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The optimized condition for the isolation and *in vitro* propagation of mouse spermatogonial stem cells

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Introduction: The ability for isolation and *in vitro* propagation of spermatogonial stem cells (SSCs) offer a base for studies on spermatogenesis, and also contribute to the development of new methods for the preservation of livestock and animal genetic modification. The aim of this study was to find the optimal isolation and culture condition for efficient propagation of SSCs. *Methods:* Three different isolation methods (mechanical, one-, and two-step enzymatic digestion) were compared to find the optimal isolation method. To find the best culture conditions for *in vitro* propagation, isolated SSCs were cultured for 7 days in three different culture conditions supplemented with 10% FBS, 0.25% BSA, and 10% KSR, respectively. *Results:* The result showed that two-step enzymatic digestion produced a significant high fraction of live cells compared the other two. Non-adhering cells collected after 48 hr and cultured in BSA- and KSR-supplemented medium had a significantly high number of SSCs clump formation compared to FBS-supplemented group. The expression of CD9 confirmed that cell clumps were SSCs clumps. Spermatogonial stem cells cultured in BSA-supplemented medium were positive for NGN3 and PLZF expressions, whereas negative for Stra8 (a meiotic-specific gene) expression, suggesting that most of the cells were undifferentiated SSCs in BSA culture system. In contrast, in FBS- and KSR-supplemented groups, the SSCs were positive for NGN3, PLZF, and Stra8. *Conclusion:* These data revealed that two-step enzymatic digestion is the best method for the isolation, and 0.25% BSA-supplemented culture condition is effective for optimal *in vitro* propagation of SSCs.

Original Article

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INTRODUCTION

Spermatogenesis is a complex process whereby spermatogonial stem cells (SSCs) divide and differentiate into haploid male germ cells. This complex developmental process consists of three phases including the mitotic division of spermatogonia, meiosis of spermatocytes, and spermiogenesis, which are supported by precise and orderly regulation of gene expression (Saitou & Miyauchi, 2016).

SSCs are the foundation of spermatogenesis because these stem cells ensure the lifelong continuous production of sperm. Although SSCs are infrequent in the testis, typically mice's testes have 0.02%–0.03% of the total germ cell population (Tegelenbosch & de Rooij, 1993). Despite their paucity, SSCs have a distinctive ability to undergo self-renewal division to duplicate themselves as well as produce progenitors that are committed to differentiate. In recent years, there has been an increasing interest in SSCs due to their unique property of being only stem cells that can transmit genetic information to the next generation of sexually inbred species (Dym et al., 2009). This distinctive property makes SSCs a good candidate for key researches in spermatogenesis mechanism studies (Hermann et al., 2012), therapy of male infertility, generation of transgenic animals (Honaramooz et al., 2003), and endangered animals protection (Silva et al., 2013). Hence, *in vitro* expansion of SSCs for future utilization is significant for application of these reproductive techniques.

The isolation and propagation of SSCs is the first step toward establishing germ stem cell lines. SSCs are a very small proportion of testicular cells, and especially, the paucity of these stem cells in the mature testis has limited their isolation for *in vitro* studies. Hence, due to the low proportion, the optimal isolation method and culture

condition for the enrichment of SSCs are very important. The number of strategies has been adopted to isolate the testicular cells including mechanical isolation method and enzymatic digestion method. After isolation for further enrichment of SSCs, different approaches have been used, such as Percoll gradient (Yoshida et al., 2007), differential plating (Dym et al., 1995), magnetic-activated cell sorting, and fluorescence-activated cell sorting (Herrid et al., 2009). Despite there are a lot of progress still, the current cell separation methods usually result in low proportions of spermatogonia in freshly isolated testis cells. Therefore, one aim of this study was to investigate different approaches for optimal testicular cell isolation, in order to maximize the proportion of live SSCs in freshly isolated mouse's testis.

