Establishment of *Etv5* gene knockout mice as a recipient model for spermatogonial stem cell transplantation

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Abstract: Transplantation of allogeneic spermatogonial stem cells (SSCs) into the testes could generate heterologous sperm and thus produce offspring by natural breeding. This has a significant impact on the conservation of economically valuable and endangered animals, as well as to the understanding of SSCs function. In this study, we report the generation of Ets-variant gene5 (*Etv5*) knockout (KO) mice using the CRISPR/Cas9 system. The testicular weight of *Etv5*$^{-/-}$ mice was significantly lower compared to wild-type (WT) mice ($P<0.01$) at 3 weeks of age. In addition, the germ cell layer of the seminiferous tubules gradually receded with age in *Etv5*$^{-/-}$ mice. At 12 weeks of age, the tubules of *Etv5*$^{-/-}$ mice lacked germ cell layers (Sertoli-cell-only syndrome) and there was a lack of sperm in the epididymis. Based on the above observations, we determined that *Etv5*$^{-/-}$ mice were suitable for SSCs transplantation. We transplanted allogeneic SSCs with or without enhanced green fluorescent protein (EGFP) into 3 week (immature) or 7 week (mature) *Etv5*$^{-/-}$ mice via efferent duct injection. Spermatogenesis was observed in all immature testes but not in mature adult testes two months post-transplantation. In addition, we measured the expression of heterologous gene *Etv5* and *EGFP* in recipient testicular tissue and epididymal sperm by PCR and confirmed that sperm originated from the transplanted donor cells. Our study provides an appropriate animal model for investigating spermatogonial stem cell function and an alternative for mammalian breeding.

Keyword: *Etv*5, SSCs, Gene knockout, Transplantation, Spermatogenesis

36

37 **Introduction**

38 Spermatogonial stem cells (SSCs) are an important component of male germ
39 cells. They form spermatocytes through mitosis and then produce sperm after two
40 consecutive meiosis cycles. SSCs are located on the basement membrane of
41 seminiferous tubules and reside in a very complex micro-environment. Although they
42 account for only 0.02 %-0.035 % of the total germ cells in the testis, they are essential
43 for maintaining a stable number of germ cells. This is via constant self-renewal and
44 differentiation throughout the reproductive cycle [1]. When allogeneic SSCs are
45 transplanted into SSCs-deficient testes, they can repair damaged tissue via
46 spermatogenesis and regeneration [2]. Based on the biological function of SSCs,
47 allogeneic germ cells when transplanted into the recipient's testicles have the
48 capability to undergo spermatogenesis, produce donor-derived sperm, and generate
49 offspring via natural conception. Using this procedure, the conservation of
50 economically valuable and endangered animals could be achieved. In addition, this
51 model will be significant for understanding the function of SSCs.

52 At present, successful SSCs-deficient animal models have been generated
53 through natural mutations, busulan intraperitoneal or testicular injections [3, 4].
54 However, natural mutants are difficult to obtain, and Busan ablation is difficult to
55 perform and has a high mortality rate [5, 6], which significantly limits its use. With
56 the advent of gene-editing tools such as the clustered regularly interspaced short
57 palindromic repeat (CRISPR)/CRISPR-associated 9 (Cas9) artificial nuclease system,

58 animal models with specific mutations could be efficiently generated. Ets-variant
59 gene5 *(Etv5*), a member of the PEA3 family, is an important transcription factor
60 encoded by E-twenty-six (ETS) genes. It is essential for self-renewal, proliferation,
61 and differentiation of SSCs [7]. Tyagi *et al*. [8] observed that *Etv5* KO mice generated
62 by homologous recombination had a degenerative loss of SSCs in the seminiferous
63 tubules, had Sertoli cell-only syndrome and were infertile. Regrettably, they did not
64 perform follow-up studies to determine whether the microenvironment of this model
65 could support the re-spermatogenesis of donor SSCs, nor did they investigate suitable
66 transplantation techniques and transplantation times. In this study, we generated *Etv5*
67 gene KO mouse models using the CRISPR/Cas9 system, and then measured relevant
68 changes in germ cells. In addition, we performed allogeneic SSCs transplantation into
69 $Etv5^{-/-}$ mice testicles. Our study provides a gene-editing method to generate receptor
70 models for SSCs transplantation. In addition, it may provide possible avenues for the
71 conservation of endangered species as well as understanding spermatogonial stem cell
72 function.
73
74 **Materials and methods**
75 **Ethics statement**
76 All animal experiments were performed in accordance with the guidelines of the
77 National Institutes of Health's Guide for the Care and Use of Laboratory Animals.
78 Our study was approved by the IACUCs at South China Agricultural University.
79 **Cell culture**

