

Progress in *in vitro* culture and gene editing of porcine spermatogonial stem cells

Research on *in vitro* culture and gene editing of domestic spermatogonial stem cells (SSCs) is of considerable interest but remains a challenging issue in animal science. In recent years, some progress on the isolation, purification, and genetic manipulation of porcine SSCs has been reported. Here, we summarize the characteristics of porcine SSCs as well current advances in their *in vitro* culture, potential usage, and genetic manipulation. Furthermore, we discuss the current application of gene editing in pig cloning technology. Collectively, this commentary aims to summarize the progress made and obstacles encountered in porcine SSC research to better serve animal husbandry, improve livestock fecundity, and enhance potential clinical use.

Gene editing technology can not only improve livestock and poultry reproduction and meat quality (Gonen et al., 2017), but can also promote the study of gene function and therapy for human disease models via precise fixed-point editing (Gori et al., 2015; Zhao et al., 2019). Existing research suggests that SSCs maintain stability through self-renewal and can differentiate into sperm in order to produce offspring (Dym, 1994). However, the number of SSCs in mammalian testis is limited, and enrichment of SSCs is usually required using *in vitro* culture. In this context, constructing a suitable *in vitro* culture system that facilitates stable passage and maintenance of undifferentiated SSCs will help to reveal the biological characteristics of SSCs. Furthermore, it will allow for exploration of their potential usage and mechanisms of self-renewal and differentiation, as well as their application in transgenic manipulation and male infertility (Kubota & Brinster, 2006). For instance, a recent study established long-term propagation of tree shrew SSCs, paving the way for genetic modification of this animal for biomedical research (Li et al., 2017).

Gene editing is a genomic manipulation process involving base deletion, insertion, or mutation of a target gene sequence by means of the cell's own DNA damage repair

mechanism (Hwang et al., 2013; Ma et al., 2018). To date, the protocols for the isolation, purification, *in vitro* culture, and transplantation of murine SSCs have been established successfully (Brinster & Avarbock, 1994; Kubota et al., 2003, 2011; Nagano et al., 1998; Shinohara et al., 1999, 2000a, 2000b), and are widely accepted and used within the field. In contrast, the lack of a reliable protocol for the establishment of porcine SSC (pSSC) lines remains challenging and a major obstacle in genetic manipulation. However, many scientists are attempting to overcome these difficulties, with particular progress reported in recent years (Park et al., 2017b). Here, we summarize the characteristics of pSSCs, difficulties in establishment of pSSC lines, and recent advances in the field, which we hope will provide a useful reference for researchers.

SSCs are a population of germline stem cells residing in the testes of male animals. They are a type of unipotent stem cell with capacities of self-renewal and differentiation, which is the basis of spermatogenesis and male reproduction (de Rooij, 1998). Due to these abilities, SSCs can be permanently maintained to continuously produce sperm over the lifetime of a male, and thus allow the transmission of genetic information to the next generation. Therefore, SSCs are a core factor of male animal fertility.

Primordial germ cells (PGCs), the precursors of germ line cells, undergo a series of changes to eventually develop into SSCs. In the embryonic stages, the pre-spermatogonia are arrested, and it is generally believed that they regain proliferative activity within one week after birth in mice (Huckins & Clermont, 1968; Sapsford, 1962). Postnatally, SSCs are located on the basement membrane of the seminiferous tubule of male testes and function as initiating cells for spermatogenesis regulated by their microenvironment. The signals in the niche microenvironment affect the fate of SSCs, which can maintain their own population through self-renewal or can be directed to differentiate and eventually produce sperm (Chiarini-Garcia et al., 2003). Glial cell line-derived neurotrophic factor (GDNF) is essential for SSCs to maintain their reserves via constant self-

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renewal (Bugeaw et al., 2005; Meng et al., 2000), with the stable pool of SSCs forming the basis of continuous spermatogenesis throughout life (Meachem et al., 2001). The production of type A spermatogonia marks the beginning of spermatogenesis (Takagi et al., 2001). According to the hierarchy of differentiation, type A cells can be subdivided into single (A_s), paired (A_{pr}), and aligned (A_{al}) spermatogonia, which are also called undifferentiated spermatogonia (de Rooij, 1998; de Rooij & Russell, 2000). Normally, about half of the A_s spermatogonial cell population divide into A_{pr} spermatogonial cells, whereas the other half undergo self-renewal and division to maintain the pool of stem cells. The A_{pr} spermatogonia then divide further to form 4, 8, or 16 A_{al} spermatogonial cell chains (Meachem et al., 2001). All of these processes are under precise control.

