Chapter 7

# Guardians of the Germline: the Origin and Regenerative Promise of Spermatogonial Stem Cells

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## Abstract

Spermatogonial stem cells form the basis of the male reproductive system and are unique cells with the ability to self-renew and differentiate, and are the only stem cells capable of transmitting genetic traits from one generation to the next. These cells differentiate into mature sperm after dividing through a special mechanism called spermatogenesis. Specific markers, including GPR125 and OCT-4, enable the isolation and identification of SSCs .The microenvironment of SSCs is located in the basement membrane of the seminiferous tubules and consists of various cell types and extracellular matrix that provide the factors and interactions necessary for the sustenance and function of the cells. Sertoli cells are one of the main components in the testicular microenvironment that are directly associated with SSCs and mediate protecting, nourishing, proliferation, and differentiation of germ cells via secreting various types of factors. Isolation, culture, and cryopreservation of SSCs are used as a method to treat some cases of infertility. Today, stem cell-based therapies such as SSCs transplantation to restore fertility in cancer patients and in vitro spermatogenesis are one of the most promising tools in improving male fertility.

**Keywords:** Spermatogonial stem cells, Spermatogenesis, Self-renewal, Differentiation, Therapeutic applications

## 1. Introduction

Undifferentiated germ cells, known as SSCs[1], continuously sustain spermatogenesis throughout adulthood [1]. This rare population of stem cells is located in the basal part of the seminiferous epithelium of testicular tissue. SSCs are essential for spermatogenesis and have a critical role in

[1]. Spermatogonial stem cells

transmitting genetic material to offspring [2]. SSCs possess two essential capacities of self-renewal to preserve the stem cell population and differentiate into progenitor cells that eventually develop into sperm [3]. They originate from PGCs[1] during embryogenesis [4]. The functionality of SSCs is critically dependent on their specialized microenvironment, known as the stem cell niche [5]. The stem cell niche is composed of multiple elements, including growth factors, different cell types, ECM[2], and other signaling factors, all of which interact to control the SSCs' proliferation and differentiation [5]. Sertoli cells are the structural basis of this niche; they not only nourish SSCs but also play a significant role in their self-renewal and differentiation by secreting various factors [6]. Several molecular markers are used to identify and study SSCs, including OCT- 4, GFRα1, PLZF, and THY1 (CD90). These markers help distinguish SSCs from other cell types and facilitate their isolation for research or clinical applications [7]. Spermatogenesis, the process by which male germ cells are generated, includes the self-renewal of SSCs and their differentiation into mature sperm [8]. This process occurs in the seminiferous epithelium of the testis and is regulated by a network of endocrine and regulatory factors [9]. Spermatogenesis is divided into three stages: spermatocytogenesis, meiosis, and spermiogenesis. During spermatocytogenesis, spermatogonia divide mitotically to form type A and B spermatogonia, which subsequently develop into primary spermatocytes. During meiosis, primary spermatocytes undergo the first meiotic division, producing secondary spermatocytes. The secondary spermatocytes progress through the second meiotic division, resulting in the formation of spermatids. Following this, spermiogenesis occurs, in which round

1. Primordial germ cells
2. Extracellular matrix

spermatids undergo morphological and biochemical changes and become spermatozoa [10]. The study of SSCs and the process of spermatogenesis is clinically significant, particularly because many forms of male infertility stem from defects in SSC function or disruptions in spermatogenesis stages. Various diseases, genetic factors, and damage caused by chemotherapy can impair the SSC function and reduce fertility [11, 12]. Research on the biology of SSCs has led to promising strategies for preserving fertility, especially in young boys undergoing cancer treatment. Advancements in stem cell science have initiated the development of methods such as SSC transplantation and in vitro spermatogenesis, which are now being intended as treatment options [2].

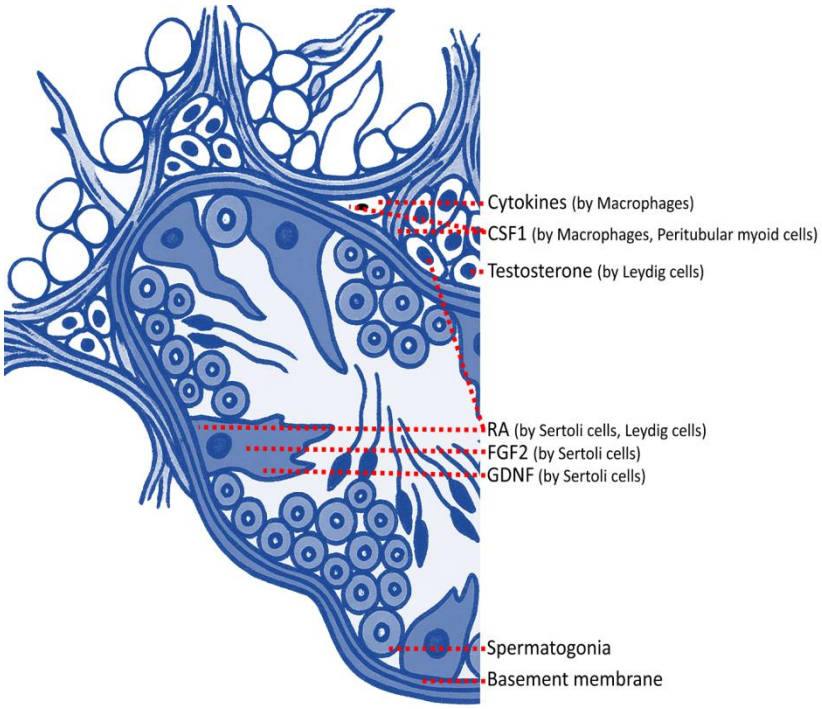
## 2. Origin of SSCs

SSCs originate from PGCs during early embryonic development [4]. In humans, PGCs arise from the epiblast, a layer of cells in the early embryo, around the second to third week of embryogenesis. Initially, PGCs form near the yolk sac and migrate through the hindgut and abdominal mesentery to the gonads, which eventually develop into the testes. After migration, PGCs proliferate and differentiate into gonocytes [13]. Following birth, gonocytes move to the basal membrane of the seminiferous tubules and transform into SSCs [14].

