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Sperm Biology

REVIEW

Recent advances in isolation, identification, and culture of mammalian spermatogonial stem cells

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Continuous spermatogenesis depends on the self-renewal and differentiation of spermatogonial stem cells (SSCs). SSCs, the only male reproductive stem cells that transmit genetic material to subsequent generations, possess an inherent self-renewal ability, which allows the maintenance of a steady stem cell pool. SSCs eventually differentiate to produce sperm. However, in an *in vitro* culture system, SSCs can be induced to differentiate into various types of germ cells. Rodent SSCs are well defined, and a culture system has been successfully established for them. In contrast, available information on the biomolecular markers and a culture system for livestock SSCs is limited. This review summarizes the existing knowledge and research progress regarding mammalian SSCs to determine the mammalian spermatogenic process, the biology and niche of SSCs, the isolation and culture systems of SSCs, and the biomolecular markers and identification of SSCs. This information can be used for the effective utilization of SSCs in reproductive technologies for large livestock animals, enhancement of human male fertility, reproductive medicine, and protection of endangered species.

INTRODUCTION

Spermatogenesis is the process by which spermatogonia, which originate from spermatogonial stem cells (SSCs), divide and differentiate into spermatocytes, which further develop into spermatozoa/sperm. It is a complex and continuous process of cell differentiation, in which different stages are precisely timed and coregulated by a range of genes and hormones.[1] SSCs are derived from gonocytes in the testes after birth; gonocytes originate from primordial germ cells at the embryonic stage.[2] SSCs are the only specialized male reproductive stem cells that serve as carriers of genetic material to subsequent generations and are located on the basement membrane of the testicular seminiferous tubule epithelium.[3]

SSCs play a pivotal role in mammalian spermatogenesis. They are distinguished from other cells by their capability of maintaining a steady stem cell pool by self-renewal, and that for further differentiating into haploid sperm cells.[4,5] The number of SSCs in mice testes is limited and difficult to determine; SSCs account for approximately 0.03% of all germ cells in the testes of mice.[6] Numerous unresolved issues remain in the study of the mechanism and function of SSCs in culture. The present review aimed to summarize recent research advances in the biology, niche, culture system, biomolecular markers, and identification of mammalian SSCs. The detailed evaluation of these points is crucial for understanding the physiological and pathological mechanisms related to mammalian reproduction. It would also provide valuable insights into SSC applications in assisted reproduction and cell therapy.

THE SPERMATOGENIC PROCESS

Testicular tissue is comprised of seminiferous tubules and interstitial tissue (**Figure 1a**). Cell types in the seminiferous tubules include spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids, and Sertoli cells (**Figure 1b** and **2**). During spermatogenesis, different types of spermatogenic cells are arranged in specific positions in the seminiferous tubules.[7] Spermatogenesis originates from the differentiation of SSCs, which depends on hormonal regulation and cell signal transduction. The precise balance between the self-renewal and differentiation of SSCs is regulated by the SSCs themselves, epigenetic factors,[8] and the SSC niche.[9–11] The SSC niche[12,13] is suggested to be comprised of Sertoli cells, Leydig cells, peritubular myoid cells, growth factors, immune cells, vascular cells, and the basement membrane. The SSC niche plays a pivotal role in the self-renewal and differentiation of SSCs[14] by providing extrinsic factors for maintaining stem cell potential.[15]

Spermatogenesis in mammals includes three phases:[16] the mitotic phase, the meiotic phase, and spermiogenesis (**Figure 3**). The mitotic phase of spermatogenic development in rodents includes undifferentiated spermatogonia (A_{single} [A_s], A_{paired} [A_{pr}], and $A_{aligned}$ [A_{al}]), differentiating spermatogonia (A1, A2, A3, A4, and intermediate), and differentiated spermatogonia (type B).[17] The population of undifferentiated spermatogonia is heterogeneous; most studies have suggested that only A_s spermatogonia possess stem cell characteristics (characteristics of self-renewal and differentiation into A_{pr} and A_{al}).[18,19] Therefore, A_s spermatogonia have been defined as SSCs. However, some recent studies have suggested that both A_{pr} and A_{al} populations show stem cell potential.[20,21] The meiotic phase of spermatogenic development in rodents involves three types of germ cells: successive type of primary spermatocytes, secondary spermatocytes, and haploid spermatids. Spermatids are transformed into spermatozoa during the

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spermiogenesis phase. The spermatogenic process in humans is similar to that in rodents. In humans, however, spermatogonia are divided into three types: A_{dark}, A_{pale}, and type B.[22] The transformation of SSCs into mature spermatozoa is vastly different across species. For instance, the duration of spermatogenesis in various mammals is as follows: 74 days

Figure 1: Composition of the SSC niche. (**a**) Histological cross-section of the seminiferous tubules from goat testes stained with hematoxylin and eosin. Scale bar = 25 μm. (**b**) Schematic diagram of the seminiferous tubules and interstitial tissue from mammalian testes. SSC: spermatogonial stem cell.