Spermatogenesis is a complex process of sperm cell development, including mitosis and meiosis, starting from SSCs to derived differentiated germ cells. Although markers for developing germ cells have been well studied in mice (Encinas et al., 2012), developmental stage-specific markers of germ cells in livestock have not yet been identified (Park et al., 2017a). Existing studies suggest that PLZF, ID4, and E-cadherin are markers of undifferentiated spermatogonia (Abbasi et al., 2013; Borjigin et al., 2010; Costa et al., 2012; Reding et al., 2010; Sun et al., 2015). In addition, c-kit is thought to be a marker for differentiated spermatozoa in porcine testis after puberty (Ran et al., 2018). These findings may contribute to future research on pig spermatogenesis (Ran et al., 2018). Moreover, the GDNF signaling pathway is essential for maintaining SSC self-renewal and replication in SSC culture systems. Excessive GDNF can lead to testicular germ cell tumors, whereas insufficient GDNF expression causes premature depletion of SSCs in testes (Ferranti et al., 2012; Hofmann, 2008). Zheng et al. (2014) identified the expression of thymus cell antigen 1 (THY1) in pig testicular tissue and subsequently used THY1 to isolate and enrich SSCs from testes of newborn piglets, showing that THY1 is a surface marker of SSCs in pre-pubertal testes and can be used for SSC identification and isolation in pigs. Moreover, THY1 has been used for the purification of SSCs in bulls (Reding et al., 2010) and goats (Abbasi et al., 2013). Notably, previous study has also reported that SSCs from tree shrews, a species closely related to primates, can be successfully enriched using THY1, with the Wnt/ β -catenin signaling pathway also identified as pivotal for their maintenance (Li et al., 2017). These observations indicate similarity in the characteristics of SSCs from different species, and that the experience gained from species with established SSC lines may be valuable for pSSC study.

An effective culture system of primary pSSCs is a very powerful tool for both research and animal husbandry and provides a good platform for exploring spermatogenesis *in vitro*. In addition, further establishment of a highly efficient *in vitro* culture system for pSSCs would be conducive for studies on biological characteristics, and also lay a foundation for the application of SSCs in transgenic animals or in the treatment of human infertility. However, pig spermatogonial stem cell

establishment is a universal problem, with many large obstacles that need to be overcome to realize the long-term culture (Lin et al., 2016). The survival of SSCs requires a specific microenvironment, called the SSC niche. Thus, SSCs can only survive transiently or spontaneously differentiate when they are detached from the microenvironment on which they depend (Brinster & Zimmermann, 1994). Therefore, a suitable culture system combining feeder cells and growth factors is required to effectively expand SSCs and maintain their undifferentiated state *in vitro* (Brinster & Zimmermann, 1994). Although rodent SSC lines have been successfully established *in vitro* (de Rooij & van Beek, 2013), there are few reports on the establishment of pSSCs. In addition to the rareness of SSCs in the testis and the lack of reliable surface markers, which hinder the isolation and purification of SSCs from livestock, the lack of stable culture systems *in vitro* is another challenging obstacle in this field. For rodent SSC culture, finite growth factors and suitable cell feeders have been identified (de Rooij & van Beek, 2013). GDNF is a well-known factor for maintenance of SSCs both *in vivo* and *in vitro*, and mammalian single-minded (SIM) mouse embryo-derived thioguanine and ouabain resistant cells (STO) or mouse embryonic fibroblasts (MEF) are feeder cells that secrete essential factors for the proliferation of rodent SSCs (Bellvé et al., 1977). However, the pSSC culture system is imperfect, and the existing culture system for rodent SSCs cannot be applied to fully realize the long-term culture of pSSCs *in vitro* (Kuijk et al., 2009). In the established system for rodent SSCs, neither the serum-containing nor serum-free system appear to work well for pSSCs, and the feeders are unsuitable for pSSC maintenance. Therefore, optimizing the *in vitro* culture system of pSSCs is necessary to lay the foundation for exploring their biological characteristics (Schlatt, 2002). Typically, SSCs should be stably passaged under an undifferentiated state.