The *in vitro* culture system is an effective method to investigate SSC/progenitor behavior as it is difficult to study the molecular mechanisms controlling the proliferation and differentiation of SSCs and progenitors *in vivo*. However, the very low proportion of SSCs in testicular cells obstructs the studies that explicate their biological characteristics. One tactic to crack this problem is to develop a culture system that could support the self-renewal of SSCs and maintains their germ cell and stem cell potentials. Glial cell line-derived neurotrophic factor (GDNF) produced by Sertoli cells (Hofmann et al., 2005) was shown to be the first molecule that regulates the self-renewal and differentiation of mouse SSCs (Meng et al., 2000). The viability and maintenance of SSCs is strongly associated with the testicular micro-environmental produced by somatic cells (e.g., Sertoli cells; Forbes et al., 2018). Sertoli cell produced growth factors, e.g., leukemia inhibitory factor (De Miguel et al., 1996), basic fibroblast growth factor (Kubota et al., 2004a), bone morphogenetic proteins, and stem cell factor (Jan et al., 2012) are showed to be crucial for SSCs maintenance. Subsequently, Kubota et al. (2004b) reported that addition of 0.2% (w/v) bovine serum albumin (BSA) with GDNF could promote the propagation of SSCs for the longer period. This development permitted SSCs to be cultured for longer periods deprived of changing their undifferentiated properties, consequently giving an indefinite source of SSCs. The aforementioned serum-free culture medium strongly depends on BSA a component with batch-to-batch quality variations similar to those of fetal bovine serum (FBS). Later on, it was found that above-reported culture condition depends on many factors including the type of species, age, quality of serum source, etc. Recently, to solve that problem, knockout serum replacement (KSR) was used as a replacement of BSA for *in vitro* culture of SSCs and the results suggested that KSR is an effective substitute for BSA for long-term *in vitro* cultures of SSCs. (Aoshima et al., 2013). However, the report of the successful production of haploid male germ cells using organ culture technique was also strongly associated with the help of KSR (Sato et al., 2011), which shows that KSR also supports the differentiation of SSCs. In this study, we compared different chemically defined culture condition supplemented with FBS, BSA, and KSR, respectively, and our aim was to find an optimal condition, which could support *in vitro* propagation of SSCs.

MATERIALS AND METHODS

Materials

The culture media [Dulbecco's Modified Eagle Medium (DMEM-12)], FBS, KSR, 100 IU/ml of penicillin, and 100 µg/ml of streptomycin were obtained from Gibco/Invitrogen (Beijing, China). Collagenase IV, GDNF, and 0.25% trypsin were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All solutions were prepared using ultra-purified water supplied by a Milli-Q system (Millipore, Billerica, MA, USA).

Animals

Mice (Kunming mice) were purchased from Gunagdong Medical University, Guangdong, P. R. China. Male mice at 6–9 days of age were killed by cervical vertebra dislocation, and the testes were collected for undifferentiated SSCs collection.

Study plan

Different SSCs isolation and purification method were investigated with at least four replicates per experiment to maximize the collection of live cells, and in every experiment two mouse pups were used. We investigated the mechanical dissociation methods and enzymatic digestions. To separate the SSCs and Sertoli cells, two-step differential plating process was adopted. To maximize the collection of SSCs, various timing was checked in the two-step differential plating processes. Finally, the isolated SSCs were cultured in BSA-, KSR-, and FBS-supplemented culture medium to find the optimized condition for the *in vitro* propagation of SSCs.

Isolation of SSCs

Testes obtained from mouse pup (6–9 days old) were washed in phosphate-buffered saline (PBS). With the help of fine forceps, the tunica albuginea was removed to expose the semiserious tubules and subsequently the semiserious tubules were dissociated (Fig. 1). The dissociated semiserious tubules were subjected for mechanical and enzymatic digestions.

Experiment 1: Mechanical dissociation of testis

Mechanical methods (teasing, mincing, grinding, and sieving) for dissociation of tissue are the commonly used method because these are less costly, easy, and less time-consuming. For mechanical dissociation, we adopted the teasing method. Briefly, the decapsulated testes tissues were torn apart into tubular fragments in 1 ml of 10% FBS–DMEM-12 using fine needles (27-gauge) attached to 1 ml syringes. Cell solution was made up to 5 ml in total by adding more 4 ml of 10% FBS–DMEM-12 and then tissue clumps were triturated and cells were filtered through 40-µm filters. Cell viability was assayed using the trypan blue exclusion method.

