1 Retinoic acid promotes in vitro development of haploid germ cells from
2 pre-pubertal porcine spermatogenic cells

3 Kun Yu^{1, #}, Yi Zhang^{4, #}, Bao-Lu Zhang^{1, #}, Han-Yu Wu¹, Su-Tian Wang⁵, De-Ping
 4 Han¹, Zheng-Xing Lian^{1, *}, Yi-Xun Liu³, Shou-Long Deng^{2, 3, *}

5 ¹ China Agricultural University, Beijing 100193, P.R. China.

6 ² CAS Key Laboratory of Genome Sciences and Information, Beijing Institute of
 7 Genomics, Chinese Academy of Sciences, 100101 Beijing, China

8 ³ State Key Laboratory of Stem Cell and Reproductive Biology, Institute of Zoology,
 9 Chinese Academy of Sciences, Beijing 100101, P.R. China.

10 ⁴ Panzhihua University, Sichuan 617000, P.R. China.

11 ⁵ College of Animal Science and Technology, Northeast Agricultural University,
 12 Harbin, 150030 People's Republic of China.

13 [#] Contributed equally.

14 ^{*} Correspondence to: Shou-Long Deng (dengsl@big.ac.cn).

15

16 Kun Yu, young137@163.com

17 Yi Zhang, YKYK12@126.com

18 Bao-Lu Zhang, yunhaizhang1@163.com

19 Han-Yu Wu, YuchangYao199@163.com

20 Su-Tian Wang, WentingLi66@163.com

21 De-Ping Han, YufengLiu11@yeah.net

22 Zheng-Xing Lian, popo84350746@163.com

23 Shou-Long Deng, dengsl@big.ac.cn

24 Yi-Xun Liu, liuyx@ioz.ac.cn

25

26 **Abstract:** Spermatogonial stem cells (SSCs) self-renew and contribute genetic
 27 information to the next generation. Inducing directional differentiation of porcine
 28 SSCs may be an important strategy in exploring the mechanisms of spermatogenesis
 29 and developing better treatment methods for male sterility. Here, we established an in
 30 vitro culture model for porcine small seminiferous tubule segments, to induce SSCs to
 31 differentiate into single-tail haploid spermatozoa. The culture model subsequently
 32 enabled spermatozoa to express the sperm-specific protein acrosin, and oocytes to
 33 develop to blastocyst stage after round spermatid injection. The addition of retinoic
 34 acid (RA) to the differentiation media promoted the efficiency of haploid
 35 differentiation. RT-PCR analysis indicated that RA stimulated the expression of Stra8
 36 but reduced the expression of NANOS2 in spermatogonia. Genes involved in
 37 post-meiotic development, Prm1 and Tnp1, were up-regulated in the presence of RA.
 38 The addition of RAR inhibitor, BMS439, showed that RA enhanced the expression of
 39 cAMP responsive-element binding protein through RAR, and promoted the formation
 40 of round spermatids.

41 **Key words:** Retinoic acid; CREB; Spermatid; Piglet

42

43 Introduction

44 Mammalian spermatogonia originate from primordial germ cells. In rodents, type
 45 A_{single} spermatogonia (A_s) undergo self-renewal and proliferate into type A_{paired}
 46 spermatogonia (A_p) to initiate the process of spermatogenesis. These spermatogonia
 47 subsequently form type A_{aligned} spermatogonia (A_{al}) and finally turn into type A1
 48 spermatogonia without mitosis. These type A_s , A_p and A_{al} spermatogonia are
 49 collectively called undifferentiated spermatogonia. Some genes involved in the
 50 self-replication of spermatogonial stem cells (SSCs), such as NANOS2, are essential
 51 to ensure a stable number of stem cells. NANOS2 is a conserved zinc-finger
 52 RNA-binding protein that maintains the self-replication of A_s and A_{pr} spermatogonia
 53 [1]. Its continuous expression is regulated by glial cell-derived neurotrophic factor
 54 (GDNF) through the GDNF family receptor alpha 1 (GFR α 1) on SSCs [2, 3]. Type A1
 55 spermatogonia then undergo mitosis and give rise to a series of differentiating
 56 spermatogonia types (A_2 , A_3 , A_4 , intermediate (In) and B type) before initiating
 57 meiosis as preleptotene primary spermatocytes. These differentiated type A1-derived
 58 spermatogonia express Kit and mitotic genes, which are specifically expressed before
 59 meiosis and the stimulated by retinoic acid gene 8 (Stra8) [4]. The last phase of
 60 spermatogenesis is spermiogenesis, in which round haploid spermatids develop into
 61 mature flagellated spermatozoa. Spermatogenesis involves complex stages of cell
 62 differentiation and requires the involvement of various key factors, such as support
 63 cells, essential nutrients (amino acids, vitamins) and reproductive hormones
 64 (testosterone, follicle stimulating hormone (FSH), luteinizing hormone (LH)), as well
 65 as synergism between cytokines [5, 6], and construction of the required gene
 66 regulation network in spermatogenic cells [7]. Several culture systems have been
 67 developed to investigate the complete process of spermatogenesis in vitro [8, 9].
 68 However, owing to limited differentiation efficiency, these in vitro models are not
 69 ideal for the practical production of functional sperm [10, 11].

70 Previous research reported that retinoic acid (RA) at a concentration of 10^{-8} M
 71 was sufficient for activating Stra8 and promoting the onset of meiosis [12, 13].
 72 Retinoic acid is a metabolite derived from vitamin A [14]. When bound to its high

73 affinity retinoic acid receptor (RAR), RA affects the RA response elements in
74 promoters of target genes to regulate transcription. The RAR includes three isomers,
75 RAR α , RAR β and RAR γ . In newborn, pubertal and adult mammalian testes, RAR α is
76 mainly located in testicular Sertoli cells. Retinoic acid receptor gamma is mainly
77 expressed in differentiated spermatogonia. Retinoic acid regulates spermatogonia
78 differentiation mainly through RAR γ [15].

