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REVIEW ARTICLE

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Biology and manipulation technologies of male germline stem cells in mammals

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Abstract

Background: Spermatogonial stem cells (SSCs) are the origin of sperm and defined by their functions of "colonization in the testis" and "spermatogenesis". In vitro manipulation techniques of SSCs contribute to a wide variety of fields including reproductive medicine and molecular breeding. This review presents the recent progress of the biology and manipulation technologies of SSCs.

Methods: Research articles regarding SSC biology and technologies were collected and summarized.

Main findings: Dr. Ralph Brinster developed the spermatogonial transplantation technique that enables SSC detection by functional markers. Using this technique, cultured SSCs, termed germline stem (GS) cells, were established from the mouse. GS cells provide the opportunity to produce genome-edited animals without using zygotes. In vitro spermatogenesis allows production of haploid germ cells from GS cells without spermatogonial transplantation. The recent advancement of pluripotent stem cell culture techniques has also achieved production of functional GS-like cells in addition to male/female germ cells.

Conclusion: Although in vitro manipulation techniques of GS cells have been developed for the mouse, it appears to be difficult to apply these techniques to other species. Understanding and control of interspecies barriers are required to extend this technology to nonrodent mammals.

KEYWORDS

genome editing, germline stem cell, in vitro spermatogenesis, spermatogonial stem cell, spermatogonial transplantation

1 | INTRODUCTION

Spermatogonial stem cells (SSCs) are the special subset of undifferentiated spermatogonia. These cells contribute to spermatogenesis by providing differentiating spermatogonia that can initiate meiotic division to produce haploid spermatids. Recent progress of SSC manipulation techniques allows not only to quantify the SSC number by transplantation assay but also to expand SSC population in vitro under specific culture condition. These techniques have greatly contributed to the elucidation of the survival and self-renewal mechanism of SSCs. In addition, it was also shown that cultured SSCs are susceptible to genome editing and in vitro spermatogenesis, representing considerable potentials for medical and industrial application. In this review, recent progress of biology and manipulation technologies of male germline stem cells in mammals is described.

2 | DEFINITION AND DETECTION OF SSC

Stem cells are defined by two particular functions: self-renewal and differentiation.[1] For example, hematopoietic stem cells (HSCs) can produce multipotent progenitor cells that proliferate rapidly to generate more committed progenitor cells, such as common lymphoid and myeloid progenitor cells, toward producing the various types of mature effector cells.[2] A HSC can be detected by a transplantation assay in which the single cell produces T-lymphoid cells, B-lymphoid cells, and myeloid lineage cells to be identified as a HSC.[3,4] Somatic stem cells cannot be recognized and isolated precisely by a prospective approach such as fluorescence-activated cell sorting (FACS). Hence, the stem cell transplantation assay is the only retrospective method to detect and count the number of stem cells based on their functional definition.

Spermatogonial stem cells are the only cell type that can produce differentiating progeny for sperm production. Therefore, SSCs are categorized as unipotent stem cells. The transplantation assay for SSCs was primarily developed by Brinster and Zimmermann.[5] In their study, a testis cell suspension containing SSCs was injected into a seminiferous tubule of an infertile mouse testis (eg, busulfan-treated mice and congenitally infertile $Kit^{W}/Kit^{W\text{-}v}$ mice). The transplanted SSCs colonized the recipient seminiferous tubule and started spermatogenesis. The generated sperms were able to produce offspring, indicating that the colonized cells were SSCs.[6] SSC injection can be performed via the efferent duct and/or rete testis (Figure 1).[7] Subsequent studies have demonstrated that one colony generated by spermatogonial transplantation is derived from a single SSC,[8,9] demonstrating that the spermatogonial transplantation assay can be used for SSC quantitation.

