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Journal Name	Reproductive Sciences	
Corresponding Author	FamilyName	**Heidari**
	Particle	
	Given Name	**Banafsheh**
	Suffix	
	Division	Department of Photo Healing and Regeneration, Medical Laser Research Center
	Organization	Yara Institute, ACECR
	Address	Tehran, Iran
	Phone	
	Fax	
	Email	ban_heidari@yahoo.com
	URL	
	ORCID	http://orcid.org/0000-0002-4131-4837
Author	FamilyName	**Shirazi**
	Particle	
	Given Name	**Abolfazl**
	Suffix	
	Division	Reproductive Biotechnology Research Center
	Organization	Avicenna Research Institute, ACECR
	Address	Tehran, Iran
	Phone	
	Fax	
	Email	a.shirazi@avicenna.ac.ir
	URL	
	ORCID	http://orcid.org/0000-0003-3212-6137
Author	FamilyName	**Akbari**
	Particle	
	Given Name	**Nazanin**
	Suffix	
	Division	Department of Biology
	Organization	Shahid Beheshti University
	Address	Tehran, Iran
	Division	Clinical Research Development Unit, Faculty of Medicine
	Organization	Ghaem Hospital, Mashhad University of Medical Sciences
	Address	Mashhad, Iran
	Phone	
	Fax	
	Email	nazaninakbarii000@gmail.com
	URL	
	ORCID	http://orcid.org/0000-0002-6830-986X
Author	FamilyName	**Barzegar-Amini**

	Particle	
	Given Name	**Maral**
	Suffix	
	Division	Clinical Research Development Unit, Faculty of Medicine
	Organization	Ghaem Hospital, Mashhad University of Medical Sciences
	Address	Mashhad, Iran
	Division	Allergy Research Center
	Organization	Mashhad University of Medical Sciences
	Address	Mashhad, Iran
	Phone	
	Fax	
	Email	Aminim1@mums.ac.ir; maral.barzegar.amini@gmail.com
	URL	
	ORCID	http://orcid.org/0000-0001-8547-1423

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Abstract	Assisted reproduction techniques for infertile men with non-obstructive azoospermia require a sufficient number of functional germ cells produced in vitro. Understanding the mechanisms that allow the resumption of spermatogenesis outside the testicular environment is crucial for fertility preservation in these patients. A review of the literature was conducted using databases such as PubMed, Scopus and Web of Science, with keywords including “spermatogonial stem cell,” “germ cells,” “male factor infertility,” and “enrichment and propagation of SSCs in vitro.” Currently, two models—“in vivo” and “in vitro”—have been developed for producing haploid germ cells. The “in vivo” models include spermatogonial stem cell transplantation and testicular xenograft techniques. In contrast, the “in vitro” models consist of conventional culture systems, organ culture, and three-dimensional culture systems, all designed to induce spermatogenesis in vitro. These culture systems enable the simulation of the seminiferous epithelium in vitro, which facilitates better regulation of cell-signaling pathways that control the self-renewal and differentiation of SSCs. This review provides up-to-date information on the organization of SSCs, focusing on the identification, proliferation, and differentiation of spermatogonia in vitro.

Keywords (separated by '-')	Germ Cells - Spermatogonial stem cell - Propagation - Spermatogenesis - Manipulation
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REPRODUCTIVE BIOLOGY: REVIEW

Identification and Manipulation of Spermatogonial Stem Cells with the Aim of Inducing Spermatogenesis in Vitro

Banafsheh Heidari[1] · Abolfazl Shirazi[2] · Nazanin Akbari[3,4] · Maral Barzegar-Amini[4,5]

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Abstract

Assisted reproduction techniques for infertile men with non-obstructive azoospermia require a sufficient number of functional germ cells produced in vitro. Understanding the mechanisms that allow the resumption of spermatogenesis outside the testicular environment is crucial for fertility preservation in these patients. A review of the literature was conducted using databases such as PubMed, Scopus and Web of Science, with keywords including "spermatogonial stem cell," "germ cells," "male factor infertility," and "enrichment and propagation of SSCs in vitro." Currently, two models—"in vivo" and "in vitro"—have been developed for producing haploid germ cells. The "in vivo" models include spermatogonial stem cell transplantation and testicular xenograft techniques. In contrast, the "in vitro" models consist of conventional culture systems, organ culture, and three-dimensional culture systems, all designed to induce spermatogenesis in vitro. These culture systems enable the simulation of the seminiferous epithelium in vitro, which facilitates better regulation of cell-signaling pathways that control the self-renewal and differentiation of SSCs. This review provides up-to-date information on the organization of SSCs, focusing on the identification, proliferation, and differentiation of spermatogonia in vitro.

Keywords Germ Cells · Spermatogonial stem cell · Propagation · Spermatogenesis · Manipulation

Introduction

Spermatogonial stem cells (SSCs) are unipotent adult stem cells responsible for maintaining male fertility throughout life. Among adult stem cells, SSCs are unique because they divide mitotically and contribute genes to subsequent generations, making them ideal targets for genetic manipulation [1]. These specific germ cells (GCs) can rapidly proliferate when the testes are damaged by chemicals or radiation; however, under normal physiological conditions in vivo, they divide slowly to produce both stem and progenitor cells [1, 2]. When cultured in vitro under appropriate conditions, SSCs can acquire pluripotency and differentiate into derivatives of the three embryonic germ layers [3]. It is well documented that the transplantation of SSCs into the seminiferous tubules of an infertile male can induce donor-derived spermatogenesis and produce spermatozoa that transmit the donor haplotype to progeny [4]. Studying SSC populations and their niches, as well as their genetic manipulation and transplantation in vitro, provides a valuable model for understanding adult stem cell biology, decoding cell-signaling pathways that control SSC functions (self-renewal or differentiation), and modifying the germline to produce transgenic