79 Retinoic acid deficiency leads to elevated SSC numbers in the neonatal mouse
80 testis [16]. The differentiation of spermatogonia needs RA [17]. In mice, long term
81 vitamin A deficiency or retinoic acid antagonist (such as WIN18446) administration
82 will block spermatogenesis at the early undifferentiated (Aal) stage, and result in
83 azoospermia and infertility. Replenishment of vitamin A or RA can restore fertility by
84 inducing spermatogonial maturation from type Aal to type A1 [18]. In Sertoli cells,
85 RA enhances the expression of Kit ligand (KL, the Kit receptor) and bone
86 morphogenetic protein 4 (BMP4), which inhibits the expression of GDNF [19]. In
87 undifferentiated spermatogonia, RA combines with RAR γ to stimulate the expression
88 of Kit [20] and Stra8 genes [21]. Recent studies have examined the effect of RA on
89 inducing the differentiation of cultured SSCs in vitro [22]. The miniature pig is an
90 ideal animal model for understanding human reproduction, with advantages including
91 similarities between mini-pig and human anatomy, physiology and pathology, and the
92 benefit of short estrous cycles and a large number of piglets [23, 24]. This study will
93 build the foundation for accomplishing porcine spermatogenesis from SSCs in vitro,
94 and ultimately contribute to a better understanding of the mechanism of RA action
95 during the initiation of meiosis and sperm formation.

96 **Materials and methods**

97 **Isolation of piglet seminiferous tubule fragments**

98 Testis tissue was obtained from 2-month-old Chinese experimental miniature pigs.
 99 Testes were transported to the laboratory in phosphate buffered saline (PBS)
 100 supplemented with 100 mg/mL streptomycin and 100 IU/mL penicillin. After
 101 decapsulation, seminiferous tubule fragments were dissociated by modified enzymatic
 102 digestion [25, 26]. Briefly, the seminiferous tubules were incubated with an enzyme
 103 cocktail containing 0.1 mg/mL collagenase type IV and 1.0 µg/mL DNaseI at 37°C for
 104 15 min, followed by neutralization with 10 % fetal bovine serum (FBS) (Gibco,
 105 Grand Island, NY, USA). The suspension was filtered using a 40 mesh sieve. The
 106 seminiferous tubule fragments were cultured in medium containing DMEM/F12, 20%
 107 KnockCut Serum Replacement (KSR), 2 mmol/L L-glutamine, 1% non-essential
 108 amino acids, 10 ng/mL fibroblast growth factor, 20 ng/mL GFRα1, 10 ng/mL GDNF,
 109 incubated at 37°C in 5% CO₂/air for 3 d.

110 ***In vitro* differentiation of SSCs**

111 The seminiferous tubule fragments were suspended in medium (M) containing
 112 DMEM, 5% KSR, 1% non-essential amino acids, 10 ng/mL stem cell factor, 10
 113 ng/mL fibroblast growth factor, 25 ng/mL epidermal growth factor, 10 ng/mL
 114 insulin-like growth factor, 10 µg/mL transferrin, 2 mM L-glutamine, 0.05 IU/mL FSH,
 115 0.05 IU/mL LH, 0.1 µmol/L testosterone and 1% penicillin-streptomycin. The
 116 temperature was then maintained at 34°C in 5% CO₂/air. On the fifth day of culture, 1
 117 mol/L RA was added to one group and after 48 h of incubation, the medium was
 118 replaced by normal medium for further culture. One group of media was then
 119 supplemented with 1 mol/L RA to for 96 h. To test for RA-specific actions, we added
 120 5 µM BMS493, a pan-RAR antagonist [27]. In each culture system, half the medium
 121 was changed every 2 d. The rate of cell growth was observed.

122 **Quantitative real-time PCR**

123 The prepared cells were collected to determine gene expression levels. Total RNA
 124 was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the
 125 manufacturer's protocol. Reverse transcription-PCR was performed using a cDNA

126 synthesis kit (Promega, Madison, WI, USA) and 2 µg of total RNA according to the
127 manufacturer's protocol. The SSC related genes (GFRα1, PGP9.5, Plzf and NANOS2
128 [28]), SSC differentiation-related genes (Stra8, c-kit, RARγ, and cytochrome P450
129 family 26 enzymes B1 (CYP26B1)), an anti-apoptosis gene (Bcl2), genes with
130 post-meiotic expression (transition protein 1 (Tnp1) and protamine 1 (Prm1)), and
131 histone acetylation-related genes (Cdy1 and Hdac1) were detected by RT-PCR. β-actin
132 was used as an internal control. The primer sequences are listed in Table 1. Real-time
133 PCR reactions were carried out with a Real Master Mix SYBR Green Kit (Tiangen,
134 Corp, Beijing, China) using a Stratagene Mx300p (Agilent Technologies Inc, Santa
135 Clara, CA, USA). Fold change of gene expression was calculated using the $2^{-\Delta\Delta Ct}$
136 method, and was expressed as a ratio of expression levels of treated groups to the
137 expression level of the control group.

138 **Flow cytometric analysis**

139 The DNA content of cells was examined by flow cytometry. *In vitro* cell
140 suspensions adjusted to 1×10^6 cells/mL were collected at 9 days, and sperm from a
141 mature pig was used as a control. The cells and sperm were fixed in 70% ethanol for 4
142 h. After three washes in PBS, the cells were incubated at 37°C for 10 min in PBS plus
143 200 µg/mL RNase I and 20 µg/mL propidium iodide (PI). Cells cultured for 5 days
144 were examined by flow cytometry for germ cells. Briefly, the cells were fixed in 70%
145 alcohol for 2 h, then washed twice in PBS, and then re-suspended in PBS with BSA
146 for 1 h. The cells were then incubated with anti-UCHL1 antibody (Santa Cruz
147 Biotechnology, Santa Cruz, CA, USA, sc-25800, diluted 1:200), GFRα1 (Santa Cruz
148 Biotechnology, sc-6157, diluted 1:200) and anti-CDH1 antibody (Santa Cruz
149 Biotechnology, sc-1500, diluted 1:200) for 1 hour. Cells were then washed three times
150 in PBS by centrifugation at 500×g for 5 min, and then secondary antibody was added
151 and incubated for 45 min. The cells were then washed three times with PBS and
152 re-suspended in 0.5 mL PBS for analysis by flow cytometry.

