Induction of goat bone marrow mesenchymal stem cells into putative male germ cells using mRNA for *STRA8*, *BOULE* and *DAZL*

Yan-Li Zhang · Pei-Zhen Li · Jing Pang · Yong-Jie Wan · Guo-Min Zhang ·
Yi-Xuan Fan · Zi-Yu Wang · Nie-Hai Tao · Feng Wang

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Abstract Bone mesenchymal stem cells (BMSCs) have the capacity to differentiate into germ cells (GCs). This study was conducted to develop a non-integrated method of using RNA transfection to derive putative male GCs from goat BMSCs (gBMSCs) in vitro by overexpressing *STRA8*, *BOULE* and *DAZL*. The gBMSCs were induced by co-transfection these three mRNAs together (mi-SBD group) or sequential transfection according to their expression time order in vivo (mi-S + BD group). After transfection, a small population of gBMSCs transdifferentiated into early germ cell-like cells and had the potential to enter meiosis. These cells expressed primordial germ cell specific genes *STELLA*, *C-KIT* and *MVH*, as well as premeiotic genes *DAZL*, *BOULE*, *STRA8*, *PIWIL2* and *RNF17*. Importantly, the expression level of meiotic marker synaptonemal complex protein 3 significantly increased in these transfected two groups compared with control cells by qRT-PCR, immunofluorescence and western blot analysis ($P < 0.05$). Moreover, the protein expression of MVH was significantly higher in

mi-S + BD group than that in mi-SBD group ($P < 0.05$). In addition, compared with control group, the methylation rate of imprinted gene *H19* decreased in these two transfected group ($P < 0.05$), and the rate was significantly lower in mi-S + BD group compared with mi-SBD group ($P < 0.05$). This study helps to understand the mechanisms of action of key genes in GCs differentiation and also provides a novel system for in vitro induction of male GCs from stem cells.

Keywords BMSCs · Germ cells · *STRA8* · *DAZL* · *BOULE* · Differentiation

Abbreviations

gBMCs	Goat bone mesenchymal stem cells
ESCs	Embryonic stem cells
GCs	Germ cells
iPSCs	Induced pluripotent stem cells
PGCs	Primordial germ cells
qRT-PCR	Quantitative real-time PCR
RA	Retinoic acid
BMP4	Bone morphogenetic protein 4
DMRs	Differentially methylated regions

Y.-L. Zhang · J. Pang · Y.-J. Wan · G.-M. Zhang ·
Y.-X. Fan · Z.-Y. Wang · N.-H. Tao · F. Wang (✉)
Jiangsu Livestock Embryo Engineering Laboratory,
Nanjing Agricultural University, No. 1 Weigang, Nanjing,
China
e-mail: caeet@njau.edu.cn

P.-Z. Li
Jiangsu Provincial Station of Animal Husbandry, Nanjing,
China

Introduction

Stem cells provide a powerful tool for studying the mechanisms of germ cell development in vitro. Several studies have shown that embryonic stem cells (ESCs) have the ability to differentiate into haploid male germ cells in vitro (Geijsen et al. 2004; Kee et al. 2009b; Kerkis et al. 2007). However, due to ethical constraints for human ESC and difficult establishment of other species ES cell lines, it limits the application of ESCs. Meanwhile, induced pluripotent stem cells (iPSCs) are another potential cells to induce differentiation of germ cells in vitro (Li et al. 2014; Easley et al. 2012), while the efficiency of iPSC induction still remains low. The BMSCs are pluripotent adult stem cells that exhibit differentiation capacity into different cell types of both mesenchymal and non-mesenchymal lineages, including germ cells (Nayernia et al. 2006; Drusenheimer et al. 2007; Hua et al. 2009; Ghasemzadeh-Hasankolaei et al. 2016). More importantly, BMSCs is relatively easier to isolate and expand extensively without losing their multipotency, which make them ideal model cells for in vitro differentiation and study gametogenesis. To date, induction methods by treatment with exogenous factors like retinoic acid (RA), transforming growth factor-beta superfamily growth factors and bone morphogenetic protein (BMP4) are reported to induce BMSCs differentiation into GC-like lineages (Shirazi et al. 2012; Ghasemzadeh-Hasankolaei et al. 2014; Yan et al. 2015). However, in both the mouse and human in vitro systems, progression of meiosis has limited the success of production of mature gametes.