✉ Banafsheh Heidari
ban_heidari@yahoo.com

Abolfazl Shirazi
a.shirazi@avicenna.ac.ir

Nazanin Akbari
nazaninakbarii000@gmail.com

Maral Barzegar-Amini
Aminim1@mums.ac.ir; maral.barzegar.amini@gmail.com

[1] Department of Photo Healing and Regeneration, Medical Laser Research Center, Yara Institute, ACECR, Tehran, Iran

[2] Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

[3] Department of Biology, Shahid Beheshti University, Tehran, Iran

[4] Clinical Research Development Unit, Faculty of Medicine, Ghaem Hospital, Mashhad University of Medical Sciences, Mashhad, Iran

[5] Allergy Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

animals [5, 6]. Despite their critical role, SSCs are difficult to study in vitro due to their low numbers in the testes and the challenges in identifying, culturing, and assaying their biological activity [1]. This review introduces the latest approaches in identifying and isolating SSCs in mammals.

Methods

A comprehensive literature search was conducted across various databases, including PubMed, ScienceDirect, Cochrane Library, ISI, and Google Scholar, using a combination of keywords such as germ cells, spermatogonial stem cell, propagation, spermatogenesis, and manipulation. The search was carried out until February 2024. The review adhered to specific inclusion and exclusion criteria, which filtered studies based on their relevance to human subjects or clinically relevant animal models, and their focus on SSC proliferation and differentiation. The initial search yielded **321** articles, from which **249** were screened by title and abstract. Subsequently, **89** articles were selected for full-text review, resulting in a final selection of **76** articles for detailed analysis.

Origin of Spermatogonial Stem Cells

Spermatogonia arise from gonocytes in the postnatal testis, which originate from primordial germ cells (PGCs) during fetal development. PGCs are transient cells that initially appear as a small cluster (20–25 cells) of alkaline phosphatase-positive cells in the fetal yolk sac at the epiblast stage, around 7-7.25 days post coitum (dpc) in mice and 3 weeks after conception in humans (Fig. 1A) [7]. During the formation of the allantois, these cells are passively swept out of the embryo to reach the indifferent gonads at 8.5–12.5 dpc in mice and 4–5 weeks in humans (Fig. 1B and C). Upon colonization, PGCs are surrounded by protrusions of the genital tract, Sertoli progenitor cells, and peritubular myoid cells around the fallopian tubes, beginning the formation of primitive sex cords (Fig. 1D) [8]. They migrate from the embryonic ectoderm to the genital ridges by amoeboid movement through the allantois and hindgut (Fig. 1D and E). These cells proliferate during the migratory phase, resulting in approximately 3,000 germ cells colonizing the genital ridge at about 13.5 dpc in mice and 5–6 weeks in humans [9]. At this stage, the gonads are bipotential in both sexes. Following colonization in the genital ridges, PGCs are surrounded by Sertoli precursor cells and peritubular myoid cells, initiating the formation of primitive sex cords (Fig. 1E and F) [8]. Sex differentiation occurs at 6–7 weeks in humans and 12.5–13.5 days in mice. The secretion of testis-determining factor (TDF), produced by the SRY gene (sex-determining region of the Y gene), converts the bipotential gonad into a testis. The primitive sex cords evolve into seminiferous tubules in the male gonad (Fig. 1D-G), and the blood-testis barrier is formed by tight junctions between adjacent Sertoli cells. From this stage onward, germ cells are