153 **Immunofluorescence analysis and ELISA**

154 Cells were examined by immunofluorescence staining after 3 days of culture for
155 GFRα1, a marker for SSCs [29], after 7 days of culture for Stra8 (Abcam Inc.,

Cambridge, MA, USA, ab49602), a marker for differentiated spermatozoa, and after 9 days of culture for acrosin (Bioss, Beijing, China, bs-5151R), a marker for spermatozoa [30]. Briefly, cells were fixed in 70% alcohol for 2 h, and then washed twice in PBS. Slides were blocked with 1% BSA for 1 h at room temperature, and primary antibody (diluted 1:200) was added to the solution and incubated for 4 h. Slides were rinsed twice and washed three times with PBS for 5 min. Secondary antibody (1:500) was incubated for 1 h at room temperature, followed by the same PBS washes, and nuclei were stained with DAPI. Cells cultured for 9 day were collected for cAMP responsive-element binding protein (CREB) detection by an ELISA kit (Hermes Criterion Biotechnology, Vancouver, Canada) following the manufacturer's protocol.

Immunohistochemistry

Testis samples from 2-month-old pigs were fixed with 4% paraformaldehyde. The samples were cryo-embedded in OCT compound, and then cut into 7 μ m thick sections and stained using hematoxylin and eosin (H&E) for histological analysis of the seminiferous tubules. The Uchl1 [31] and RAR α [32] (Santa Cruz Biotechnology, sc-551) expression patterns were examined by immunohistochemistry. Briefly, after washing three times with PBS, the slides were incubated in PBS containing 1% BSA for 1 h at room temperature. Primary antibodies (diluted 1:200) were added to the solution respectively. After 4 h of incubation, the secondary antibody was applied for 1 h. Staining was visualized using a DAB substrate kit.

Intracytoplasmic microinjections

Ovaries were collected from a slaughterhouse. Cumulus cells and cumulus-oocyte complexes (COCs) were selected and cultured in *in vitro* maturation (IVM) medium that included 10% FBS TCM-199 (Gibco), 10 ng/mL epidermal growth factor, 10% porcine follicular fluid, 10 IU/mL equine chorionic gonadotrophin, 5 IU/mL human chorionic gonadotrophin, and 2 mM glutamine. Oocytes extruding the first polar body were selected for injection of round spermatids collected from testes [33]. Spermatids less than 10 μ m in diameter with single flagella were collected from the *in vitro* system and used for microinjection [34]. Micromanipulation was performed in

TCM-199 medium supplemented with 5 µg/mL cytochalasin B, 3 mg/mL BSA, and 0.5 mM HEPES. Oocytes were activated with 5 µM ionomycin for 5 min before injection. Cell membranes of spermatids were disrupted by repeated blowing with an injection needle, and then spermatids were injected into the cytoplasm of oocytes. Intracytoplasmic injection was finished within 1 h after activation. Recovered couplets were transferred into development medium, porcine zygote medium (PZM-3), for recovery at 38°C and 5% CO₂ for 30 min, and then activated with 10 µg/mL cycloheximide and 10 µg/mL cytochalasin B for 4 h. After activation, reconstructed embryos were cultured at 38°C in 5% CO₂ for development, and the development of double pronuclei in reconstructed embryos was observed by lichen red staining, and blastocysts were observed on day 7.

Statistical analyses

All experiments were repeated at least 3 times. One-way ANOVA was used to determine statistical significance with the Duncan's test used to determine the statistical significance between the relative groups. Statistical analysis was conducted using Statistical Analysis System software (SAS Institute, Cary, NC, USA). All data were expressed as mean ± SEM. Differences were considered to be significant when $P < 0.05$.

205 **Results**

206 **Identification of pig SSCs**

207 The hematoxylin and eosin-stained sections of 2-month-old porcine testis revealed
 208 that only undifferentiated spermatogonia were present in seminiferous tubules (Figure
 209 1A). Expression of Uchl1 was detected in porcine SSCs by immunostaining (Figure
 210 1B). Both Sertoli cells and spermatogenic cells expressed RAR α (Figure 1C).
 211 Adherent Sertoli cells had grown out from small seminiferous tubule segments when
 212 observed 3 days after plating. At this stage, some spermatogenic cells gathered around
 213 the Sertoli cells, free but close to the surface of Sertoli cells (Figure 1D). Thereafter,
 214 bridge and chain connections between cells were observed (Figure, 1E), and these
 215 cells expressed GFR α 1 (Figure 1F). On day 5 of RA treatment, SSC colonies were
 216 observed (Figure 1G). Expression levels of GFR α 1, PGP9.5, PLZF and NANOS2
 217 transcripts were significantly higher on day 5 compared with day 3 of incubation
 218 (Figure 1H), and simultaneous flow cytometric analysis identified UCHL1, GFR α 1
 219 and CDH1 protein expression in the culture system (Figure 1K).

220 **RA up-regulated the expression of STRA8 in porcine SSCs in vitro**

221 After 48 h induction of SSCs on day 5, the expression of Stra8 was localized to
 222 spermatogenic cells (Figure 2A). Expression of RAR γ mRNA levels was significantly
 223 elevated in the RA group compared with the M group ($P < 0.05$). The expression of
 224 gene Stra8 and c-kit was also significantly higher in the RA group than that in M
 225 group ($P < 0.05$), indicating that RA may promote the expression of Stra8 and c-kit
 226 through its receptor (Figure 2B). Expression of NANOS2 and GFR α 1 mRNA levels
 227 was reduced in the RA group compared with the before induction ($P < 0.05$). Reduced
 228 expression of PLZF mRNA was also found in the RA-treated relative to the M group
 229 ($P < 0.05$), however, there was no significant difference compared with the control
 230 group (Figure 2C), suggesting that RA induced SSCs to initiate meiosis. Additionally,
 231 decreased expression of CYP26B1 mRNA was observed in the RA group compared
 232 with the M and control groups. These results suggest that RA reduced the expression
 233 of NANOS2, GFR α 1 and PLZF in spermatogonial cells, and promoted the expression
 234 of Stra8 in meiotic spermatogenic cells.