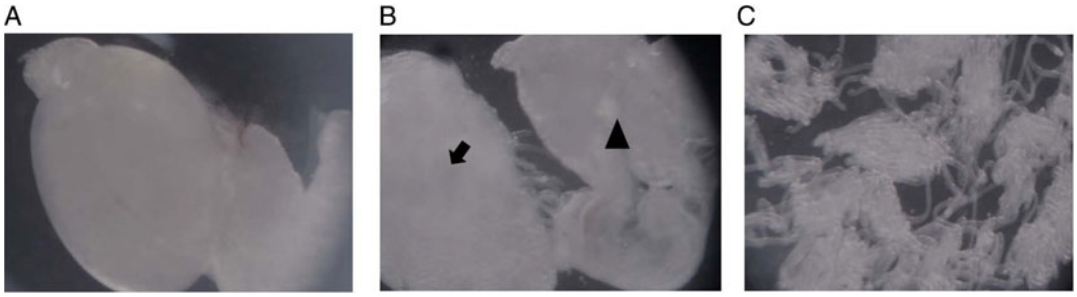

Fig. 1. Isolation of semiserious tubules. (A) Freshly isolated testis of 8-day-old mouse pup. (B) Removal of tunica albuginea (arrow head) to expose the semiserious tubules (arrow). (C) Dissociated semiserious tubules

Experiment 2: Enzymatic digestions of testis

For enzymatic dissociation of testis tissue, we investigated two different methods. In the first method, testes tissues were treated with 2 mg/ml of collagenase (Type IV) digestion for 15 min, followed 0.25% trypsin for 6 min at 37 °C. On the other hand, in the second method, the tissues were treated with the 0.25% trypsin for 15 min at 37 °C. The reaction was stopped by adding 10% FBS–DMEM-12 and cells were filtered through 40-µm filters. Cell viability was assayed using the trypan blue exclusion method.

Experiment 3: Purification of SSCs

To isolate the SSCs from other somatic cells, the two-step differential plating processes were adopted. In order to investigate the optimal timing for purification, we collected the cells at 12, 24, and 48 hr and transferred the cells to mitomycin C-treated mouse embryonic fibroblast (MEF). Cells were cultured for 7 days and then SSCs growth condition was observed.

Experiment 4: Culture of SSCs

The isolated testes cells (0.2×103/well, 24-well plate) were resuspended into three different culture conditions group (Table 1) and incubated for 24 hr in dishes coated with 0.2% (w/v) gelatin to remove fibroblasts and Sertoli cells that adhere to the base. Following this, non-adhered cells were cultured for 7 days on mitomycin C-treated MEF, at 37 °C and 5 CO_2. After 7 days of culture, the number of cell clumps was counted.

Immunofluorescence cell analysis

SSCs of cultured mouse were confirmed via the CD9-marker. Briefly, the cells were fixed in 4% paraformaldehyde for 15 min and then rinsed three times in PBS. Subsequently, the cells were resuspended in PBS with BSA for 90 min at room temperature. Then, the cells were incubated with primary antibodies of CD9 (1:250 in PBS, Abcam, Cambridge, UK), for 12 hr at room temperature and after washing thrice with PBS, secondary antibody was added for 1 hr.

Real-time PCR

In order to investigate the stage of cells after 7 days of culture, the expression of Ngn3, PLZF, and Stra8 was analyzed. Total RNA was isolated by the Trizol reagent. The cDNA was synthesized by PrimeScript^{TM} RT reagent kit (Takara, Japan) according to the manufacturer's instruction. The cDNA amplification was carried out in a total volume of 20 µl using PCR Premix (Takara) according to the manufacturer's instruction. Nucleotide sequences were obtained from GenBank and primer pairs were designed by Primer 5 program (Premier Biosoft International, Palo Alto, CA, USA). The marker was DNA marker I (TianGen Co. Ltd., Beijing, China). PCR products were run on 1% agarose gels and visualized by ethidium bromide staining. List of primers used in this study is present in Table 2.

Statistical analysis

The results were expressed as mean ± standard deviation. The statistical significance between the mean values was determined by one-way analysis of variance, Tukey's, and Duncan's post-test. The $p \leq .05$ was considered significant. For cell numbers, the values are given per testes of mouse pup.

RESULTS

Effect of different cell dissociation method on cell viability

Effect of different cell dissociation method on cell viability is shown in Fig. 2. The result showed that mechanical

Table 1. Different culture conditions of SSCs

Group	Culture medium
1	DMEM-F12 + 10% FBS + 15 ng/ml GDNF + 3 ng/ml bFGF + 100 U/ml penicillin + 100 µg/ml streptomycin
2	DMEM-F12 + 0.2% BSA + 15 ng/ml GDNF + 3 ng/ml bFGF + 100 U/ml penicillin + 100 µg/ml streptomycin
3	DMEM-F12 + 10% KSR + 15 ng/ml GDNF + 3 ng/ml bFGF + 100 U/ml penicillin + 100 µg/ml streptomycin

Note. SSCs: spermatogonial stem cells; DMEM: Dulbecco's Modified Eagle Medium; FBS: fetal bovine serum; BSA: bovine serum albumin; KSR: knockout serum replacement; GDNF: glial cell line-derived neurotrophic factor; bFGF: basic fibroblast growth factor.