Based on the efforts of many scientific teams, we have learned that *in vitro* culture of pSSCs requires efficient enrichment of SSCs, identification of key growth factors, component finite medium, and appropriate feeder cells. Zhang et al. (2017b) optimized *in vitro* culture conditions for undifferentiated pig spermatogonia, in which germ cells were isolated and enriched from 7-d-old pig testes by optimized differential plating. They tested the effects of several different growth factors and feeder layers to maintain spermatogonia for at least two months *in vitro* without losing stem cell characteristics (Zhang et al., 2017b). Moreover, they found PLD6 to be a surface marker of undifferentiated spermatogonia in pre-adolescent boar testes, which could be used to enrich undifferentiated spermatogonia in an unprecedented way (Zhang et al., 2017a). Liu et al. (2017) identified SETDB1 as a novel epigenetic regulator of male porcine germ cells, which helps maintaining germ cell survival under regulation of H3K27me3. These findings provide a sufficient theoretical basis for the future epigenetic regulation of spermatogenesis (Liu et al., 2017). Zhao et al. (2018) attempted to establish a culture system for spermatogenesis of Bama mini-pig SSCs. They co-cultured dissociated

testicular cells from 30-d-old pigs to simulate spermatogenesis, confirming that SSCs can differentiate in α -MEM-supplemented knockout serum replacement medium and develop to the post-meiosis stage (Zhao et al., 2018). Park et al. (2017b) developed a 3D culture microenvironment to promote the self-renewal of pig SSCs. In brief, pSSCs were cultured in agarose-based 3D hydrogels and 2D culture plates, followed by analysis of cell colony formation, morphology, alkaline phosphatase activity, transcription and translational regulation of self-renewal related genes, cell viability, etc. Proliferation was determined by the effect of 3D culture on the maintenance of undifferentiated SSCs, with the final results indicating that self-renewal of pSSCs was more

effectively maintained in the 3D than in the 2D culture microenvironment (Park et al., 2017b). These findings will play an important role in the future development of new SSC culture systems for different species, and thus help in advancing SSC research (Park et al., 2017b). The aforementioned studies indicate a step forward for the maintenance of pSSCs *in vitro*. Accordingly, we constructed a summary schematic to briefly delineate the establishment of porcine SSCs in Figure 1. Although the current system for pSSC isolation and *in vitro* culture remains imperfect, we believe that continued progress will play an important role in the future development of new culture systems for SSCs from different species.

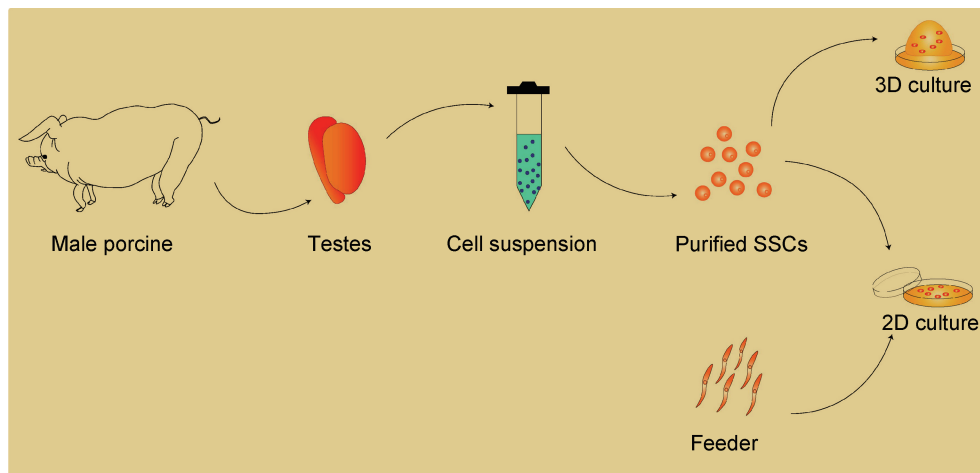


Figure 1 Schematic of pSSC isolation and culture

Testes harvested from male porcine were enzymatically digested and sorted, and the purified pSSCs were plated on feeders or maintained with 3D matrix gel for *in vitro* culture.