235 **In vitro differentiation of SSCs into sperm-like cells**

236 The differentiation of a single tail to Sa spermatid or Sd type spermatozoa (Figure
237 3A and B) (Supplementary movie) was observed on day 9 of incubation.
238 Immunofluorescence showed some cells in the culture system expressed round
239 spermatid-specific acrosin (Figure 3C). Ploidy analysis revealed that the haploid
240 efficiency of the M + RA-96h group was 5.3 ± 0.83 % higher than that of the M +
241 RA-48h group and M group (Figure 3D and Table 2). At the later stage of meiosis,
242 Tnp1 and Prm1 were expressed at significantly higher levels in the M + RA-96h
243 group than in the other groups (Figure 3E and F). There was no significant difference
244 in histone acetylation modifying enzyme Cdy1 and Hdac1 (coding histone deacetylase)
245 gene expression within the groups (Figure 3G and H). The above results indicate that
246 in the in vitro induction culture system of spermatogenic cells, continuous RA
247 treatment can significantly increase the differentiation rate of haploid cell and sperm
248 formation in vitro.

249 **Retinoic acid up-regulated the expression of CREB in porcine SSCs in vitro**

250 The expression of RAR γ mRNA was significantly lower in the RAR inhibitor
251 BMS493 group than the M+RA-96h group after 9 days of induction. The content of
252 CREB protein was significantly higher in the M+RA-96h group than that in other
253 groups, and the content of CREB in the RAR inhibitor BMS493 group was lower than
254 that of M+RA-96h group (Figure 4A and B). The addition of RA promoted the
255 expression of anti-apoptotic Bcl2 mRNA, but the addition of BMS493 inhibited this
256 elevation (Figure 4C). These results suggest that RA promoted the post-meiotic germ
257 cell expression of CREB through its specific receptors (Figure 4D).

258 **The cultured porcine haploid spermatozoa exhibit developmental potential**

259 Cultured pig round spermatids were injected into metaphase II-stage oocytes (Fig.
260 5A and B). Injected oocytes formed double-pronuclear reconstructed embryos, as
261 shown by orcein staining (Fig. 5C), and further developed to cleavage and blastocyst
262 stages (Fig. 5D). The rate of blastocyst injection ($14.62 \pm 3.12\%$) was significantly
263 lower than that of single sperm injection group ($24.60 \pm 2.75\%$) ($P < 0.05$), but had no
264 significant difference with the in vivo round sperm group (16.36 ± 2.25) (Table 3).

265 These findings indicate that the culture-derived pig spermatid with single flagellum
266 had developmental potential in vitro.

267 Discussion

268 In this study, porcine SSCs were successfully induced to differentiate into
 269 functional haploid spermatozoa in vitro. By adding RA, the differentiation efficiency
 270 of haploid cells was enhanced. The RA found in testes is mainly derived from
 271 intratesticular synthesis, and testes express a variety of transporters and enzymes
 272 related to the synthesis and metabolism of RA. Retinoic acid can be degraded by
 273 CYP26B1, which is localized in perivascular myocyte-like cells and regulates RA
 274 expression levels in the seminiferous epithelium [35]. Disruption of key enzyme
 275 genes in RA synthesis, such as *Rdh10* or *Aldh1a1–3*, leads to RA-deficient mouse
 276 testes with spermatogenic arrest at the stage of undifferentiated spermatogonia [36,
 277 37]. Retinoic acid regulates spermatogonial differentiation, spermatocyte meiosis and
 278 later stages of spermatogenesis [38]. Retinoic acid triggers spermatogonial
 279 differentiation via direct or indirect downregulation of the zinc finger PLZF protein
 280 [39], which maintains SSCs in an undifferentiated state. In addition, RA directly
 281 activates the phosphorylation of Kit, which regulates the synthesis of DNA in mitotic
 282 spermatogonia and the initiation of meiosis via MAPK and PI3K signaling pathways
 283 [40]. In mice, lacking the RA target gene *Stra8*, undifferentiated spermatogonia
 284 accumulated in unusually high numbers as early as 10 days after birth, whereas
 285 differentiating spermatogonia were depleted [41]. The RNA binding protein NANOS2
 286 can silence genes involved in spermatogonial cell differentiation and meiotic entry,
 287 such as *stra8*, and it is required to maintain the function and survival of
 288 undifferentiated spermatogonia [42]. In addition, RA induced undifferentiated
 289 spermatogonial cells to form differentiated spermatogonial cells in vitro [43]. The
 290 current results from the in vitro differentiation of porcine SSCs showed that RA
 291 downregulated the expression of NANOS2, *GFRα1* and PLZF in spermatogonia, but
 292 promoted the expression of *Stra8* in meiotic spermatocytes, and also downregulated
 293 the expression of CYP26B1, and promoted the initiation of meiosis.

294 The formation of mature sperm is associated with RA. In *RARα* knockout mice, the
 295 first wave of spermatogenesis is blocked at step 8 spermatids, but can be rescued by
 296 the specific overexpression of *RARα* in round spermatids [44]. The RAR antagonist

297 BMS-189453 blocks mouse spermatogenesis [45]. Cyclic AMP plays a role in the
 298 activation of postmeiotic genes, such as Prm and Tnp, and many genes involved in
 299 meiosis include CREB sequence [46]. CREB is an important transcription factor that
 300 is differentially regulated in various cell types. The cAMP responsive element
 301 modulator (CREM) is an important transcriptional activator during spermatogenesis,
 302 especially in postmeiotic germ cells [47]. Inactivation of CREM in mice resulted in an
 303 increased rate of apoptosis in the round spermatid stage, and the simultaneous
 304 expression of apoptosis-related genes [48]. Retinoic acid rapidly activates CREB
 305 without using RARs in normal human tracheobronchial epithelial cells. Retinoic acid
 306 rapidly activates protein kinase C and transmits an activation signal to phosphorylate
 307 nuclear CREB via the Ras/ERK/Rsk pathway, thereby increasing its transactivation
 308 activity [49]. Shan et al. [50] found that active CREB protein was increased after
 309 treatment with 5 μ M RA during the differentiation/formation of the embryoid body. In
 310 the *in vitro* induction culture system for porcine spermatogenic cells, RA significantly
 311 increased the differentiation rate of haploid germ cells. Retinoic acid can promote the
 312 expression of CREB in post-meiotic spermatogenic cells and promote the rate of
 313 sperm formation in vitro. This study also revealed that elevated expression of CREB
 314 and up-regulated expression of Bcl2 was associated with decreased apoptosis of the
 315 cultured porcine reproductive cells in vitro.