Table 2. The primer sequence

Gene (accession no.)	Primer sequence	Product size (bp)
NGN3 (NM_009719.6)	F-CACTCAGCAAACAGCGAAGAAG R-CAGATGTAGTTGTGGGCGAAGC	173
PLZF (BC_138775.1)	F-CGCCACCTTCGCTCACATACAG R-CTTCTTGCCACAGCCGTTACAC	144
Stra8 (NM_009292.1)	F-GGCAAGTTTCCTGGACAAGAGT R-GGTTCCTGGTTTAATGGAGTGT	165
β-Actin (NM_007393.5)	F-AAATCGTGCGTGACATCAAAGA R-CCCAAGAAGGAAGGCTGGAAAA	184

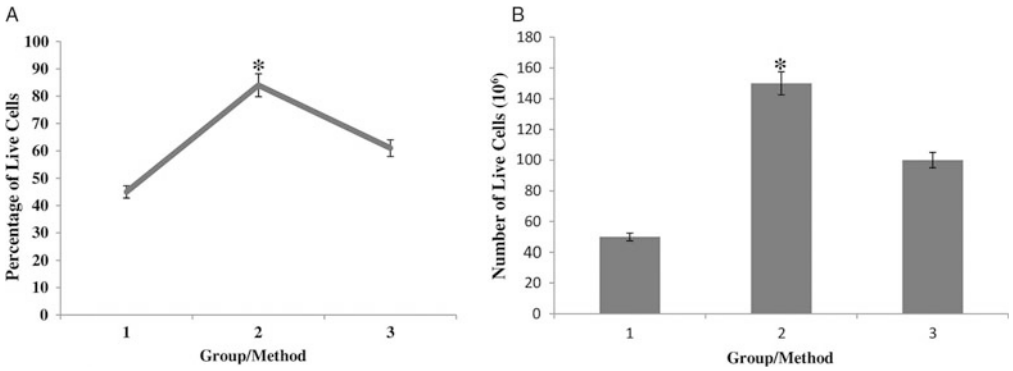

Fig. 2. Effect of different cell dissociation method on cell viability, 1: Teasing, 2: Collagenase + trypsin, 3: Trypsin (*$p < .05$)

methods produced the lowest number of live cells. One-step enzymatic digestion method had twofold more number of live cells compared to mechanical method but the difference was not significant, whereas the two-step enzymatic digestion (collagenase + trypsin) method produced more number of live cells compared to that both mechanical method and one-step enzymatic digestion method. Two-step enzymatic digestion method yielded about fivefold more number of live cells compared to mechanical dissociation and the difference was significant. Overall, the results showed that enzymatic digestion is better compared to mechanical isolation, and two-step enzymatic digestion is the best method for the maximum isolation of live cells.

The effect of timing and culture medium on SSCs clump formation

The non-adhering cells (mostly SSCs) collected at 12, 24, and 48 hr were cultured in 24-well plates and after 7 days of culture, the cell clumps were counted (group of cells more the four was considered as clump). The results showed that the group cultured with 10% FBS-supplemented medium had the lowest number of SSCs clumps formation compared to both other groups cultured with 0.25% BSA- and 10% KSR-supplemented medium, respectively (Fig. 3). The BSA-supplemented group (A-2) had the higher number of cell clumps formation compared to FBS (A-1)- and KSR (A-3)-supplemented groups. The difference between BSA- and KSR-supplemented groups was not significant but the BSA group had significant high number of clumps formation compared to FBS-supplemented group. The numbers of SSCs clumps in KSR-supplemented groups were fewer than BSA-supplemented group but significantly higher than FBS-supplemented group. We also observed that the non-adhering cell collected at 48 hr has the highest proportion of SSCs. We observed less number of colony formation in the same culture condition when cells were collected at 12 and 24 hr compared to 48 hr. As a whole, the result showed that the non-adhering cell collected at 48 hr and cultured in BSA-supplemented group had the highest number of SSCs colonies formation.