80 Testes were harvested from 6 to 8 days postpartum C57BL/6 male pups, and then
81 were digested using a two-step enzymatic digestion protocol. Briefly, testes were
82 washed in DPBS with 2% penicillin-streptomycin (Gibco) and then the tunica
83 albuginea and convoluted epididymis were removed. Afterward, the seminiferous
84 tubules were digested in 5 mLs of DPBS solution I consisting of 1 mg/mL collagenase
85 type IV (Gibco, 17104019) and 20 U/mL DNase I (Takara, 2212) at 37 °C for 10 min
86 with intermittent agitation every 5 min. Samples were then centrifuged at 300 g for 5
87 min and the supernatants discarded. The precipitates were washed in DPBS and then
88 incubated in 5 mLs of solution II consisting of 20 U/mL DNase I and 0.25%
89 trypsin/EDTA at 37 °C for 5 min until cells were completely dispersed. Digestion was
90 terminated using 10% fetal bovine serum in DMEM/F12 medium (Gibco). The
91 samples containing single cells and clumps were filtered through a nylon cell strainer
92 with 40 μm pore size (B&D). Single-cell suspensions were collected in the filtrate and
93 then centrifuged. Supernatants were discarded and the cell pellet was washed three
94 times with DPBS, and then resuspended in complete medium. The number and
95 viability of the resulting dissociated single cells had a density of 1.06×10^{7} cells/mL
96 with viability greater than 99%.

97 **Lentivirus infection**

98 Cells were infected with LPP-EGFP-Lv156-400 lentivirus expressing enhanced
99 green fluorescent protein (EGFP) into the genome of donor mice SSCs prior to
100 transplantation. Briefly, 30 μL lentivirus packaging vector (1×10^{8} TU/mL) was added
101 into 1 mL of cell suspension and then seeded into 12-well plates. The cells were

102 incubated at 37 °C and 5% CO_2 atmosphere for up to 12 h. Subsequently, cells were
103 washed once in DPBS and then resuspended gently in DPBS using a Pasteur pipette.
104 Cell suspensions were then transferred into a new 15 mL centrifuge tube and
105 centrifuged. Supernatants were discarded and cells were re-suspended in 300 μL of
106 Hanks' Balanced Salt Solution (HBSS, Gibco, 14170112). The cell suspension was
107 then transferred to a designated transplantation suite for transplantation.

108 **Embryo injections for KO mice production**

109 gRNA sequences are shown in Fig. 1A. The mouse (C57BL/6) *Etv5* gene
110 (GenBank accession number: NM_023794.2; Ensembl: ENSMUSG00000013089) is
111 located on chromosome 16, with thirteen identifiable exons, with an ATG start codon
112 on exon 2 and a TAA stop codon on exon 13. The introns on both sides of exon 1 to 5
113 were selected as the target sites for CRISPR/Cas9-mediated genome editing. A pair of
114 gRNA targeting vectors (pRP[CRISPR]-hCas9-U6) was constructed and confirmed
115 by sequencing: gRNA1 (matching the forward strand of the gene):
116 GAACGGCCATTGTCGGTGGCAGG, and gRNA2 (matching the forward strand of
117 the gene): CTTCTATGCTAATAACGGGTGGG were selected. These two targeting
118 vectors were used for embryo injections, together with Cas9 mRNA. gRNA generated
119 by *in vitro* transcription was then co-injected into fertilized eggs for the generation of
120 KO mice.

121 **Western blot and PCR analysis**

122 CRISPR-induced mutation assays. Female and male heterozygous edited ($Etv5^{+/-}$)
123 mice were bred to generate homozygous edited ($Etv5^{-/-}$) offspring. DNA was extracted

124 from the toes of pups from the same litter at 2 days post-birth for PCR analysis and
125 sequencing. The following two primers were used for genotype identification by PCR;
126 Mouse ETV5 Primer-Forward: 5'-CAACTGGTGCCCTTCCCAGTCT-3', Mouse
127 ETV5 Primer-Reverse: 5'-GCCGCTCTTAAACCTGTTCATTCG-3'. Wild-type (WT)
128 mice were designated as the control group and *Etv5*$^{-/-}$ mice as the experimental group.
129 Polyclonal anti-ERM/ETV5 antibody targeting amino acids 244 of the human
130 ERM/ETV5 was used to measure ETV5 protein expression in *Etv5*$^{-/-}$ male and WT
131 mice by western blotting. The anti-β-actin monoclonal antibody was used as the
132 loading control. *Etv5* expression in male mice was measured by gene and protein
133 expression levels.

134 **Monitoring body and testicular weight**

135 Animal care and use protocols were approved by our institutional review board.
136 Animals were provided sufficient food and water and were housed in appropriate
137 conditions to safeguard their welfare. Bodyweight of 5 male littermates during the
138 development period from 4 postnatal days to adulthood were measured and recorded.
139 Testes weights were determined at weekly intervals from 1 to 12 weeks of age.