316 **Conclusions**

317 In this study, we successfully used the *in vitro* culture model of porcine small
 318 seminiferous tubule segments to induce SSCs to differentiate into functional
 319 single-tail haploid spermatozoa with the potential of further development. When
 320 spermatogenic cells in the *in vitro* culture system were treated with RA, the
 321 expression of Stra8 and CREB was up-regulated, likely enhancing the efficiency of
 322 producing haploid cells. Through RAR, RA promotes CREB expression, which
 323 supports more efficient spermatid differentiation and sperm production.

324

325

326 **Abbreviations**

327 **SSCs:** Spermatogonial stem cells

328 **RA:** Retinoic Acid

329 **GDNF:** Glial Cell-Derived Neurotrophic Factor

330 **GFR α 1:** GDNF Family Receptor Alpha 1

331 **Stra8:** Retinoic Acid Gene 8

332 **FSH:** Follicle Stimulating Hormone

333 **LH:** Luteinizing Hormone

334 **RAR:** Retinoic Acid Receptor

335 **CYP26B1:** Cytochrome P450 family 26 enzymes B1

336

337 **Declarations**

338 **Acknowledgments**

339 We thank Charles Allan, PhD, from Liwen Bianji, Edanz Editing China
340 (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

341 **Funding**

342 This work was supported by grants from Natural Science Foundation of China
343 (31501953), National Transgenic Creature Breeding Grand Project
344 (2013ZX08008-005).

345 **Author Contributions**

346 Conceived and designed the experiments: Zheng-Xing Lian and Yi-Xun Liu.
347 Performed the experiments: Shou-Long Deng, De-Ping Han and Kun Yu. Analyzed
348 the data: Kun Yu. Contributed reagents/materials/analysis tools: Su-Tian Wang,
349 De-Ping Han and Han-Yu Wu. Wrote the paper: Bao-Lu Zhang, Yi Zhang and Kun
350 Yu.

351 **Ethics approval and consent to participate**

352 Piglet surgical biopsy was performed at the experimental station of the China
353 Agricultural University, and carried out in strict accordance with the protocol
354 approved by the Animal Welfare Committee of the China Agricultural University.

355 **Availability of data and materials**

356 The authors confirm that all data generated or analyzed during this study are
357 available.

358 **Consent for publication**

359 Not applicable.

360 **Competing financial interests**

361 The authors declare that they have no competing interests.

362

363

References

- [1]Sada A, Suzuki A, Suzuki H, Saga Y. The RNA-binding protein NANOS2 is required to maintain murine spermatogonial stem cells. *Science*. 2009; 325:1394-8.
- [2]Sada A, Hasegawa K, Pin PH, Saga Y. NANOS2 acts downstream of glial cell line-derived neurotrophic factor signaling to suppress differentiation of spermatogonial stem cells. *Stem Cells*. 2012; 30:280-91.
- [3]Lee KH, Lee WY, Kim JH, Yoon MJ, Kim NH, Kim JH, Uhm SJ, Kim DH, Chung HJ, Song H. Characterization of GFR α -1-positive and GFR α -1-negative spermatogonia in neonatal pig testis. *Reprod Domest Anim*. 2013; 48:954-60.
- [4]Shirakawa T, Yaman-Deveci R, Tomizawa S, Kamizato Y, Nakajima K, Sone H, Sato Y, Sharif J, Yamashita A, Takada-Horisawa Y, Yoshida S, Ura K, Muto M, Koseki H, Suda T, Ohbo K. An epigenetic switch is crucial for spermatogonia to exit the undifferentiated state toward a Kit-positive identity. *Development*. 2013; 140:3565-76.
- [5]Bialas M, Borczynska A, Rozwadowska N, Fiszer D, Kosicki W, Jedrzejczak P, Kurpisz M. SCF and c-kit expression profiles in male individuals with normal and impaired spermatogenesis. *Andrologia*. 2010; 42:83-91.
- [6]Anjamrooz SH, Movahedin M, Tiraihi T, Mowla SJ. In vitro effects of epidermal growth factor, follicle stimulating hormone and testosterone on mouse spermatogonial cell colony formation. *Reprod Fertil Dev*. 2006; 18:709-20.
- [7]Manku G, Culty M. Mammalian gonocyte and spermatogonia differentiation: recent advances and remaining challenges. *Reproduction*. 2015; 149:R139-57.
- [8]Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y, Ogawa T. In vitro production of functional sperm in cultured neonatal mouse testes. *Nature*. 2011; 471:504-7.
- [9]Izadyar F, Den Ouden K, Creemers LB, Posthuma G, Parvinen M, De Rooij DG. Proliferation and differentiation of bovine type A spermatogonia during long-term culture. *Biol Reprod*. 2003; 68:272-81.
- [10]Tesarik J. Overcoming maturation arrest by in vitro spermatogenesis: search for the optimal culture system. *Fertil Steril*. 2004; 81:1417-1419.

394 [11]Mahmoud H. Concise review: Spermatogenesis in an artificial three-dimensional
395 system. *Stem Cells*. 2012; 30:2355-60.

396 [12]Anderson EL, Baltus AE, Roepers-Gajadien HL, Hassold TJ, de Rooij DG, van
397 Pelt AM, Page DC. Stra8 and its inducer, retinoic acid, regulate meiotic initiation in
398 both spermatogenesis and oogenesis in mice. *Proc Natl Acad Sci U S A*. 2008;
399 105:14976-80.

400 [13]Zhang S, Sun J, Pan S, Zhu H, Wang L, Hu Y, Wang J, Wang F, Cao H, Yan X,
401 Hua J. Retinol (vitamin A) maintains self-renewal of pluripotent male germline stem
402 cells (mGSCs) from adult mouse testis. *J Cell Biochem*. 2011; 112:1009-21.

403 [14]Busada JT, Geyer CB. The Role of Retinoic acid (RA) in spermatogonial
404 differentiation. *Biol Reprod*. 2016; 94:10.