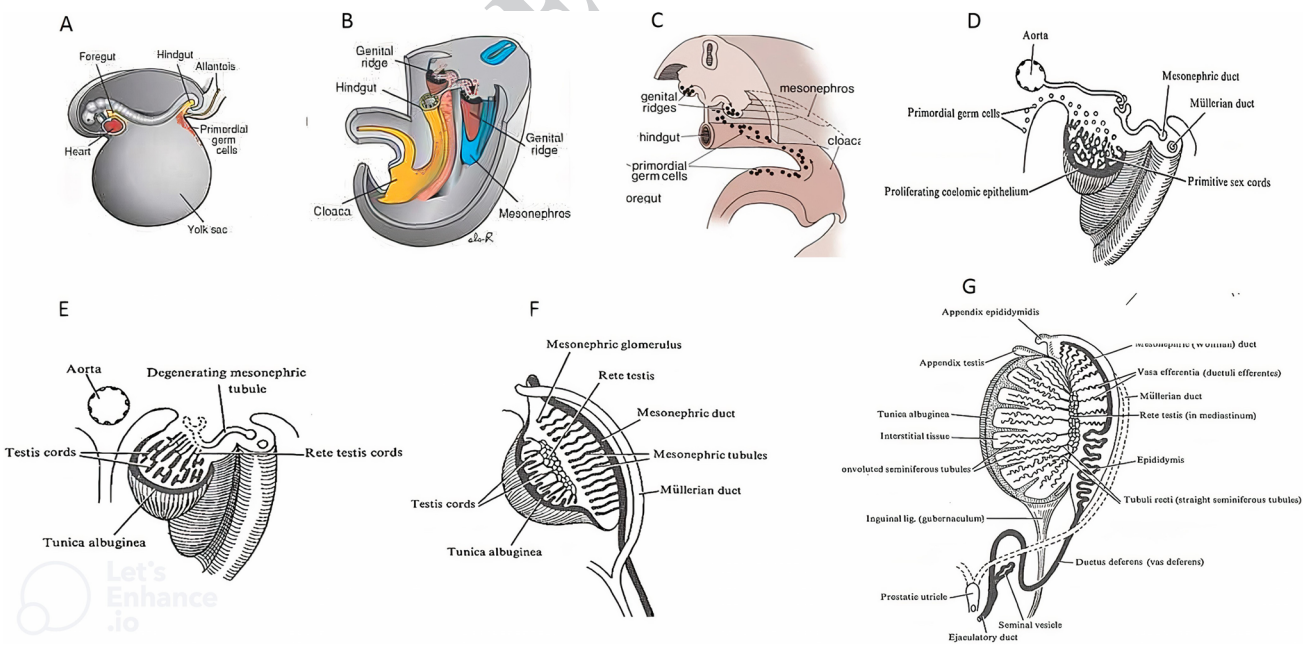

Fig. 1 Schematic representation of testis formation. Accumulation of primordial germ cells as a small cluster in the wall of yolk sac, near the junction of hindgut and allantois at week 4 in humane (**A**). Migration path of germ cells along the wall of hindgut and dorsal mesentery into the genital ridge in 5 weeks (**B**, **C**). Colonization of cells in the genital ridge and formation of primitive sex cords in 6 weeks (**D**) to 8 weeks (**E**). Late development of testis at month 2–3 (**F**) and 4 (**G**) in human testis

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referred to as gonocytes, which differ morphologically from PGCs [10]. Gonocyte is a general term that includes mitotic (M)-prospermatogonia, T1-prospermatogonia, and T2-prospermatogonia. M-prospermatogonia are located in the center of testicular cords, away from the basement membrane, and reproduce up to 16-16.5 dpc in mice. These cells subsequently differentiate into T1-prospermatogonia around 16.5 dpc [11]. In late gestation, T1-prospermatogonia become arrested in the G0/G1 phase of the cell cycle and remain in this state until birth [10]. Low-level expression of meiosis-associated genes such as Sycp3 and Dmc1 in arrested germ cells indicates their potential to enter meiosis upon increased expression of meiosis-related genes [12]. It is hypothesized that meiosis arrest is induced by meiosis-inhibitory agents from the testes. One of the most significant meiosis-inhibiting factors is Cyp26b1, produced by Sertoli cell precursors on day 11.5 in mice, before sex differentiation. This factor is present throughout fetal development and regulates retinoic acid (RA) function in the gonads before birth [13]. Recent studies have shown that exposure to RA during embryonic development (12.5–16.5 dpc in mice) completes mitotic divisions and initiates meiosis in XX female gonocytes. However, in the male XY embryonic gonad, Sertoli cell precursors inactivate RA through Cyp26b1 expression, preventing the completion of mitotic divisions and initiation of meiosis during embryonic development [14]. In rodents, during the first week after birth, T1-prospermatogonia begin to proliferate, differentiate into T2-prospermatogonia, and migrate to the basement membrane of seminiferous tubules [10]. T2-prospermatogonia establish the initial pool of type A spermatogonia, which maintain spermatogenesis throughout post-pubertal life [10, 15].In normal human testes, fetal spermatogonia, originating from gonocytes between 10 and 22 weeks post-conception, differentiate into adult dark spermatogonia (Adark). These cells are known as diploid SSCs with adult stem cell characteristics. They self-renew through asymmetric cell divisions and produce more differentiated progenitor cells, known as adult pale spermatogonia (Apale). Although both Adark and Apale are often referred to as spermatogonial stem cells, their biological functions differ significantly. Adark spermatogonia, as true SSCs, regenerate testicular tissue and germ cells, whereas Apale acts as progenitors, differentiating into type B spermatogonia, which serve as a functional reserve [16].

The Spermatogenic Cycle

The development of the spermatogenic lineage, referred to as the seminiferous epithelium cycle, is a complex and asynchronous process that occurs regularly [10]. The time interval required for one complete series of cell associations to appear at a specific point within the seminiferous tubule is known as the duration of the seminiferous epithelium cycle. This duration is generally constant for a given species, although variations can occur between different strains or breeds of the same species. The timing of the cycle is primarily controlled by the germ cell genotype and is not affected by exposure to gonadotropic hormones in different species [17]. The durations of the seminiferous epithelium cycles and the complete spermatogenesis process for various species, including pigs, sheep, goats, cows, boars, horses, humans, rabbits, dogs, monkeys, buffalo, and rodents, are presented in Table 1 [7, 9, 10, 17–22].

In humans, the entire development process from stem cell to spermatozoa takes approximately 64 days and is divided into four phases: 16 days for mitotic divisions (up to the primary spermatocyte stage), 24 days for the first meiosis (up to the secondary spermatocyte stage), a few hours for the second meiosis (up to the spermatid stage), and 24 days for spermiogenesis (resulting in mature spermatozoa) [18, 28].