140 **H.E. staining and immunohistochemistry**

141 Whole testes were fixed in 4% paraformaldehyde and then paraffin sectioned for
142 hematoxylin-eosin (H.E.) staining and immunohistochemistry. For H.E. staining,
143 paraffin sections were counterstained with hematoxylin and eosin and then observed
144 under an electron microscope for comparing spermatogonium between *Etv5*$^{-/-}$ mice
145 and WT littermates. Immunohistochemistry was performed after antigen retrieval with

146 EDTA (pH9.0), 3% hydrogen peroxide was used for blocking endogenous
147 peroxidase, and 3% BSA was used to block non-specific binding. Afterward, sections
148 were incubated with specific antibodies. Anti-promyelocytic leukemia zinc-finger
149 (PLZF) mouse antibody (sc-28319), a marker of undifferentiated spermatogonia, was
150 used at 1:100 dilution. HRP labeled goat anti-mouse IgG diluted at 1:200 was used as
151 the secondary antibody. The sections were then washed and developed using DAB
152 color rendering and nuclear staining. Hematoxylin staining of the nucleus was in blue
153 and brownish yellow color indicated positive expression of DAB.

154 **Real time-PCR analysis**

155 Total RNA samples were extracted using Eastep Super Total RNA Extraction
156 Kit (LS1040, Promega) according to the manufacturer's instructions. Total RNA was
157 then converted to cDNA using PrimeScript RT reagent Kit with gDNA Eraser
158 (Perfect Real, Takara). mRNA expression levels of *Ccl9*, *Prim2*, and *Cyp17a1* genes
159 were then measured by quantitative PCR using PowerUp™ SYBR™ Green Master
160 Mix (A25742). *β-actin* served as an internal control. Each tested gene from control
161 and experimental samples were performed in triplicate. Relative gene expression was
162 calculated using the $2^{-\Delta\Delta CT}$ method. Primer sequences used for RT-qPCR are shown in
163 Supplementary Table 1.

164 **Measurement of Testosterone levels**

165 Blood was collected from mice via the orbital vein and then centrifuged for serum
166 collection. Testosterone levels in *Etv5*$^{-/-}$ male and WT control littermates at 12 weeks of
167 age were determined using the mouse testosterone ELISA kit. Brief, 50 μL of standard

168 or sample were added to each well, with blank wells left empty. Then 50 μL of
169 HRP-conjugate was added to each well except for the blank well. Afterward, 50 μL of
170 antibody was added to each well. The wells were mixed and then incubated for 1 h at
171 37°C. Contents from each well were then aspirated and washed 3 times. The assay plate
172 was blotted dry and 50 μL of substrate A and 50 μL of substrate B was added to each
173 well and incubated for 15 min at 37°C in the dark. After incubation, 50 μL of stop
174 solution was added to each well, and the optical density (OD) of each well was
175 recorded within 10 min using a microplate reader set to 450 nm.

176 **Semen collection and analysis**

177 Mice were euthanized by cervical dislocation. The unilateral epididymis was
178 then surgically removed, cut into pieces and immersed in 1 mL of semen diluter. After
179 incubation at room temperature, 10 μL sperm diluent was transferred to a
180 hemocytometer to determine the number and vitality of sperm. The average of 3 data
181 records from different mice in each group was then determined. The sperm smear test
182 was used to compare the sperm concentrations between *Etv5*$^{-/-}$ male and WT mice.
183 Briefly, approximately 1 mL of semen diluter (mentioned above) was centrifuged at
184 2000 rpm for 10 min. The resulting precipitate was re-suspended with 500 μL semen
185 diluter followed by the addition of trypan blue (1:2 in volume). Slides were sealed
186 using glycerol gelatin after 10 μL of the sample was placed onto glass slides.

187 **Transplantation**

188 Mice with homozygous mutations of *Etv5* gene were used as recipients for
189 transplantation. Mice were anesthetized intraperitoneally (M2910, 0.2 mL/10g body

190 weight) and then were placed dorsally under a stereomicroscope for the
191 transplantation procedure. Briefly, the cuticular layer at the midline of the abdomen,
192 not far from the genitals, was lifted using small forceps. Then a transverse 0.3 cm
193 incision was made followed by an incision to the peritoneum and abdominal muscle
194 layer until the peritoneal cavity was visible. The lateral fat pad attached to the testis
195 was gently pulled until the testis was exteriorized. The efferent duct that connected
196 the testis to the epididymis was identified and fat tissues around the duct were gently
197 removed. The SSCs suspension was then carefully transferred to a 100 μL volume
198 microinjection syringe. The syringe was then connected into a capillary-glass tube
199 with an inner diameter of 40-50 μm at the tip. The cell suspension was gently forced
200 into the seminiferous tubules of the testis via the efferent duct by applying pressure to
201 the syringe. The injection pipette was held parallel to the ordinate axis of the efferent
202 duct. The injection rate and cell suspension flow rate were controlled manually by
203 monitoring the movement of the cell suspension in the tubules. Approximately 5-10
204 μL of the donor cell suspension was injected into each recipient testis. The other testis
205 was not surgically manipulated and served as a control. After transplantation, the
206 testis was placed back into the peritoneal cavity. The incision was closed with one
207 stitch of a 7.0 absorbable suture from the inside out, with the abdominal muscle layer
208 first, followed by the peritoneum layer, and finally the cuticular layer. Mice were then
209 returned back to their cages. Mice were monitored for distress and assessed regularly
210 until testis samples were collected.