## 3. SSCs Niche

The term "niche" refers to a specific microenvironment where stem cells are located and stored. Within this niche, stem cells interact with other cells and factors that regulate their self-renewal, differentiation, and overall cell fate [15]. SSC niche resides in the basal part of the seminiferous tubules [16]. The main components of the SSC niche include Sertoli cells, peritubular myoid cells, Leydig cells, and macrophages [17]. Sertoli cells

play a critical role in the SSC niche. These somatic cells are directly connected to SSCs and are involved in nourishing and protecting them [18]. Moreover, Sertoli cells are involved in controlling the self-renewal and differentiation of SSCs via secreting various growth factors such as GDNF[1] and FGF2[2], along with cytokines and proteins [19]. Peritubular myoid cells contribute to the contractile activity of testicular tubules and help form the ECM. They also regulate SSC function by secreting growth factors [20]. Leydig cells primarily secrete testosterone [21]. While macrophages in the microenvironment play a role in SSC self-renewal and differentiation by secreting CSF1[3] and retinoic acid, respectively (**Figure 1** and **Table 1**) [22]




[1]. Glial cell line-derived neurotrophic factor
[2]. Fibroblast Growth Factor 2
[3]. Colony Stimulating Factor 1

**Figure 1**. SSC niche atlas (human/mouse cross-walk). Schematic of the basal compartment showing SSCs, Sertoli junctions (BTB), peritubular myoid cells, Leydig cells, macrophages, and key ligands (GDNF, FGF2, CSF1, RA). Cellular and molecular architecture of the SSC niche integrating paracrine and ECM cues that balance self-renewal and differentiation.

**Table 1**. Niche-derived factors regulating spermatogonial stem cell (SSC) self-renewal and differentiation

| *Source cell* | *Secreted factor* | *SSC receptor/target* | *Activated pathway(s)* | *Primary biological effect* | *Key references* |
|---|---|---|---|---|---|
| ***Sertoli cell*** | **GDNF**[1] | GFRA1 + RET | PI3K–AKT, RAS–ERK | Promotes SSC self-renewal and proliferation | [19], [36], [38] |
| ***Sertoli cell*** | **FGF2**[2] | FGFR1/2 | RAS–MAPK, PI3K–AKT, WNT/β-catenin | Supports SSC proliferation, synergistic with GDNF | [39], [40] |
| ***Sertoli / interstitial cells*** | Retinoic acid (RA) | RARα/γ → STRA8/MEIOSIN | RA signaling cascade | Induces spermatogonial differentiation and meiotic entry | [44], [45], [62] |
| ***Peritubular myoid cell*** | **CSF1**[3] | CSF1R | CSF1R–MAPK/PI3K | Enhances SSC survival and self-renewal | [20], [36] |
| ***Peritubular myoid cell*** | **LIF**[4] | LIFR–gp130 | JAK–STAT3 | Stimulates SSC proliferation in vitro with GDNF | [40] |
| ***Leydig cell*** | **Testosterone** | Androgen receptor (AR) in Sertoli cells | AR-mediated transcription | Indirectly maintains Sertoli support of SSCs, regulates BTB | [21], [74] |
| ***Macrophage (interstitial)*** | **CSF1, cytokines** | CSF1R and various receptors | MAPK, STAT | Modulates SSC niche homeostasis, immune-paracrine support | [22], [36] |
| ***Basement membrane / ECM*** | **Laminin, collagen IV, fibronectin** | ITGA6/ITGB1 integrins | FAK–PI3K | Provides adhesion, polarity, and positional cues to SSCs | [2], [16] |

[1]. Glial-cell-derived neurotrophic factor
[2]. Basic fibroblast growth factor: FGF
[3]. Colony-stimulating factor 1
[4]. Leukemia inhibitory factor

## 4. SSCs Markers

SSCs are a rare and heterogeneous subpopulation of undifferentiated spermatogonia. To facilitate the enrichment and characterization of these cells from a variety of testicular tissue cell types, numerous specific molecular markers have been introduced [23]. One such marker is GPR125[1], which has been reported as a marker for both mouse and human SSCs [24]. Furthermore, OCT- 4 has been known as a common marker for SSCs in humans and mice [25]. Other markers for human SSCs include SSEA4[2], GFRα1R[3], and EPCAM[4] [26]. However, the expression of GFRα1R, GPR125, and EPCAM also been proven in adult testicular stromal cells and cannot be solely used for identifying SSCs [27]. Additionally, Thy-1 (CD90) is a marker that helps in the identification and isolation of mouse and rat SSCs, with approximately 95% of SSCs being Thy1-positive Based on FACS analysis [26, 28, 29]. Testicular tissues from adult rhesus are also positive for the expression of DDX4 (VASA), DAZL, GFRα1, and PLZF [7]. In mice, common markers of SSCs and progenitor cells include α6-integrin (CD49f), β1-integrin (CD29), and Thy-1 [2]. Markers such as α6-integrin, CD90, GFRα1, and CD133 have been successfully applied to isolate human spermatogonia using MACS[5] [30]. While advances in the study of molecular markers and biomarkers for germline stem cells have improved the ability to enrich SSCs, a definitive and unique marker for SSCs has yet to be identified [2]. Furthermore, additional molecular markers for SSCs have been proposed, including

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1. G protein-coupled receptor 125
2. Stage-specific embryonic antigen-4
3. GDNF family receptor alpha-1
4. Epithelial cell adhesion molecule
5. Magnetic-activated cell sorting

transcription factors such as PLZF, Lin28, and Ngn3[1] [31]. PLZF and Lin28 are expressed in As, Apr, and Aal spermatogonia [32, 33]. While Ngn3 is mainly detected in Aal spermatogonia (**Table 2**) [32].

