donors that express a marker gene (e.g., LacZ or GFP) are used. In particular is the use of Rosa mice as donors which contain the LacZ gene within the Rosa26 locus, thereby providing ubiquitous expression in all germ cell types. Spermatogenesis arising from transplanted Rosa SSCs is easily identifiable in testes of non-Rosa recipient mice following incubation with the X-Gal substrate, which is converted to a blue product by the β -galactosidase enzyme produced from the LacZ gene. Because each reformed colony is clonally derived from an individual SSC, this system provides a quantifiable measure of SSC number in the injected cell suspension. Also, the number of reformed colonies reflects (a measure of) the number of accessible niches within recipient testes that can be colonized by injected donor SSCs.

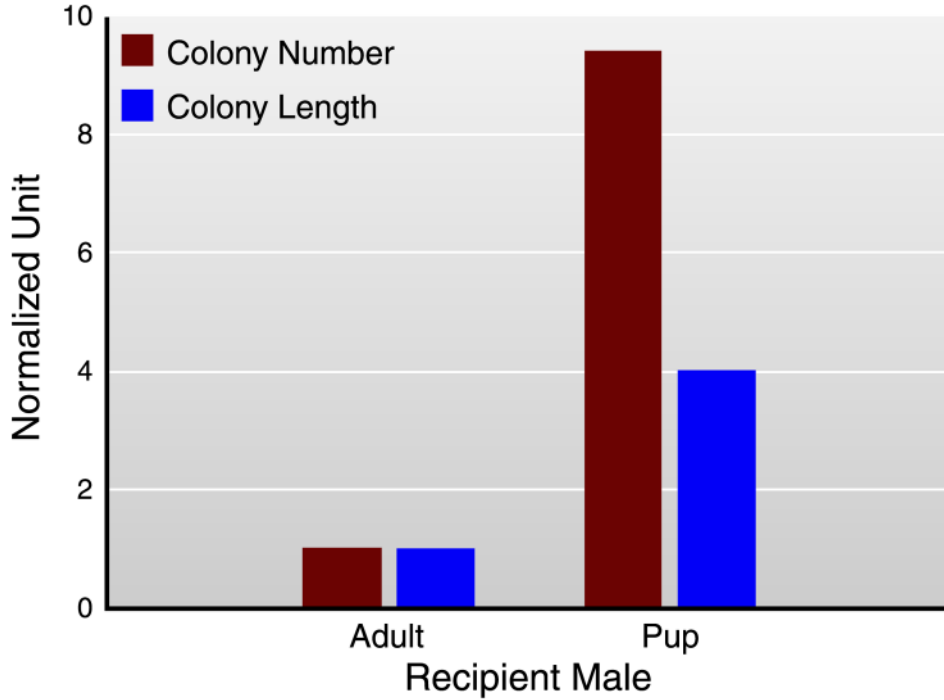
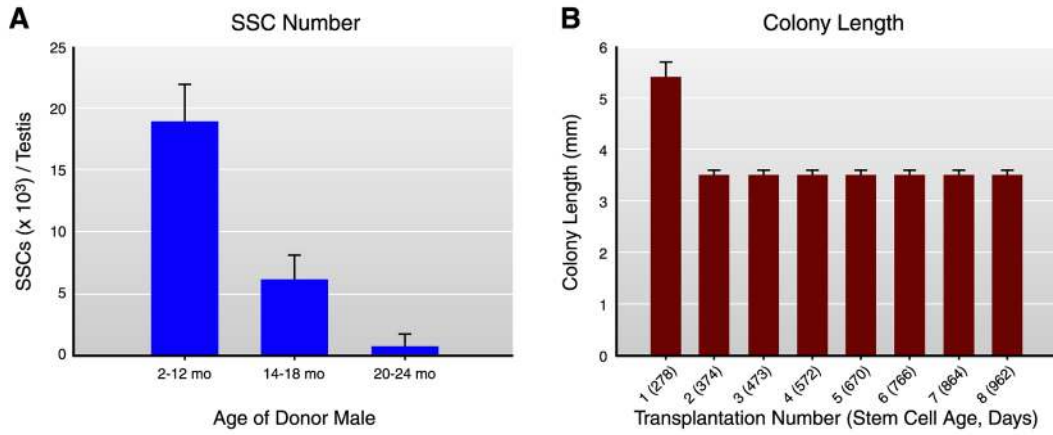