Traditional selective breeding methods in animals are limited by the low efficiency of species source and cell screening. The birth and development of gene editing technologies have broken this technical barrier, and allowed for improvements in livestock and poultry production, including growth and development, meat quality, and disease resistance (Hampton, 2017). Typically, gene editing technology can greatly shorten the time required to construct modern pig breeding and disease models, which should increase the relevance of porcine models in agricultural science and biomedical research (Whitworth & Prather, 2017). Recently developed DNA targeting endonuclease technologies, including ZFN (Zinc-finger nucleases), TALEN (transcription activator-like effector nucleases), and CRISPR (clustered regularly interspaced short palindromic repeats)-Cas9, have enabled researchers to specifically edit any chromosomal sequence for genetic operation. For the above three gene editing approaches, the cell initiates an endogenous mechanism to repair the site cleaved by nucleases or Cas9, with a mutation, deletion, or insertion introduced during the repair process; furthermore, a homologous recombination mechanism can be induced in the presence of a homologous sequence in the cell to obtain a specific fixed point for

mutation, deletion, or insertion (Polejaeva et al., 2000). In recent years, these technologies have been widely used in gene function research and genetically modified breeding (Li et al., 2016; Luo et al., 2014; Proudfoot et al., 2015), disease resistance breeding (Wu et al., 2015), biological disease model establishment (Whitworth et al., 2014), and other biological research. For example, Zhao et al. (2016) applied TALEN and CRISPR-Cas9 technology to humanize pig insulin by replacing the nucleotides that encode the one amino acid that differs between porcine and human insulin. As such, they successfully obtained gene-edited cloned pigs that expressed human insulin using somatic cell nuclear transfer (SCNT), thus providing the basis for the mass production of human insulin and treatment of diabetes. In addition, Park et al. (2017c) used CRISPR/Cas9 technology combined with SCNT technology to prepare Nanos2 mutant large white pigs, which may serve as a potential model for pSSC transplantation. Thus, genetically modified breeding has opened up a whole new field. With the development of gene editing technology, its application in livestock breeding such as that of pigs, cattle, and sheep is increasing, especially to improve the traits of species with no stem cell lines currently available (Park et al., 2017c). A recent study reported that knockout of the MSTN

(myostatin) gene in pigs using ZFN technology can significantly increase skeletal muscle fibers and lean meat mass (Zou et al., 2015). Polyunsaturated fatty acids (such as n-3 PUFAs), which are important nutritionally, can also exhibit anti-inflammatory and anti-coagulation activity as well as alleviate cardio-cerebral vascular diseases and influence immune regulation to improve atherosclerosis status (Belluzzi, 2004; Weber et al., 2006). However, under natural conditions, PUFAs are only found in a few plants and seafood, with very low yield. The *cbr-fat-1* gene derived from *Caenorhabditis elegans* encodes n-3 PUFAs and catalyzes the formation of n-3 PUFAs from n-6 PUFAs. Based on this principle, Zhou et al. (2014a) constructed a transgenic pig that highly expressed *cbr-fat-1*, thus laying the foundation for the production of pork rich in n-3 PUFAs. In short, the application of gene editing technology has greatly shortened the breeding period and increased the potential of pigs and other livestock in agricultural production.

In addition to gene editing methods based on SCNT or embryonic operation, germline transmission is an alternative strategy. Production of genetically modified spermatozoa could enhance the efficiency of transgenic pig production, although technical obstacles continue to impede the application of pSSC transplantation in animal cloning. Recently, however, some remarkable achievements have been made. Increased efficiency of gene delivery in pSSCs may eliminate one of barricades to livestock transgenic operation, which has hampered the development of animal cloning for many years (Kim et al., 2019; Park et al., 2019). Success in cryopreservation of pSSCs has also facilitated the application of porcine cloning using pSSCs (Lee et al., 2014). Genetically edited pSSCs with a purity higher than 90% have been obtained recently using TALEN (Tang et al., 2018). Furthermore, the busulfan-induced SSC recipient model has been established in porcine (Lin et al., 2017). Such progress indicates that the essential conditions for pSSC editing and transplantation are ready, and we believe that gene-edited pigs derived from pSSCs will be generated soon.