Figure 2: Immunohistochemical staining of DDX4, SOX9, and WT1 in the seminiferous tubules of adult goat testes. (**a**) The negative control section used non-immune rabbit serum. (**b**) DDX4 localized in germ cells. (**c**) SOX9 and (**d**) WT1 localized in Sertoli cells. Scale bars = 25 μm. SOX9: SRY-related high mobility group-box gene 9; WT1: Wilms tumor protein 1; DDX4: DEAD-box polypeptide-4.

in humans,[23] 35 days in mice,[19] 41 days in boars,[24] 57 days in stallions,[25] 63 days in cattle,[26] and 47.7 days in goats.[27]
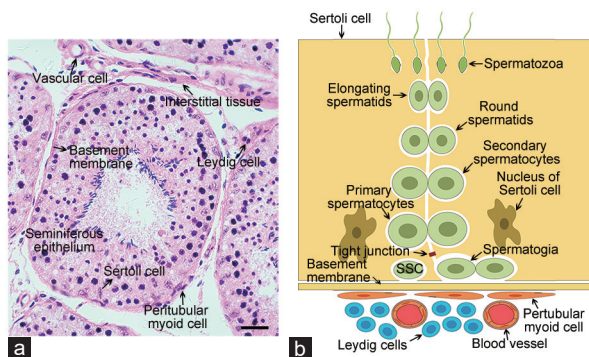
ISOLATION OF SSCS

The number of SSCs in immature and adult testes is limited.[6] Thus, the isolation and distinction of SSCs remain challenging. In immature testes, spermatogonia are the main cell type found in the seminiferous tubules. Therefore, immature testes are generally preferred for the isolation of SSCs. The optimal age of different animals for the isolation of their SSCs is as follows: 4.5 days to 7.5 days for mice;[28] 9 days for rats;[29] 5 months to 7 months for cattle;[30] 4 months for goats;[31] and 1 month for pigs.[32] Two-step enzymatic digestion, which was first proposed by Davis and Schuetz,[33] is widely used for the isolation of SSCs from humans,[34] mice,[35] monkeys,[36] sheep,[37] pigs,[38] and cattle[39] (**Table 1**). In this approach, the testicular tissue is digested using collagenase and trypsin. The enzyme concentration and digestion time affect the activity and quantity of the collected SSCs.[40]

For further enrichment of SSCs, differential plating,[41] Percoll gradient,[41,42] magnetic-activated cell sorting (MACS),[43] or fluorescence-activated cell sorting (FACS)[44] is typically performed (**Figure 4**). The theory behind the differential plating method is that the adhesion rates of SSCs and somatic cells are different. Somatic cells have a higher affinity for gelatin than SSCs. Compared to other enrichment methods, the differential plating method yields the best results for SSC enrichment, although it has a lower specificity for SSCs.[45] Izadyar *et al.*[46] used a Percoll gradient to concentrate a suspension of testis cells containing 25.5% bovine type A spermatogonia to 51%. This method is also widely used for the enrichment of SSCs from humans,[47] pigs,[48] sheep,[49] goats,[50] and monkeys.[51] FACS and MACS yield SSCs with higher purity than the other methods. For instance, Liu *et al.*[47] enriched human SSCs to an 86.7% concentration by FACS using the SSC surface marker octamer-binding transcription factor-4 (OCT4). Human SSCs were also purified by MACS using another SSC surface marker called integrin alpha 6 (ITGA6).[44] However, the use of MACS and FACS is limited by specific SSC surface markers. SSC isolation and enrichment methods for livestock species have been developed; however, their development has occurred at a slower pace than SSC isolation and enrichment methods for other species, which may be attributable to species differences. Moreover, no unique and specific SSC molecular markers have been determined in livestock species.
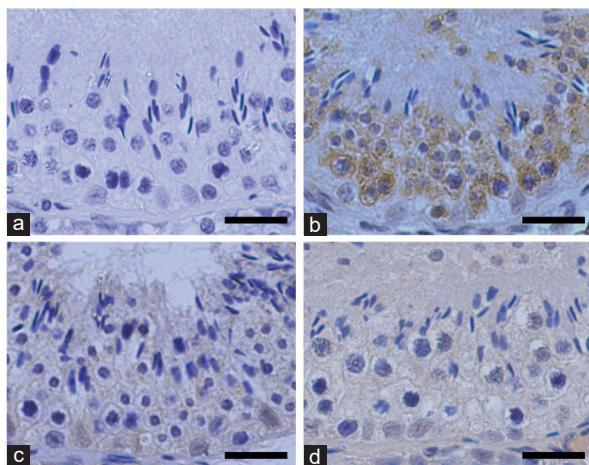
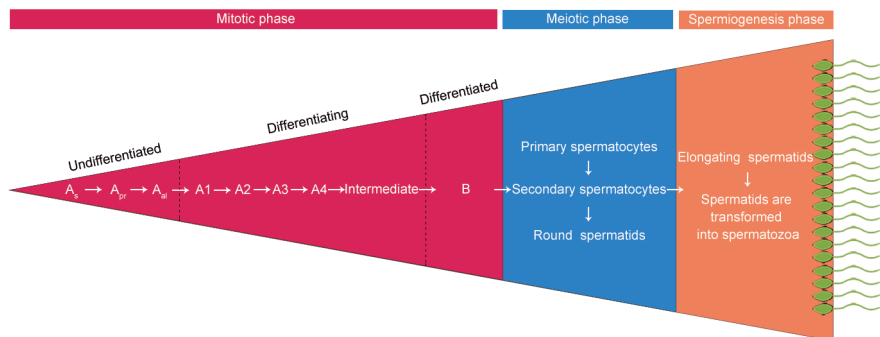## IDENTIFICATION OF SSCS
Biomolecular markers of SSCs
Currently, there are two methods for the identification of SSCs: (1) biomolecular marker-based identification (identification using
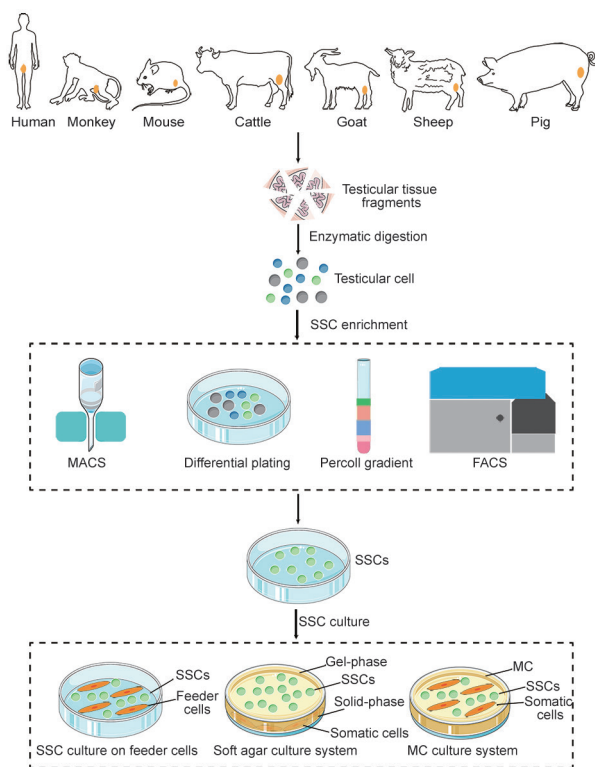