Identification of SSCs

There are several methods to distinguish different types of spermatogonia from other testicular cells, including morphological characteristics, molecular markers, and functional assays. Morphologically, SSCs can be identified based on nuclear morphology, cell location in the seminiferous epithelium, the amount of heterochromatin in the nucleus, and its relationship to the nuclear membrane. In rodents, these criteria allow for the identification of three types of spermatogonia: types A, Intermediate, and B [6, 7, 26, 38]. In humans and rhesus monkeys, there are two subtypes of type A spermatogonia, Adark and Apale, followed by one

Table 1 The length of spermatogenesis and the seminiferous epithelium cycles in different species

Species	Spermatogenic cycle length	Overall rate of spermatogenesis	References
Pig	8.6-9	40	[23]
Boar	8.3	36-40.6	[23]
Human	16	64	[6, 7, 18]
Cow	13.5	55–61	[21, 24]
Stallion	12.2	55–59	[25]
Goat	10.6	47.7–49	[21, 25–27]
Rat	12.9	45–48	[28–30]
Mouse	8.6–8.9	44	[2, 11, 31–34]
Rabbit	10.7	39	[35]
Monkey	9.5–9.8	42	[22, 77]
Buffalo	8.6–8.7	64–74	[18]
Ram	10.5–10.6	45–49	[36, 37]
Dog	13.6	51	[35]

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generation of type B spermatogonia in humans and four generations (B1, B2, B3, and B4) in monkeys (Fig. 2) [26, 28, 39]. In rodents, seven subtypes of type A spermatogonia have been reported: Asingle (As), Apair (Ap), Aaligned (Aal), A1, A2, A3, and A4. The spermatogenic lineage in rodents includes four divisions in the undifferentiated spermatogonia pool (As, Apr, Aal), followed by 6–7 divisions in the differentiated spermatogonia pool (Aal (16 cell-chain) A1–A4, Intermediate, and B), leading to the formation of primary spermatocytes (Fig. 2) [36]. Thus, there are typically 14 divisions in rodents, 10 in monkeys, and 7 in humans between undifferentiated type A spermatogonia and sperm production (Fig. 2).

In ruminants, three subtypes of type A spermatogonia have been identified: basal, aggregated, and committed spermatogonia. Basal and some aggregated (small chains of cells) spermatogonia are described as undifferentiated type A SSCs, while long chains of aggregated SSCs and committed spermatogonia are considered differentiated type A SSCs. Basal stem cells, equivalent to As and Ap in rodents, appear as small round or oval cells with a thin rim of cytoplasm, a large central nucleolus, and one to three irregular nucleoli. Aggregated spermatogonia (Aal), varying in size, typically contain one or two nucleoli. Committed spermatogonia, the largest type, include different subpopulations of differentiating type A spermatogonia (A1-A4). Intermediate spermatogonia are usually large cells with ovoid nuclei and more heterochromatin near the nuclear envelope. Type B spermatogonia have round nuclei with significant amounts of heterochromatin concentrated at the nuclear periphery [5, 28, 38, 40]. In general, type A spermatogonia have little or no heterochromatin, intermediate spermatogonia display a moderate amount, and type B spermatogonia have an abundant amount of heterochromatin [28].

Although morphological evaluation is a useful tool for identifying SSCs, relying solely on this approach can lead to errors, especially when analyzing cell suspensions outside the seminiferous tubules. Therefore, it is essential to complement morphological characterization with functional methods or molecular techniques to accurately identify the types of testicular cells.