211 **Identification of Allogeneic SSCs**

212 Testes of recipient mice were harvested 2 months after transplantation to
213 determine the functional recovery of spermatogenesis. Recipient testes were fixed in
214 4% paraformaldehyde and embedded in paraffin wax for histomorphology and
215 immunohistochemistry analysis. Heterogenous spermatogenesis was observed under a
216 fluorescence stereomicroscope using EGFP-positive sperm obtained from the
217 epididymis of recipient mice. In addition, sperm smear slides were generated.
218 Expression of exogenous genes in the testes and spermatozoa, i.e., *Etv5* and *EGFP*
219 were measured by PCR (primer sequences are shown in Supplementary Table 1).

220 **Statistical Analysis**

221 PASW Statistics 21 (IBM SPSS, Chicago, IL, USA) was used to determine
222 statistical significance and standard deviation. Bodyweight, testes weight, gene
223 expression, testosterone levels, and cell counts were analyzed using the student *t*-test.
224 Differences were considered significant at $P<0.05$ (*) and $P<0.01$(**).

225

226 **Results**

227 **Generation of *Etv5* KO mice using CRISPR/Cas9**

228 We designed two gRNAs targeting the introns on both sides of exon 1 (gRNA1)
229 to exon 5 (gRNA2) to delete about 5610 bp of the *Etv5* gene to generate $Etv5^{-/-}$ mice
230 by embryo injection of Cas9 mRNA and gRNAs(Fig. 1A). The genotypes of the
231 founder and their offspring were identified using primers F/R (Fig. 1A). DNA gel
232 electrophoresis results demonstrated that homozygous KO mice generated a 710bp
233 band, while heterozygotes had two bands, 710bp, and 6326bp, and wild type mice had

234 a single band of 6326bp (Fig. 1B). PCR and DNA sequencing demonstrated that
235 homozygous KO mice had a 5610 bp deletion (Fig. 1C). In addition, western blot
236 analysis confirmed a lack of ETV5 expression in homozygous *Etv5*$^{-/-}$ KO mice (Fig.
237 1D). Interestingly, *Etv5*$^{-/-}$ mice at 3 weeks of age were obviously thinner compared to
238 WT mice, and this was more pronounced at 12 weeks of age (Sup-Fig. 1A). The body
239 weights of *Etv5*$^{-/-}$ mice were markedly lower at each point from 14 postnatal days to
240 84 days ($P < 0.05$) compared to WT controls, with the magnitude of bodyweight
241 decline between 34.3% to 45.7% (Sup-Fig. 1B).

242 **Lack of SSCs in the seminiferous tubules of *Etv5* KO mice**

243 We measured the weight of testes in *Etv5*$^{-/-}$ mice from day 4 to 12 weeks of age
244 and compared them to WT mice of the same age. The weights were almost similar in
245 mice aged 4 days to 1 week. However, the testes of *Etv5*$^{-/-}$ mice were much smaller
246 compared to WT mice at 3 weeks of age (reduced by as much as 43.1%). The
247 difference became more evident with age, with up to a 67.4% reduction at 12 weeks
248 of age (Fig. 2A and B). H.E. staining demonstrated a remarkable decrease in the
249 number and class of testicular cells in the seminiferous tubules of *Etv5*$^{-/-}$ mice at 3
250 weeks of age. Testicular cells were absent in the tubules of *Etv5*$^{-/-}$ mice and
251 Sertoli-cell-only syndrome was observed at 12 weeks of age (Fig. 2C). Additionally,
252 we measured the protein expression levels of PLZF, which is a marker for SSCs. We
253 observed that PLZF was still expressed in KO mice in some seminiferous tubules at 3
254 weeks of age. However, by 12 weeks of age, SSCs in *Etv5*$^{-/-}$ had completely
255 disappeared, while they were present in WT mice (Fig. 2D). In addition, the number

256 of premeiotic germ cells, meiotic germ cells, and round spermatids gradually declined
257 in *Etv5*$^{-/-}$ mice, and by 12 weeks of age, were undetectable (Fig. 2E). Additionally, the
258 percentage of vacuolation observed in seminiferous tubules both in 3 and 12 week old
259 *Etv5*$^{-/-}$ mice were higher compared to WT control mice, reaching 100% at 12 weeks of
260 age (Fig. 2F). There were no differences in the number of seminiferous tubules
261 between *Etv5*$^{-/-}$ and WT mice at 3 weeks of age ($P>0.05$), however, there was a
262 significant difference at 12 weeks of age ($P<0.01$) (Fig. 2G).