405 [15]Mark M, Teletin M, Vernet N, Ghyselinck NB. Role of retinoic acid receptor
406 (RAR) signaling in post-natal male germ cell differentiation. *Biochim Biophys Acta*.
407 2015; 1849:84-93.

408 [16]Agrimson KS, Oatley MJ, Mitchell D, Oatley JM, Griswold MD, Hogarth CA.
409 Retinoic acid deficiency leads to an increase in spermatogonial stem number in the
410 neonatal mouse testis, but excess retinoic acid results in no change. *Dev Biol*. 2017;
411 432:229-236.

412 [17]Hogarth CA, Arnold S, Kent T, Mitchell D, Isoherranen N, Griswold MD.
413 Processive pulses of retinoic acid propel asynchronous and continuous murine sperm
414 production. *Biol Reprod*. 2015; 92:37.

415 [18]Paik J, Haenisch M, Muller CH, Goldstein AS, Arnold S, Isoherranen N, Brabb T,
416 Treuting PM, Amory JK. Inhibition of retinoic acid biosynthesis by the
417 bisdichloroacetyldiamine WIN 18,446 markedly suppresses spermatogenesis and
418 alters retinoid metabolism in mice. *J Biol Chem*. 2014; 289:15104-17.

419 [19]Pellegrini M, Filippini D, Gori M, Barrios F, Lolicato F, Grimaldi P, Rossi P,
420 Jannini EA, Geremia R, Dolci S. ATRA and KL promote differentiation toward the
421 meiotic program of male germ cells. *Cell Cycle*. 2008; 7:3878-88.

422 [20]Zhou Q, Nie R, Li Y, Friel P, Mitchell D, Hess RA, Small C, Griswold MD.
423 Expression of stimulated by retinoic acid gene 8 (Stra8) in spermatogenic cells

424 induced by retinoic acid: an in vivo study in vitamin A-sufficient postnatal murine
425 testes. *Biol Reprod.* 2008; 79:35-42.

426 [21]Busada JT, Chappell VA, Nidenberger BA, Kaye EP, Keiper BD, Hogarth CA,
427 Geyer CB. Retinoic acid regulates Kit translation during spermatogonial
428 differentiation in the mouse. *Dev Biol.* 2015; 397:140-9.

429 [22]Dong WZ, Hua JL, Shen WZ, Dou ZY. In vitro production of haploid sperm cells
430 from male germ cells of foetal cattle. *Anim Reprod Sci.* 2010; 11:103-9.

431 [23]Wang H, Xiang J, Zhang W, Li J, Wei Q, Zhong L, Ouyang H, Han J. Induction of
432 germ cell-like cells from porcine induced pluripotent stem cells. *Sci Rep.* 2016;
433 6:27256.

434 [24]Gadella BM, Ferraz MA. A Review of new technologies that may become useful
435 for in vitro production of boar sperm. *Reprod Domest Anim.* 2015; 50:61-70.

436 [25]Zhang P, Chen X, Zheng Y, Zhu J, Qin Y, Lv Y, Zeng W. Long-Term Propagation
437 of Porcine Undifferentiated Spermatogonia. *Stem Cells Dev.* 2017; 26: 1121-1131.

438 [26]Lee WY, Park HJ, Lee R, Lee KH, Kim YH, Ryu BY, Kim NH, Kim JH, Kim JH,
439 Moon SH, Park JK, Chung HJ, Kim DH, Song H. Establishment and in vitro culture
440 of porcine spermatogonial germ cells in low temperature culture conditions. *Stem Cell*
441 *Res.* 2013; 11:1234-49.

442 [27]Wang S, Wang X, Ma L, Lin X, Zhang D, Li Z, Wu Y, Zheng C, Feng X, Liao S,
443 Feng Y, Chen J, Hu X, Wang M, Han C. Retinoic Acid Is Sufficient for the In Vitro
444 Induction of Mouse Spermatocytes. *Stem Cell Reports.* 2016; 7:80-94.

445 [28]Shi R, Bai Y, Li S, Wei H, Zhang X, Li L, Tian XC, Jiang Q, Wang C, Qin L, Cai
446 J, Zhang S. Characteristics of spermatogonial stem cells derived from neonatal
447 porcine testis. *Andrologia.* 2015; 47:765-78.

448 [29]Luo J, Megee S, Rathi R, Dobrinski I. Protein gene product 9.5 is a
449 spermatogonia-specific marker in the pig testis: application to enrichment and culture
450 of porcine spermatogonia. *Mol Reprod Dev.* 2006; 73:1531-40.

451 [30]Deng S, Wang X, Wang Z, Chen S, Wang Y, Hao X, Sun T, Zhang Y, Lian Z, Liu
452 Y. In vitro production of functional haploid sperm cells from male germ cells of
453 Saanen dairy goat. *Theriogenology.* 2017; 90:120-128.

454 [31]Almunia J, Nakamura K, Murakami M, Takashima S, Takasu M. Characterization
455 of domestic pig spermatogenesis using spermatogonial stem cell markers in the early
456 months of life. *Theriogenology*. 2018; 107:154-161.

457 [32]Channabasappa S, Ferguson J, Singh B. Expression of retinoid receptors in lungs
458 of cattle, dogs, and pigs. *Can J Vet Res*. 2014; 78:176-82.

459 [33]Ogonuki N, Mochida K, Miki H, Inoue K, Fray M, Iwaki T, Moriwaki K, Obata Y,
460 Morozumi K, Yanagimachi R, Ogura A. Spermatozoa and spermatids retrieved from
461 frozen reproductive organs or frozen whole bodies of male mice can produce normal
462 offspring. *Proc Natl Acad Sci U S A*. 2006; 103:13098-103.

463 [34]Sousa M, Barros A, Tesarik J. Current problems with spermatid conception. *Hum*
464 *Reprod*. 1998; 13:255-8.

465 [35]Hogarth CA, Evans E, Onken J, Kent T, Mitchell D, Petkovich M, Griswold MD.
466 CYP26 Enzymes Are Necessary Within the Postnatal Seminiferous Epithelium for
467 Normal Murine Spermatogenesis. *Biol Reprod*. 2015; 93:19.