This technique led to the possibility of in vitro SSC manipulation. The primary application was developed by Nagano et al who infected SSCs in vitro with a retroviral vector carrying a *LacZ* transgene, which colonized infertile mice.[10,11] This study demonstrated the possibility of in vitro SSC manipulation. However, simultaneously, it was strongly suggested that the SSC culture system is beneficial for further advancement of SSC manipulation.

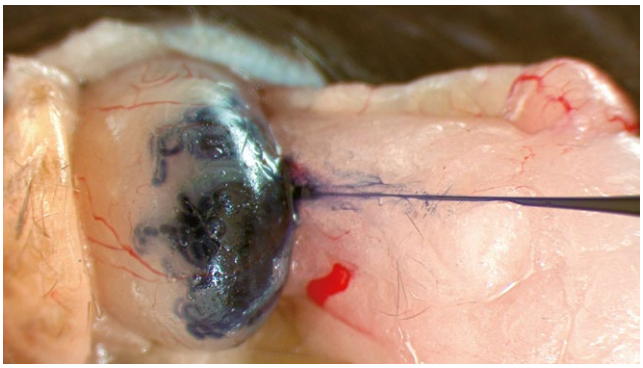

FIGURE 1 Transplantation of SSCs via the efferent duct. In this procedure, a glass capillary is inserted into the rete testis via the efferent duct. This photo demonstrates injection of a trypan blue solution into seminiferous tubules, instead of SSCs/GS cells. The image was obtained from a previous review with permission from the Japanese Journal of Embryo Transfer[129]

3 | SELF-RENEWAL FACTORS FOR SSCS AND ESTABLISHMENT OF GERMLINE STEM (GS) CELLS

Maintenance and expansion of SSCs are supported by several soluble factors. Thus far, multiple cytokines, such as colony stimulating factor 1 (CSF1), wingless-type MMTV integration site family (WNT) 5A, WNT3A, vascular endothelial cell growth factor A, fibroblast growth factor (FGF) 8, and WNT6, are reported to be a functional in SSC maintenance and expansion.[12-18] Among these cytokines, glial cell line-derived neurotrophic factor (GDNF) is the primary factor that is indispensable for SSCs. Meng et al reported that haploinsufficiency of *Gdnf* results in gradual loss of spermatogenesis, whereas *Gdnf* overexpression causes hyperproliferation of undifferentiated spermatogonia.[19] Mutation in the *Ret* proto-oncogene also resulted in a similar phenotype of spermatogonia.[20,21]

Discovery of GDNF allowed establishment of SSC lines. The first report of in vitro SSC culture was published by Nagano et al, in which testis cells were cultured on mitomycin-treated STO feeder cells with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Although the testis cells maintained SSC activity even after 111 days of culture in the best case, obvious expansion of SSCs was not observed.[22] Long-term culture and expansion of SSCs in vitro were achieved by Kanatsu-Shinohara et al. using epidermal growth factor (EGF), leukemia inhibitory factor (LIF), FGF2, GDNF, and mitomycin C-treated mouse embryonic fibroblasts as feeder cells.[23] In their culture system, testis cells derived from a pup of the DBA/2 strain formed grape-like clumps of cells and proliferated for more than 4 months in a logarithmic manner without losing colonization activity in testes of infertile mice. Moreover, haploid male germ cells could produce offspring, demonstrating that the cultured cells possessed the proper SSC activity. Hence, these cells were named GS cells (Figure 2). Subsequently, some studies reported comparable results regarding GS cell derivation from other mouse strains under similar conditions.[24,25] These results suggested that the combination of mouse strain and age, feeder cells used, and serum concentration affected the in vitro expansion of SSCs.