Confirmation of SSCs

Immunofluorescence analysis of CD9 was performed to confirm that cultured cells clumps were SSCs. CD9 is considered as a surface marker of undifferentiated SSCs (Kanatsu-shinohara et al., 2004). The cultured colonies were positive for CD9 expression (Fig. 4). In contrast, MEF cells (control group) barely expressed the CD9 expression.

Effect on different culture condition on cell stage

For constant and long-term multiplication of SSCs, it is important that cells should not go toward differentiation, since the differentiating cells are no more stem cell and such cells cannot further propagate because they lose their self-renewal efficiency. Therefore, our aim was to find the culture condition in which the SSCs could have highest rate of proliferation. We observed that the cells cultured in BSA-supplemented medium formed very tight clumps and were positive for expression of NGN3 and PLZF, whereas negative for Stra8 expression. It suggests that in BSA-supplemented group, most of the cells were in undifferentiated. Therefore, the cells cultured in both KSR and FBS were positive for Stra8 expression, suggesting that some cells were in the

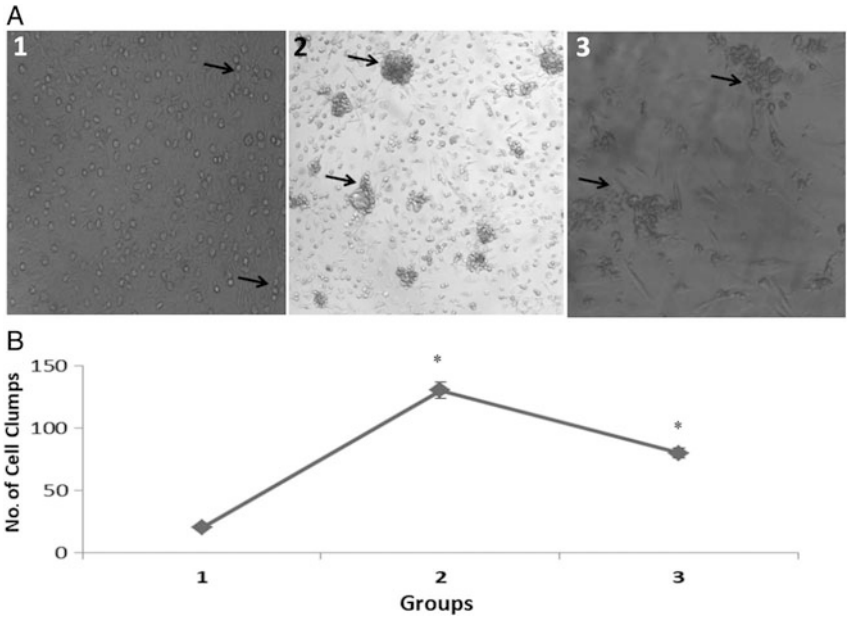

Fig. 3. (A) Effect of different culture medium on clump formation, SSCs cultured in FBS-supplemented groups (A-1) showed lower number of cell clumps (arrow) comparatively to BSA (A-2) and KSR (A-3) culture. (B) Cells cultured with KSR-supplemented medium had significant high number of cell clumps (*$p < .05$)

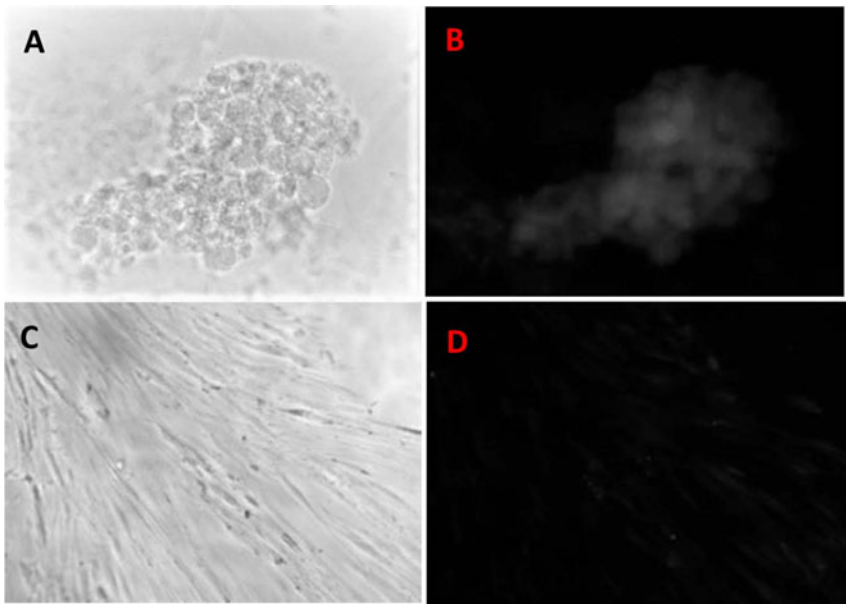

Fig. 4. Identification of SSCs. (A) A clump of SSCs (bright field). (B) CD9 expression of SSCs clump. (C) MEF cells (bright field). (D) CD9 staining of MEF cells