Figure 3: Schematic representation of the three phases of spermatogenesis in rodents. A_s: type A_{single} spermatogonia; A_{pr}: type A_{paird} spermatogonia; A_{al}: type $A_{aligned}$ spermatogonia; intermediate: intermediate spermatogonia; B: type B spermatogonia.

Table 1: Culture conditions for spermatogonial stem cells in human, monkey, mouse, cattle, goat, sheep, and pig

Subject, reference	Donor age	Isolation method	Enrichment method	Medium	Serum	Growth factors	Feeder cells	Identification method	Duration of culture (day)
Human, Sadri-Ardekani et al.[108]	NA	Two-step enzymatic digestion	Differential plating	StemPro-34 serum-free medium	10% FCS	20 ng ml⁻¹ EGF, 10 ng ml⁻¹ GDNF, 10 ng ml⁻¹ LIF	NA	Immunostaining	Approximately 196
Human, Guo et al.[109]	22–35 years	Two-step enzymatic digestion	Differential plating + MACS for GPR125	StemPro-34 serum-free medium	2.5 mg ml⁻¹ lipid-rich BSA, 1% FBS	50 ng ml⁻¹ GDNF, 20 ng ml⁻¹ EGF, 10 ng ml⁻¹ bFGF, 10 ng ml⁻¹ LIF	NA	Immunostaining	Approximately 60
Monkey, Langenstroth et al.[110]	3.7–4.6 years	Two-step enzymatic digestion	Differential plating	α-MEM	10% FBS	NA	Somatic cells	Immunostaining	Approximately 11
Mouse, Kubota et al.[28]	4.5–7.5 days	Two-step enzymatic digestion	MACS for Thy1	α-MEM	0.2% BSA	40 ng ml⁻¹ GDNF, 300 ng ml⁻¹ GFRα1, 1 ng ml⁻¹ bFGF	STO	Immunostaining	Approximately 180
Mouse, Kanatsu-Shinohara et al.[111]	8 days	Two-step enzymatic digestion	MACA for CD9	StemPro-34 serum-free medium	3 mg ml⁻¹ of lipid-rich BSA, 1:100 lipid mixture 1, 1:1000 lipoprotein-cholesterol concentrate	20 ng ml⁻¹ EGF, 10 ng ml⁻¹ FGF2, 15 ng ml⁻¹ GDNF	NA	Flow cytometry	Approximately 178
Cattle, Oatley et al.[93]	4–5 months	Two-step enzymatic digestion	Percoll gradient + differential plating	α-MEM	0.5% BSA	20 ng ml⁻¹ GDNF, 2 ng ml⁻¹ FGF2, 100 ng ml⁻¹ LIF	Bovine fetal fibroblasts	Immunostaining	Approximately 60
Cattle, Herrid et al.[30]	5–7 months	Two-step enzymatic digestion	Differential plating	DMEM	5% FBS	NA	NA	Immunostaining	NA
Goat, Ren et al.[112]	2–2.5 months	Two-step enzymatic digestion	Differential plating	DMEM	5% FBS	25 ng ml⁻¹ EGF, 20 ng ml⁻¹ PDGF-BB, 5 ng ml⁻¹ bFGF	Sertoli cells	Immunostaining	Approximately 28
Goat, Pramod and Mitra[50]	3–4 months	Two-step enzymatic digestion	Percoll gradient	DMEM	10% FBS	NA	Sertoli cells	Immunostaining	Approximately 45
Sheep, Binsila et al.[37]	Prepubertal	Two-step enzymatic digestion	Differential plating	DMEM	5% FBS	NA	NA	Immunostaining	NA
Sheep, Binsila et al.[113]	Prepubertal	Two-step enzymatic digestion	Ficoll density gradient + differential plating	StemPro-34 serum-free medium	5% FBS	40 ng ml⁻¹ GDNF, 20 ng ml⁻¹ EGF, 100 ng ml⁻¹ IGF1	NA	Immunostaining	Approximately 36
Pig, Goel et al.[114]	2–4 days	Two-step enzymatic digestion	Percoll gradient + differential plating	DMEM	10% FBS	10 µg ml⁻¹ insulin	NA	Immunostaining	Approximately 7
Pig, Zheng et al.[115]	7 days	Two-step enzymatic digestion	FACS for PLD6	DMEM	5% FBS + 5% KSR	20 ng ml⁻¹ GDNF, 40 ng ml⁻¹ GFRα1, 10 ng ml⁻¹ bFGF	NA	Immunostaining	Approximately 210