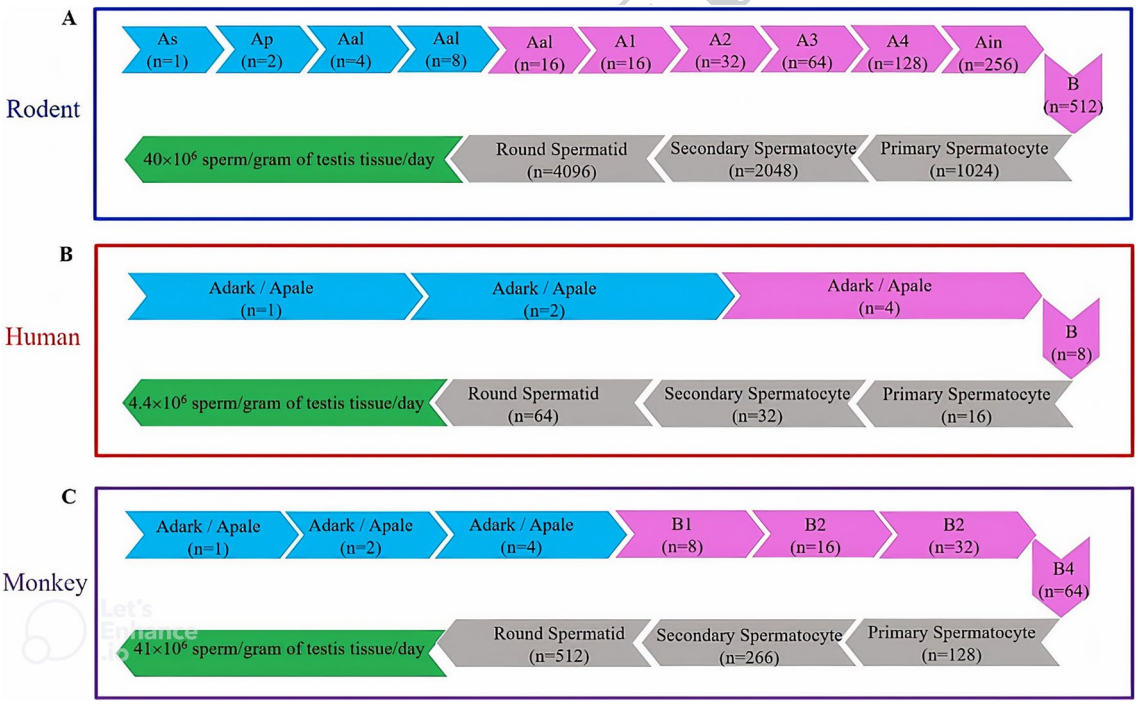

Fig. 2 The spermatogenic lineages in rodents (**A**), humans (**B**), and monkeys (**C**). Undifferentiated spermatogonia are described as As, A_p, or A_{al} in the rodents and A_{dark} or A_{pale} in monkeys and humans. During spermatogenic development, A_{single} (A_s) and A_{dark} and/or A_{pale} undergo mitotic divisions to give rise to cells of larger chains of interconnected cells sizes through transit-amplifying mitotic divisions. The spermatogenic lineages in rodents have 4 divisions in the pool of undifferentiated A_s, A_p, and A_{al} spermatogonia, followed by 6–7 divisions in the pool of differentiated spermatogonia (A_{al} (16 cell-chain, A1–A4, Intermediate, and B), that give rise to primary spermatocytes. Two additional meiotic divisions produce round spermatids that undergo spermiogenesis to produce sperm (A). The spermatogenic lineages in humans consist of 2 divisions in the pool of undifferentiated $A_{dark/Apale}$ spermatogonia, followed by a single division in differentiated B spermatogonia that give rise to primary spermatocytes (B). The spermatogenic lineages in monkeys consist of 3 divisions in the pool of undifferentiated $A_{dark/Apale}$ spermatogonia, followed by 4 divisions of differentiated spermatogonia (B1–B4), which give rise to primary spermatocytes (C)

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Bimolecular Markers of Spermatogenic Cells

Identifying the molecular signatures of testicular cells, including spermatogonial stem cells (SSCs), is a complex process that requires accurate detection of premeiotic, meiotic, and postmeiotic molecular markers. These markers are highly specific, being expressed exclusively on certain cells at specific stages of differentiation. A summary of some specific molecular markers used to identify different types of spermatogenic cells, particularly undifferentiated and differentiated type A SSCs, as well as testicular somatic cells, is provided in Table 2.

In Vitro Enrichment

To study the regenerative and biological properties of SSCs, an adequate population of pure spermatogonia and an in vitro system that supports this process in biologically enriched populations of cells are necessary. In ruminants, testicular cell suspensions isolated from seminiferous tubules contain a mixture of different somatic cells (Sertoli, Leydig, and peritubular myoid cells) and about 1.33% germ cells. Among these, only a small fraction consists of undifferentiated type A spermatogonia (0.02–0.03% of total germ cells) [37, 38]. Therefore, efficient purification methods that minimally impact SSC survival and proliferation are crucial for evaluating the clonogenic properties of these cells.

Several methods for enriching male germline stem cells exist, including elutriation [25], differential plating [29], velocity sedimentation [24], discontinuous Percoll density gradient [23, 40], Magnetic-Activated Cell Sorting (MACS) [31], and Fluorescence-Activated Cell Sorting (FACS) [23, 50]. Among these methods, Percoll density gradient and differential plating are preferred due to their speed, ease, safety, cost-effectiveness, and minimal negative impact on SSC viability and morphology [29, 40]. The low viscosity and osmolality of Percoll, ideal for isolating different cell types, have led to its widespread use for separating adult and prepubertal SSCs in goats [40], bovines [27], chickens [51], and pigs [20].

In the differential plating method, somatic cells adhere to the culture plate during incubation due to their anchorage dependence, allowing for the rapid removal of most Sertoli and Leydig cells from the cell suspension [38, 40]. This method has been shown to yield significant and acceptable purity levels of undifferentiated type A spermatogonia in goats [40], mice [32], rats [41], and pigs [48].

Table 2 Specific molecular markers of different subtypes of spermatogenic cells

Types of spermatogenic cells	Molecular markers	References
Undifferentiated type A Spermatogonia (A_s, A_p, A_{al})	EGR3, Ngn3, RBM, Nanos3, CD9, Thy1 (CD90), CD24, GFR-a1, ID4, PLZF, CSFR, GCNA1, Lin28 (Tex17), Numb, Bcl6b, VASA (MvH), UTF1, CDH1, Nucleostemin, EE2 antigen, Ret, Lrp4, GPR125, TAF4B, Sox-3, DAZL, Sohlh2, Stra8, a6-integrin (CD49f), b1-integrin (CD29), Epcam, Pou5f1 (Oct4)	[11, 31]
Differentiated type A Spermatogonia (A1-A4)	c-kit, GCNA1, RBM, VASA (MvH), EE2 antigen, DAZL, Nucleostemin, Numb, TAF4B, Stra8, a6-integrin (CD49f), Sohlh1, Lrp4, b1-integrin (CD29), Epcam, CD9	[41]
Type In and type B Spermatogonia	c-kit, GCNA1, VASA (MvH), EE2 antigen, DAZL, Stra8, a6-integrin (CD49f), b1-integrin (CD29), Epcam, CD9, TAF4B, Numb, Lrp4, Sohlh1, Nucleostemin	[38, 42]
Spermatocyte	c-kit, GCNA1, VASA (MvH), EE2 antigen, TAF4B, DAZL, Stra8, a6-integrin (CD49f), Lrp4, Sohlh1, Numb, b1-integrin (CD29), Ngn3, Nucleostemin, HSP60, MAGEA-4, UCHL1, ITGA6, ZBTB16, HLA class I	[43]
Round and Elongated Spermatid	c-kit, GCNA1, TAF4B, Lrp4, VASA (MvH), Stra8, CRES, Protamin 2 (PRM2), GRP78, Transition protein T1 and T2, HLA class I	[44, 45]
Spermatozoa	Other Dense Fibers (ODF-2), SPANX, GRP78, CRES, DAZ2, Tissue Specific Protein 2 (TPX-1) LDHC	[46]
Immature Sertoli cells	AMH (Anti-mulleraim Hormone), WT1 (Wilms tumor gene)-transcription factor, Aromatase (P450 enzyme), NCAM (Neural cell adhesion molecule), Cytokeratin 15, M2A	[7, 19]
Mature Sertoli cells	Occludin, Wimentin, P27 (Cyclin-dependent kinase inhibitor), WT1, Dmrt1, Gata 4, Gata 1, AR (Androgen receptor), Transferrin, ITGA6 (Alpha 6 integrin)	[47]
Leydig cells	ITG alpha 6 (alpha 6 integrin), RLF, 3 beta-HSD, TGF alpha	[48]
Peritubular myoid cells	Alpha-smooth muscle actin	[49]

