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Current Biotechnological Advances and Challenges in Culture and Transplantation of Spermatogonial Stem Cells in Animals

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ABSTRACT

The spermatogonial stem cells (SSCs) are unique stem cells of the adult body having the power of self-renewal and differentiation into spermatozoa. Due to their distinct advantages over other stem cell types, the field of cell based regenerative medicine is exploring the potential of SSCs for its various applications in biotechnology and animal reproduction. Here we discuss about several approaches for SSC isolation, enrichment and characterization. Given the low percentage of SSCs in the testis, it is difficult to obtain an enriched population of SSC for further research. Differentiation and apoptosis over time is another challenge in establishing a long term SSC culture system. Hence, optimization of the culture media and culture conditions along with cryopreservation is a prerequisite for the downstream application of SSC technology in livestock. Although several markers have been identified for SSCs in animals, an appropriate species specific marker is still lacking. Suitable recipients for SSC transplantation are generated through surrogate sire technology in livestock. SSC transplantation has been experimentally successful in numerous livestock species with donor derived spermatogenesis and production of offspring. Such advancement coupled with genome editing may open new avenues for augmenting livestock production in near future. We emphasize that a deeper understanding of the SSCs characteristics and the factors that influence their differentiation and stemness is essential for the wider application of SSC technology in animals.
Key words: spermatogonial stem cell, isolation, enrichment, culture, transplantation

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INTRODUCTION

The spermatogonial stem cells (SSCs) are unique stem cells of the adult body having the power of self-renewal and differentiation into spermatozoa. They originate from primordial germ cells which then differentiate to form gonocytes in the postnatal testes and later on forming SSCs in the prepubertal and adult animals. The SSCs estab-

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lish a niche in the basement membrane of seminiferous tubules, and the signals from the microenvironment dictate whether the cells divide into stem cells, develop into sperm, or undergo apoptosis. A single (*As*) spermatogonia are single cells and divide into interconnected A paired (*Apr*) cells, which further divide to produce four A aligned (*Aal*) cells, subsequently divide to produce a chains of eight and 16 cells. During the *Aal-A1* transition as a result of retinoic acid stimulation, KIT expression is activated, which marks the initiation of spermatogonial differentiation (Hermann and Oatley, 2023). Since they exhibit the majority of SSC markers and have stem cell characteristics, these are collectively referred to as undifferentiated A type spermatogonia. However, the factors that drive the self-renewing population capable of differentiation through spermatogenesis are yet to be elucidated in animals.

The field of cell based regenerative medicine is gradually shifting its attention towards exploring the potential of SSCs, due to their distinct advantages over other stem cell types like ESCs and adult stem cells, one of the major advantages being their reduced immunogenicity and comparatively lesser ethical issues (Chen *et al*., 2020). Furthermore, promising investigations have demonstrated that SSCs can be effectively differentiated into other cell types under various stimulated conditions (Shinohara *et al*., 2004).

SSC IN ANIMAL REPRODUCTION

SSCs have various applications in livestock such as improving the fertility of low fertile animals, production of elite animals, understanding the process and the pathways of self-renewal and differentiation, mining factors regulating male fertility and preservation of the genetic material of valuable males and production of sperm from superior animals (Binsila *et al*., 2021). A few promising other applications of SSCs are cross-breeding in harsh environments, conservation of endangered species etc.,(Hill and Dobrinski, 2005). SSC transplantation in rodent models resulted in donor-derived spermatogenesis, production of sperm and offspring, and this has been considered a milestone in SSC technology (Brinster *et al*., 1994). Stem cell technology especially, SSC could facilitate the genetic modification in agriculturally important animals through the introduction of desired genes and for the production of transgenic animals with superior productive and reproductive traits. SSCs mediated transgenic animal production may result in permanent modification of the germ line. Hence, SSC technology can be a feasible technology in the near future for male germ line modification and fertility research.

In vitro spermatogenesis though successful in rodent models (Sato *et al*., 2011), has not become a reality today in livestock and humans. This poses a challenge in standardizing SSC research in livestock and humans for the production of elite animals.