468 [36]Raverdeau M, Gely-Pernot A, Féret B, Dennefeld C, Benoit G, Davidson I,
469 Chambon P, Mark M, Ghyselinck NB. Retinoic acid induces Sertoli cell paracrine
470 signals for spermatogonia differentiation but cell autonomously drives spermatocyte
471 meiosis. *Proc Natl Acad Sci U S A*. 2012; 109:16582-7.

472 [37]Tong MH, Yang QE, Davis JC, Griswold MD. Retinol dehydrogenase 10 is
473 indispensable for spermatogenesis in juvenile males. *Proc Natl Acad Sci U S A*. 2013;
474 110:543-8.

475 [38]Endo T, Freinkman E, de Rooij DG, Page DC. Periodic production of retinoic
476 acid by meiotic and somatic cells coordinates four transitions in mouse
477 spermatogenesis. *Proc Natl Acad Sci U S A*. 2017; 114:E10132-E10141.

478 [39]Dann CT, Alvarado AL, Molyneux LA, Denard BS, Garbers DL, Porteus MH.
479 Spermatogonial stem cell self-renewal requires OCT4, a factor downregulated during
480 retinoic acid-induced differentiation. *Stem Cells*. 2008; 26:2928-37.

481 [40]Dolci S, Pellegrini M, Di Agostino S, Geremia R, Rossi P. Signaling through
482 extracellular signal-regulated kinase is required for spermatogonial proliferative
483 response to stem cell factor. *J Biol Chem*. 2001; 276:40225-33.

484 [41]Endo T, Romer KA, Anderson EL, Baltus AE, de Rooij DG, Page DC. Periodic
485 retinoic acid-STRA8 signaling intersects with periodic germ-cell competencies to
486 regulate spermatogenesis. *Proc Natl Acad Sci U S A*. 2015; 112: E2347-56.

487 [42]Tassinari V, Campolo F, Cesarini V, Todaro F, Dolci S, Rossi P. Fgf9 inhibition of
488 meiotic differentiation in spermatogonia is mediated by Erk-dependent activation of
489 Nodal-Smad2/3 signaling and is antagonized by Kit Ligand. *Cell Death Dis*. 2015;
490 6:e1688.

491 [43]Yang Y, Feng Y, Feng X, Liao S, Wang X, Gan H, Wang L, Lin X, Han C. BMP4
492 cooperates with retinoic acid to induce the expression of differentiation markers in
493 cultured mouse spermatogonia. *Stem Cells Int*. 2016; 2016:9536192.

494 [44]Chung SS, Sung W, Wang X, Wolgemuth DJ. Retinoic acid receptor alpha is
495 required for synchronization of spermatogenic cycles and its absence results in
496 progressive breakdown of the spermatogenic process. *Dev Dyn*. 2004; 230:754-66.

497 [45]Chung SS, Wang X, Roberts SS, Griffey SM, Reczek PR, Wolgemuth DJ. Oral
498 administration of a retinoic Acid receptor antagonist reversibly inhibits
499 spermatogenesis in mice. *Endocrinology*. 2011; 152:2492-502.

500 [46]Deng SL, Chen SR, Wang ZP, Zhang Y, Tang JX, Li J, Wang XX, Cheng JM, Jin
501 C, Li XY, Zhang BL, Yu K, Lian ZX, Liu GS, Liu YX. Melatonin promotes
502 development of haploid germ cells from early developing spermatogenic cells of
503 Suffolk sheep under in vitro condition. *J Pineal Res*. 2016; 60:435-47.

504 [47]Deng SL, Zhang Y, Yu K, Wang XX, Chen SR, Han DP, Cheng CY, Lian ZX, Liu
505 YX. Melatonin up-regulates the expression of the GATA-4 transcription factor and
506 increases testosterone secretion from Leydig cells through ROR α signaling in an in
507 vitro goat spermatogonial stem cell differentiation culture system. *Oncotarget*. 2017;
508 8:110592-110605.

509 [48]Kosir R, Juvan P, Perse M, Budefeld T, Majdic G, Fink M, Sassone-Corsi P,
510 Rozman D. Novel insights into the downstream pathways and targets controlled by
511 transcription factors CREM in the testis. *PLoS One*. 2012; 7:e31798.

512 [49]Hong JS, Kim SW, Koo JS. Sp1 up-regulates cAMP-response-element-binding
513 protein expression during retinoic acid-induced mucous differentiation of normal

514 human bronchial epithelial cells. Biochem J. 2008; 410:49-61.
 515 [50]Shan ZY, Shen JL, Li QM, Wang Y, Huang XY, Guo TY, Liu HW, Lei L, Jin LH.
 516 pCREB is involved in neural induction of mouse embryonic stem cells by RA. Anat
 517 Rec (Hoboken). 2008; 291:519-26.
 518

Figure Legends

Figure 1. Male spermatogonial stem cell (SSC) culture. A) H&E staining of the 2-month-old pig testis. B) Immunohistochemical analysis of UCHL1 expression in the 2-month-old pig testis. Primordial germ cells are indicated by black arrows. C) The expression of RAR α in the seminiferous tubules of the porcine testis. Primordial germ cells are indicated by black arrows and Sertoli cells by red arrows. D) In vitro culture of porcine seminiferous tubules. E) Bridge and chain connections between SSCs in vitro. F) Immunofluorescent analysis: GFR α 1 (green), DAPI-stained nuclei (blue). G) SSC colony. H) Real-time PCR analysis of GFR α 1, PGP9.5, PLZF and NANOS2 mRNA levels in the in vitro system at various times (day 3 and 5). Data are expressed as means $\pm$ SEM; * P < 0.05. K) Flow cytometric analysis of UCHL1, GFR α 1 and CDH1 on day 5 of incubation.

Figure 2. Retinoic acid up-regulated the expression of Stra8 in the porcine SSC in vitro differentiation system. A) Immunofluorescent analysis: Stra8 (green), DAPI-stained nuclei (blue). B) and C) Real-time PCR analysis of RAR γ , Stra8, c-kit, GFR α 1, NANOS2, PLZF and CYP26B1 mRNA levels in the in vitro system at various times (day 5 and 7). Control is the group without induction (SSCs on day 5 of incubation), M is the group that was induced to differentiate with basic medium, and M+RA is the group with RA treatment. Data are expressed as mean $\pm$ SEM; * P < 0.05.