[1]. Neurogenin 3

**Table 2.** SSC markers across species and compartments

| *Marker (gene/protein)* | *Type* | *Species evidence* | *Main compartment/pattern* | *Specificity & typical use* | *Key caveats* | *Refs* |
|---|---|---|---|---|---|---|
| ***GFRA1 (GDNF family receptor α1)*** | Surface | Mouse, human, rhesus | Undifferentiated spermatogonia (As/Apr/Aal); basal compartment | Enrichment of SSCs/ undifferentiated pool; often combined with ITGA6/THY1 | Also present on some stromal/interstitial cells in human tissue; not SSC-exclusive | [7, 23,26, 27] |
| ***RET*** | Surface (RTK) | Mouse, human | Co-expressed with GFRA1 in undifferentiated spermatogonia | Part of the GDNF receptor complex for SSC maintenance | Broader expression during development; not ideal alone | [2, 25,36,38 |
| ***ITGA6 (CD49f)*** | Surface (integrin) | Mouse, human | Basal spermatogonia; cell–ECM interface | Useful for FACS/MACS enrichment (often with ITGB1/THY1) | Also, on Sertoli/basal cells in some contexts, not SSC-specific | [2, 25,26] |
| ***ITGB1 (CD29)*** | Surface (integrin) | Mouse | Basal spermatogonia | Frequently paired with ITGA6 for enrichment | Broad integrin; requires combinatorial gating | [2] |
| ***THY1 (CD90)*** | Surface | Mouse, rat | Undifferentiated spermatogonia enriched in the $THY1^{+}$ fraction | Robust enrichment of rodent SSCs by FACS | Limited human specificity; stromal contamination possible | [26, 28] |
| ***EPCAM*** | Surface | Human | Basal spermatogonia | Positive selection marker in some human protocols | Also expressed by adult testicular stromal/epithelial cells—use with other markers. | [26, 27] |
| ***GPR125*** | Surface (GPCR) | Mouse, human | Undifferentiated spermatogonia | Reported as SSC/ Undifferentiated marker, aids isolation | Expression in other cell types reported; validation varies by antibody | [24, 25,27] |

| | | | | | | |
|---|---|---|---|---|---|---|
| ***SSEA4*** | Surface glycolipid | Human | Subset of undifferentiated spermatogonia | Used to enrich human SSC-like populations | Not exclusive to SSCs; developmental stage-dependent | [26] |
| ***KIT (CD117)*** | Surface (RTK) | Mouse | Differentiating type A1–A4/B spermatogonia | Negative selection for Undifferentiated SSCs ($KIT^-$ fraction) | Up-regulated upon differentiation; species and stage-dependent | [44] |
| ***CD133 (PROM1)*** | Surface | Human | Reported in spermatogonia | Occasionally used with ITGA6/GFRA1 | Heterogeneous/variable expression; not SSC-specific | [30] |
| ***PLZF (ZBTB16)*** | Intracellular (TF) | Mouse, human | As/Apr/Aal undifferentiated spermatogonia nuclei | Hallmark TF for undifferentiated Pool/SSC maintenance | Intracellular—cannot be used for live sorting without permeabilization; present in some progenitors | [31, 32,41, 43] |
| ***LIN28A*** | Intracellular (RNA-binding) | Mouse | As/Apr/Aal undifferentiated spermatogonia | Marks undifferentiated pool; developmental regulation | Not strictly SSC-exclusive; decreases with differentiation | [33] |
| ***NGN3 (NEUROG3)*** | Intracellular (TF) | Mouse | Predominantly Aal (undifferentiated progenitors) | Distinguishes SSC vs progenitor states in mouse | Limited human data; mainly progenitor marker | [31, 32] |
| ***DDX4 (VASA)*** | Intracellular (RNA helicase) | Human, rhesus | Pan-germ cell cytoplasm (all stages) | Germline identifier for histology; not for SSC enrichment | Not specific to SSCs; absent from somatic cells but present across the germline | [7, 23] |
| ***DAZL*** | Intracellular (RNA-binding) | Human, rhesus | Broad germ cell expression | Confirms germ cell identity | Not specific; overlaps with differentiating cells | [7] |
| ***OCT4 (POU5F1)*** | Intracellular (TF) | Mouse, human (variable) | Reported in undifferentiated spermatogonia in some studies | Occasionally used as a pluripotency-related marker in SSC literature | Antibody-specific and stage-dependent; controversial in adult humans | [25] |

## 5. SSCs Self-renewal and Differentiation

Stem cells that can self-renew and differentiate. These cells ensure the continuous generation of mature sperm throughout the life of the organism. These two properties are unique to stem cells [34]. Self-renewal refers to the process by which stem cells divide mitotically to form daughter cells, while differentiation is the process through which undifferentiated cells become specialized cells [22]. The balance between self-renewal and differentiation is essential for supporting cell homeostasis, and spermatogenesis relies on the self-renewal capacity of SSCs. Numerous factors and signaling pathways regulate the self-renewal and differentiation of SSCs [35]. GDNF, an important element for preserving self-renewal of SSC, is secreted by Sertoli cells. It binds to the tyrosine kinase receptors Ret and GDNF α1 family on spermatogonial cells, activating two signaling pathways: PI3K/Akt and SFK[1], which promote SSC self-renewal [22, 36]. Among the targets of these signaling pathways are the transcription factors MYCN and FOS [37]. The activation of RET and SRC-kinase proteins leads to the phosphorylation of PI3K, activating the AKT pathway and ultimately inducing the expression of the Mycn gene. Simultaneously, the binding of GDNF to GFRA1 and RET triggers the RAS-ERK1/2 pathway, which regulates SSC proliferation by activating the FOS transcription factor [38]. Sertoli cells also control the self-renewal of SSCs by secreting FGF2 (**Figure 2**). The activation of various signaling pathways, including WNT/β-Catenin, RAS/MAPK, PI3K/AKT, and TGF-β, due to the phosphorylation of tyrosine kinase receptors following the binding of FGF2 to FGFR. This controls the expression of downstream genes, influencing cellular growth and reproduction of distinct sperm [39].