FGF2 was thought to be a supportive factor for GS cells. However, we found that GS cells can be expanded with GDNF or FGF2 alone in vitro. This finding suggested that GDNF is dispensable for SSC maintenance and self-renewal.[26] Intriguingly, FGF2-cultured spermatogonia have a morphology, doubling time, and SSC activity distinct from those of GDNF-cultured spermatogonia. In addition, the former cells survive and proliferate without MAP2K1 (dual specificity mitogen-activated protein kinase 1) activation, whereas the latter cells require its activation, suggesting that FGF2 promotes survival and proliferation of SSCs

via signals distinct from GDNF signals. In fact, we found that FGF2-stimulated undifferentiated spermatogonia form colonies with characteristics distinct from those of GDNF-induced undifferentiated spermatogonia in vivo.[27] In this study, forced input of strong FGF2/GDNF signals was applied directly in mouse testes using intelligent biomaterials. Although we found that both factors induced hyperproliferation of GDNF family receptor alpha-1 (GFRA1)-expressing undifferentiated spermatogonia, FGF2-induced GFRA1$^+$ spermatogonial colonies exhibited a morphology distinct from those induced by GDNF (Figure 3). In addition, we found that FGF2-induced spermatogonia were prone to express retinoic acid receptor γ (RARG). Considering that expression of RARG is sufficient for retinoic acid-mediated differentiation of undifferentiated spermatogonia into differentiating spermatogonia,[28] FGF2 is quite unique because this molecule contributes to spermatogonial differentiation despite the fact that it is a *bona fide* self-renewal factor for SSCs. These observations raise the possibility that FGF2 plays a role distinct from GDNF in controlling the fate of SSCs/undifferentiated spermatogonia in vivo. Our most recent studies revealed expression of *Fgf2* in the germ cell population, while *Gdnf* is expressed in Sertoli cells and peritubular myoid cells.[27,29-31] In these studies, germ cell depletion increased the relative expression of *Gdnf*, while *Fgf2* was relatively suppressed.[27,29] Considering that germ cell-depleted conditions are relatively appropriate for expansion rather than differentiation of transplanted undifferentiated spermatogonia/SSCs,[32, 33] the *Gdnf*/*Fgf2* ratio might affect fate determination of undifferentiated spermatogonia/SSCs (Figure 3).

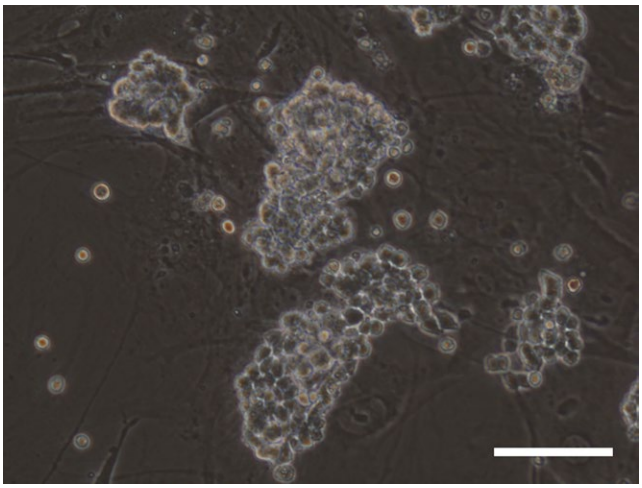

FIGURE 2 Morphology of mouse GS cells. GS cells form grape-like cellular clusters on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts in the presence of GDNF and FGF2. Scale bar = 100 μm

Although our recent studies demonstrate that FGF2 acts as a differentiation factor by expanding the RARG$^+$ subset of undifferentiated spermatogonia, FGF2 is still a promising factor to expand SSCs in vitro. Indeed, FGF2-cultured spermatogonia possess considerable SSC activity even after in vitro culture for more than 4 months under GDNF-free conditions.[26] Moreover, it has been demonstrated that FGF2 supports GDNF-mediated expansion of undifferentiated spermatogonia in vitro.[23] Although CSF1 and WNT5A were confirmed to support expansion of an SSC subset in vitro,[12,13,18] it should be determined whether the other candidate factors mentioned above can support GS cell proliferation.