Continued advances in pSSC research will be reported in the next decade as the considerable advantages of pSSCs are recognized by researchers. We look forward to the development of a rapid and stable protocol for the establishment of pSSCs and their application in transgenic operation. We believe this achievement will benefit many fields, including biomedical research, regenerative medicine, and agricultural research and production, in the near future.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

Y. Z. S. and S. T. L. wrote the manuscript. X. M. L. and K. Z. reviewed and corrected the manuscript. All authors read and approved the final version of the manuscript.

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REFERENCES

- Abbasi H, Tahmoorespur M, Hosseini SM, Nasiri Z, Bahadorani M, Hajian M, Nasiri MR, Nasr-Esfahani MH. 2013. THY1 as a reliable marker for enrichment of undifferentiated spermatogonia in the goat. *Theriogenology*, **80**(8): 923–932.
- Belluzzi A. 2004. Polyunsaturated fatty acids (n-3 PUFAs) and inflammatory bowel disease (IBD): pathogenesis and treatment. *European Review for Medical and Pharmacological Sciences*, **8**(5): 225–229.
- Bellvé AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M. 1977. Spermatogenic cells of the prepubertal mouse: Isolation and morphological characterization. *Journal of Cell Biology*, **74**(1): 68–85.
- Borjigin U, Davey R, Hutton K, Herrid M. 2010. Expression of promyelocytic leukaemia zinc-finger in ovine testis and its application in evaluating the enrichment efficiency of differential plating. *Reproduction, Fertility and Development*, **22**(5): 733–742.
- Brinster RL, Avarbock MR. 1994. Germline transmission of donor haplotype following spermatogonial transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, **91**(24): 11303–11307.
- Brinster RL, Zimmermann JW. 1994. Spermatogenesis following male germ-cell transplantation. *Proceedings of the National Academy of Sciences of the United States of America*, **91**(24): 11298–11302.
- Buageaw A, Sukhwani M, Ben-Yehudah A, Ehmcke J, Rawe VY, Pholpramool C, Orwig KE, Schlatt S. 2005. GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes. *Biology of Reproduction*, **73**(5): 1011–1016.
- Chiarini-Garcia H, Raymer AM, Russell LD. 2003. Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. *Reproduction*, **126**(5): 669–680.
- Costa GMJ, Avelar GF, Rezende-Neto JV, Campos-Junior PHA, Lacerda SMSN, Andrade BSC, Thomé RG, Hofmann M-C, Franca LR. 2012. Spermatogonial stem cell markers and niche in equids. *PLoS One*, **7**(8): e44091.
- de Rooij DG. 1998. Stem cells in the testis. *International Journal of Experimental Pathology*, **79**(2): 67–80.
- de Rooij DG, Russell LD. 2000. All you wanted to know about spermatogonia but were afraid to ask. *Journal of Andrology*, **21**(6): 776–798.
- de Rooij DG, van Beek MEAB. 2013. Computer simulation of the rodent spermatogonial stem cell niche. *Biology of Reproduction*, **88**(5): 131.
- Dym M. 1994. Spermatogonial stem cells of the testis. *Proceedings of the National Academy of Sciences of the United States of America*, **91**(24):

11287–11289.