bFGF: basic fibroblast growth factor; BSA: bovine serum albumin; DMEM: Dulbecco's modified Eagle's medium; EGF: epidermal growth factor; FBS: fetal bovine serum; FCS: fetal calf serum; FGF2: fibroblast growth factor 2; GDNF: glial cell line-derived neurotrophic factor; GFRα1: glial cell line-derived neurotrophic factor receptor alpha 1; IGF1: insulin-like growth factor 1; KSR: knockout serum replacement; LIF: leukemia inhibitory factor; MACS: magnetic-activated cell sorting; FACS: fluorescence-activated cell sorting; MEM: minimum essential medium; NA: not available; PDGF-BB: platelet-derived growth factor-BB; STO: SIM mouse embryonic fibroblasts; GPR: G-protein receptor; THY1: Thy1 cell surface antigen; CD9: CD9 molecule; PLD6: phospholipase D family, member 6

Figure 4: Schematic representation of isolation, enrichment, and culture methods of SSCs. The testicular tissues is digested using the enzyme. Then, MACS, differential plating, Percoll gradient, or FACS is used for the enrichment of SSCs. In two-dimensional cell culture system, SSCs are cultured on feeder cells. Soft agar and MC are the most commonly used media in the three-dimensional cell culture system. SSC: spermatogonial stem cell; MACS: magnetic-activated cell sorting; FACS: fluorescence-activated cell sorting; MC: methylcellulose.

multiple SSC markers) and (2) spermatogonial transplantation (SSC colonization of recipient testes). Thus far, the understanding of the morphological characteristics and markers of SSCs has not been comprehensive. SSC markers in mice have been intensively searched for; however, SSC markers in humans and certain livestock species have not been identified. Previously, human SSCs were identified using markers of rodent SSCs. However, studies have demonstrated that several mouse SSC markers used for the identification of human SSCs are also expressed in nongerm cells in the human testes.[52,53] As previously mentioned, SSC markers differ between species (**Table 2**). OCT4 and ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) have been successfully identified as shared markers among the SSCs of humans, mice, cattle, goats, sheep, and pigs. Glial cell line-derived neurotrophic factor receptor alpha 1 (GFRα1) is widely used for SSC identification in humans, monkey, mice, cattle, and pigs. Whether GFRα1 can be specifically localized in the SSCs of goats and sheep still requires further study. Currently, BMI1 proto-oncogene, polycomb ring finger (BMI1), cadherin 1 (CDH1), forkhead box O1 (FOXO1), nanos C2HC-type zinc finger 2 (NANOS2), integrin beta 1 (ITGB1), paired box 7 (PAX7), and RET proto-oncogene (RET) are unique SSC markers in mice. The specific expression of fibroblast growth factor receptor 3 (FGFR3) is only found in human SSCs. Furthermore, the expression of SSC markers in livestock species has been shown to be heterogeneous. As some markers are not unique to SSCs, it is necessary to simultaneously use multiple markers for the identification of SSCs

in a species. Additionally, specific molecular markers need to be continuously discovered and canonically used to identify the SSCs of various species.