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[1]. Src family kinase

Csf1[1], secreted by Leydig and peritubular myoid cells, enhances SSC self-renewal by binding to its receptor (Csf1r) on undifferentiated spermatogonia [36]. Additionally, LIF[2], secreted by peritubular myoid cells, plays a role in stimulating SSCs' proliferation alongside GDNF [40].

The transcription factor PLZF is the first identified factor essential for the self-renewal of germinal stem cells in the testis. Studies indicate that PLZF controls the self-renewal of SSCs via suppressing the mTORC1 pathway, a mechanism thought to be crucial in preserving the balance between SSC self-renewal and differentiation [41]. Activated mTORC1 can reduce the expression of the GDNF receptor [42]. PLZF is involved in inducing the expression of Redd1, an inhibitor of the mTORC1 signaling pathway [43].

In addition to factors promoting SSC self-renewal, some agents facilitate the differentiation of these cells. RA signaling has been reported to significantly influence spermatogonia differentiation [31]. In conditions of vitamin A deficiency, the differentiation of undifferentiated spermatogonia into type A1 spermatogonia is impaired [44], highlighting the critical role of RA receptors in the differentiation of spermatogonia [45].

[1]. Colony-stimulating factor 1
[2]. Leukemia inhibitory growth factor

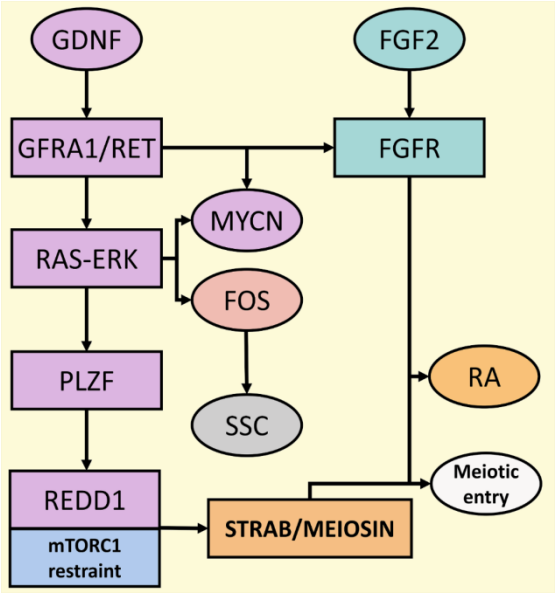


**Figure 2.** Signaling pathways governing SSC fate. Pathways from GDNF → GFRA1/RET → PI3K–AKT & RAS–ERK (MYCN, FOS); FGF2 → FGFR; PLZF → REDD1 → mTORC1 restraint; RA → STRA8/MEIOSIN → meiotic entry. Core signaling circuits controlling SSC maintenance versus differentiation, highlighting convergence on proliferation and meiotic licensing.

## 6. Culture of SSCs

The culture of SSCs in vitro is essential for studying biological processes such as spermatogenesis, fertility preservation, and clinical applications of these cells [2]. Due to the scarcity of SSCs available, one of the critical steps in their culture involves isolating and enriching these cells from other somatic cells present in testicular tissue [46]. The isolation process typically entails a two-step enzymatic digestion using collagenase and trypsin. Initially, the testicular tissue is cut into smaller pieces, and collagenase Type 1 is utilized to dissociate the seminiferous tubules. Following this, trypsin is used to achieve a single-cell suspension [47]. However, due to the cellular heterogeneity of testicular tissue and the scarcity of SSCs, additional enrichment methods are necessary [48].

Techniques such as differential plating, MACS, and FACS[1] are commonly used to enhance the purity of SCCs [25]. Differential culture is based on the greater adhesion of somatic cells to gelatin compared to SSCs [49]. MACS and FACS utilize surface markers of SSCs, along with specific antibodies like OCT-4, CD90, and GFRα1, allowing for a higher purity of isolation compared to other methods [50]. FACS is often preferred over MACS due to its efficiency and lower requirement for cell numbers [2]. Successful cultivation of SSCs relies on supportive cells and supplements in the culture medium. Sertoli cells serve as feeder and supportive cells, providing nourishment and mechanical support to SSCs [51]. These Sertoli cells also have a crucial role in regulating SSCs' self-renewal and differentiation by secreting growth factors [52]. In testicular cell cultures, feeder cells can either naturally exist within the cellular suspension derived from the testicular biopsy or be introduced from an external source [53]. Key growth factors such as GDNF and bFGF are used to promote cell proliferation [53]. Studies have shown that culture medium supplemented with these factors helps maintain SSCs' self-renewal [54]. Additionally, LIF is known to inhibit stem cell differentiation *in vitro* [25]. The content of the culture medium is a vital factor in cell culture, with various studies reporting different formulations, including StemPro-34 SFM, DMEM/F-12 and αMEM[2], often supplemented with varying percentages of FBS[3] and other components [53]. 2D[4] culture systems have traditionally been used for the proliferation and differentiation of SSCs; however, these systems have the disadvantage of failing to provide adequate cell-to-cell interactions and

1. Fluorescence-activated cell sorting
2. Minimum essential medium alpha modification
3. Fetal bovine serum
4. Two-Dimensional

mimic in vivo conditions [55]. In contrast, 3D[1] culture systems have been established, which better replicate the testicular tissue niche. For example, culturing testicular cells on collagen-based matrices mimics the context of the seminiferous tubules and enhances the survival and development of testicular cells by regulating cell-to-cell interactions [56]. A SACS[2] consists of two distinct parts determined by agar concentration- one gel part for SSCs and a solid part containing supporting cells- developed to generate haploid germ cells (**Figure 3**) [57]. Furthermore, an MCS[3] has been reported as effective for differentiating immature germ cells into haploid male germ cells [58]. To simulate the seminiferous epithelium environment and mimic somatic and germ cells connections, a 3D eBTB[4] system has been established. In this system, immature testicular cells aggregated under 3D conditions to form tubule-like structures [59]. Later, a modified 3D culture method called the 3-LGS[5] was developed for the formation of testicular organoids in vitro. In this method, rat testicular cells are suspended in Matrigel and positioned between two acellular Matrigel layers, leading to the development of testicular organoids after seven days [60].