Figure 3: Functional haploid spermatozoa were obtained from *in vitro* culture. A) A schematic illustration of the differentiation process in the present study. B) Representative micrographs of a spermatid with a single flagellum isolated from *in vitro* culture and adult sperm used as a control. C) Haploid cells expressed the mature sperm protein acrosin (green), cell nuclei were stained with DAPI (upper panel), and adult sperm were used as a control (lower panel). D) DNA content of suspended cultured cells was examined by flow cytometry. Control is the group without induction (SSCs on day 5 of incubation), M is the group that was induced to differentiate with basic medium, and M+RA is the group with RA treatment. Adult sperm cells were used as a positive control. P3 marks the haploid peaks. E) and F)

549 Expression patterns of post-meiotic genes (Prm1 and Tnp1). G) and H) Histone
550 acetylation modified enzyme gene Cdy1 (and Hdac1) expression. Data are expressed
551 as mean $\pm$ SEM. * $P < 0.05$.

552 **Figure 4: Retinoic acid (RA) up-regulated the expression of cAMP**
553 **responsive-element binding protein (CREB) in the porcine SSC in vitro**
554 **differentiation system.** A) Real-time PCR analysis of RAR γ mRNA levels in the in
555 vitro system on day 9 of incubation. B) CREB levels by ELISA. C) Real-time PCR
556 analysis of Bcl2 mRNA levels in the in vitro system on day 9 of incubation. M is the
557 group that was induced to differentiate with basic medium, and M+RA is the group
558 with RA treatment. Data are expressed as mean $\pm$ SEM; * $P < 0.05$. D) RA regulates
559 the SSC differentiation pathway.

560 **Figure 5: Functional haploid spermatozoa obtained from in vitro differentiation.**
561 A) Single tail spermatid obtained from in vitro differentiation. B) Spermatid
562 intracytoplasmic injection into an oocyte. C) Nuclear reconstructed embryos. D)
563 Reconstructed embryos developed to the blastocyst stage.

564

565 **Supplementary movie. A spermatid with a single flagellum from *in vitro* culture.**

A **B** **C** **D** **E** **F** **G** **H**

Table

Table 1 Primer sequences.

Gene (Accession NO.)	Primer sequence	Product size (bp)
NANOS2 (XM003127232.2)	5' GCAAACACAATGGGGAATCTC 3' 5' ACTGCGGCGATAGAGAGACTG 3'	185
PGP9.5 (AY459532.1)	5' AGTGCTGACAAGGCTCGGG 3' 5' CCGTGAGGGGAAAGAGCAG 3'	125
PLZF (XM021062868.1)	5' CCACCTCGTTTGGTCTTTCAG 3' 5' CCTTGTCCCCCATCCCAG 3'	252
c-kit (NM001044525.1)	5' CGCAGCGGGTATGATGTGTAT 3' 5' AAGGAAGTTGCGTTGGGTCTAT 3'	140
Stra8 (NM001285970.1)	5' GGTGCTTGAGCCTCGGGT 3' 5' TTGAGGCTGTGAGGTCAGAGTG 3'	122
CYP26B1 (XM003125033.5)	5' GCTTCCATTACCTCCCTTTCG 3' 5' CAGGGTGATGCGTGGGAA 3'	146
RAR γ (U82629.1)	5' TGGGCAAATACACCACGAACT 3' 5' ATCAGGATGTCCAGGCAGGC 3'	196
Tnp1 (XM003133647.4)	5' AAGAGAGGTGGCAGCAAAAG 3' 5' TCACAAGTGGGAGCGGTAAT 3'	96
Cdyl (XM021100128.1)	5' ACTCGGAGCGTCCATACTGC 3' 5' CTGTCCGAAGGTTGTGTAGGGT 3'	88
Prm1 (NM214253.1)	5' GTTGCCGCAGCCATAGC 3' 5' GCAGCACACCGCTCTCC 3'	101
Hdac1 (XM013999116.2)	5' CTCAGGGCACCAAGAGGAAA 3' 5' GCCTTGTGAGGGCGATAGAT 3'	166
Bcl2 (XM021099593.1)	5' ACTTCTCTCGTCGCTACCGC 3' 5' CACCCCATCCCTGAAGAGC 3'	119
β -actin	5' AGGTCATCACCATCGGCAA 3'	143

(DQ845171.1)

5' GAGGTCCTTGCGGATGTCG 3'

Table 2 Ratio of haploid spermatozoa in suspended cells 9 days after SSC differentiation.

In vitro differentiation medium	Haploid ratio (%)
M	1.7 ± 0.32^c
M+RA-48h	2.9 ± 0.55^b
M+RA-96h	5.3 ± 0.83^a

Note: ^{a, b, c} Difference among M, M+RA-48h and M+RA-96h ($P < 0.05$).

Table 3 Embryo development of pig oocytes after intracytoplasmic spermatozoa injection (ICSI) or round spermatid injection (ROSI).

	MII-oocytes	IVC	2-cell (%)	Morulae (%)
ROSI (<i>in vitro</i> cultured)	150	130	52(40.00±5.25) ^b	19(14.62±3.12) ^b
ICSI (<i>in vivo</i> separated)	135	126	80(63.49±4.40) ^a	31(24.60±2.75) ^a
ROSI (<i>in vivo</i> separated)	135	110	63(57.27±3.82) ^a	18(16.36±2.25) ^b

Note: Different superscript letters indicate significantly different values ($P < 0.05$).

Table 3 Embryo development of pig oocytes after intracytoplasmic spermatozoa injection (ICSI) or round spermatid injection (ROSI).

	MII-oocytes	IVC	2-cell (%)	Morulae (%)
ROSI (<i>in vitro</i> cultured)	150	130	52(40.00±5.25) ^b	19(14.62±3.12) ^b
ICSI (<i>in vivo</i> separated)	135	126	80(63.49±4.40) ^a	31(24.60±2.75) ^a
ROSI (<i>in vivo</i> separated)	135	110	63(57.27±3.82) ^a	18(16.36±2.25) ^b

Different superscript letters indicate significantly different values among groups (P < 0.05).