SSC ISOLATION AND CHARACTERIZATION

The testis consists of different types of cells like Sertoli cells, Leydig cells, SSCs and different stages of differentiating germ cells. SSCs constitute only about less than 1% of those cells. Attempts have been made to isolate these SSCs with high viability. Testicular cell dissociation can often be achieved by the enzymatic technique. Collagenase IV, trypsin, DNase I, and hyaluronidase are some of the several enzymes used for enzymatic digestion. Two, three, or sequential enzymatic digestion involving these enzymes is utilized because a single enzyme is insufficient to separate SSCs. Seminiferous tubules are mechanically separated and filtered using 70 and 40 micron nylon filters after tunica albuginea and visible connective structures are removed. Though mechanical dissociation of the testis yields spermatogonial cells, enzymatic digestion of testis tissues proved nine fold recovery of live cells. The velocity sedimentation germ cell separation system (STA-PUT) is a method that combines initial isolation by enzymatic methods followed by velocity gradient separation. STA-PUT separation is less expensive than using a cell sorter. Combinations of enzymes required for the isolation are different for prepubertal and adult testes. Studies from our lab revelaed that the prepubertal stage is optimal for isolation of a large number of SSCs for culture (Binsila *et al.,* 2018).

SSCs which have been isolated, proliferated in culture, or transplanted are characterized using molecular, biochemical, or morphological markers. Morphological and biochemical characterization may be used for the initial screening of spermatogonial stem cells but is not confirmatory. Though, species-specific SSC markers have been identified in cattle (PLZF, DBA, PGP 9.5, THY1), buffalo (PLZF, PGP 9.5, THY1), goat (α6 integrin, PLZF, β1 integrin, OCT-4, THY1), sheep (PLZF, PGP 9.5, CDH1) and pig (PLZF, DBA, PGP 9.5) (Goel *et al.,* 2010; Zheng *et al.,* 2014; Binsila *et al.,* 2021), the appropriate confirmatory marker is still lacking, especially in livestock.

SSC ENRICHMENT AND PURIFICATION

As SSCs are very rare cells in the testis, a sufficient quantity of enriched population is a prerequisite for culture work. There are several approaches for obtaining enriched SSCs from livestock species. Some of the techniques are magnetic-activated cell sorting (MACS), fluorescence-activated cell sorting (FACS), differential plating, the selection with extracellular matrix (ECM), velocity sedimentation or density gradient centrifugation. The combination of enrichment techniques significantly improved the purity of spermatogonia. The best efficient enrichment method of selection is crucial for the improved SSC culture.

The enrichment process using percoll density gradient centrifugation yields 3.65 fold more α6 integrin positive cells (SSCs). The combination of enrichment methods, differential plating and percoll and ficoll density-gradient centrifugation, yielded approximately 55-61% spermatogonia enriched cell population from prepubertal bovine testes. However, the purity percentage differed between studies may be attributed to the developmental stage of testes, selection of the isolation protocol and the markers used to determine the purification (Yang and Honaramooz, 2011). Laminin, an adhesion molecule that helps form the basement membrane, is secreted by Sertoli cells. Differential plating is utilized to purify the SSCs because laminin binds to ITGα6/β1 receptors on the spermatogonial cell surface. Laminin in combination with BSA is also used to enrich putative SSC population from testicular cell isolate (Binsila *et al.,* 2019). Gelatin and lectin obtained from matrix components assist in cell attachment and non-adherent cells are enriched in SSCs (Binsila *et al.,* 2021).

SSC CULTURE IN LIVESTOCK

The establishment of SSC technology in livestock may be achieved by the development of a suitable SSC culture medium that can sustain stemness, sustenance, and proliferation. Although SSCs play a significant role in animal reproduction, little is known about the mechanisms governing their self-renewal, proliferation, and destiny. Therefore, in order to provide the best species-specific culture medium, researchers are optimizing the right additives, growth factors, matrix substrates, and serum-free supplements to promote SSC self-renewal and proliferation *in vitro*.

In case of livestock, the long-term culture has not been standardized yet. Short-term culture of SSC in livestock was carried out to a certain extent. There is a gradual decrease in the proliferation of putative SSCs during sub-culture. Over a period of time, stemness and functional capability of SSC for donor-derived spermatogenesis declines, and differentiation and apoptosis dominate the cellular events, the duration of decline could be extended by modulating the SSC culture using a hypoxic microenvironment (Helsel *et al*., 2017). A few research have documented a long-term culture method that facilitates the ongoing *in vitro* proliferation of bovine gonocytes. However, compared to rodent models, the culture duration is shorter Guan *et al*., 2006).