[1]. Three-dimensional
[2]. Soft-agar culture system
[3]. Methylcellulose culture system
[4]. Engineered blood-testis barrier
[5]. Three-layer gradient system

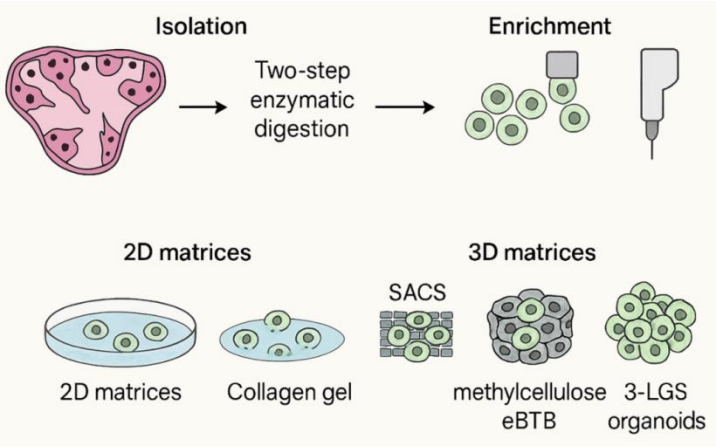


**Figure 3.** Culture workflows for in vitro spermatogenesis. (A) Isolation of SSCs from testicular tissue through two-step enzymatic digestion (collagenase, trypsin) followed by enrichment using differential plating, MACS, or FACS. (B) Comparison of 2D and 3D matrices for SSC culture, including collagen gel, SACS, MCS, eBTB, and 3-LGS organoids. Expected molecular readouts include KIT, SYCP3, PRM1, and acrosin. From tissue to testicular organoids: experimental platforms to model and induce spermatogenesis in vitro.

Tissue engineering methods have been explored in the field of testicular tissue regeneration. In this context, 3D acellular scaffolds of testicular tissue have been combined with hyaluronic acid and chitosan and have been designed to induce both differentiation and proliferation of mouse SSCs. Culturing mouse SSCs on a 3D scaffold made of alginate hydrogel, along with Sertoli cells, has promoted their proliferation and self-renewal, supporting long-term survival and differentiation of the cells [61]. These findings emphasize the importance of considering the testis tissue microenvironment to achieve optimal *in vitro* spermatogenesis [2].

## 7. Spermatogenesis

Spermatogenesis, the process crucial for male fertility, occurs within the seminiferous tubules of the testes and plays an essential role in fertility. It occurs in three main stages: the proliferation of spermatogonia (spermatocytogenesis), spermatocyte meiotic divisions, and finally, the

development of spermatids into mature sperm cells (spermiogenesis). This process involves various cell types and numerous genes with different expression patterns, and it is mediated by a combination of external signals, cell-to-cell communications, and intrinsic pathways (**Figure 4**).

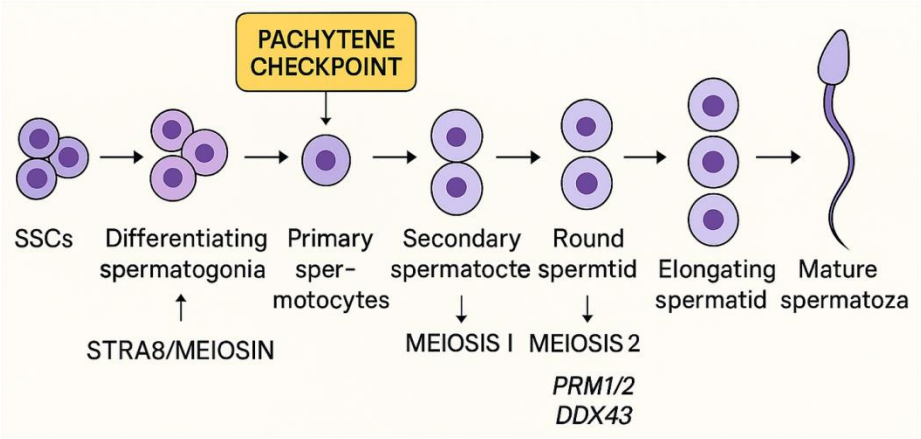


**Figure 4**. Spermatogenesis timeline. Developmental progression from spermatogonial stem cells (SSCs) to mature spermatozoa with stage-specific regulatory checkpoints. SSCs → differentiating spermatogonia → primary and secondary spermatocytes (meiosis I/II) → round → elongating spermatids; annotated checkpoints include pachytene checkpoint and MSCI, with hallmark genes STRA8/MEIOSIN, PRM1/2, and DDX43.

In mammals, the classification of spermatogonia differs between primates and rodents. Primates have two types of spermatogonia: Type A and Type B. Type A spermatogonia are stem cells that can self-renew and proliferate; they can be further divided into two subtypes: A dark and A pale cells. Type B spermatogonia undergo multiple cycles of cell divisions and differentiation processes that result in the formation of mature spermatids. Research indicates that true SSCs are Adark spermatogonia, while the other type is regarded as precursors to male germ cells [5].