FIGURE 4. Impact of niche developmental stage on support of SSC activity in mice. The functional transplantation assay was used to measure niche activity in testes of adult and prepubertal pup recipient male mice. The number of colonies of reestablished spermatogenesis (red bars) is a reflection of the number of accessible niches for colonization. The length of reestablished colonies of spermatogenesis (blue bars) is a reflection of niche support of SSC expansion upon colonization. Regardless of donor SSC age, a greater number of colonies are formed (9.4 \times), and each colony is longer (4 \times) in recipient testes of young prepubertal pups than adults. These findings indicate a greater number of accessible niches and greater niche support of SSC expansion in seminiferous tubules of pups during development of the germline compared with adults in which steady-state spermatogenesis has occurred (131, 132).

**FIGURE 5.**

Impact of aging on niche support of SSC activity in testes of mice. *A*: assessment of SSC number in testes of mice at different time periods of postpubertal aging. These findings suggest an age-related decline in SSC number, which could be a result of impaired SSC function or niche support. *B*: effect of age on the ability of SSCs to expand in number and regenerate spermatogenesis when serially transplanted into testes of young mice. These findings indicate that aging does not impair SSC activity when the SSCs are continually exposed to niches in testes of young males. Collectively, these findings indicate a decline in niche support of SSC functions in aged males leading to impaired SSC maintenance. For *A* and *B*, data were obtained using the functional germ cell transplantation assay to measure SSC numbers in donor testes (*A*) and biological activity of aged colonizing SSCs within niches of testes from young recipient mice. Colony length is longer in the first transplant because the cells are taken from cryptorchid mice in the original transplantation of the series. These stem cells are likely in a different status than those taken for subsequent transplantations, which came from full spermatogenesis (119).

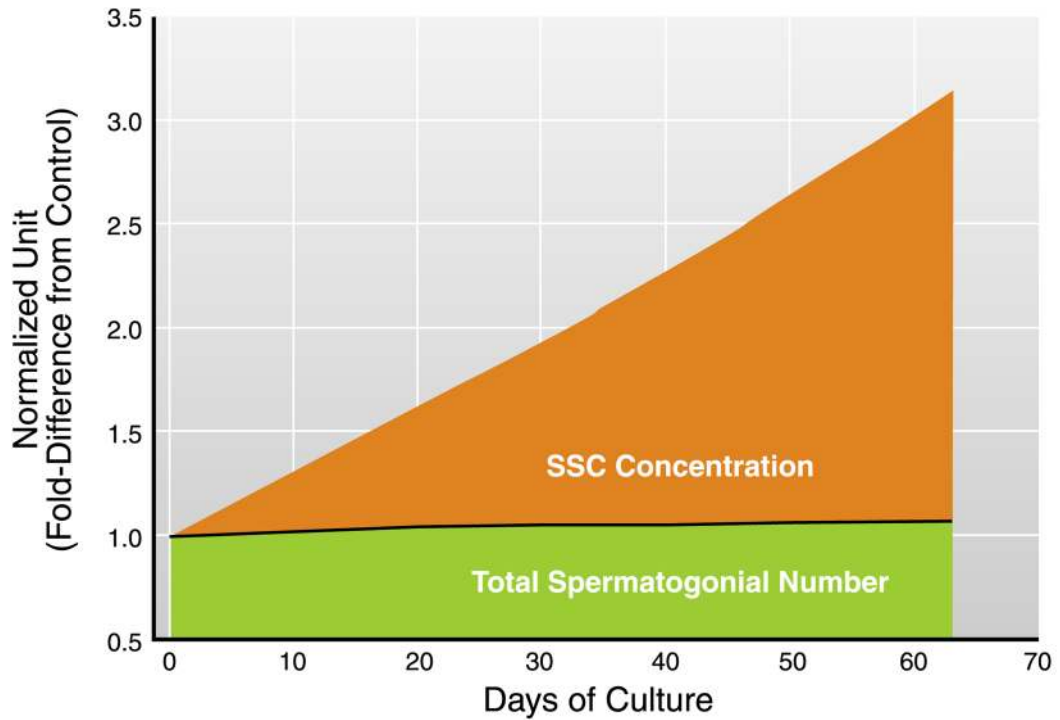


FIGURE 6.