Germ cell development is controlled by unique gene expression programs. Some genes have been shown to be critical for germ cell development, with several demonstrating conservation across species. *STAR8* (stimulated by retinoic acid gene 8) is a specific expression gene in mammalian germ cells transition from mitosis to meiosis, which is indispensable for premeiotic DNA replication and subsequent entry into the prophase of meiosis I (Mark et al. 2008). *STAR8* was also usually used to construct the germ cell specific reporting system (Anderson et al. 2008). The RNA-binding protein *DAZL* and *BOULE*, highly conserved between species, play important roles in animal germ cell development. *DAZL* is a master gene controlling germ cell differentiation and ectopic expression of *DAZL* promotes the dynamic

differentiation of mouse ES cells into gametes in vitro (Yu et al. 2009). Forced expression of *DAZ* gene family (*DAZL*, *DAZ* and *BOULE*) induces human ESCs to differentiate to PGCs and haploid gametes in vitro (Kee et al. 2009a). Also, co-overexpression of *DAZL* and *VASA* could promote meiotic progression of human-derived germ cells from human ESCs and iPSCs in vitro (Medrano et al. 2012). Taken together, co-overexpression of multiple germ cell special genes might be able to improve in vitro germ cell differentiation. However, most of the research about in vitro derivation germ cells from BMSCs focused on mice and human, other species are still limited.

In fact, we previously demonstrated that ectopic expression of *DAZL* gene enhanced goat BMSCs trans-differentiation into male germ cells compared to the RA treated cells (Yan et al. 2015). Our recent study also has revealed the co-transfection with plasmids of *STAR8*, *BOULE*, and *DAZL* was able to promote gBMSCs trans-differentiate into to early goat germ cell-like cells in vitro (Li et al. 2017). However, compared with plasmid DNA based method, RNA does not require cell proliferation for expression of the encoded protein, on the other hand, mRNAs are translated within minutes following entry into the cytoplasm, whereas plasmids require time-consuming nuclear import and transcription, which indicate more efficient transduction of primary cells than DNA (McLennan et al. 2013). Therefore, here we sought to determine whether co-overexpression of *STAR8*, *BOULE* and *DAZL* using RNA synthesized in vitro would efficiently induce gBMSCs to trans-differentiate into germ cells and also compare the induction efficiency of co-transfection these three mRNAs together or sequential transfection according to their expression time order in vivo. To our knowledge, this is the first study using a non-integrated method of RNA transfection to derive putative male GCs from BMSCs.

Methods and materials

Ethics statement

This study was approved by the ethical committee of the College of Animal Science and Technology, Nanjing Agricultural University, China. All protocols were conducted in strict accordance with the Animal Research Committee guidelines of the College of

Animal Science and Technology, Nanjing Agricultural University, China.

STRA8, *BOULE* and *DAZL* mRNA preparation by in vitro transcription

The plasmids pEX-4-*STRA8*, pEX-4-*DAZL* and pEGFP-*DAZL* were constructed in our previous study (Li et al. 2017) (Fig. 1a). Their primers including the T7 promoter sequence were designed and used amplified *STRA8*, *BOULE* and *DAZL*, the PCR product were purified and followed by in vitro RNA transcription (IVT) using the mMESSAGE mMACHINE T7 kit (Applied Biosystems/Ambion, TX, USA) that allows 5' cap formation. The transcribed RNA was recovered on MEGAclear™ Transcription Clean-Up Kit according to the RNA Cleanup protocol of the manufacturer. RNA integrity and quantity was

monitored by agarose gel electrophoresis and Nanodrop, respectively. PCR primers were as follows:

T7-*STRA8*-F: TAATACGACTCACTATAGGGA
TGGAGACTGCAGGAGAAGGTG
T7-*STRA8*-R: TCACAGATCTTCATCGTCAAA
T
T7-*BOULE*-F: TAATACGACTCACTATAGGGA
TGGAGACCGAGTCCGGGGCGC
T7-*BOULE*-R: TTAATAATGAATGCTCCACACT
T7-*DAZL*-F: TAATACGACTCACTATAGGGAT
GGCGCCCTCCTCTCTGAACC
T7-*DAZL*-R: TCAACAGACTTAAGCACTGCC

In vitro induction of gBMSCs to putative germ cells by transfection

One day prior to transfection, the in vitro isolated and cultured goat BMSCs at passage 4 were seeded in a