The culture medium with appropriate growth factors, serum, and feeder cells are required for the long-term culture of SSCs. Mitotically inactivated feeder monolayers such as Sandoz Inbred Swiss Mouse (SIM), thioguanine-resistant ouabain-resistant (STO) cell line, mouse embryonic fibroblasts (MEF), or Sertoli cells are used for propagating SSCs. When bull SSCs were co-cultured using Sertoli cell monolayer as feeder layer, the self-renewal process has been increased. Similarly, serum was also used as an important component in the culture medium for survival and self-renewal of culturing cells (Zheng *et al.,* 2014). However, some undefined factors in serum induce cell differentiation, or serum at higher concentrations had detrimental effects on SSC expansion in culture. Hence, serum free media avoids unknown variable factors in the culture systems. The addition of growth factors like glial cell line-derived neurotrophic factor (GDNF), epidermal growth factor (EGF) and insulin like growth factor (IGF) enhanced the proliferation and self-renewal of the SSCs (Binsila *et al*., 2020). As the culture conditions are unique for each species, it needs to be standardized and many recent research works are oriented towards this direction (Sahare *et al*., 2015; Xi *et al*., 2022). Recently emerged three-dimensional (3D) culture systems are used for *in vitro* culture of the SSCs. These mimic the testicular architecture and provide ECM support for the proliferation *in vitro*. Various ECM such as collagen IV, laminin, fibronectin and vitronectin are used for the culture of SSCs in livestock (Binsila *et al*., 2020; Singh *et al*., 2021).

CRYOPRESERVATION AND TRANSPLANTATION OF SSC

Cryopreservation is essential for the preservation of the genetic diversity of livestock, particularly endangered species, as well as for enhancing SSC transplantation studies. Cryopreservation of semen and spermatozoa is a frequently employed technique for genome conservation and further research. Remarkably, testis tissue and cell

suspension including germ cells have also been preserved using this method. The cryopreservation process for SSCs can generally be of two types, slow freezing and vitrification. In the former, cell suspension with an appropriate freezing mix is gradually cooled to subzero temperatures, allowing ice crystal formation while reducing the cryoinjury. In the latter, the aqueous milieu of the cells or tissues were allowed to solidify into a noncrystalline glassy phase. Several mammalian SSCs have been cryopreserved successfully including rodents (Lee *et al*., 2014), porcine (Abrishami *et al*., 2010), cattle (Cai *et al*., 2016), and even humans (Mirzapour *et al*., 2013). Cryoinjury of cells may result in apoptosis and cell death. Hence, it is important to standardize SSC cryopreservation media and protocol for maximizing the post-thaw transplantation efficiency. Commonly used cryoprotectants in SSC cryopreservation are DMSO and 2.5% PEG (Mol. Wt 1000) (Lee *et al*., 2013). To increase the efficiency of cryopreservation, various additives are added to the cryomedia including antioxidants, apoptotic inhibitors, sugars etc. The post-thaw viability of SSCs using different methods of freezing and cryoprotectants ranges from 40-70% (Izadyar *et al*., 2002; Jung *et al*., 2021).

Successful transplantation of SSCs has been reported in cattle, sheep and goat, and live progeny was produced from sheep, and goat (Izadyar *et al*., 2000; Honaramooz *et al*., 2003; Herrid *et al*., 2009). *NANOS2* gene knock models were produced in species such as cattle, pig, and goat which can be used as an ideal surrogate recipients for SSC transplantations. The knock out animals lacked the SSCs and other germ cell populations but had intact seminiferous tubules and other somatic cell populations (Park *et al*., 2017). The applications of SSC technology will be more effective and feasible once the cryopreservation and transplantation technology of SSC is established.

GENETIC MODIFICATION AND GENE EDITING OF SSC

SSC gene editing in livestock holds significant potential for various applications in animal breeding and agriculture. Breeders can use genome editing as a tool to enhance animal performance, welfare, and efficiency, unlocking the possibilities for a more sustainable future for livestock production. The traditional breeding techniques are laboriously a slow approach, that takes a lot of time even to accumulate the smallest variation in management across several generations in a breeding program and calls for a large commitment of time, space, and money. Genome editing is precise technology and offers controlled modifications to produce animals with agriculturally important traits such as milk and meat (Sun *et al*., 2019; Laible *et al*., 2015). Traditional techniques of gene editing often face challenges like genetic mosaicism, where only a certain percentage of cells carry the desired alteration (Webster *et al*., 2021). Through surrogate sire technology (SST), researchers were able to create animals lacking native SSC and so the mature sperm. Upon transplantation of genetically modified mouse and rat SSCs to recipient males, birth of progenies with modified characteristics have been reported (Chapman *et al*., 2015; Wang *et al*., 2017). The use of gene modification approaches like CRISPR-Cas9 and HDRTs (Homologous DNA Repair Template) provides a possibility to produce animals with superior productive, reproductive and thermotolerant traits in the near future.