Influence of colony stimulating factor-1 (CSF-1) on self-renewal of mouse SSCs. Cultures of undifferentiated spermatogonia were maintained in serum-free medium with supplementation of GDNF and FGF2, which supports self-renewal of SSCs and generation of A_{pr}/A_{al} -like spermatogonia for long periods of time. Exposure to CSF-1 in addition to GDNF and FGF2 for longer than 2 mo increased SSC number specifically (orange area) without affecting the expansion of total spermatogonial number (green area). Data are fold-difference of SSC and spermatogonial number in CSF-1-treated cultures compared with control cultures exposed to GDNF and FGF2 only, and SSC numbers were determined by transplantation analyses. These findings indicate that exposure to CSF-1 selectively promoted a greater frequency of SSC self-renewal to generate more SSCs at the expense of differentiation to produce A_{pr}/A_{al} spermatogonia (102).

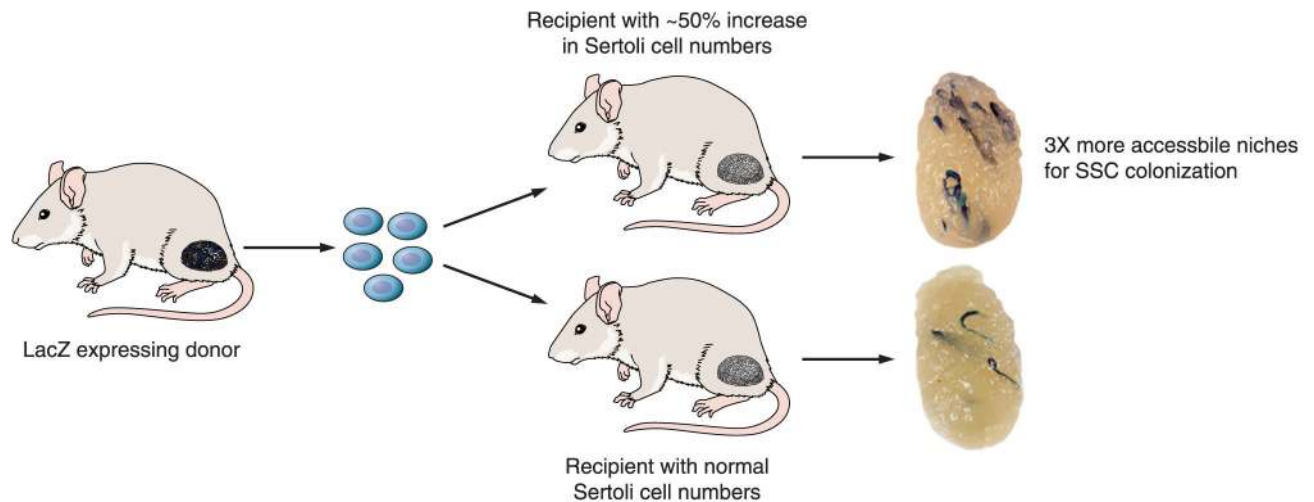


FIGURE 7.

Impact of Sertoli cell number on SSC niches in testes of adult mice. Transplantation analyses were used to investigate whether an alteration in Sertoli cell number influences the number of niches accessible for colonization by transplanted SSCs in testes of mice. These experiments utilized Rosa mice as SSC donors which express the LacZ marker transgene in all germ cell types. Thus colonies of spermatogenesis arising from transplanted SSCs within accessible niches of testes from non-LacZ expressing recipient mice could be easily quantified. An increase of ~50% in Sertoli cell number resulted in greater than a 3-fold increase in accessible niches for colonization by transplanted SSCs from LacZ-expressing Rosa donor mice. These findings indicate that Sertoli cells influence SSC niche function in seminiferous tubules of mice (104).