263 We next examined sperm production in KO mice at different ages. The results
264 were consistent with H.E. staining and immunohistochemistry. At the time of sexual
265 maturity, there were numerous motile sperm in WT mice compared to age-matched
266 *Etv5*$^{-/-}$ mice, whose sperm numbers were extremely low (Table 1, Sup-Fig. 2A). At 12
267 weeks of age, numerous motile sperm were observed in the epididymis WT mice,
268 however, there was a lack of spermatozoa in age-matched *Etv5*$^{-/-}$ mice (Table 1,
269 Sup-Fig. 2C). In addition, we found that the *Etv5* target gene *Ccl9,* spermatid-specific
270 gene *Prim2* and interstitial gland-specific gene *Cyp17a1* expression levels were
271 significantly decreased ($P<0.01$, $P<0.01$ and $P<0.05$ respectively) in *Etv5*$^{-/-}$ mice
272 (Sup-Fig. 3A, B and C). We also tested testosterone levels in WT and *Etv5*$^{-/-}$
273 littermates at 6, 8 and 12 weeks. There was a significant reduction of testosterone
274 levels in *Etv5*$^{-/-}$ mice, and at 12 weeks of age reached a maximum difference
275 compared to WT mice ($P<0.01$) (Sup-Fig. 3D).

276 **Transplantation of allogeneic SSCs through the efferent duct**

277 In order to determine whether the *Etv5*$^{-/-}$ mouse model could serve as a

278 ‘‘surrogate’’, we transplanted allogeneic SSCs (expressing or not expressing EGFP)
279 through the efferent duct into immature (3 weeks of age) and mature (7 weeks of age)
280 mice (Fig. 3A). Two months post-transplantation, complete spermatogenesis was
281 observed in all post-transplanted immature testes with spermatozoa observed in the
282 epididymides (approximately $9.58 \pm 5.05 \times 10^4$) (Table 2, Fig. 3B), however, no
283 spermatozoa were detected in adult testes (Table 2, Sup-Fig. 4A). The proportion of
284 tubules with spermatogenesis was 29.51%±0.08 and epididymides with spermatozoa
285 were as high as 77.78% (7/9) (Table 2). All germ cell layers could be observed in
286 these tubules by H.E. staining (Fig3. C) and spermatogonia were positive for PLZF
287 protein expression (Fig. 3D) in KO mice after SSCs transplantation. To determine the
288 origin of the sperm, the expression of heterologous genes *Etv5* and *EGFP* were
289 measured in recipient epididymal sperm by PCR. *Etv5* and *EGFP* expression were not
290 detected in KO mice, however, they were expressed in posttransplant *Etv5*$^{-/-}$ mice
291 spermatozoa (Fig. 3E). This indicated that the sperm were derived from donor cells.
292

293 **Discussion**

294 The mitogen-activated protein kinase 1 (Map2k1) signaling pathway is vital for
295 SSC self-renewal and is activated by fibroblast growth factor 2 (Fgf2). Activated
296 Map2k1 induces the expression of ETV5 which in turn up-regulates the expression of
297 Bcl6b that is critical for SSC self-renewal [9, 10]. Chen et al. [11] demonstrated the
298 presence of multiple germ cell layers in the tubules of 4 week old ERM$^{-/-}$ mice, with
299 gradual loss of spermatogonia. However, by 10 weeks of age, most tubules were

300 devoid of any germ cells, with only the presence of Sertoli cells. In this study, we
301 successfully generated *Etv5* KO mice using CRISPR/Cas9. These KO mice gradually
302 lost SSCs and only retained Sertoli cells, indicating that they were suitable models for
303 SSCs transplantation. This is because the loss of endogenous SSCs facilitates the
304 transplantation of exogenous SSCs [12].

305 Knockout of the *Etv5* gene affected the differentiation of SSCs and the formation
306 of sperm. Based on our results, only a small region of the tubules retained multilayer
307 germ cells, which included premeiotic germ cells, meiotic germ cells, and round
308 spermatids. This suggested that differentiated germ cells were lost in the majority of
309 tubules. The testicular sizes of 3-week-old *Etv5*$^{-/-}$ mice were slightly smaller
310 compared to age-matched WT controls. In addition, 3-week-old Etv5-/- mice had
311 partial germ cell loss in the seminiferous tubules. By 12 weeks of age, the testicular
312 sizes of 3-week-old *Etv5*$^{-/-}$ mice were much smaller compared to WT controls, with
313 germ cell layers lost in the tubules. These results suggest that testicular size may
314 reflect the extent of germ cell loss. In addition, several previous studies have
315 demonstrated that the maintenance of spermatogenesis was associated with
316 testosterone levels. Testosterone, secreted by Leydig cells in the interstitial space of
317 the testis, is a sex hormone responsible for maintaining spermatogenesis in the testis
318 [13]. The lack of testosterone in males leads to infertility. This is because
319 spermatogenesis does not progress beyond meiosis. In the absence of testosterone,
320 germ cells, even if they underwent meiosis, would detach from Sertoli cells and
321 undergo apoptosis [14]. Lack of testosterone in males leads to two major reproductive