differentiating stage (Fig. 5). Sertoli cells served as a control group for RT-PCR analysis and the Sertoli cells only showed the expression of β-actin (Fig. 5). In conclusion, our results showed that the medium supplemented with BSA is suitable for optimal *in vitro* propagation of SSCs.

DISCUSSION

Spermatogenesis is a complicated process that starts with division and differentiation of the SSCs on the basement membrane of the seminiferous tubule of the testis. SSCs characterize a small fraction of cells in the testes of any animal but SSCs are the cells of most interest due to their ability to reproduce the whole haploid male germ cells, which are important for the survival of species.

As SSCs are the only stem cells in adults that divide to contribute genes to successive generations, they are valuable for biological experimentation, medical research, and biotechnology. In particular, human SSCs population is of great interest to find the answer of some basic research focusing on SSCs self-renewal and differentiation. Furthermore, the capability of isolating and proliferation of SSCs will also help to preserve the fertility of adult cancer patients

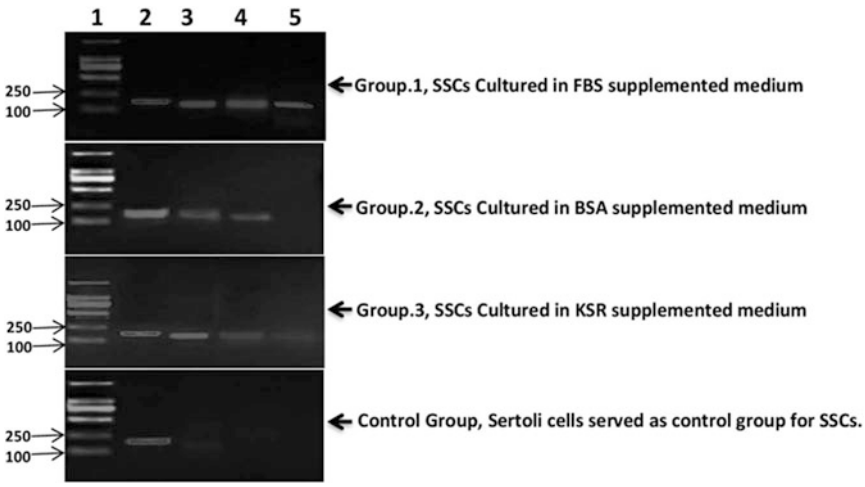

Fig. 5. RT-PCR analysis for the expression of cell stage-specific gene, *lane 1*: Marker, *lane 2*: β-Actin, *lane 3*: Ngn3, *lane 4:* PLZF, *lane 5:* Stra8