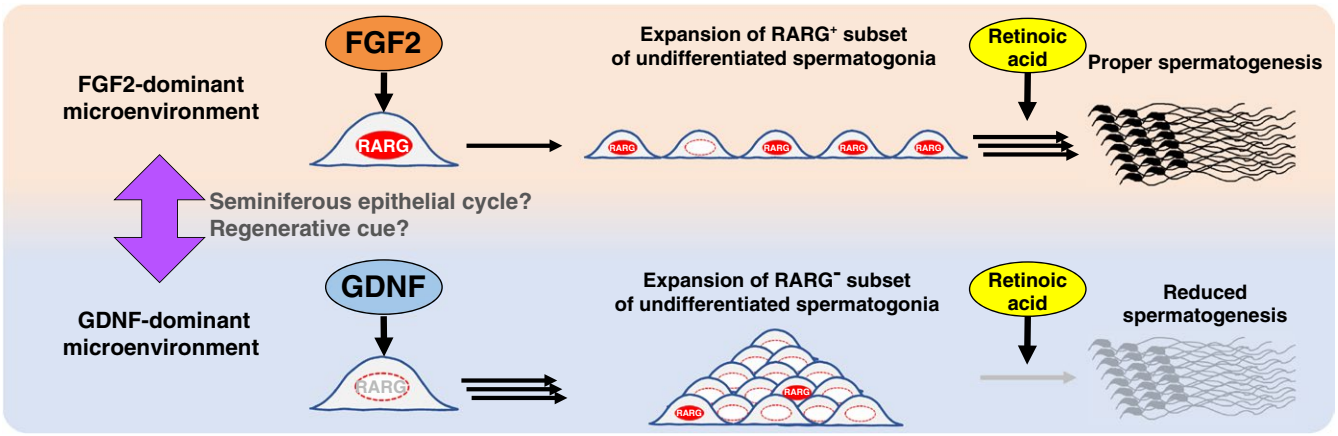

FIGURE 3 Functional differences between FGF2 and GDNF in the testicular microenvironment. FGF2-induced GFRA1$^+$ spermatogonia are a differentiation-prone subset because these cells tend to express RARG, the receptor for retinoic acid that induces spermatogonial differentiation. However, GDNF-induced GFRA1$^+$ spermatogonia tend to be negative for RARG, suggesting that these cells are a differentiation-resistant subset.[27] The *Gdnf*/*Fgf2* ratio decreases along with postnatal testicular development and increases during regeneration.[29] During testicular development or regeneration (eg, after busulfan-mediated germ cell depletion), *Gdnf* expression upregulates in the testis, while undifferentiated spermatogonia expand their population without differentiation.[29,32,33] Considering these observations, the *Gdnf*/*Fgf2* ratio in the testicular microenvironment might regulate the behavior of undifferentiated spermatogonia via RARG expression. Under the GDNF-dominant microenvironment, the RARG$^-$ subset of undifferentiated spermatogonia expand their population without differentiation, while RARG$^+$ undifferentiated spermatogonia are susceptible to retinoic acid-mediated spermatogonial differentiation toward proper spermatogenesis in the FGF2-dominant microenvironment

4 | GENOME EDITING VIA GS CELLS

Germline stem cells are distinct from embryonic stem (ES) cells by their remarkably stable epigenetic/genetic properties. SSCs maintain a normal karyotype, normal genomic imprinting status, and spermatogenic activity to generate offspring for 2 years in vitro, whereas ES cells cannot maintain genomic stability and their ability for germline transmission.[34] Additionally, these cells are susceptible to drug selection.[35] Therefore, GS cells are considered to be more suitable than ES cells for genome editing of germline lineages.