Fig. 1 The general experimental procedure. **a** The scheme of the in vitro synthesis of *STRA8*, *BOULE* and *DAZL* mRNA. **b** The transfection method scheme. **c** The morphology of non-transfected and transfected gBMSCs

12-well plate at 70% confluency. The detailed culture information on gBMSCs was described in our previous study (Li et al. 2017; Yan et al. 2015). The transfection method was followed the protocol of Lipofectamine® MessengerMAX (Thermo Fisher Scientific, USA). To optimize the induction efficiency of *STRA8*, *BOULE* and *DAZL* mRNA, gBMSCs were transfected with these three mixture together (mi-SBD group, the ratio was 1:1:1), or with the mRNA according their chronological expression order in vivo (named mi-S + BD group, the ratio was 1:1:1). For mi-SBD group, the mRNA mixtures of *STRA8*, *BOULE* and *DAZL* were transfected three times on Day 1, Day 3 and Day 5, respectively. For mi-SBD group, only *STRA8* mRNA were first transfected on Day 1, followed by *STRA8*, *BOULE* and *DAZL* mRNA on Day 3, and finally *BOULE* and *DAZL* mRNA were transfected on Day 5. The transfected BMSCs on Day 8 were collected for further analysis. The experiment design was shown as Fig. 1b.

Quantitative RT-PCR analysis

Total RNA was isolated from the cells and the goat testis using PureLink RNA Mini Kit and TRIzol reagent (Invitrogen Life Technologies), respectively. The cDNA was then synthesized using a reverse transcription reagent kit with gDNA Eraser (Takara, Dalian, China). qRT-PCR was performed to detect the expression of genes on a StepOnePlus Real-Time PCR System (Life Technologies, USA) using SYBR Green Master mix (Roche Applied Science, Mannheim, Germany). Comparative quantification of the expression levels of targeted genes was done by the $2^{-\Delta\Delta C_t}$ method with the expression of the target genes normalized to glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*). The gene primers were designed as our previous paper (Li et al. 2017).

Immunofluorescence analysis

Cells were seeded in glass-bottom dishes and fixed with ice-cold methanol for 10 min at room temperature (RT) and permeabilized (0.25% Triton X-100) (Sigma) for 10 min. Then they were blocked with Immunol Staining Blocking Buffer (Beyotime) for 60 min at RT on a rocking platform. The primary rabbit anti-*DAZL* antibody (1:100 dilution, ab34139; Abcam) and rabbit anti-SCP3 antibody (1:100

dilution; ab150292, Abcam) were added to cells and incubated for overnight at 4 °C. Afterward, the cells were washed with PBS for 3 times, then the secondary antibody, 594-conjugated donkey anti-rabbit antibody (1:200 dilution, ab96921; Abcam) was added to cells and incubated in dark for 2 h at RT. Finally, the nuclei of samples were stained with 4',6-diamidino-2-phenylindole (DAPI) (Beyotime) for 10 min and samples were examined with a confocal laser-scanning microscope (Zeiss LSM 710 META, Germany).

Western blotting

Total protein was extracted from the cells with RIPA lysis buffer (Beyotime Biotechnology, China) and quantified by BCA method. The Western blot standard protocol were used as our previously described (Li et al. 2017). The primary rabbit anti-MVH antibody (1:300 dilution, ab13840; Abcam) and rabbit anti-SCP3 antibody (1:300 dilution, ab150292; Abcam) were used. Horseradish peroxidase-labeled goat antirabbit (1:1000 dilution, A0208; Beyotime) was used as a secondary antibody. Samples were also incubated for ACTIN (β -actin, internal protein) (1:200 dilution, AA128; Beyotime) with secondary antibody (1:1000 dilution, A0216; Beyotime). Band intensities were estimated by densitometry and normalized to ACTIN. Bands were quantified using Image J software (Wayne Rasband, Maryland, USA).