Spermatogonial transplantation

Spermatogonial transplantation, which can be divided into autologous transplantation, allogeneic transplantation, and xenotransplantation, depending on the recipient, has been demonstrated to restore the fertility of male individuals with damaged testes. The spermatogonial transplantation technique was developed to identify SSCs in 1994.[54] Endogenous germ cells must be eliminated to ensure that only donor-derived sperm are produced after transplantation.[55] As such, recipient mice are treated with busulfan (1,4-butanediol dimethanesulfonate) to deplete endogenous SSCs. Spermatogenic colonies derived from donor germ cells are identified in recipient testes through the expression of reporter genes. The donor-derived germ cells are labeled with β-galactosidase, green fluorescent protein, or other fluorescent proteins. Thereafter, they are transplanted into the seminiferous tubules of recipient mice. Busulfan treatment is known to deplete endogenous germ cells in a dose-dependent manner. However, busulfan has also been reported to have side effects, including toxicity, in recipient animals and may damage organs and Sertoli cells.[56] Hence, specific mutant mice lacking spermatogenesis can be used as recipients. For instance, knocking out *NANOS2* in mice[57] and pigs[58] has been shown to reduce the number of endogenous germ cells; these animals can then be employed as ideal transplant recipients for SSCs. After microinjection, the donor SSCs colonize the seminiferous tubules of the recipient. Consequently, spermatogenesis is restored. Fertilization of eggs by donor-derived spermatozoa can lead to the production of fertile offspring.[59] Therefore, the germ cell transplantation technique can be used for SSC identification. The success rate of homologous transplantation is higher than that of other types of transplantation. Ablation of endogenous germ cells is easier in rodents than that in livestock. To obtain more effective and reliable recipient males, the ablation of endogenous germ cells in livestock needs better strategies. SSC transplantation methods and recipient males for livestock SSC transplantation still need to be further explored. Currently, the colonization efficiency of SSCs in recipient testes is approximately 12.5%.[60] The reason for this low colonization efficiency after transplantation is unclear. Thus, it is important to elucidate the underlying mechanism and improve the colonization efficiency in SSC transplantation.

Complete spermatogenesis was observed after cross-species mouse-hamster[61] and mouse-rat[62] transplantations, and healthy offspring were produced. However, whether SSCs from nonrodent species could be identified by transplantation has been questioned.[63] In 2002, Nagano *et al.*[64] transplanted human SSCs into immunodeficient mice for the first time. Human SSCs were found to colonize the seminiferous tubules in mice and survived there for up to 6 months.[64] However, spermatogonial development did not proceed to the meiotic phase. The absence in the mice testes, of certain unique growth factors that are necessary for the spermatogenic process in nonrodent species may explain the inability of mice testes to support complete spermatogenesis following transplantation of SSCs from these species. SSCs from several nonrodent species have been transplanted into the testes of mice. SSC transplantation may be used as a breeding tool for livestock. However, complete spermatogenesis was observed only in the case of SSC transplantation from monkeys and sheep (**Table 3**). The reason for these interspecies differences may be the requirement for blood–testis barrier and Sertoli cells for the colonization of SSCs

Table 2: Overview of spermatogonial stem cell markers in human, monkey, mouse, cattle, goat, sheep, and pig

Marker	Human	Monkey	Mouse	Cattle	Goat	Sheep	Pig
BMI1	ND	ND	Komai et al.[116]	ND	ND	ND	ND
CD9	Zohni et al.[117]	ND	Kanatsu-Shinohara et al.[118]	Cai et al.[119]	Kaul et al.[120]	ND	ND
CDH1	ND	ND	Tokuda et al.[121]	ND	ND	ND	ND
DBA	ND	ND	ND	Izadyar et al.[122]	ND	Borjigin et al.[123]	Goel et al.[114]
FOXO1	ND	ND	Goertz et al.[124]	ND	ND	ND	ND
FGFR3	von Kopylow et al.[125]	ND	ND	ND	ND	ND	ND
GFRα1	He et al.[43]	Hermann et al.[126]	Meng et al.[70]	Oatley et al.[127]	ND	ND	Lee et al.[128]
GPR125	He et al.[43]	ND	Seandel et al.[129]	ND	ND	ND	ND
ID4	Sachs et al.[130]	ND	Helsel et al.[131]	ND	ND	ND	ND
ITGB1	ND	ND	Kanatsu-Shinohara et al.[132]	ND	ND	ND	ND
ITGA6	Valli et al.[44]	ND	Shinohara et al.[133]	De Barros et al.[134]	ND	ND	ND
LIN28	Aeckerle et al.[135]	Aeckerle et al.[135]	Zheng et al.[136]	ND	ND	ND	ND
NANOS2	ND	ND	Suzuki et al.[137]	ND	ND	ND	ND
NGN3	ND	Hermann et al.[138]	Yoshida et al.[42]	ND	ND	ND	ND
OCT4	Bhartiya et al.[139]	ND	Pesce et al.[140]	Borjigin et al.[123]	Pramod and Mitra[50]	Qasemi-Panahi et al.[141]	Goel et al.[114]
PAX7	ND	ND	Aloisio et al.[142]	ND	ND	ND	ND
PLZF	ND	Hermann et al.[126]	Costoya et al.[143]	Reding et al.[144]	ND	Borjigin et al.[123]	Goel et al.[114]
RET	ND	ND	Naughton et al.[145]	ND	ND	ND	ND
SALL4	Eildermann et al.[146]	Eildermann et al.[146]	Hobbs et al.[147]	ND	ND	ND	ND
SOHLH1	ND	Ramaswamy et al.[148]	Ballow et al.[149]	ND	ND	ND	ND
THY1	He et al.[43]	ND	Kubota et al.[28]	Reding et al.[144]	Abbasi et al.[150]	ND	ND
UCHL1	He et al.[43]	ND	Kwon et al.[151]	Herrid et al.[152]	Heidari et al.[153]	Rodriguez-Sosa et al.[49]	Luo et al.[154]
UTF1	ND	ND	van Bragt et al.[155]	ND	ND	ND	Lee et al.[156]