CHALLENGES FOR SSC TECHNOLOGY IN LIVESTOCK

The challenges are many in front of SSCs technologies for application. Isolation of small pool of SSCs and further propagation of SSCs *in vitro* without compromising stemness is essential for SSC transplantation. Reduction of stemness and dominance of processes such as differentiation and death is the main obstacle in establishing a long-term SSC culture system. Modifying the *in vitro* culture system for SSC self-renewal requires an understanding of the stemness pathways. For the long-term maintenance of SSC culture, an understanding of biomolecules involved in SSC differentiation and apoptotic pathways are essential. A few SSC pathways associated with stemness have been identified in the recent past. For example, specific pathway inhibitors studies revealed that p38 MAPK pathway have a key role in maintaining self-renewal capacity of mouse mGSCs (Niu *et al*., 2017). Myc-mediated glycolysis increases SSC self-renewal, as Myc/Mycn deficiency significantly reduces the proliferation rate of germline stem cells (Shinohara *et al*., 2016). Blocking Ras/ERK1/2 pathway by MEK1/2 inhibitor (PD0325901) inhibits the proliferation of goat SSCs. Several microRNAs, genes and proteins that regulate the pathways towards maintenance of SSCs stemness in testicular stem cells have been identified. So far the complete mechanisms involved in SSC stemness and differentiation, and elucidating the pathways have not been detailed yet. In depth knowledge of stemness regulators might help to alter SSCs fate in culture conditions towards self-renewal, differentiation or apoptosis. Production of an ideal surrogate sire is the challenge for SSC transplantation. The chemical and irradiation methodology have the possibility of contamination with the endogenous SSC population.

FUTURE PERSPECTIVE IN SSC RESEARCH

SSCs being the precursor cells for spermatids hold a promising application in regenerative medicine to treat male infertility. This includes SSC transplantation, testicular tissue grafting and techniques for *in vivo* or *ex vivo* spermatogenesis (Sanou *et al*., 2022). Advancements in single-cell transcriptomics and 3D culture techniques can offer a comprehensive understanding on the gene expression patterns in SSCs at various stages of growth and differentiation. This can assist in discovering important regulatory processes and provide insights into the variety of SSC populations.

However, in livestock management SSC research has significant implications in breeding and conservation. Having a better understanding of SSC can help in the production of animals using gene editing techniques with desired traits for improved production, reproduction and climate resilience. Also *in vitro* differentiation enables to understand the molecular pathway and biomolecules regulating spermatogenesis process, to test the effect of chemicals/drugs on germ cell development more effectively without the need for *in vivo* manipulation (Figure 1).