Using this property, transgenic/knockout mice have been produced via lipofection, electroporation, and retroviral vector infection of GS cells.[35,36] In addition, lentivirus-, adenovirus-, and adeno-associated virus-mediated gene transductions are suitable for GS cells.[37–40] Moreover, GS cell-mediated transfer of an ectopic chromosome was achieved by Shinohara et al.[41] Since editing of the animal genome has become more common with the application of clustered regularly interspaced short palindromic repeat (CRISPR)-CRISPR-associated protein 9 technology to mammalian zygotes,[42-44] GS cells have also been demonstrated to be an alternative platform for CRISPR-Cas9-mediated genome editing.[45,46] Zygote-mediated genome editing is superior to produce genome-edited animals in a short period. However, the editing accuracy cannot be verified until production of the offspring. Although accuracy verification of ES cell-mediated genome editing can be performed before producing genome-edited offspring,[47,48] GS cell-mediated genome editing is thought to be more superior because of its genomic stability.[34]

5 | IN VITRO SPERMATOGENESIS OF GS CELLS

Although GS cells primarily produce mature sperm by spermatogonial transplantation into infertile recipient testes, an in vitro spermatogenesis technique is also available for GS cells to produce haploid male germ cells in vitro. Sato et al developed an organ culture system for testis tissue, in which pup testis tissues were placed on an agarose gel block for culture at the liquid-air interface.[49] In this system, some tissues show completion of spermatogenesis and the resultant haploid sperm can fertilize an oocyte to produce offspring. This technique is also applicable to adult and cryopreserved tissues.[50,51] Moreover, their group applied a microfluidic device system to improve the frequency and maintenance period of in vitro spermatogenesis.[52,53] These techniques are also applicable to sperm production from GS cells in vitro.[54]

6 | GERM CELL INDUCTION FROM PLURIPOTENT STEM CELLS

Pluripotent stem cells, such as ES cells and induced pluripotent stem (iPS) cells, are a source for haploid male germ cells, because these cells show germline contribution in chimeric mice.[55-57] In vitro induction of male and female germ cells from ES/iPS cells was achieved by Saitou and colleagues. First, they identified genes that can trace the state of differentiation toward the germ cell lineage.[58,59] Subsequently, a transgenic mouse stain and ES cell line were established, which showed germ cell commitment by dual fluorescence reporter genes (*Blimp1-Venus*::*Stella-Cfp* reporter mouse/ES cells).[60] Simultaneously, they also explored cytokines essential for in vitro induction of the germ cell fate in primary epiblast tissue.[61] By combining these achievements, they succeeded to induce functional male germ cells from ES and iPS cells.[62] In these reports, they primarily induced epiblast-like cells (EpiLCs) from ES cells using activin A and FGF2, and then primordial germ cell-like cells (PGCLCs) were derived from aggregated EpiLCs in suspension culture by stimulation with bone morphogenic protein (BMP) 4, BMP8b, stem cell factor, LIF, and EGF. The resultant PGCLCs were then transplanted into infertile mouse testes to produce haploid male germ cells. Following these studies, they also succeeded to complete induction of oocytes in vitro by coculture of PGCLCs with sex-matched embryonic gonadal cells.[63,64] These achievements conceived the idea to derive GS cells from ES/iPS cells.[65]

7 | APPLICATION OF GS CELL TECHNOLOGIES TO NONRODENT SPECIES

As described above, in vitro manipulation techniques for GS cells, including transplantation, in vitro expansion, genome editing, and sperm production, were established in the mouse. These techniques can contribute to the fields of biological science, agriculture, and medicine. Therefore, it is required to apply these techniques to nonrodent mammals. However, there are some remaining issues to be resolved as discussed below.