322 disorders. First, the integrity of the blood-testis barrier (BTB) is compromised, which
323 exposes germ cells to autoimmune attack and cytotoxicity [15, 16]. This can be
324 explained by the classical testosterone signaling pathway: Testosterone induces gene
325 expression that alters the function of Sertoli cells. Second, defects in cell adhesion not
326 only make round sperm cells separate from Sertoli cells before they convert into long
327 sperm cells but also prevent mature sperm from being released by being engulfed by
328 Sertoli cells. This can be explained by the nonclassical kinase activation pathway [17,
329 18], i.e., kinase activation especially Erk and Src kinases. Testosterone functions in
330 promoting adhesion between germ cells and Sertoli cells, maturation, and release,
331 which are crucial to maintaining spermatogenesis. However, the direct link between
332 *Etv5* and testosterone levels have not been demonstrated. Our results demonstrated
333 that continuous loss of germ cells was observed with a decrease in testosterone levels
334 in *Etv5*$^{-/-}$ mice.

335 Interestingly, 3-week-old *Etv5*$^{-/-}$ mice were thinner compared to WT mice. At 12
336 weeks of age, this trend was even more obvious. The reduced body weights in *Etv5*$^{-/-}$
337 mice indicated that ETV5 had an influence on overall growth. *Etv5* mRNA has been
338 detected in a variety of tissues, including the heart, lungs, thymus, lymphocytes,
339 kidneys, and skeletal muscles [19, 20]. The widespread expression of ETV5 during
340 development may be crucial for growth. In a previous study that investigated the
341 viability of *Etv5*$^{-/-}$ mice, it was observed that *Etv5* mRNA was abundantly expressed
342 in the brain, lungs, and colon, but was the most abundantly expressed in the testes
343 [21].

344 We next investigated whether our *Etv5*$^{-/-}$ mouse model would support sperm
345 generation after allogeneic SSC transplantation. Although complete spermatogenesis
346 was visible in all post-transplantation immature *Etv5*$^{-/-}$ mouse testes, the number and
347 sperm motility in the epididymes were not ideal. Possible reasons could be attributed
348 to the following: Immunologic rejection. It is well known that the testis is one of the
349 immune-privileged organs. Leydig and Sertoli cells have immunosuppressive activity
350 to inhibit the proliferation of lymphocytes [22], and testicular macrophages also play
351 an immunosuppressive role [23]. These immune-privileged conditions in the testis
352 may still make the transplant of donor SSCs differentiate to a certain extent even in
353 immunocompetent recipients. This is because the blood-testis barrier protects
354 allogeneic sperm from an attack from the immune system [24]. A previous study
355 reported that the percentage of tubules with spermatogenesis in recipient testes
356 increased by as much as 48.1% compared to 9.4% in control mice after treatment with
357 monoclonal anti-CD4 antibody [25]. Complete spermatogenesis takes approximately
358 34.5 days from type a- single (As) spermatogonia to mature spermatozoa in WT mice
359 [26]. Colonization and establishment of two-dimensional colonies are usually
360 complete within 2 weeks and post-2 weeks respectively after transplantation. Meioses
361 usually initiates within the second month, with several spermatids being observed
362 after 2 months post-transplantation. The degree of germ cell differentiation will
363 continue to increase, with normal spermatogenesis being observed 3 months
364 post-transplantation [27]. In addition, it takes at least 10 days for sperm to travel from
365 the testes to the epididymides to mature [28]. With regard to the environment in the

366 epididymis, a previous study [29] reported that flagellar motion is crucial for the
367 reproductive function of sperm. Flagellar movement depends on several factors,
368 including intracellular pH, sperm membrane, flagellar elements, and external factors
369 such as matrix availability, ionic signals, and physical elements in the sperm
370 microenvironment. It is not clear what factors reduce the motility of sperm in
371 transplant recipients.

372 In summary, we successfully generated *Etv5*$^{-/-}$ mice suitable for SSCs
373 transplantation and verified that donor spermatogonial stem cells transplanted into
374 recipient testes could restore heterogenous spermatogenesis and produced
375 donor-derived sperm. However, spermatogenesis and production of donor-derived
376 sperm were not optimal and may have been due to factors, such as immune rejection,
377 sperm recovery time, etc., resulting in unsatisfactory sperm quantity and vitality.
378 Additional studies are required to investigate factors that contribute to sperm quality
379 and vitality after SSC transplantation.

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384 **Author Contributions**

385 Conceived and designed the experiments: Xianyu Zhang, Huaqiang Yang, Zhenfang
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387 Performed the experiments: Mao Zhang, Xianyu Zhang, Xin Zhao, Pingping Xing.