## 8. Spermatocytogenesis

The first phase of sperm production during puberty is called spermatocytogenesis. In this phase, spermatogenic cells in the seminiferous tubules increase in number through mitosis, enabling self-renewal. During these mitotic divisions, some gonocytes convert directly into spermatogonia and continue spermatogenesis, while others differentiate to maintain self-renewal. Typically, these mitotic divisions result in incomplete cytokinesis, leading to the formation of cell syncytia [62]. Recent studies have identified the transcription factor FOXC2 as a specific marker for SSCs, indicating that FOXC2+ cells are normally quiescent and function to maintain the SSC population [63]. Research also shows that extracellular factors, such as GDNF, FGF, CSF, IGF, and CXCL12, which are secreted by testicular somatic cells like Sertoli cells, peritubular myoid cells, interstitial cells, and cells from the bloodstream, play a crucial role in sustaining SSC reserves [64]. Ultimately, the outcome of these mitotic divisions is a notable rise in the population of SSCs and primary spermatocytes [65].

## 9. Meiosis

Meiosis is a vital mechanism for transmitting diploid cells to haploid gametes. The initiation of meiosis begins with the differentiation of spermatocytes and continues through meiotic prophase, which is responsible for the completion of key chromosomal events specific to meiosis. Recent research has identified a protein known as myosin[1] that binds to DNA and triggers the start of meiosis [62]. Additionally, the STRA8[2] gene, stimulated by retinoic acid, activates meiosis and sets in motion the prophase meiotic program during the S phase prior to meiosis. Myosin also activates

[1]. The meiosis initiator
[2]. Retinoic acid-stimulated 8

downstream genes that are essential for specific events during meiosis prophase, particularly regarding chromosome dynamics and gene expression programming [62]. Studies have shown that male meiotic prophase is monitored by various regulatory mechanisms, such as the pachytene checkpoint and MSCI1, both of which lead to significant alterations in gene expression and epigenetic status [66]. During meiotic prophase, chromosomes reorganize into axis-loop structures that create a framework for meiosis-specific events. Homologous chromosomes pair, undergo synapsis, and engage in meiotic recombination, leading to crossovers. The dynamic movements required for homologous pairing and synapsis are guided by telomeres attached to the nuclear envelope [66]. Sex chromosomes undergoing MSCI occupy a heterochromatin region known as the sex body (or XY body). This region is enriched with various histones and histone modifications, as well as promoters involved in DNA damage response, epigenetics, and transcriptional regulation. MSCI serves many functions during spermatogenesis, including the prevention of DNA DSBs[2] from engaging in ectopic recombination, which can have a profound effect on the development of future generations [67]. Subsequently, the two secondary spermatocytes undergo a second division similar to mitosis to produce haploid spermatids (**Table 3**) [66].

[1]. Meiotic sex chromosome inactivation
[2]. Double-strand breaks

**Table 3**. Key genes and checkpoints in meiosis and spermiogenesis.

| *Stage / Checkpoint* | *Key gene(s) / protein(s)* | *Main function / molecular role* | *Effect on spermatogenesis* | *Representative references* |
|---|---|---|---|---|
| ***Pre-meiotic (Licensing)*** | **STRA8**, **MEIOSIN** | Transcription factors that initiate meiotic entry; MEIOSIN partners with STRA8 to activate meiosis-specific gene expression | Required for transition from spermatogonia to primary spermatocytes; loss leads to meiotic arrest. | [62, 66] |
| ***Meiotic prophase I*** | **Pachytene checkpoint regulators** | Ensure synapsis and recombination fidelity; monitor chromosomal pairing and DSB repair. | Defects cause meiotic arrest and apoptosis of primary spermatocytes | [66] |
| ***MSCI (Meiotic Sex Chromosome Inactivation)*** | **ATR, BRCA1, γH2AX**, and histone modifiers | Silence X and Y chromosomes during pachytene; form a sex body with repressive chromatin marks | Protects genomic integrity by preventing ectopic recombination; MSCI failure leads to infertility. | [66, 67] |
| ***Post-meiotic (Spermiogenesis initiation)*** | **DDX43** | RNA helicase regulating chromatin remodeling and mRNA dynamics during spermiogenesis | Facilitates histone–protamine exchange; knockout impairs chromatin condensation. | [69] |
| ***Chromatin remodeling & nuclear condensation*** | **PRM1**, **PRM2** | Protamines replace histones to achieve dense chromatin packing in sperm nuclei. | Essential for nuclear shaping and sperm head integrity; deficiency causes abnormal sperm morphology and infertility. | [69, 70] |
| ***Histone–protamine transition regulator*** | **FBXO24** | F-box protein modulating MIWI degradation and alternative splicing during spermiogenesis | Mutation leads to defective histone removal, enlarged chromatin bodies, and male sterility. | [71] |
| ***Late spermatid differentiation*** | **SPEM2** | Testis-enriched gene critical for cytoplasmic remodeling and flagellar maturation | Required for proper sperm tail formation and fertilization capacity | [72] |
| ***Mitochondrial & autophagic homeostasis*** | **DNAJC5B** | Chaperone-like protein maintaining mitochondrial integrity and autophagy balance | Loss leads to mitochondrial damage, oxidative stress, and decreased sperm motility. | [73] |

## 10. Spermiogenesis

Spermiogenesis is the post-meiotic maturation of haploid cells, characterized by morphological changes such as the development of specialized organelles, including flagella and the acrosome, nuclear condensation, and the loss of most of the cytoplasm. Immediately following meiosis, the expression of various genes begins. Among these genes, a specific group is responsible for exiting the MSCI state [68]. Histones are exchanged by protamines, resulting in the formation of DNA supercoils that are subsequently resolved through DNA DSBs and repair processes, ultimately leading to highly condensed sperm heads [69]. Research indicated that retinoic acid plays a significant role in promoting spermiogenesis by enabling the transcription of key coding factors necessary for this process. In the absence of retinoic acid, morphological abnormalities in sperm are more likely to occur [70]. Further studies have highlighted the significance of the FBXO24 gene in spermiogenesis; its deficiency leads to abnormal retention of histones, incomplete axonemes, enlarged chromatid bodies, and irregular mitochondrial twisting along sperm flagella, ultimately leading to male sterility [71]. Other genes implicated in this process include Spem2[1], which is essential for both spermiogenesis and male fertility [72]. Additionally, DNAJC5B[2] is involved in spermatogenesis and male fertility. A deficiency in DNAJC5B expression leads to mitochondrial damage, including oxidative stress and reduced sperm mitochondrial membrane potential [73].