7.1 | Functional assessment of GS cells

Spermatogonial transplantation is critical for SSC manipulation. Xenotransplantation using germ cell-depleted immunodeficient mice has been used to measure the number of SSCs from mammalian species other than mouse.[66-73] SSCs derived from the rat and hamster can colonize testes of germ cell-depleted nude mice to produce mature spermatozoa.[66,67] In contrast, although SSCs derived from rabbits, porcine, bovine, canine, equine, nonhuman primates, and humans can also colonize recipient mouse testes, these cells cannot differentiate beyond the stage of spermatogonial expansion.[68-73] These results demonstrate the existence of a species barrier. Considering that rabbit testis tissues transplanted into the testes of nude mice generate mature sperm to produce offspring,[74] an interspecies difference regarding molecules that participate in the cellular communication might hamper spermatogenesis. Indeed, the spermatogonial JAG2-Sertoli NOTCH-mediated interaction is essential for proper spermatogenesis.[75,76] Cell adhesion molecules NECTIN2 and NECTIN3 also contribute to Sertoli-spermatid interactions.[77,78] Tight junction molecules occludin and claudin (CLDN)-11

are indispensable for the blood–testis barrier (BTB) that is required for spermatogenesis.[79,80] It was also demonstrated that RAC1 (Ras-related C3 botulinum toxin substrate 1)-mediated CLDN-3 expression in SSCs is indispensable for passage through the BTB in mice.[81] Considering these observations, cellular communication defects attributed to the interspecies barrier might prevent xenotransplanted SSCs from undergoing spermatogenesis. Additionally, other conditions, such as temperature, hormones, nutrition, and retinoic acid concentrations, might affect colonization of xenogeneic SSCs.

7.2 | GS cell derivation from nonrodent mammals

Establishment of GS cells was achieved only in rodents including the mouse, rat, hamster, and rabbit.[23,82-86] Since establishment of mouse GS cells, many trials have been reported to establish GS cells from other mammalian species by following the mouse GS cell culture method in combination with spermatogonial transplantation. However, SSCs derived from bovines, pigs, and humans, have not been expanded in vitro.[72,73,87-91] In the most recent report, Oatley et al developed a culture condition for cattle undifferentiated spermatogonia to form germ cell colonies with a grape-like morphology that resembles mouse GS cell morphology.[92] Although obvious expansion of GS cell-like cells was not observed, their study suggested that the choice of basal medium, cultivation temperature, and species matching between SSCs and feeder cells for culture might be essential to establish stable GS cell lines from testes of mammals other than rodents. To establish a universal culture method for mammalian GS cells, a serum- and feeder-free culture system might be valuable. The first report regarding successful GS cell culture under serum-free conditions was published by Kubota et al.[25] Moreover, a feeder-free culture system was primarily established by Kanatsu-Shinohara et al.[93] They also succeeded to expand GS cells in suspension culture.[94] As a combined approach, a serum- and feeder-free long-term culture system was established in 2011.[95] Furthermore, they eliminated the chemically undefined supplements from the serum- and feeder-free culture system.[96] In the case of pluripotent stem cells, chemically defined culture systems contribute to maintaining the ground state of pluripotency under which pluripotent stem cells can highly contribute to forming chimeric offspring after injection into a blastocyst.[97,98] This concept was expanded to human pluripotent stem cells.[99] Improvement of chemically defined culture for GS cells will contribute to not only establishment of animal/human GS cells, but also understanding the self-renewal mechanism of SSCs.