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In Vitro Propagation

Propagating male germline stem cells in vitro offers a valuable approach to investigating the molecular mechanisms underlying spermatogenesis and the cell-signaling pathways regulating SSC function (self-renewal and differentiation). In 2009, Sadri-Ardekani et al. established a long-term culture (up to 28 weeks) of human spermatogonial stem cells from small testicular biopsies, achieving a 53-fold increase in SSC numbers within 19 days and an 18,450-fold increase within 64 days [47].

Typically, testicular cultures begin with a mixed population of cells, including spermatogonia and somatic cells. Within two or three days, somatic cells form a confluent monolayer serving as a feeder layer, to which 90% of SSCs attach. SSCs, as colony-forming cells, cluster together in some areas of the culture plate, forming various types of type A spermatogonia (single, paired, multi-cell chains, or small clusters) (Fig. 3). Over time, the number and size of spermatogonia colonies increase, displaying forms such as single, paired, aligned, and rosette configurations after one week of culture.

Using a chemically defined medium, researchers have explored the growth factor-regulated molecular mechanisms involved in SSC proliferation and self-renewal [33, 42, 52, 53]. Key growth factors used for SSC propagation in vitro include glial cell line-derived neurotrophic factor (GDNF), GDNF receptor-α1 (GFR-α1), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), and leukemia inhibitory factor (LIF) [33, 34, 52–54]. GDNF, secreted by Sertoli cells under the influence of FSH and cytokines [53], is particularly crucial for SSC regeneration and proliferation, both in vivo [52, 55] and in vitro [49, 52, 53, 56, 57].