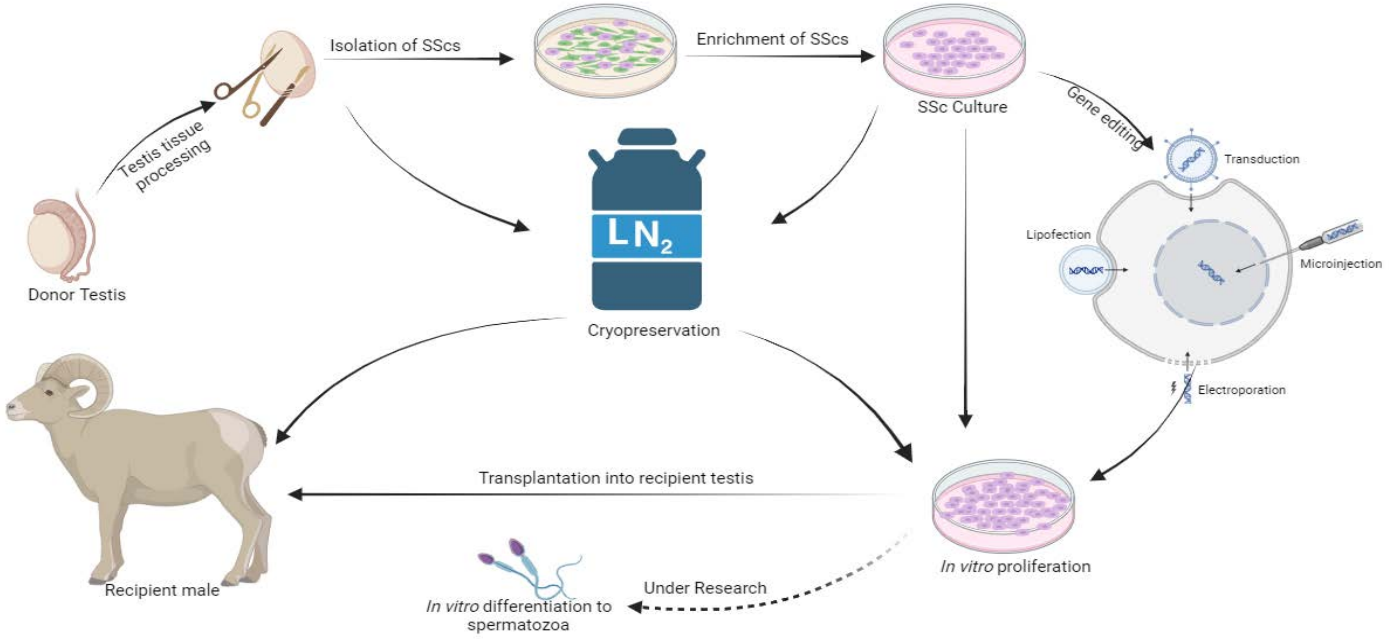

Fig. 1: Present scenario and future perspectives in the application of SSC technology in livestock advancement (Image created using BioRender.com).

Future research endeavors may involve exploring the medicinal potential of stem cells especially SSCs in the treatment of reproductive diseases or in enhancing livestock reproduction. Investigating the influence of environmental elements, including diet and pollution exposure, on SSCs can shed light on how these elements affect livestock reproductive function and manage animal husbandry.

CONCLUSION

Though SSCs research started a few decades back, the pace of advancement of the technology is very slow, especially in livestock. In-depth knowledge of species-specific factors affecting stemness and differentiation of SSC are required for the development of long-term culture systems in livestock. The use of SSC markers and culture conditions need to be applied with caution as they are species-specific to certain extent and not applicable as such in livestock. The identification of stemness regulators that may alter the SSCs fate in terms of self-renewal, differentiation or apoptosis in the culture system are very much essential. Epigenetic changes definitely can happen during the culture and differentiation of SSCs *in vitro;* however the impact of such alterations on fertility need to be investigated. Germ-line ablated suitable recipients (surrogate sire) are generated in livestock and considered as ideal for SSC transplantation. The success rate of SSCs transplantation using the cultured and cryopreserved SSCs is low. *In vitro* differentiation of sperm from cultured SSCs though developed in rodent models, remains a challenge in human and farm animals.

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CONFLICT OF INTEREST

The authors declare no conclict of interest.

REFERENCES

Abrishami, M., Anzar, M., Yang, Y. and Honaramooz, A. (2010). Cryopreservation of immature porcine testis tissue to maintain its developmental potential after xenografting into recipient mice. *Theriogenology*, **73**(1): 86-96.

Binsila, B.K., Selvaraju, S., Ranjith K.R., Archana, S.S., Balaganur, Krishnappa, Ghosh, S.K., Kumar, H., Raghavendra, B.S., Arangasamy, A. and Bhatta, R. (2021). Current scenario and challenges ahead in application of spermatogonial stem cell technology in livestock. *J. Assist. Reprod. Genet.*, **38**: 3155-3173.

Binsila, B.K., Selvaraju, S., Ghosh, S.K., Ramya, L., Arangasamy, A., Ranjithkumaran, R. and Bhatta, R. (2020). EGF, GDNF, and IGF-1 influence the proliferation and stemness of ovine spermatogonial stem cells in vitro. *J. Assist. Reprod. Genet.*, **37**: 2615-2630.

Binsila, B. K., Selvaraju, S., Ghosh, S. K., Prasad, J. K., Ramya, L., Ravindra, J. P. and Bhatta, R. (2019). Purification of spermatogonial stem cells from ram testicular isolate using ficoll density gradient separation. *Indian J. Anim. Reprod.*, **40**(1): 7-11.