BMI1: BMI1 proto-oncogene, polycomb ring finger; CD9: CD9 molecule; CDH1: cadherin 1; DBA: dolichos biflorus agglutinin; FOXO1: forkhead box O1; FGFR3: fibroblast growth factor receptor 3; GFRα1: glial cell line-derived neurotrophic factor receptor alpha 1; GPR125: G-protein receptor 125; ID4: inhibitor of differentiation 4; ITGB1: integrin beta 1; ITGA6: integrin alpha 6; LIN28: Lin-28 homolog A; NANOS2: nanos C2HC-type zinc finger 2; ND: not determined; NGN3: neurogenin 3; OCT4: octamer-binding transcription factor-4; PAX7: paired box 7; PLZF: promyelocytic leukemia zinc-finger; RET: RET proto-oncogene; SALL4: spalt like transcription factor 4; SOHLH1: spermatogenesis and oogenesis-specific basic helix-loop-helix 1; THY1: Thy1 cell surface antigen; UCHL1: ubiquitin carboxyl-terminal hydrolase isozyme L1; UTF1: undifferentiated embryonic cell transcription factor 1

in recipient testes. The immunoprotection against germ cells offered by Sertoli cells ensures normal spermatogenesis. The number of Sertoli cells in the seminiferous tubules has also been demonstrated to affect the colonization efficiency of transplanted SSCs in mice.[65] Sertoli cells are a category of mesenchymal stem cells (MSCs) that have begun to differentiate.[66] Therefore, co-transplanting SSCs and MSCs has been shown to significantly improve the colonization efficiency of SSCs.[67] Co-transplantation of Sertoli cells and SSCs has also been reported to improve the efficiency of SSC transplantation in recipient testes.[68,69] Before SSC transplantation, SSCs need to expand *in vitro* to reach a sufficient number. SSC culture conditions of rodents have been used to culture in livestock SSCs. Obviously, expansion efficiency of livestock SSCs *in vitro* is still lower than that of rodents. There are still difficulties in SSC transplantation for livestock.

CULTURE OF SSCS

The limited number of SSCs in the testes hampers biological and applied research on these cells. However, this obstacle may be overcome by establishing an *in vitro* culture system that maintains the stem cell potential of SSCs. The culture of SSCs is a challenge at the outset, as growth factors, serum, and the feeder layer may affect the SSC state during *in vitro* culture (**Table 1**).

In 2000, glial cell line-derived neurotrophic factor (GDNF) was demonstrated to regulate the fate of undifferentiated spermatogonia in mice,[70] even though it was initially identified as a neurotrophic factor. Currently, GDNF and fibroblast growth factor 2 (FGF2) are known to be essential factors for the maintenance of SSC self-renewal in culture,[71–73] with GDNF having been shown to regulate the fate of SSCs in a dose-dependent manner.[70] Wang et al.[74] suggested that a high concentration of GDNF (20 ng ml^{-1}) is conducive to the early

proliferation of mouse SSCs; conversely, a low concentration of GDNF (4 ng ml^{-1}) was conducive to stable culture in the later stage of SSC development. Leukemia inhibitory factor (LIF) has also been reported to inhibit the differentiation of stem cells *in vitro*, with 15 mg ml^{-1} LIF promoting the stable proliferation of SSCs.[75] Wu et al.[76] demonstrated that the addition of 25 mg ml^{-1} basic fibroblast growth factor (bFGF) and other factors could continuously promote the proliferation of mouse SSCs *in vitro* for more than 120 days.

In addition to growth factors, fetal bovine serum (FBS) has been shown to be crucial for the survival and self-renewal of SSCs *in vitro* (**Table 1**). Goat SSCs were cultured under different serum concentrations (1%, 5%, 10%, and 15%).[77] After 7 days, the number of goat SSC colonies was observed to be higher in the presence of 1% serum compared to the number of colonies in the presence of 5%, 10%, and 15% serum.[77] A higher concentration of serum was found to inhibit the proliferation of SSCs.[77] However, various undefined factors in FBS may affect the culture status of SSCs *in vitro* and induce their differentiation. Therefore, knockout serum replacement (KSR) has been attempted for culturing SSCs *in vitro*. SSCs from immature bovine testes have also been cultured in serum-free medium containing GDNF, bovine leukemia inhibitory factor (bLIF), and KSR,[78] with SSC colonies being formed and identified based on morphological characteristics and molecular markers.