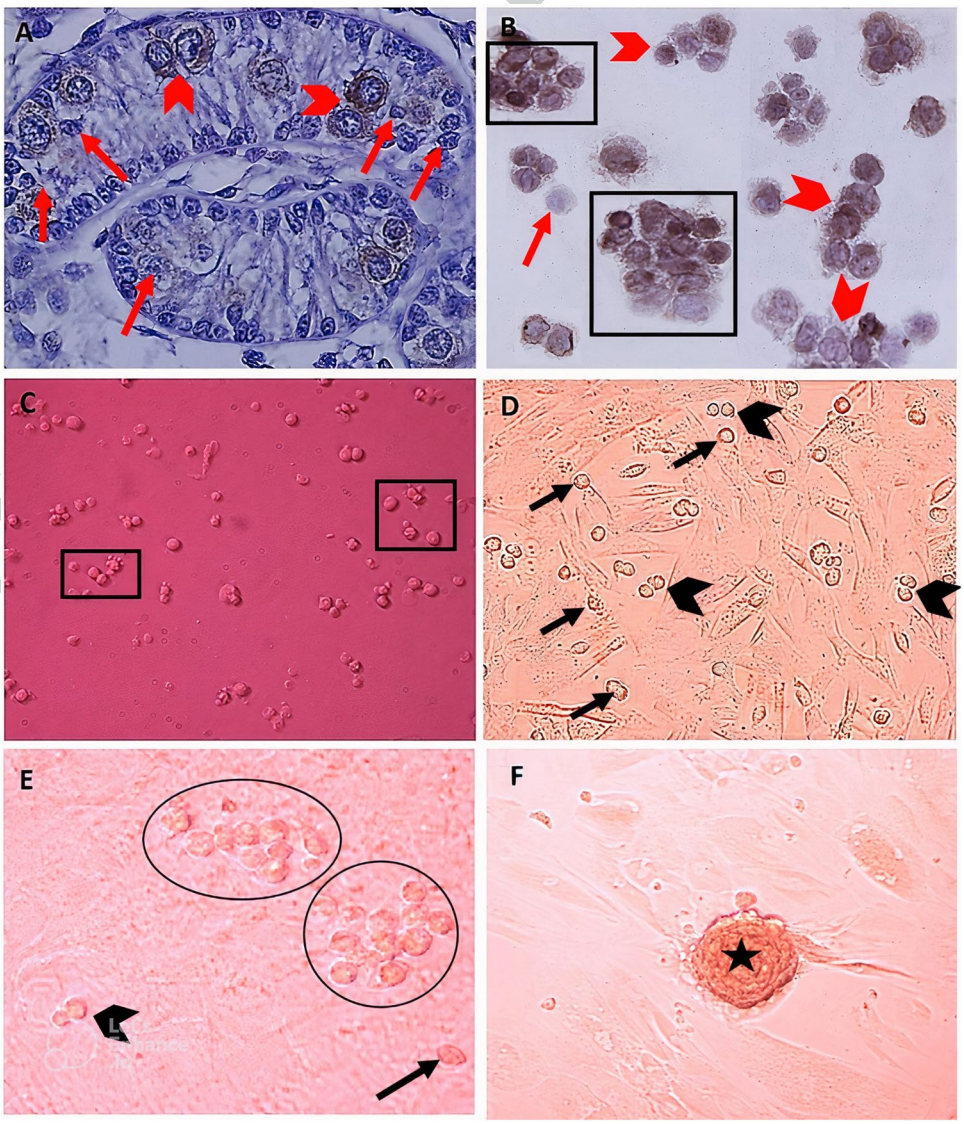

Fig. 3 The immunohistochemical (**A**) and immunocytochemical (**B**) identification of type A spermatogonia in prepubertal goat seminiferous tubules using an antibody against c-kit. Three groups of spermatogonia with different sizes were determined. Basal spermatogonia (red arrows, A, B) were negative for c-kit. These cells were comparable to A_s and A_p spermatogonia. Aggregated spermatogonia resembled the A_{al} spermatogonia (red arrowhead, A, B) were c-kit positive. Committed spermatogonia, comparable to A1–A4 differentiating spermatogonia, were positive for c-kit (square, B, C). The testicular cells, including somatic and SSCs after 1 day (**C**), 2–3 days (**D**) and 1 week (**E**, **F**) culture. After a few days of culture, the spermatogonia were seen in different forms: single (black arrow, D, E), paired (black arrowhead, D, E), aligned (circle, E), and cluster (asterisk, F)

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Studies have shown that GDNF alone or in combination with other growth factors significantly increases SSC viability and colony formation [2, 56, 57]. Withdrawal of GDNF leads to decreased expression of key genes like bcl6b and Sox2 in SSCs, reducing SSC numbers and colony size [58]. In goats, higher GDNF concentrations have been found to induce logarithmic divisions of type A spermatogonia, forming denser and larger clusters [33]. Similar effects have been observed in mouse, rabbit, and bovine SSCs [49, 52, 55].

In addition to GDNF, bFGF plays a vital role in maintaining spermatogenesis and supporting SSC growth, particularly during germ cell cluster formation. EGF also directly affects the growth of Sertoli cells and spermatogonia, as demonstrated in various species [33, 34, 53, 54, 59]. LIF, another Sertoli cell-derived factor, inhibits SSC differentiation and supports SSC propagation [3, 43].

Classification of Culture Systems for Induction of Spermatogenesis in Vitro

In recent decades, techniques for the long-term maintenance of spermatogonial stem cells (SSCs) in vitro have significantly improved by removing somatic cells from culture, which otherwise promote germ cell differentiation, enabling continuous subculture on feeder cells, and using chemically defined media containing various growth factors [52, 53]. Currently, two models—"in vivo" and "in vitro"—have been developed to preserve genetic material such as SSCs in vitro. "In vitro" models include organ culture, conventional (two-dimensional), and three-dimensional (3D) culture systems, while spermatogonial stem cell transplantation (SSCT) and testicular xenograft techniques are considered "in vivo" models.

Organ/Tissue Culture System

Increasing knowledge about tissue culture systems has helped elucidate the molecular mechanisms underlying spermatogenesis and the development of diagnostic and therapeutic techniques. Organ culture systems manage tissue environmental conditions more easily than in vivo systems. In the 1960s, research into spermatogenesis in vitro began by culturing testicular organs from neonatal rodents [60, 61]. This technique was improved in the 1970s by Steinberger and colleagues, who were able to induce rat gonocytes to differentiate into the pachytene stage of primary spermatocytes [44]. The main advantage of this approach is that germ cells maintain their spatial arrangement and microenvironmental composition in vitro, similar to in vivo conditions, with many endogenous factors being produced and released by the intact seminiferous epithelium and associated hormone-producing cells.

Sato et al. (2011) reported the production of functional mouse sperm in vitro by modifying the tissue culture medium. They demonstrated that cryopreserved fragments of neonatal testicular tissue could undergo spermatogenesis using the standard gas-liquid interface culture method. In this procedure, small fragments of neonatal mouse testis containing primitive spermatogonia were placed on an agarose gel half-soaked and incubated in a modified medium supplemented with knockout serum or lipid-rich bovine serum albumin. They observed mature spermatozoa approximately 27–45 days after culture initiation. After collecting round spermatids and mature spermatozoa from the cultured fragments and injecting them into mature oocytes via intracytoplasmic sperm injection, live offspring were born [45].

In humans, Pendergraft et al. (2017) developed a three-dimensional (3D) in vitro testicular organoid culture system as a novel tool for testicular toxicity screening and as a laboratory model for human spermatogenesis. They produced multicellular human testicular organoids consisting of spermatogonia, Sertoli, Leydig cells, and cells around the Wolffian ducts. These organoids were evaluated for morphology, viability, androgen production, and their ability to support germ cell differentiation. The upregulation of postmeiotic genes, including PRM1 and Acrosin, indicated a transition of a small percentage of diploid to haploid germ cells. These 3D organoids showed a dose-dependent response and maintained IC50 values significantly higher than 2D cultures, highlighting their potential as a reproductive toxicity screening tool [62].While organ culture models have great potential for elucidating the regulatory mechanisms of spermatogenesis and simulating natural environments, their clinical applications are limited. Tissue preservation in organ culture systems is challenging for long periods due to disrupted oxygen and nutrient supply, which often results in arrested or inefficient spermatogenesis, typically halting at the pachytene stage of the first meiotic division [30, 44, 45, 61–63].