388 Analyzed the data: Xianyu Zhang, Guoling, Li.

389 Contributedreagents/materials/analysis tools: Xianyu Zhang, Zicong Li.

390 Wrote the paper: Xianyu Zhang, Guoling, Li, Huaqiang Yang.

391 **Legend**

392 **Fig. 1. Generation of *Etv5* KO mice by CRISPR/Cas9.** (A) Schematic diagram
393 depicting the strategy for generation of knockout *Etv5* mice, which includes gRNA1
394 and gRNA2 targeting sites. Primer-F and primer-R used to determine the genotype of
395 the mice. (B) Genotype determined by PCR for newborn male mice littermates. WT,
396 *Etv5*$^{+/-}$ and *Etv5*$^{-/-}$ represent the wild type, heterozygote, and homozygote, respectively.
397 Blue asterisks indicate complete sequence and red asterisks indicate remaining
398 sequence after deleting. (C) Sequencing the PCR products of *Etv5*$^{-/-}$ mice and WT
399 controls in figure B. (D) ETV5 protein expression in WT and *Etv5*$^{-/-}$ mice measured
400 by western blotting.

401 **Fig. 2. Absence of SSCs in the seminiferous tubules of *Etv5* KO mice.** (A) The
402 phenotype of testes of WT and *Etv5*$^{-/-}$ mice at 3 weeks and 12 weeks of age. (B)
403 Weight of Testes in *Etv5*$^{-/-}$ mice compared to WT littermates at periodic points during
404 development. For all time points, n=4 per group for both WT and *Etv5*$^{-/-}$ mice. (C and
405 D) Representative figures for H.E. staining and immunohistochemistry of testes in
406 WT and *Etv5*$^{-/-}$ mice littermates at 3 weeks and 12 weeks. Bars=50 μm. Red arrows
407 indicate spermatogonial stem cells with brown positive staining. Pentacles reveal
408 vacuolation in some seminiferous tubules in *Etv5*$^{-/-}$ mice. (E) The number of testicular
409 cells were mainly composed of premeiotic germ cells, meiotic germ cells, round

410 spermatids, and elongated spermatids in seminiferous tubules, (F) the percentage of
411 vacuolation of seminiferous tubules, and (G) number of seminiferous tubules from
412 WT and *Etv5*$^{-/-}$ mice at 3 weeks and 12 weeks of age at different time points, n=3 per
413 group for both WT and *Etv5*$^{-/-}$ mice. A total of 9 slices per group was prepared and
414 data from each slice was collected at least from 3 different regions. ** represents
415 $p<0.01$.

416 **Fig. 3. Transplantation of allogeneic SSCs through the efferent duct.** (A)
417 Transplantation of allogeneic SSCs through the efferent duct. (B) The epididymis of
418 3-week-old recipient mice was observed using a stereomicroscope 2 months
419 post-transplantation. NT and AT represent no transplantation and after transplantation.
420 AT-1 and AT-2 represent allogeneic SSCs with and without *EGFP* gene expression,
421 respectively. The insets represent higher magnification of cells. Blue arrows indicate
422 sperm under visible light (left) and fluorescence (right). (C and D) H.E. staining and
423 PLZF immunohistochemistry of testes for NT and AT. Bars=50 μm. Red arrows
424 indicate spermatogonial stem cells with brown positive staining. (E) Heterogeneous
425 expression of *Etv5* and *EGFP* were measured to determine the origin of epididymal
426 sperm. M1 and M2 represent testis and epididyme transplanted with donor cells on the
427 same side, and M3 represents donor cells.

428 **Supplementary fig. 1. Body weighs in KO mice.** (A) The phenotype of WT and
429 *Etv5*$^{-/-}$mice at 3 weeks and 12 weeks of age. Arrows indicate *Etv5* gene KO and WT
430 mice. (B) Bodyweight of WT and *Etv5*$^{-/-}$ mice at periodic timepoints during
431 development from 4 postnatal days to adulthood. For all time points, n=5 for both WT

432 and *Etv5*$^{-/-}$ mice. Data presented as mean±*SD*. *p<0.05, **p<0.01.

433 **Supplementary fig. 2. Sperm density in the epididymis at postpartum 6 to**
434 **12-week WT and *Etv5*$^{-/-}$ mice.** Arrows indicate sperm and the insert represents the
435 higher magnification of sperm. For all time points, n=3 per group, Bars=50 μm.

436 **Supplementary fig. 3. Changes in gene expression related to spermatogenesis and**
437 **testosterone levels.** mRNA expression levels of *Etv5* target gene *Ccl9* (A),
438 spermatids-specific gene *Prim2* (B) and interstitial gland-specific gene *Cyp17a1* (C)
439 in postnatal week 12 testes of WT and *Etv5*$^{-/-}$ mice littermates. WT values were set as
440 100% in the histogram. Testosterone levels of WT and *Etv5*$^{-/-}$ littermates at 6, 8 and
441 12 weeks (D). n=3 per group, *p<0.05, **p<0.01. Data presented as mean±*SD*.