Binsila, K.B., Selvaraju, S., Ghosh, S.K., Parthipan, S., Archana, S.S., Arangasamy, A., Prasad, J.K., Bhatta, R. and Ravindra, J.P. (2018). Isolation and enrichment of putative spermatogonial stem cells from ram (Ovis aries) testis. *Anim. Reprod. Sci.*, **196**: 9-18.

Brinster, R.L. and Zimmermann, J.W. (1994). Spermatogenesis following male germ-cell transplantation. *Proc. Natl. Acad. Sci.* U.S.A., **91**(24): 11298-11302.

Cai, H., Wu, J.Y., An, X.L., Zhao, X.X., Wang, Z.Z., Tang, B. and Zhang, X.M. (2016). Enrichment and culture of spermatogonia from cryopreserved adult bovine testis tissue. *Anim. Reprod. Sci.*, **166**: 109-115.

Chapman, K.M., Medrano, G.A., Jaichander, P., Chaudhary, J., Waits, A.E., Nobrega, M.A. and Hamra, F.K. (2015). Targeted germline modifications in rats using CRISPR/Cas9 and spermatogonial stem cells. *Cell Reports*, **10**(11): 1828-1835.

Chen, Z., Hong, F., Wang, Z., Hao, D. and Yang, H. (2020). Spermatogonial stem cells are a promising and pluripotent cell source for regenerative medicine. *Am. J. Transl. Res.*, **12**(11): 7048.

Goel, S., Reddy, N., Mandal, S., Fujihara, M., Kim, S.M. and Imai, H. (2010). Spermatogonia-specific proteins expressed in prepubertal buffalo (Bubalus bubalis) testis and their utilization for isolation and in vitro cultivation of spermatogonia. *Theriogenology*, **74**: 1221-1232.

Guan, K., Wolf, F., Becker, A., Engel, W., Nayernia, K. and Hasenfuss, G. (2006). Isolation and cultivation of stem cells from adult mouse testes. *Nature Protocols*, **1**(3): 1344-1351.

Helsel, A.R., Oatley, M.J. and Oatley, J.M. (2017). Glycolysis-optimized conditions enhance maintenance of regenerative integrity in mouse spermatogonial stem cells during long-term culture. *Stem Cell Rep.*, **8**(5): 1430-1441.

Hermann, B.P. and Oatley, J.M. (2023). Introduction: The Why's and How's for Studying Spermatogenesis and Spermatogonial Stem Cells. *Methods Mol. Biol.*, **2656**: 1-6.

Herrid, M., Olejnik, J., Jackson, M., Suchowerska, N., Stockwell, S., Davey, R., Hutton, K., Hope, S. and Hill, J.R. (2009). Irradiation enhances the efficiency of testicular germ cell transplantation in sheep. *Biol. Reprod.*, **81**: 898-905.

Hill, J. and Dobrinski, I. (2005). Male germ cell transplantation in livestock. *Reprod. Fertil. Dev.*, **18**: 13-18.

Honaramooz, A., Behboodi, E., Megee, S.O., Overton, S.A., Galantino, Homer, H., Echelard, Y. and Dobrinski, I. (2003). Fertility and germline transmission of donor haplotype following germ cell transplantation in immunocompetent goats. *Biol. Reprod.*, **69**: 1260-1264.

Izadyar, F., Matthijs, Rijsenbilt, J.J., Den Ouden, K., Creemers, L. B., Woelders, H. and de Rooij, D. G. (2002). Development of a cryopreservation protocol for type A spermatogonia. *J. Androl.*, **23**(4): 537-545.

Izadyar, F., Creemers, L.B., van Dissel-Emiliani, F.M., van Pelt, A.M., and de Rooij, D.G. (2000). Spermatogonial stem cell transplantation. *Mol. Cell. Endocrinol.*, **169**(1-2): 21-26.

Jung, S. E., Jin, J. H., Ahn, J. S., Kim, Y. H., Yun, M. H., Kim, S. H. and Ryu, B. Y. (2021). Effect of serum replacement on murine spermatogonial stem cell cryopreservation. *Theriogenology*, **159:** 165-175.

Kanatsu, Shinohara, M., Inoue, K., Lee, J., Yoshimoto, M., Ogonuki, N., Miki, H. and Shinohara, T. (2004). Generation of pluripotent stem cells from neonatal mouse testis. *Cell*, **119**(7):1001-1012.

Laible, G., Wei, J. and Wagner, S. (2015). Improving livestock for agriculture–technological progress from random transgenesis to precision genome editing heralds a new era. *Biotechnol. J.*, **10**(1):109-120.