- Encinas G, Zogbi C, Stumpp T. 2012. Detection of four germ cell markers in rats during testis morphogenesis: Differences and similarities with mice. *Cells Tissues Organs*, **195**(5): 443–455.
- Ferranti F, Muciaccia B, Ricci G, Dovere L, Canipari R, Magliocca F, Stefanini M, Catizone A, Vicini E. 2012. Glial cell line-derived neurotrophic factor promotes invasive behaviour in testicular seminoma cells. *International Journal of Andrology*, **35**(5): 758–768.
- Gonen S, Jenko J, Gorjanc G, Mileham AJ, Whitelaw CBA, Hickey JM. 2017. Potential of gene drives with genome editing to increase genetic gain in livestock breeding programs. *Genetics Selection Evolution*, **49**(1): 3.
- Gori JL, Hsu PD, Maeder ML, Shen S, Welstead GG, Bumcrot D. 2015. Delivery and specificity of CRISPR-Cas9 genome editing technologies for human gene therapy. *Human Gene Therapy*, **26**(7): 443.
- Hampton T. 2017. Gene editing could help pave the way for pig-to-human transplantations. *Circulation*, **136**(21): 2083–2084.
- Hofmann MC. 2008. Gdnf signaling pathways within the mammalian spermatogonial stem cell niche. *Molecular and Cellular Endocrinology*, **288**(1–2): 95–103.
- Huckins C, Clermont Y. 1968. Evolution of gonocytes in the rat testis during late embryonic and early post-natal life. *Archives D'anatomie, D'histologie et D'embryologie Normales et Experimentales*, **51**(1): 341–354.
- Hwang WY, Fu Y, Reyon D, Maeder ML, Tsai SQ, Sander JD, Peterson RT, Yeh JR, Joung JK. 2013. Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nature Biotechnology*, **31**(3): 227–229.
- Kim MS, Park MH, Park JE, Yun JI, Choi JH, Lee E, Lee ST. 2019. Establishment of an electroporation-mediated gene delivery system in porcine spermatogonial stem cells. *In Vitro Cellular & Developmental Biology - Animal*, **55**(3): 177–188.
- Kubota H, Brinster RL. 2006. Technology Insight: in vitro culture of spermatogonial stem cells and their potential therapeutic uses. *Nature Clinical Practice Endocrinology & Metabolism*, **2**(2): 99–108.
- Kubota H, Avarbock MR, Brinster RL. 2003. Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, **100**(11): 6487–6492.
- Kubota H, Wu X, Goodyear SM, Avarbock MR, Brinster RL. 2011. Glial cell line-derived neurotrophic factor and endothelial cells promote self-renewal of rabbit germ cells with spermatogonial stem cell properties. *The FASEB Journal*, **25**(8): 2604–2614.
- Kuijk EW, Colenbrander B, Roelen BAJ. 2009. The effects of growth factors on *in vitro*-cultured porcine testicular cells. *Reproduction*, **138**(4): 721–731.
- Lee YA, Kim YH, Ha SJ, Kim KJ, Kim BJ, Kim BG, Choi SH, Kim IC, Schmidt JA, Ryu BY. 2014. Cryopreservation of porcine spermatogonial stem cells by slow-freezing testis tissue in trehalose. *Journal of Animal Science*, **92**(3): 984–995.
- Li CH, Yan LZ, Ban WZ, Tu Q, Wu Y, Wang L, Bi R, Ji S, Ma YH, Nie WH, Lv LB, Yao YG, Zhao XD, Zheng P. 2017. Long-term propagation of tree shrew spermatogonial stem cells in culture and successful generation of transgenic offspring. *Cell Research*, **27**(2): 241.
- Li H, Wang G, Hao Z, Zhang G, Qing Y, Liu S, Qing L, Pan W, Chen L, Liu G, Zhao P, Jia B, Zeng L, Guo J, Zhao L, Zhao H, Lv C, Xu K, Cheng W, Li H, Zhao H, Wang W, Wei H. 2016. Generation of biallelic knock-out sheep via gene-editing and somatic cell nuclear transfer. *Scientific Reports*, **6**: 33675.