## 11. Regulation of Spermatogenesis

[1]. SPEM family member 2
[2]. DnaJ/heat shock protein family member C5 beta

For spermatogenesis to occur successfully, a set of hormonal agents operates through endocrine, paracrine, and autocrine pathways. Among the most crucial hormones in this process are gonadotropins, FSH[1], LH[2], and androgens. Testosterone, one of the principal androgens, is secreted by steroidogenic Leydig cells and is crucial for regulating the differentiation of germ cells. Research indicates that maintaining the normal functioning of testicular somatic cells is one of the primary roles of gonadotropins. Myeloid cells provide physical support and contractile movement during the spermatogenesis process, which diploid Sertoli cells within the spermatogenic tubule help protect and feed the germ cells. Each of these cell types is directly targeted by one or more of the hormones vital for unimpaired male fertility. The anterior pituitary gland secretes two glycoprotein hormones: FSH and LH. Their target cells are the somatic cells of the testis, where they regulate the spermatogenesis process. The FSH-R[3] gene is expressed in Sertoli cells, contributing to an increase in the population of Sertoli cells in the testis. The hormone LH also acts on its receptors in Leydig cells, causing the production and secretion of testosterone from these cells [74].

## 12. Cryopreservation of Testicular Tissue

Cryopreservation is a long-term storage method to preserve biological samples. This process encompasses the freezing of gametes (sperm and ova), embryos, and reproductive cells (TCS[4]), and tissues (such as ovarian tissue and testicular tissue [75]. In reproductive medicine, freezing semen before gonadotoxic treatment is a viable option; however, this method is

[1]. Follicle-stimulating hormone
[2]. Luteinizing hormone
[3]. FSH receptor
[4]. Testicular cell suspensions

not effective for prepubescent individuals. For these patients, frozen TT[1] or TCS is utilized to help preserve their chance of having children in the future. After thawing frozen testicular biopsies, several treatment options are available, including (1) autologous SSCT[2]; (2) TT transplantation to the testis or a heterotopic site, and (3) IVS[3]. The cryopreservation of testicular tissue presents several challenges due to the diverse cell types contained within the tissue, each varying in size, complexity, and water permeability. Over the years, various protocols have been developed to minimize freezing damage and maximize cell recovery. Research has identified that one of the most critical factors in the freezing process is the size and structure of the testicular biopsy sample [76]. There are three primary freezing methods: slow freezing (controlled or uncontrolled), vitrification, and rapid freezing. Of these, rapid freezing is considered the most effective. The other two methods differ in terms of concentrations of CPA[4] and cooling rates. Slow freezing, with its controlled freezing rates, aims to reduce the risk of damaging ice crystal generation by allowing the soaking temperature to be adjusted. Among the freezing solutions used in research, notable CPAs include DMSO[5] (0.7-3 M), ethylene glycol (EG, 1.5 M), and glycerol (1 M), often used with additional sucrose (0.05-0.1 M) or serum (5-80%). In the glass freezing method, ice crystal formation is effectively minimized by employing ultrafast cooling rates and increased CPA levels. Typically, a combination of different CPAs is used to reduce their cytotoxic effects. Additionally, the methods used for thawing and CPA removal are also crucial for the survival of tissue after freezing. Typically,

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1. Testicular tissue
2. SSC transplantation
3. In vitro spermatogenesis
4. Cytoprotective agents
5. Dimethyl sulfoxide

tissues are thawed through rapid warming, followed by the gradual removal of loaded CPAs (**Figure 5**) [76].

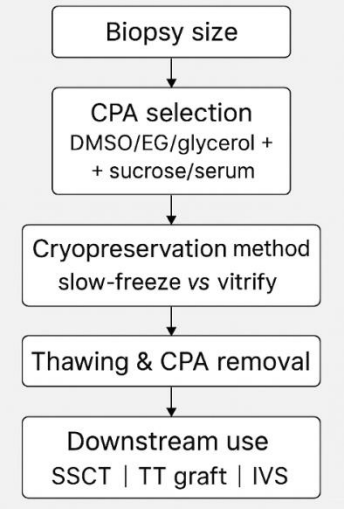


**Figure 5**. Cryopreservation decision tree. Key variables in testicular tissue/cell cryopreservation and downstream fertility restoration options. Biopsy size → CPA selection (DMSO/EG/glycerol ± sucrose/serum) → slow-freeze vs. vitrify → thawing & CPA removal → downstream use (SSCT, TT graft, IVS).

## 13. Oogonial Stem Cells

Mature ovaries contain OSCs[1], which are germ cells capable of sustaining neooogenesis, thereby replenishing the pool of primordial follicles. In lower vertebrates, multiple OSCs generate new oocytes via mitotic and meiotic division in every reproductive cycle. However, OSCs are relatively rare in more advanced mammals. The mature ovaries of many non-mammalian vertebrates, such as fish, amphibians, and reptiles, contain a large number of OSCs. Notably, the presence of OSCs in birds has yet to be explored. It was previously supposed that the mammalian ovary contains a limited number of follicles initially, which decreases over

[1]. Oogenic stem cells

time as germ cells enter meiosis and terminate dividing. Despite this widely accepted concept, recent studies have shown that mitotically active germ cell precursors can be found in postnatal mammalian ovaries, particularly in mouse models [77].