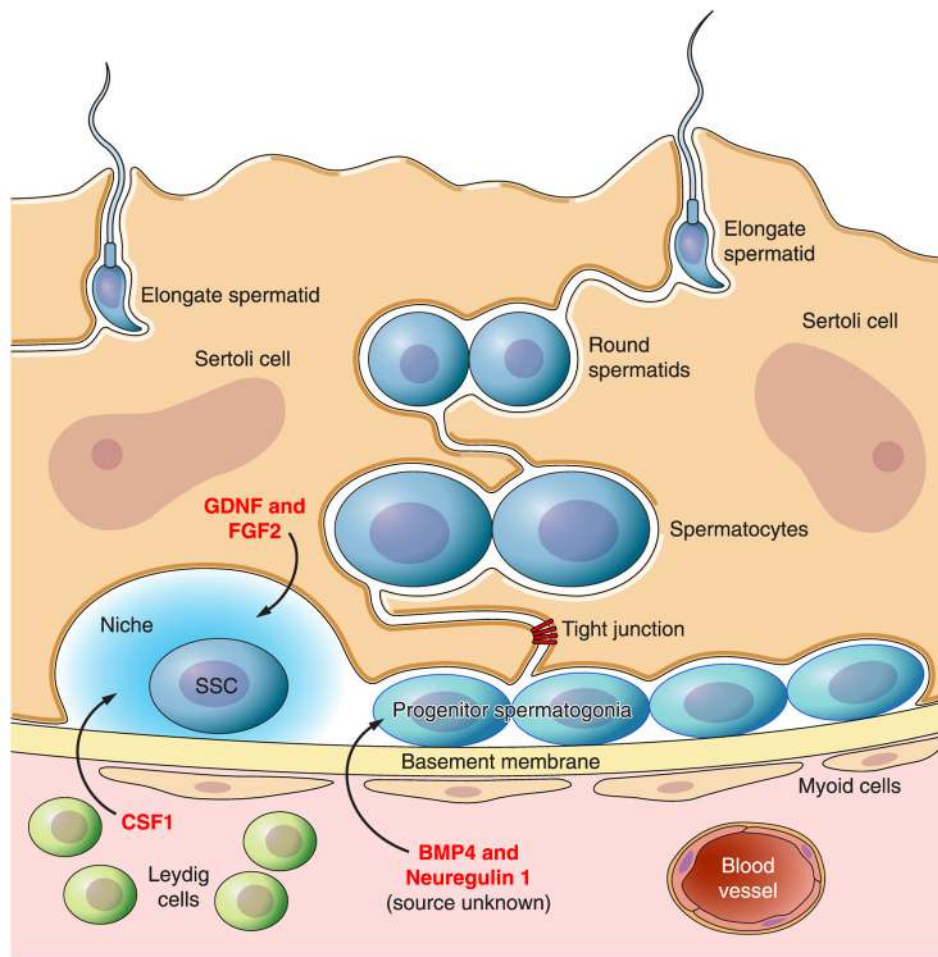


FIGURE 8. Schematic depicting the current understanding of determinants of the spermatogonial stem cell (SSC) niche in mammalian testes. Sertoli cells are known to dictate the formation of niche microenvironments and have been shown to produce the growth factors GDNF and FGF2 which regulate SSC proliferation and survival. Leydig cells are a source of CSF-1 which specifically regulates self-renewal of SSCs. The differentiation of SSCs is influenced by BMP4 and Neuregulin 1; however, the source of these factors is currently unknown. It is believed that upon differentiation from SSCs the resulting progenitor spermatogonia (i.e., A_{pr}/A_{al}) migrate away from the niche and continue to develop as a cohort of maturing germ cells.