- Lin Z, Bao J, Kong Q, Bai Y, Luo F, Zhou S, Wu Y, Huang J. 2017. Effective production of recipient male pigs for spermatogonial stem cell transplantation by intratesticular injection with busulfan. *Theriogenology*, **89**: 365–373.e2.
- Liu TT, Zhang P, Li TJ, Chen XX, Zhu ZS, Lyu YH, Li XL, Tian X, Zeng WX. 2017. SETDB1 plays an essential role in maintenance of gonocyte survival in pigs. *Reproduction*, **154**(1): 23–34.
- Luo J, Song Z, Yu S, Cui D, Wang B, Ding F, Li S, Dai Y, Li N. 2014. Efficient generation of myostatin (MSTN) biallelic mutations in cattle using zinc finger nucleases. *PLoS One*, **9**(4): e95225.
- Ma X, Wong SY, Tam HY, Tsui YK, Chung LS, Feng B. 2018. *In vivo* genome editing thrives with diversified CRISPR technologies. *Zoological Research*, **39**(2): 58–71.
- Meachem S, Schönfeldt VV, Schlatt S. 2001. Spermatogonia: stem cells with a great perspective. *Reproduction*, **121**(6): 825–834.
- Meng X, Lindahl M, Hyvönen ME, Parvinen M, de Rooij DG, Hess MW, Raatikainen-Ahokas A, Sainio K, Rauvala H, Lakso M, Pichel JG, Westphal H, Saarma M, Sariola H. 2000. Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. *Science*, **287**(5457): 1489–1493.
- Nagano M, Avarbock MR, Leonida EB, Brinster CJ, Brinster RL. 1998. Culture of mouse spermatogonial stem cells. *Tissue and Cell*, **30**(4): 389–397.
- Park HJ, Lee R, Lee WY, Kim JH, Do JT, Park C, Song H. 2017a. Stage-specific expression of Sal-like protein 4 in boar testicular germ cells. *Theriogenology*, **101**: 44–52.
- Park JE, Park MH, Kim MS, Park YR, Yun JI, Cheong HT, Kim M, Choi JH, Lee E, Lee ST. 2017b. Porcine spermatogonial stem cells self-renew effectively in a three dimensional culture microenvironment. *Cell Biology International*, **41**(12): 1316–1324.
- Park K, Kim MS, Kang M, Kang T, Kim B, Lee ST. 2019. Successful genetic modification of porcine spermatogonial stem cells via an electrically responsive Au nanowire injector. *Biomaterials*, **193**: 22–29.
- Park KE, Kaucher AV, Powell A, Waqas MS, Sandmaier SE, Oatley MJ, Park CH, Tibary A, Donovan DM, Blomberg LA, Lillico SG, Bruce C, Whitelaw A, Mileham A, Telugu BP, Oatley JM. 2017c. Generation of germline ablated male pigs by CRISPR/Cas9 editing of the *NANOS2* gene. *Scientific Reports*, **7**: 40176.
- Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KHS. 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature*, **407**(6800): 86–90.
- Proudfoot C, Carlson DF, Huddart R, Long CR, Pryor JH, King TJ, Lillico SG, Mileham AJ, McLaren DG, Whitelaw CBA, Fahrenkrug SC. 2015. Genome edited sheep and cattle. *Transgenic Research*, **24**(1): 147–153.
- Ran L, Lee WY, Park HJ, Ha WT, Woo JS, Chung HJ, Lee JH, Hong K, Song H. 2018. Stage-specific expression of DDX4 and C-kit at different developmental stages of the porcine testis. *Animal Reproduction Science*, **190**: 18–26.
- Reding SC, Stepnoski AL, Cloninger EW, Oatley JM. 2010. THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis. *Reproduction*, **139**(5): 893–903.
- Sapsford CS. 1962. Changes in the cells of the Sex Cords and