Lee, Y.A., Kim, Y.H., Ha, S.J., Kim, B.J., Kim, K.J., Jung, M.S. and Ryu, B.Y. (2014). Effect of sugar molecules on the cryopreservation of mouse spermatogonial stem cells. *Fertil Steril*, **101**: 1165-1175.

Lee, Y.A., Kim, Y.H., Kim, B.J., Jung, M.S., Auh, J.H., Seo, J.T. and Ryu, B.Y. (2013). Cryopreservation of mouse spermatogonial stem cells in dimethylsulfoxide and polyethylene glycol. *Biol. Reprod.*, **89**(5): 109-1.

Mirzapour, T., Movahedin, M., Tengku Ibrahim, T.A., Haron, A.W. and Nowroozi, M.R. (2013). Evaluation of the effects of cryopreservation on viability, proliferation and colony formation of human spermatogonial stem cells in vitro culture. *Andrologia*, **45**(1): 26-34.

Niu, Z., Mu, H., Zhu, H. and Wu, J. (2017). p38 MAPK pathway is essential for self-renewal of mouse male germline stem cells (mGSCs). *Cell Proliferation*, 50.

Park, K.E., Kaucher, A.V., Powell, A., Waqas, M.S., Sandmaier, S.E., Oatley, M.J., Park, C.H., Tibary, A., Donovan, D.M. and Blomberg, L.A. (2017). Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene. *Scientific Rep.*, **7**: 40176.

Sahare, M., Otomo, A., Komatsu, K., Minami, N., Yamada, M. and Imai, H. (2015). The role of signaling pathways on proliferation and self-renewal of cultured bovine primitive germ cells. Reprod. *Med. Biol.*, **14**: 17-25.

Sanou, I., van Maaren, J., Eliveld, J., Lei, Q., Meißner, A., de Melker, A.A. and Mulder, C.L. (2022). Spermatogonial stem cell-based therapies: taking preclinical research to the next level. *Front. Endocrinol.*, **13**: 850219.

Sato, T., Katagiri, K., Yokonishi, T., Kubota, Y., Inoue, K., Ogonuki, N., Matoba, S., Ogura, A. and Ogawa, T. (2011). In vitro production of fertile sperm from murine spermatogonial stem cell lines. *Nature Comm.*, **2**: 472.

Shinohara, M.K., Tanaka, T., Ogonuki, N., Ogura, A., Morimoto, H., Cheng, P.F., Eisenman, R.N., Trumpp, A. and Shinohara, T. (2016). Myc/Mycn-mediated glycolysis enhances mouse spermatogonial stem cell self-renewal. *Genes Dev.*, **30**(23): 2637-2648.

Singh, S.P., Kharche, S.D., Pathak, M., Ranjan, R., Soni, Y.K., Saraswat, S., Singh, M.K. and Chauhan, M.S. (2021). Differential effects of extracellular matrix proteins on in vitro culture and growth characteristics of caprine male germ cells. *In Vitro Cell.Dev.Biol. Animal*, **57**: 373-380.

Sun, Y. Z., Liu, S. T., Li, X. M. and Zou, K. (2019). Progress in in vitro culture and gene editing of porcine spermatogonial stem cells. *Zool. Res.*, **40**(5): 343–348.

Wang, Y., Ding, Y. and Li, J. (2017). CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. RNAi Small Regul. *RNAs Stem Cells Methods Protoc.*, 293-305.

Webster, D., Bondareva, A., Solin, S., Goldsmith, T., Su, L., Lara, N. D. L. E. M. and Dobrinski, I. (2021). Targeted gene editing in porcine spermatogonia. *Front. Genet*, **11**: 627673.

Xi, H.M., Ren, Y.J., Ren, F., Li, Y., Feng, T.Y., Wang, Z., Du, Y.Q., Zhang, L.K. and Hu, J.H. (2022). Recent advances in isolation, identification, and culture of mammalian spermatogonial stem cells. *Asian J. Androl.*, **24**(1): 5.

Yang, Y. and Honaramooz, A. (2011). Efficient purification of neonatal porcine gonocytes with Nycodenz and differential plating. *Reprod. Fertil. Dev.*, **23**(3): 496-505.

Zheng, Y., Zhang, Y., Qu, R., He, Y., Tian, X. and Zeng, W. (2014). Spermatogonial stem cells from domestic animals: progress and prospects. *Reproduction*, **147**: R65-R74.