In addition to the culture conditions, several putative factors are considered to hamper the establishment of animal/human GS cells. First, it must be noted that testicular somatic cells from large animals proliferate more rapidly than those from rodents. In the case of mice, the majority of testicular somatic cells can be excluded by differential plating with residual cells overwhelmed by proliferating SSCs.[23] This procedure was thought to be applicable to large mammals.[90,92] However, testis somatic cells from piglets show a much higher proliferation activity than those from mouse pups. In my experience, piglet somatic cells overwhelm SSCs in vitro in the present culture condition even after differential plating (unpublished data). Instead, it was found that serum reduction suppresses somatic cell proliferation (unpublished data). These circumstances suggest that SSC purification in combination with SSC-selective medium is a prerequisite for expansion of SSCs from large mammals. In this regard, SSC purification using specific antibodies and/or stem cell dyes in combination with FACS/magnetic activated cell sorting is available. Previous reports have demonstrated that several cell surface markers are applicable to SSC purification. Integrin alpha 6 (ITGA6), ITGB1, THY1, CD9, epithelial cell adhesion molecule (EPCAM), and melanoma cell adhesion molecule (MCAM) are applicable to SSC purification in a single use manner, whereas traditional spermatogonial marker KIT does not permit effective purification.[100-104] Although $CD9^+$, $EPCAM^+$, and $MCAM^+$ subsets in the mouse testis achieve SSC enrichment to some extent, the combination of these markers enhances the efficiency of enrichment, suggesting that multiparametric separation is effective for SSC purification.[104,105] Moreover, stem cell-specific dyes are applicable to SSC purification. Actually, efflux activity of Hoechst 33342 dye is reported to be applicable to enrich several types of somatic stem cells and ES cells.[106,107] However, the applicability of this technique is still controversial. Some reports have succeeded to enrich SSCs in the population negative for Hoechst 33342 staining, termed the "side population", whereas others have demonstrated the insufficiency of this technique.[101,108-112] Aldefluor reagent and CDy1 have been reported to be effective for SSC purification. The former visualizes the cellular activity of aldehyde dehydrogenase (ALDH). In fact, mouse SSCs are found within the $ALDH^-$ subset of $CD9^+$ or $CDH1^+$ testis cells in mice.[113] The latter dye positively stains an SSC-enriched subset of both testicular and GS cells.[114] Recent reports have also demonstrated that the magnitude of *Tert* expression coincides with cell surface expression of GFRA1, suggesting that TERT expression/activity can be a marker for mouse SSCs.[115,116] Although it is interesting to apply these techniques to purify SSCs from nonrodent animals, there is a concern regarding quantification of the SSC number in the purified subset. Therefore, improvement of the SSC transplantation assay should be pursued simultaneously.

7.3 | GS cell derivation from human/animal pluripotent stem cells

Primordial germ cell-like cells have also been derived from human and monkey pluripotent stem cells.[117-121] In addition, GS cell-like cells can be established from PGCLCs derived from mouse ES/iPS cells.[65] In this regard, ES/iPS cells were also established from humans, monkeys, porcine, and bovine.[122-125] Although divergence of the pluripotent stem cell phenotype and derivation mechanism of primordial germs cells between mammalian species might hamper technical development, such progress strongly suggests that animal and human GS cells can be derived from pluripotent stem cells.[121,126,127]

7.4 | In vitro spermatogenesis from GS cells

In vitro spermatogenesis to produce animal and human sperm is one of the topics in germ cell biology and its application. However, this technique is still limited to the mouse, probably because of the interspecies barrier described above. In particular, it should be noted that the volume of mesodermal tissue around the seminiferous tubule in the rodent testis is quite smaller than that in other mammals. Considering the efficient exchange of gas and medium in the organ culture system, testis tissue of rodents might be exceptionally suitable for the present technology. Furthermore, Kanatsu-Shinohara et al. succeeded in partial reconstitution of SSC-Sertoli cell interactions for long-term maintenance of SSC activity in vitro.[128] Improvement of this culture system in combination with screening of small chemicals that facilitate spermatogenesis will contribute to develop in vitro spermatogenesis technique without using an organ culture system.

8 | CONCLUSION

Because of the technical development of transplantation and in vitro culture, manipulation techniques of GS cells are expected to contribute to a wide variety of fields, such as treatment of male fertility and molecular breeding of livestock animals for industrial use. The most important issues in this field should be the interspecies barrier that is difficult to overcome. Therefore, establishment of nonrodent GS cells and development of assays that can evaluate SSC activity without transplantation will be significant contributions.

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DISCLOSURE

Conflict of interest: The author declares no conflict of interest. *Human rights statement and informed consent*: In this review, the author did not conduct any experiments using human-derived materials. *Animal studies*: In this review, the author did not conduct any animal experiments.

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