Methylation analyses of H19 imprinting gene

After 8 days transfection, total genomic DNA was extracted from the gBMSCs using TIANamp Genomic DNA kit (Tiangen, Beijing, China). First, the putative differentially methylated regions (DMRs) of H19 were identified using the online software MethyPrimer with the following restrictive conditions: GC percentage $\geq 50.0\%$ and CpG observe/expect ≥ 0.6 . Bisulfite PCR specific primers were M-H19-F: 5'-GTA TTAGGTTTTTGGTGGTATAGAG-3'; M-H19-R: 5'-CTCTCCTCTCCCAACTTCAAC-3'. Second, bisulfite treatment and recovery of DNA samples were performed using the EZ DNA Methylation-Direct kit (Zymo Research, Irvine, CA, USA) following the instructions. Finally, bisulfite PCR and sequencing was performed. The Methylation patterns were analyzed using the BIQ Analyzer software and sequences derived from greater than 10 clones with

99% cytosine conversions only. The methylation percentage of every sample was obtained using BIQ Analyzer software.

Statistical analyses

Statistical analyses were done using SPSS software (version 19.0). Values were expressed as Mean $\pm$ standard error of mean (SEM). Data were analyzed with analysis of variance and Fisher protected least significant difference tests. The difference of methylation rate was analyzed by Chi square test. For all analysis, $P < 0.05$ was considered statistically significant. All experiments were repeated at least 3 times.

Results

Morphological changes of goat BMSCs after induction with *STRA8*, *BOULE* and *DAZL* mRNA

The *STRA8*, *BOULE* and *DAZL* gene CDS fragments were amplified from the plasmids pEX-4-*STRA8*, pEX-4-*BOULE* and pEGFP-*DAZL* by primers containing the T7 promoter sequence, which was 1104 bp, 840 bp and 966 bp, respectively. After in vitro transcription, gBMSCs were induced for 3 times every 3 days by transfection of *STRA8*, *BOULE* and *DAZL* mRNA. After 8 days of induction, some of the gBMSCs in mi-SBD and mi-S + BD groups manifested as long spindle and irregular scale shaped, and the nucleoplasm ratio increased, but no obvious morphological change differences were seen the mi-SBD and mi-S + BD groups (Fig. 1c).

Expression of germ cell-specific genes in goat BMSCs after induction with *STRA8*, *BOULE* and *DAZL* mRNA

After induction gBMSCs with *STRA8*, *BOULE* and *DAZL* mRNA, we used qRT-PCR to quantitate the expression of early and late germ cell-specific genes (Fig. 2). First, we observed that mi-SBD and mi-S + BD groups showed upregulation in two pluripotency genes *OCT4* and *NANOG* compared with non-transfected gBMSCs, and the *OCT4* expression levels significantly increased ($P < 0.05$). Next, to test whether the transfected cells undergo the progress of

PGCs stage. we analyzed three PGCs markers, *STELLA*, *C-KIT* and *MVH*. As expected, the expressions of these three genes were notably increased in both mi-SBD and mi-S + BD groups. *STELLA* and *MVH* increased significantly in the miS + BD group compared with the control group ($P < 0.05$). To further investigate whether meiosis happens in the case of our differentiation process, we then focused on the gene expression levels for several genes *STRA8*, *DAZL*, *BOULE*, *PIWIL2*, *RNF17* and *SCP3*, all these 6 genes showed significant higher levels in the mi-SBD and mi-S + BD group than that in control group ($P < 0.05$). *SCP3* is required for synapsis phase of meiosis. Importantly, we also observed the expression level of the meiotic gene *SCP3* was elevated in mi-S + BD group compared with mi-SBD and control group ($P < 0.05$).

The germ cell-specific proteins exhibited expression in transdifferentiated gBMSCs

Although the gBMSCs in mi-SBD and mi-S + BD groups expressed some germ cell markers in mRNA levels, whether the translation level was concomitantly regulated remained to be studied. Therefore, we investigated the differentiation by immunofluorescence and western blot to detect the protein expression of early germ cell and meiotic germ cell marker *DAZL*, *MVH*, and *SCP3*. Immunofluorescence results showed the mi-SBD and mi-S + BD groups revealed a strong positive fluorescence signal for the *DAZL* protein, while negative signal staining was detected in the control gBMSCs (Fig. 3a). On the other hand, signaling for anti-*SCP3* antibodies was also positive in the mi-SBD and mi-S + BD groups compared with the control (Fig. 3b). Furthermore, western blot analysis showed that *MVH* and *SCP3* protein levels were significantly higher in mi-SBD and mi-S + BD groups ($P < 0.05$, Fig. 3c, d). As shown, the mi-S + BD group had the highest expression level of *MVH*, compared with control gBMSCs ($P < 0.05$).