## 14. Clinical Applications

Infertility in men can be caused by anatomical defects, exposure to gonadotoxins, genetic factors, and medical treatments such as chemotherapy [78]. Common treatment methods include hormone therapy, surgical procedures, gene therapy, ART[1] like ICSI[2], as well as the cryopreservation of sperm and preservation of testicular tissue [79]. However, current methods often have limited therapeutic effects, prompting researchers to seek new and effective strategies in the treatment of infertility [80]. Recently, cell-based treatments, particularly stem cell therapy, have gained significant interest in the field of male infertility [81]. With the rise in cancer cases and the increased use of ionizing radiation in the treatment, many patients suffer sterility as a result of radiation therapy. For adult patients, storing sperm before undergoing radiation therapy can be beneficial, but this is not an option for individuals who have not yet gone through puberty. Therefore, storing SSCs could be one viable way to preserve fertility [82, 83]. The possibility of isolating and culturing SSCs in vitro, freezing them, and then auto-transplantation of these cells post-cancer treatment presents a potential clinical method for addressing male infertility. However, safety concerns, such as the risk of tumor cell transmission to the patients, must be taken into account [84]. Another approach involves IVS of stored SSCs to produce haploid germ

[1]. Assisted reproductive technologies
[2]. Intracytoplasmic sperm injection

cells, which can then be used for ICSI or ROSI[1] [85]. Additionally, autologous grafting of testicular biopsies is being considered as a method to restore fertility in childhood cancer survivors, utilizing either orthotopic or ectopic grafting techniques [86]. For adult patients facing infertility, the conventional method typically involves using sperm from ejaculated semen or sperm retrieved via microsurgical TESE[2] for assisted reproductive techniques such as ICSI or ROSI. Although this approach is only feasible if the patient's testes contain haploid male germ cells, including cases of obstructive azoospermia [87]. In situations where haploid germ cells are absent, one strategy is in vitro spermatogenesis using SSCs to generate haploid male germ cells, which may also be combined with genetic modification to restore spermatogenesis [88]. Moreover, iPSCs[3] and ESCs[4] offer new therapeutic possibilities. In this context, iPSCs can be created from a patient's somatic cells and differentiated into SSCs, which can be used for in vitro spermatogenesis or autologous transplantation to restore spermatogenesis [2]. Despite this potential, the effectiveness of iPSC-based therapies is often limited by challenges, including immune rejection, risks of tumor formation, and possible genetic or epigenetic alterations that may occur during the reprogramming and expansion of iPSC [79]. The use of ESCs for therapeutic purposes is restricted due to ethical issues associated with gamete manipulation and the potential for inducing abortion [89]. Consequently, MSCs[5] have emerged as the most widely utilized stem cell type in medicine, especially in reproductive therapies. MSCs play a key

[1]. Round spermatid injection
[2]. Testicular sperm extraction
[3]. Induced pluripotent stem cells
[4]. Embryonic stem cells
[5]. Mesenchymal stem cells

role in tissue repair and have the potential to differentiate into germ-like cells [90, 91]. They offer several advantages, including ease of access and isolation, high proliferation rates, low immunogenicity, and the capacity to differentiate into numerous cell lineages [90]. Numerous studies have reported the positive effects of AT-MSCs[1], BM-MSCs[2], and UC-MSCs[3] in improving infertility [90-92]. It is crucial to note that stem cells are involved in cell proliferation, angiogenesis, and cell viability through their paracrine activity. This includes the secretion of signaling factors, cytokines, growth factors such as GDNF, FGF2, BMPs[4], and TGF-β[5], as well as EVs[6] within the testicular microenvironment [79]. Additionally, MSCs injected into testicular tissue may influence SSCs by releasing growth factors, presenting a novel approach to restoring spermatogenesis and promoting the differentiation of these cells into germ cells [93].

Studies have also indicated that SC-EVs[7] can help improve fertility [94]. These nano-sized structures, secreted by various cell types, contain a set of biological molecules including proteins, DNA, growth factors, lipids, and other agents. They are involved in multiple biological procedures, such as cell-to-cell communication, control of signaling pathways, cell proliferation, migration, and gene expression [95, 96]. Positive effects of EVs derived from mesenchymal stem cells on sperm parameters, including improved sperm quality and function, have been reported in various studies [97]. Additionally, studies on the effects of Sertoli cell-derived exosomes in

[1]. Adipose Tissue-derived mesenchymal stem cells
[2]. Bone marrow mesenchymal stem cells
[3]. Umbilical Cord-derived mesenchymal stem cells
[4]. Bone morphogenetic proteins
[5]. Transforming growth factor beta
[6]. Extracellular vesicles
[7]. Stem cell-derived extracellular vesicles

infertility suggest that these exosomes enhance intercellular communication among Sertoli cells, SSCs, and Leydig cells [98].

## 15. Conclusion

SSCs form the foundation of spermatogenesis and are crucial for passing genetic information to future generations. The process of spermatogenesis comprises three main stages: the proliferation of spermatogonia, the meiosis of spermatocytes, and the differentiation of spermatids into spermatozoa, all of which are controlled by complex molecular mechanisms and intricate cellular interactions. Established methods for isolating and cultivating SSCs in vitro present opportunities to develop therapeutic strategies for patients with infertility. Moreover, various cryopreservation methods have been developed to preserve testicular cells and tissues, particularly for young patients and those undergoing gonadotoxic treatments. Clinically, given the multiple causes of male infertility, various therapeutic approaches-including hormone therapy, surgery, assisted reproductive technologies, and notably, stem cell-based therapies- are emerging. Autologous storage and transplantation of SSCs, along with their in vitro proliferation and differentiation, provide promising strategies for fertility restoration. Furthermore, MSCs, due to their regenerative potential, differentiation capacity, and paracrine effects, present hopeful approaches for improving sperm quality and function. Advances in understanding the molecular, cellular, and hormonal mechanisms underlying spermatogenesis, along with the development of innovative therapeutic technologies, are paving the way for improving fertility preservation and treatment of male infertility. However, challenges remain, such as ensuring the safety of cell-based therapies, addressing ethical concerns related to ESCs, and the need for further research to optimize protocols and enhance efficacy. Therefore, continued research

efforts are essential to translate basic scientific knowledge into effective clinical applications, ultimately improving the quality of life for individuals facing infertility and increasing the success rates of fertility treatments.

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