Conventional/Two-Dimensional (2D) Culture System

Considering the culture conditions and microenvironment necessary for germ and testicular cell development is crucial for enhancing the process of spermatogenesis in vitro. Therefore, producing an experimental model that simulates seminiferous tubules for sperm production in vitro is challenging. Signals produced in a 2D culture of testicular cells differ significantly from those in vivo, where conditions necessary for SSC survival, propagation, differentiation, and recapitulation are optimally provided [53]. These differences in cell signaling pathways in conventional culture systems are due to the disturbance of spatial arrangement of testicular cells and disintegration of seminiferous niches. Nevertheless, conventional culture systems have become

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a common approach to study the influence of milieu and identify the biomolecular factors involved in the regulation of SSC proliferation and differentiation [18, 64]. Since the early 1990s, this method has been used extensively for SSC propagation in vitro, involving coated or uncoated plastic culture vessels where germ cells and/or somatic cells are cultured alone or with feeder layers [65, 66]. This method, using different types of supportive cells with specific physiological and evolutionary characteristics, has enabled the establishment of SSC lines and provided a tool to achieve male germ cells at different stages of spermatogenesis [46, 49, 56]. However, completion of spermatogenesis in vitro and access to SSC lines by this method is contingent upon overcoming several obstacles, such as accurately identifying in vitro propagation of low numbers of spermatogonia and regulating the SSC differentiation process [46].

In 2D culture systems, co-culture techniques using various types of cells cannot be standardized. However, co-culture with Vero cells, an immortalized cell line derived from kidney epithelial cells of the green monkey, has been beneficial for the maturation of spermatocytes and spermatids. These cells act as a feeder layer by contributing trophic growth factors and, due to their embryological similarity to genital epithelial cells, remove toxic compounds from the culture medium [46]. Tanaka et al. (2003) showed that a single human primary spermatocyte could undergo meiosis and differentiate into round spermatids when co-cultured with Vero cells [67]. However, despite the attractiveness of co-culturing with Vero cells, concerns remain about their use in clinical applications due to the theoretical risk of transmitting infectious agents and inducing tumorigenesis [30].

Three-Dimensional (3D) in Vitro Culture Systems

Developing efficient methods for culturing and propagating SSCs in vitro is crucial for achieving high concentrations of germ cells for medical and agricultural applications. Therefore, understanding the spatial arrangement of testicular cells is essential for completing germ cell maturation and regulating signaling pathways that control SSC self-renewal and differentiation. Three-dimensional culture systems were initially established for clonogenic assays to detect the complex mechanisms involved in SSC proliferation, providing unequivocal evidence that germ cells can routinely develop outside the body to the stage of elongated spermatids [68–71]. These systems define the optimal temporal and spatial conditions required for maintaining SSC self-renewal capacity [72].Using this culture system enhances our understanding of interactions between hormone-producing cells and germ cells, as well as between germ cells and the extracellular matrix, and their effects on spermatogenesis [68, 71, 72]. This culture system prevents ischemia, which hinders the long-term culture of testicular tissue and maintains the normal organization of germ cells in densely packed clusters [70]. Generally, 3D culture systems are organized to simulate the microenvironment of seminiferous epithelium in vitro. Several studies have provided unequivocal evidence that male germ cells in three-dimensional culture systems have grown to the stage of elongated spermatids [68–70]. Stockenburg et al. (2008) showed that embedding somatic cells in the solid bottom phase of a soft agar culture system (SACS) increased colony formation of mouse germ cells and improved spermatogenic differentiation of mouse SSCs in the upper phase of the gel layer. They showed complete maturation of germ cells into normal sperm by co-culturing premeiotic germ cells with somatic testicular cells, including Leydig, Sertoli, and peritubular myoid cells, in the presence of gonadotropin. The results confirmed that an appropriate ratio of somatic cells to germ cells, along with suitable environmental conditions, culture temperature, and gonadotropin/hormone concentrations, are essential for efficient proliferation and differentiation of juvenile and adult mouse germ cells in vitro [71].

In 2009, the same researchers compared SACS and the methylcellulose culture system (MCS), confirming that the three-dimensional simulation of mouse SSC niches in vitro could be achieved by spatial arrangement of the seminiferous epithelium in three compartments: basal, intraepithelial, and adluminal. They described that MCS and SACS, as alternative matrices for germ cell culture, provided suitable 3D structures for colony formation and differentiation of premeiotic germ cells into postmeiotic stages and subsequently normal mouse spermatozoa [70]. However, Sato et al. (2011) noted that SACS and MCS form a thick layer due to matrix properties, making it difficult to identify mouse spermatozoa directly during culture [45]. Eslahi et al. (2013) investigated the effect of a poly-L-lactic acid (PLLA) nanofiber scaffold on frozen-thawed neonate mouse SSCs, indicating the scaffold's efficacy in promoting germ cell proliferation and tissue engineering [35].

Spermatogonial Stem Cell Transplantation (SSCT)

Studies of spermatogenesis were hampered by a lack of efficient in vitro and in vivo assay systems until a method was established for transplanting germ cells from one animal to another. SSCT, a procedure in which testicular cells are harvested from a fertile male and microinjected into the seminiferous tubules of an infertile.

Conclusions

The successes in both in vivo and in vitro germ cell maturation offer hope that, in the future, we will be able to induce spermatogenesis under in vitro conditions, potentially

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eradicating male infertility, even in patients with non-obstructive azoospermia. Recent advancements in SSC cryopreservation, transplantation, and three-dimensional culture systems pave the way for developing new cell-based therapies, advancing germline gene therapy, and treating many currently incurable diseases. However, extensive basic, translational, and clinical research is still necessary before these techniques can be safely and effectively applied in human clinical settings.

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Declarations

Conflict of Interest The authors declare no competing interests.

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