- Seminiferous Tubules during the development of the Testis of the rat and mouse. *Australian Journal of Zoology*, **10**(2): 178–192.
- Schlatt S. 2002. Spermatogonial stem cell preservation and transplantation. *Molecular and Cellular Endocrinology*, **187**(1–2): 107–111.
- Shinohara T, Avarbock MR, Brinster RL. 1999. β 1- and α 6-integrin are surface markers on mouse spermatogonial stem cells. *Proceedings of the National Academy of Sciences of the United States of America*, **96**(10): 5504–5509.
- Shinohara T, Avarbock MR, Brinster RL. 2000a. Functional analysis of spermatogonial stem cells in steel and cryptorchid infertile mouse models. *Developmental Biology*, **220**(2): 401–411.
- Shinohara T, Orwig KE, Avarbock MR, Brinster RL. 2000b. Spermatogonial stem cell enrichment by multiparameter selection of mouse testis cells. *Proceedings of the National Academy of Sciences of the United States of America*, **97**(15): 8346–8351.
- Sun F, Xu Q, Zhao D, Chen CD. 2015. Id4 marks spermatogonial stem cells in the mouse testis. *Scientific Reports*, **5**: 17594.
- Takagi S, Itoh N, Kimura M, Sasao T, Tsukamoto T. 2001. Spermatogonial proliferation and apoptosis in hypospermatogenesis associated with nonobstructive azoospermia. *Fertility and Sterility*, **76**(5): 901–907.
- Tang L, Bondareva A, González R, Rodríguez-Sosa JR, Carlson DF, Webster D, Fahrenkrug S, Dobrinski I. 2018. TALEN-mediated gene targeting in porcine spermatogonia. *Molecular Reproduction and Development*, **85**(3): 250–261.
- Weber HS, Selimi D, Huber G. 2006. Prevention of cardiovascular diseases and highly concentrated n-3 polyunsaturated fatty acids (PUFAs). *Herz*, **31**(Suppl 3): 24–30.
- Whitworth KM, Prather RS. 2017. Gene editing as applied to prevention of reproductive porcine reproductive and respiratory syndrome. *Molecular Reproduction and Development*, **84**(9): 926–933.
- Whitworth KM, Lee K, Benne JA, Beaton BP, Spate LD, Murphy SL, Samuel MS, Mao J, O'gorman C, Walters EM, Murphy CN, Driver J, Mileham A, McLaren D, Wells KD, Prather RS. 2014. Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos. *Biology of Reproduction*, **91**(3): 78–78.
- Wu H, Wang Y, Zhang Y, Yang M, Lv J, Liu J, Zhang Y. 2015. TALE nickase-mediated *SP110* knockin endows cattle with increased resistance to tuberculosis. *Proceedings of the National Academy of Sciences of the United States of America*, **112**(13): E1530–E1539.
- Yang Y, Wang K, Wu H, Qin J, Ruan D, Zhen O, Zhao B, Liu Z, Zhao Y, Zhang Q, Fan N, Liu Q, Guo S, Bu L, Fan Y, Sun X, Li X, Lai L. 2016. Genetically humanized pigs exclusively expressing human insulin are generated through custom endonuclease-mediated seamless engineering. *Journal of Molecular Cell Biology*, **8**(2): 174–177.
- Zhang P, Qin Y, Zheng Y, Zeng W. 2017a. Phospholipase D family member 6 (PLD6) is a surface marker for enrichment of undifferentiated spermatogonia in the pre-pubertal boars. *Stem Cells and Development*, **27**(1): 55–64.
- Zhang P, Chen X, Zheng Y, Zhu J, Qin Y, Lv Y, Zeng W. 2017b. Long-term propagation of porcine undifferentiated spermatogonia. *Stem Cells and Development*, **26**(15): 1121–1131.
- Zhao H, Nie J, Zhu X, Lu Y, Liang X, Xu H, Yang X, Zhang Y, Lu K, Lu S. 2018. *In vitro* differentiation of spermatogonial stem cells using testicular cells from Guangxi Bama mini-pig. *Journal of Veterinary Science*, **19**(5): 592–599.
- Zhao J, Lai L, Ji W, Zhou Q. 2019. Genome editing in large animals: Current status and future prospects. *National Science Review*, **6**(3): 402–420.
- Zheng Y, He Y, An J, Qin J, Wang Y, Zhang Y, Tian X, Zeng W. 2014. THY1 is a surface marker of porcine gonocytes. *Reproduction Fertility & Development*, **26**(4): 533–539.
- Zhou Y, Lin Y, Wu X, Feng C, Long C, Xiong F, Wang N, Pan D, Chen H. 2014. The high-level accumulation of n-3 polyunsaturated fatty acids in transgenic pigs harboring the n-3 fatty acid desaturase gene from *Caenorhabditis briggsae*. *Transgenic Research*, **23**(1): 89–97.
- Zou Q, Wang X, Liu Y, Ouyang Z, Long H, Wei S, Xin J, Zhao B, Lai S, Shen J, Ni Q, Yang H, Zhong H, Li L, Hu M, Zhang Q, Zhou Z, He J, Yan Q, Fan N, Zhao Y, Liu Z, Guo L, Huang J, Zhang G, Ying J, Lai L, Gao X. 2015. Generation of gene-target dogs using CRISPR/Cas9 system. *Journal of Molecular Cell Biology*, **7**(6): 580–583.