Feeder layer cells provide a variety of necessary cytokines for the proliferation of SSCs. Various feeder layer cells, including mouse embryonic fibroblast (MEF) feeder cells,[28,79] SIM mouse embryonic fibroblasts (STO) feeder cells,[80,81] yolk sac-derived endothelial cells (C166),[82] and Sertoli cells,[50,83] have been applied in the culture of SSCs to promote their proliferation. Among them, the MEF feeder layer cells exerted the best effect on the colonization and proliferation of

Table 3: Spermatogonial transplantation in human, mouse, monkey, cattle, goat, sheep, and pig

Donor species	Transplant type	Recipient species	Spermatogenesis of recipient	Reference
Human	Xenotransplantation	Mouse	Incomplete	Izadyar *et al*.[122]
Mouse	Autologous	Mouse	Complete	Koruji *et al*.[157]
Mouse	Allogeneic	Mouse	Complete	Ma *et al*.[158]
Monkey	Autologous	Monkey	Complete	Hermann *et al*.[51]
Monkey	Allogeneic	Monkey	Complete	Hermann *et al*.[51]
Monkey	Xenotransplantation	Mouse	Complete	Ntemou *et al*.[97]
Cattle	Autologous	Cattle	Complete	Izadyar *et al*.[46]
Cattle	Allogeneic	Cattle	Complete	Izadyar *et al*.[46]
Cattle	Xenotransplantation	Mouse	Incomplete	Izadyar *et al*.[159]
Goat	Allogeneic	Goat	Complete	Honaramooz *et al*.[31]
Goat	Xenotransplantation	Mouse	Incomplete	Shirazi *et al*.[160]
Sheep	Allogeneic	Sheep	Complete	Herrid *et al*.[68]
Sheep	Xenotransplantation	Mouse	Complete	Pukazhenthi *et al*.[98]
Pig	Autologous	Pig	Not detected	Honaramooz *et al*.[32]
Pig	Allogeneic	Pig	Complete	Zeng *et al*.[161]
Pig	Xenotransplantation	Mouse	Incomplete	Dobrinski *et al*.[162]

SSCs.[84] The preparation of feeder layer cells for subculture is a tedious process; therefore, a Matrigel-based feeder-free culture system was developed.[85] This feeder-free culture system was able to maintain the biological function of SSCs in an *in vitro* culture (**Table 1**). For the culture of SSCs from livestock, the commonly used feeder layer cells are their own Sertoli cells.[50,83] However, Sertoli cells secrete certain growth factors that promote the differentiation of SSCs; therefore, the long-term culture of SSCs *in vitro* cannot be sustained.

To simulate the growth environment of SSCs *in vivo*, a three-dimensional cell culture system was developed. To this end, various types of cells in the seminiferous tubules were separated and implanted into a semi-solid medium to maintain the niche function. Currently, soft agar and methylcellulose (MC) are the most commonly used three-dimensional cell culture media (**Figure 4**). Mouse SSCs were cultured for 15 days in a soft agar culture system, and specific markers of haploid sperm cells were found to be expressed by them.[86] Similarly, SSCs from prepubertal male testes were cultured in MC and haploid sperm-like cells were subsequently identified.[87] Sertoli cells, Leydig cells, and SSCs from rats were also co-cultured in the extracellular matrix to establish an *in vitro* toxicity test system for rat testes.[88] The three-dimensional culture system provides a research model for communication and interactions between cells in the body. However, it cannot sustain the culture of SSCs for a long period and is incapable of recycling all cells.

Culture systems for neonate SSCs differ from those for adult SSCs.[39] Using a neonatal culture system to culture SSCs from adult mammals revealed that the system was unable to maintain the SSCs for a long period.[39,78] Adult bovine SSCs were cultured for a maximum of three passages, and the stem cell potential of SSCs derived from immature bovine testes was greater than that of stem cells derived from adult bovine testes.[78] It appears that the successful establishment of an SSC culture system depends on the age of the animal (neonate or adult). SSCs from rats, hamsters, and rabbits were established using species-specific culture components.[82,89–91] SSCs from pigs,[83,92] goats,[50] and cattle[39,78,93] were also cultured successfully *in vitro*. However, most of these studies only carried out a short-term culture. The long-term culture of SSCs from livestock species is still in its infancy.[94] Some reports have suggested that SSCs from pigs[83] and cattle[78] have been successfully cultured *in vitro* for a prolonged period. However, long-term culture systems for SSCs from other livestock

have not been established, which may be due to the lack of proper SSC culture conditions and necessary cytokines for proliferation. The culture conditions for rodent SSCs cannot be fully applied to SSCs from livestock species.[95] Although reports have indicated that SSCs or germline stem cells could be continuously cultured over months, the stem cell potential of these cultured cells remains controversial. Therefore, it is necessary to rigorously evaluate the function of SSCs after long-term culture. Transplantation of SSCs cultured into the testes of mice lacking endogenous germ cells was reported to produce offspring originating from donor SSCs, indicating that SSCs cultured *in vitro* are capable of differentiating into sperm *in vivo*, leading to the production of offspring.[96] Currently, only SSCs from monkeys[97] and sheep[98] cultured *in vitro* have been demonstrated to exhibit complete spermatogenesis after transplantation into immunodeficient mice. The establishment of an *in vitro* long-term culture system for livestock SSCs would help to further elucidate the biology and application of SSCs. Thus, further study of the conditions for long-term *in vitro* culture of livestock SSCs is urgently required.