The methylation status of H19 imprinted gene in transdifferentiated gBMSCs

Erasure of epigenetic imprints and their subsequent sex-specific re-establishment is a critical and diagnostic process in germ cell development that is necessary for embryo development. To determine if ectopic

Fig. 2 The expression level of germ cell markers in transfected gBMSCs by qRT-PCR

Fig. 3 Immunofluorescence analysis for cellular localization of DAZL and SCP3 in transfected gBMSCs. **a** Some of the gBMSCs in mi-SBD and mi-S + BD groups showed DAZL-positive clusters (green) in the cytoplasm. **b** Some of the gBMSCs in mi-SBD and mi-S + BD groups showed SCP3-

positive clusters (green) in the nuclear. Normal rabbit serum was used in place of primary antibody in control groups, bar = 20 μ m. **c, d** Western blot analysis for MVH and SCP3 proteins in different transfected gBMSCs. (Color figure online)

Fig. 4 DNA methylation status of H19 DMR in gBMSCs. **a** Diagrams represent methylation status of each CpG dinucleotides in individual DNA clones of mi-S + BD group, mi-SBD group and control (untreated gBMSCs). Unfilled (white) and filled (black) circles represent unmethylated and methylated CpGs, respectively. Horizontal line of circles represent one single clone that was sequenced. **b** Percentage of methylated CpGs found in each condition at the DMR of H19 (* $P < 0.05$, ** $P < 0.01$)

expression of *STRA8*, *BOULE* and *DAZL* mRNA alters epigenetic programs, we analyzed methylation status at the CpG islands of the imprinted, maternally expressed gene H19 by BIQ software. We observed that the H19 locus was 78.8% methylated in undifferentiated control cells. Overexpression of *STRA8*, *BOULE* and *DAZL* mRNA led to a significant decrease in the methylation ratio of H19 in mi-SBD and mi-S + BD groups compared with the control, which was 62.8% and 70%, respectively ($P < 0.05$, Fig. 4).

Discussion

Previous studies demonstrated that BMSCs can form germ cells in vitro, albeit with different efficiencies observed between different induction methods (Shirazi et al. 2012; Yan et al. 2015; Ghasemzadeh-

Hasankolaei et al. 2016; Li et al. 2017). Here we examined the effect of ectopic expression of *STRA8*, *BOULE* and *DAZL* on goat germ cell formation and progression through meiosis. The administration of synthetic mRNA has proven more efficient to reprogram cell fate (Rosa and Brivanlou 2010). Although mRNA transfection of fibroblasts (Yakubov et al. 2010) and iPS cells (Preskey et al. 2016) have been described, mRNA delivery to bone marrow stem cells to make them trans-differentiation has not been previously reported. We speculated that prolonged transfection of the multiple genes mRNA probably enhanced the trans-differentiation to a greater extent. Therefore, this study was conducted to develop a non-integrated method of using RNA transfection to derive putative male GCs from gBMSCs by overexpressing *STRA8*, *BOULE* and *DAZL*. Meanwhile, we also optimized the transfection system in case of

degradation of mRNA. In this study, we observed that gBMSCs with ectopic expression of *STRA8*, *BOULE* and *DAZL* mRNA expressed most of the germ cell markers that we analyzed, including marker characteristic of germ cells in both early and later stages of maturation, similar to our previous results reported by DNA-based methods (Li et al. 2017) and other reports in different model system.

OCT4 is primarily expressed in pluripotent lineages and regarded to have a role in germ cell development. *OCT4* expression is increased in PGCs and gradually decreased during development towards SSCs and A1–A4 spermatogonia (Kehler et al. 2004). qRT-PCR showed an enhanced expression of the *OCT4* in gBMSCs derived GC cells. A low level of *OCT4* expression was observed in untreated gBMSCs, which was consistent with previous study on mouse BMSCs (Nayernia et al. 2006). Furthermore, we analyzed three PGCs markers, *STELLA*, *C-KIT* and *MVH*. *STELLA* is involved in initiating germ cell competence and specification, and in the demarcation of PGCs from their somatic neighbors (Saitou et al. 2002). *C-KIT* has a similar effect, which is also essential to the process of germ cell migration, proliferation and survival in the gonadal anlage, and it is also involved in the reprogramming of PGCs (Høyer et al. 2005). Our findings indicated that *STELLA* and *C-KIT* expression levels increased in mi-SBD and mi-S + BD groups compared with non-transfected group. *MVH* is an important germ cell marker that expresses from migratory PGCs in the genital ridge to post meiotic germ cells. Loss of *MVH* function causes a deficiency in the proliferation and differentiation of male germ cells (Tanaka et al. 2000). In our study, *MVH* showed upregulated in mRNA and protein expression levels compared with the control group, which showed that cells with the highest expression in mi-S + BD group.