undergoing gonadotoxic treatment. This technique could be very effective to preserve the fertility of prepubertal cancer patient, who often lose the entire germ line peripubertallly (Jurewicz et al., 2018) due to gonadotoxic cancer treatment. Fertility of such patients can be preserved by taking the testicular biopsies before gonadotoxic cancer treatment for *in vitro* isolation and proliferation of SSCs and following retransplantation of SSCs into testis after cancer therapy (Galuppo, 2015). A limited number of SSCs can be obtained from small testicular biopsies of the cancer patient; therefore, finding the optimal condition for the isolation and *in vitro* propagation of SSCs are critical to ensure the maximum collection of viable SSCs. Similar to humans, in livestock, the optimal isolation and proliferation conditions are theoretically limiting steps in the study and manipulation of SSCs. In this research, we compared three different most commonly used cells for isolation method including the mechanical isolation, one-step enzymatic digestion, and two-step digestion method. Our results showed that enzymatic digestion method yielded high number of live cells compared to mechanical isolation method. Two-step enzymatic digestion yielded a significant high number of live cells compared to mechanical isolation and our results were in agreement with a previous observation where two-step digestion yield more number of healthy cells (Izadyar et al., 2002). Like two-step enzymatic digestion method, the one-step digestion produced a high number of live cells compared to mechanical isolation. Our result was in agreement with a previous observation where enzymatic digestion of testis cell isolation yield more number of live cells compared to mechanical isolation (Cherny et al., 1994). Most enzymes used in cell isolations target specific components within the tissue and have optimal working temperatures. In this study, we found that the only use of trypsin for a longer period of time had decreased the number of live cells. It shows that the treatment of cells for the longer period could damage the cell surface. In two-step digestion, the collagenase first disperses the seminiferous tubules and then the treatment of trypsin could easily break the seminiferous tubules in to single cell suspension without damaging the cell surface. In this study, we found that SSCs collected after two-step enzymatic digestion had high proportion of SSCs recovery. Because two-step digestion method eliminates the high proportion of intestinal cells (Bellve et al., 1977), the proportion of germ cell recovery increases. In this study, we found that the two-step enzymatic digestion method is the optimal approach for maximum isolation of testicular cells. Our results were in contrast with the report of Rodriguez Casuriaga et al. (2009), who reported that mechanical isolation method was better compared to enzymatic digestion methods. For enrichment of SSCs, we used different planting method, which is the most commonly used method to isolate the SSCs from somatic cells. We found that non-adhering cells collected after 48 hr and cultured in BSA-supplemented medium multiplied in high number compared to KSR- and FBS-supplemented culture groups.

Finally, the successful culture of SSCs and their *in vitro* propagation represents a further milestone in the transfer of SSCs culture to clinical application. Generally, for many years, it had been believed that SSCs were difficult to culture, but with recent advancement in regenerative medicine, at present, it is possible to culture the SSCs of mice (Martin & Seandel, 2013), human (He et al., 2010), and some other species (Pramod & Mitra, 2014). Serum-free culture system is considered as an important method to study the biological properties of mammalian cells *in vitro* (Barnes & Sato, 1980). Once mammalian cells were shown to proliferate in serum-free hormonally defined medium without altering the cell type-specific characteristics (Hayashi & Sato, 1976) and later serum-free culture became a major resource to study cells *in vitro* and to identify novel growth factors or regulatory mechanisms for proliferation. However, to date, the culture conditions do not seem optimal for successful maintenance and proliferation of SSCs. In this study, to find the best optimal culture condition for *in vitro* propagation of SSCs, we analyzed three different culture conditions supplemented with FBS, BSA, and KSR, respectively. The result showed that the number of SSCs clump formation in BSA- and KSR-supplemented medium was significantly high compared to FBS. Our result was in accordance with the already reported study where KSR showed the high rate of self-renewal of SSCs

(Aoshima et al., 2013). The SSCs clump formation in BSA-supplemented group was also higher than the KSR-supplemented group but the difference was not significant. In order to confirm the cell stage in different culture condition, we did PCR. The PCR data showed that the SSCs cultured in BSA-supplemented medium were positive only for the NGN3 and PLZF genes, which are specific for undifferentiated SSCs, whereas negative for Str8, a gene required for meiotic initiation in mouse (Anderson et al., 2008), suggesting that most of the cells were undifferentiated. The SSCs cultured in KSR- and FBS-supplemented mediums were positive for Stra8, which suggests that in both KSR and FBS medium, the cells were differentiating and many of cells were in mitosis stage. On the whole, our results suggest that the basic culture medium supplemented with 0.25% BSA is the optimal condition of *in vitro* propagation of murine SSCs.

CONCLUSION FOR FUTURE BIOLOGY

We concluded that two-step enzymatic digestion is best to yield the maximum number of viable cells, and isolated cells cultured in BSA-supplemented medium after 48 hr of