SSC fates are also regulated by epigenetic factors. For epigenetic factors, DNA methylation, histone methylation, and noncoding RNAs (ncRNAs) are involved in regulation of SSC fates. DNA (cytosine-5-)-methyltransferase 3-like (DNMT3L; DNA methylation regulator) precisely regulates the proliferation and quiescence of SSCs.[99] Tet oncogene 1 (TET1) participates in DNA methylation and histone modification to regulate SSC self-renewal.[100] NcRNAs, as the novel epigenetic regulator, play crucial roles in regulating SSC fates. Long ncRNAs (lncRNAs) transcription is important for the self-renewal of SSCs. In the testis, lncRNA AK015322, mainly expressed in SSCs, regulates SSC proliferation by competitively binding miR-19b-3p and reducing the inhibitory effect of miR-19b-3p on ETS translocation variant 5 (Etv5).[101] Li *et al*.[102] have demonstrated that circular RNAs (circRNAs) play a role in mammalian SSCs. MicroRNAs (miRNAs) are a kind of nonprotein-coding short sequence RNA. He *et al*.[103] reported for the first time that miRNA-20 and miRNA-106a are highly and specifically expressed in SSCs of mice, which promoted SSC proliferation and DNA synthesis. And miR-31 regulated meiosis of SSCs by targeting stimulated by retinoic acid gene 8 (*Stra8*) *in vivo* and *in vitro* to inhibit spermatogenesis.[104] Huang *et al*.[105] reported that miR-100 is mainly expressed in mouse SSCs, which indirectly regulated signal transducer and activator of transcription 3 (*Stat3*) to promote

SSCs proliferation. By isolating high-purity SSCs, Zhang *et al*.[106] found that P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs) account for 47% of the total number of small RNAs. Dong *et al*.[107] found that ubiquitin-like, containing PHD and RING finger domains 1 (Uhrf1) regulates retrotransposon silencing in male germ cells and cooperates with the PIWI pathway during spermatogenesis. LncRNAs, circRNAs, miRNAs, and piRNAs have formed complicated regulatory networks to modulate the SSCs fate. However, only a small number of ncRNAs have been verified functionally. It is very urgent to further uncover the regulatory effect of more ncRNAs (especially piRNAs and circRNAs) on SSC fates.

CONCLUSIONS

The unique biological characteristics of SSCs determine their importance in spermatogenesis. Any biological dysfunction in SSCs can cause male infertility. Investigation of the methods of isolation, identification, and culture of SSCs would help us better understand the processes of normal spermatogenesis and male infertility. Several SSC markers have been identified in rodents. There are numerous differences in spermatogenic processes between rodents and nonrodents; however, certain SSC markers and features of spermatogenesis are conserved among species. For the identification of SSCs from livestock, the source and quality of antibodies used are critical. Many SSC antibodies have limited specificity in livestock, and there are currently no specific antibodies against SSCs of various livestock species.

Researchers are encouraged to devote more attention to conduct detailed research into the regulatory effects of specific marker genes on SSC fates and spermatogenesis in different livestock species, which is currently lacking. Currently, research on spermatogenesis in livestock is limited and lacks depth. There have been no recent significant breakthroughs in the field of livestock SSCs and spermatogenic processes. The long-term culture of SSCs *in vitro* to produce sperm provides a novel method for the production of transgenic livestock. In addition, SSCs of superior livestock can be cultured *in vitro* and then transplanted into the testis of recipient, which can produce a large number of sperm carrying superior livestock genes for fertilization and promote the reproduction of superior livestock. However, the feasibility, safety, and bioethics of applying this technology have yet to be considered. Currently, our knowledge of SSC biology is still limited, and we have not fully developed the full potential of SSCs *in vitro*. Thus, it is necessary to further explore the role of SSCs in reproductive biology. A deeper understanding of the similarities and differences between the reproductive biology of various mammals would be conducive to the development of SSC culture and transplantation and applications of SSCs in human medicine, livestock improvement, and protection of endangered species.

AUTHOR CONTRIBUTIONS
HMX was a major contributor in writing the manuscript. HMX, YJR, FR, YL, TYF, ZW, YQD, and LKZ performed the literature search and data analysis. JHH helped to revise the manuscript. All authors read and approved the final manuscript.

COMPETING INTERESTS
All authors declare no competing interests.

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