The data above suggested that overexpression of *STRA8*, *BOULE* and *DAZL* mRNA promotes PGC formation. We next examined if overexpression of these genes promotes germ-cell differentiation beyond the PGC stage. Furthermore, we observed *PIWIL2* and *RNF17* expression greatly upregulated in the mi-S + BD and mi-SBD groups compared with the control group. The Piwi-like 2 (*PIWIL2*) gene, a member of the AGO/PIWI gene family, which function in development and maintenance of germline stem cells. *PIWIL2* has a low expression in pre-migratory and migratory stages and an almost

increased expression during the development of GCs from post-migratory PGCs to spermatogonia stages (Deng and Lin 2002). *RNF17*, a component of the mammalian germ cell nuage, is essential for spermatogenesis. *SCP3* is a specific meiosis marker that indicates synaptonemal complex formation in meiotic prophase (Fraune et al. 2016). Immunohistochemistry showed that *SCP3* is highly expressed in in goat BMSC-GC cells, but not in untreated BMSCs. Furthermore, both *SCP3* mRNA and protein expression levels significantly increased in the mi-SBD and mi-S + BD groups, and especially in the mi-S + BD group, which had the highest expression level. More importantly, we observed punctate and elongated *SCP3* staining foci using mRNA transfection methods in this present study, indicating the initiation of meiosis, which were not observed when using plasmids transfection in our previous pulished study (Li et al. 2017). Taken together, these results suggested that maturation and progression through meiosis of a small subpopulation of transfected gBMSCs were enhanced, but it has not yet been determined whether these cells can complete meiosis and form functional spermatozoa.

Importantly, erasure of the epigenetic marks at imprinted loci is a process that occurs specifically during mammalian germ cell development just prior to, or shortly after, germ cells arrive to the gonadal ridges. Erasure and re-establishment of epigenetic signatures of imprinted loci is a germline-specific process (Clark 2015). Medrano et al. (2012) found that overexpression of *VASA* in human ESCs and iPSCs led to a significant decrease in the methylation ratio of H19 (Medrano et al. 2012). Nolte et al. (2010) showed the mouse spermatogenic cells induced by RA induction differentiate into haploid male germ cells, whose H19 methylation level also appeared to decline (Nolte et al. 2010). Similar to those previous research, in this study, we also observed that imprinted H19 locus methylation level showed a significant decrease when cells were subjected to ectopic expression of *STRA8*, *BOULE* and *DAZL* mRNA. The gBMSCs in mi-SBD group showed lower H19 methylation level than that of gBMCs in mi-S + BD group. These results suggested that erasure of imprints may be a specialization of *VASA* in direct or indirect regulation of RNAs implicated in DNA methylation/demethylation. Further studies should examine the role of possible

targets of VASA that may be implicated in germline epigenetic reprogramming.

To summarize, after co-transfected or sequential transfected mRNA of *STRA8*, *BOULE*, and *DAZL* into gBMSCs, these cells showed increased expression levels of early- and late-stage germ cell markers and lower H19 methylation level, which indicated that gBMSCs had the ability of differentiation into putative germ cells in vitro. Furthermore, these cells had the potential to enter meiosis. Moreover, the mi-S + BD method of sequential transfection was more efficient than the miSBD co-transfection, indicating that the gene regulation in time order is essential for gametogenesis. These results suggested that maturation and progression through meiosis of a small subpopulation of transfected gBMSCs were enhanced, but it has not yet been determined whether these cells can complete meiosis and form functional spermatozoa. This study provides a novel system for in vitro induction of male GCs from stem cells, and also contributes to understanding the study of molecular mechanisms for spermatogenesis.

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Author's contribution YLZ and PZL conceived and conducted the study and wrote the manuscript, JP, WYJ, WZY, FYX and ZGM helped to perform the experiments, NHT revised the manuscript, FW supervised the project. All authors contributed to discuss the results.

Compliance with ethical standards

Competing interests The authors declare that there are no competing interests associated with the manuscript.

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