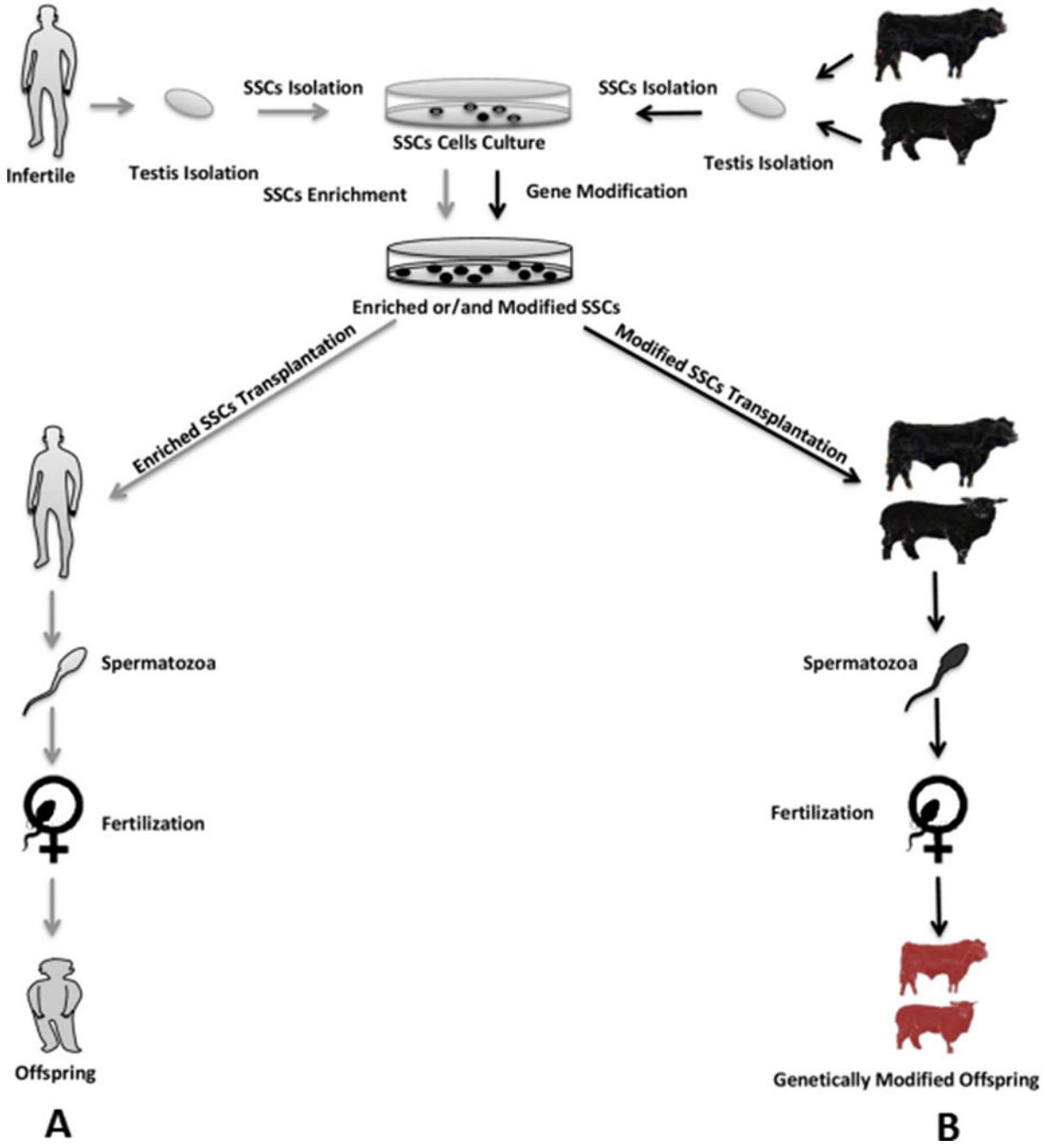

Fig. 6. A schematic representation of the use of SSCs in humans and animals. (A) Infertility due to less number of germ cells can be cured with *in vitro* enrichment of SSCs and following transplantation to the donor. (B) A schematic representation of the use of SSCs of the production of genetically modified animals

pipetting could produce the highest number of SSCs colonies without affecting the properties of SSCs. Because in adults, SSCs are the only stem cells that are capable to transfer genetic information to subsequent generations; therefore, SSCs offer a substitute efficient method to produce transgenic animal (Fig. 6B). In addition, modifying culture conditions that regulate the fate of SSCs for self-renewal and differentiation will produce a valuable model for investigating the molecular and cellular biology of SSCs cell differentiation, and may allow development of new therapeutic strategies for infertility (Fig. 6A). Our findings provide an efficient method to isolate and stable culture conditions for *in vitro* propagation of SSCs, which could facilitate various studies using SSCs including therapeutic applications.

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Ethical Statement: All animal experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the Guangdong Ocean University.

Data Accessibility: All data are included within this article.

Competing Interests: The authors declare no competing interests.

Authors' Contributions: FI has conceived the idea, conducted the experiments, analyzed the data, and drafted the manuscript. YZ, AN, and GHL contributed in experiments and data analysis. JW, XM, and LA revised the manuscript.

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