442 **Supplementary fig. 4. Transplantation of allogeneic SSCs into mature mice.** (A)
443 The epididymis of 7-week-old recipient mice was observed using a stereomicroscope
444 2 months post-transplantation. Semen containing epididymal tissue (left) and removal
445 of epididymal tissue (right). NT and AT represent no transplantation and
446 post-transplantation. AT-1 and AT-2 represent allogeneic SSCs with or without
447 *EGFP* gene expression, respectively. The insert depicts the higher magnification of
448 cells. (B and C) H.E. staining and PLZF immunohistochemistry of the testes for NT
449 and AT. Bars=50 μm.

450

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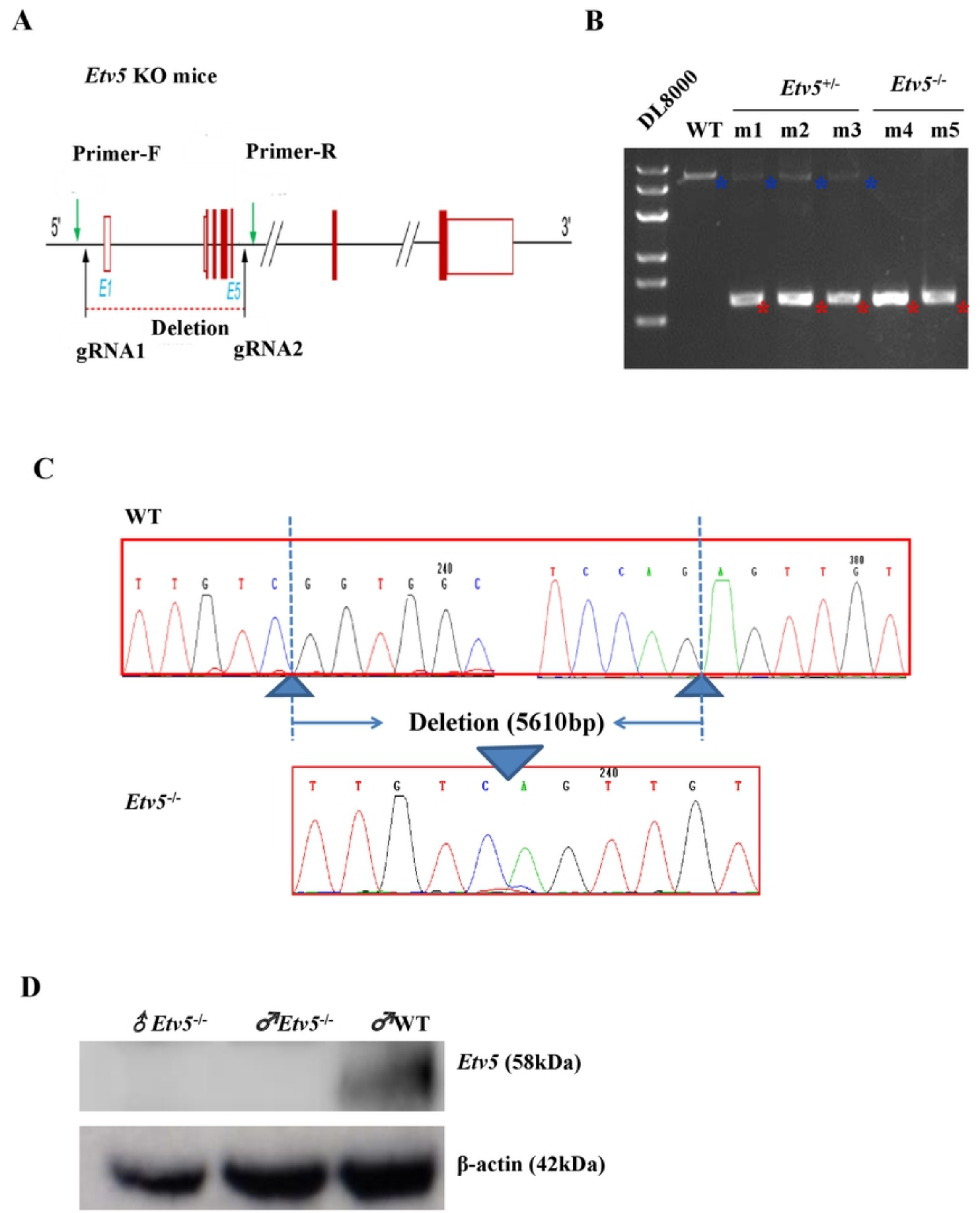
A
Etv5 KO mice
Primer-F
Primer-R
5'
3'
E1
E5
Deletion
gRNA1
gRNA2
B
DL8000
WT
Etv5+/-
Etv5-/-
m1
m2
m3
m4
m5
C
WT
Deletion (5610bp)
Etv5-/-
D
♂Etv5-/-
♂Etv5-/-
♂WT
Etv5 (58kDa)
β-actin (42kDa)

Fig.1

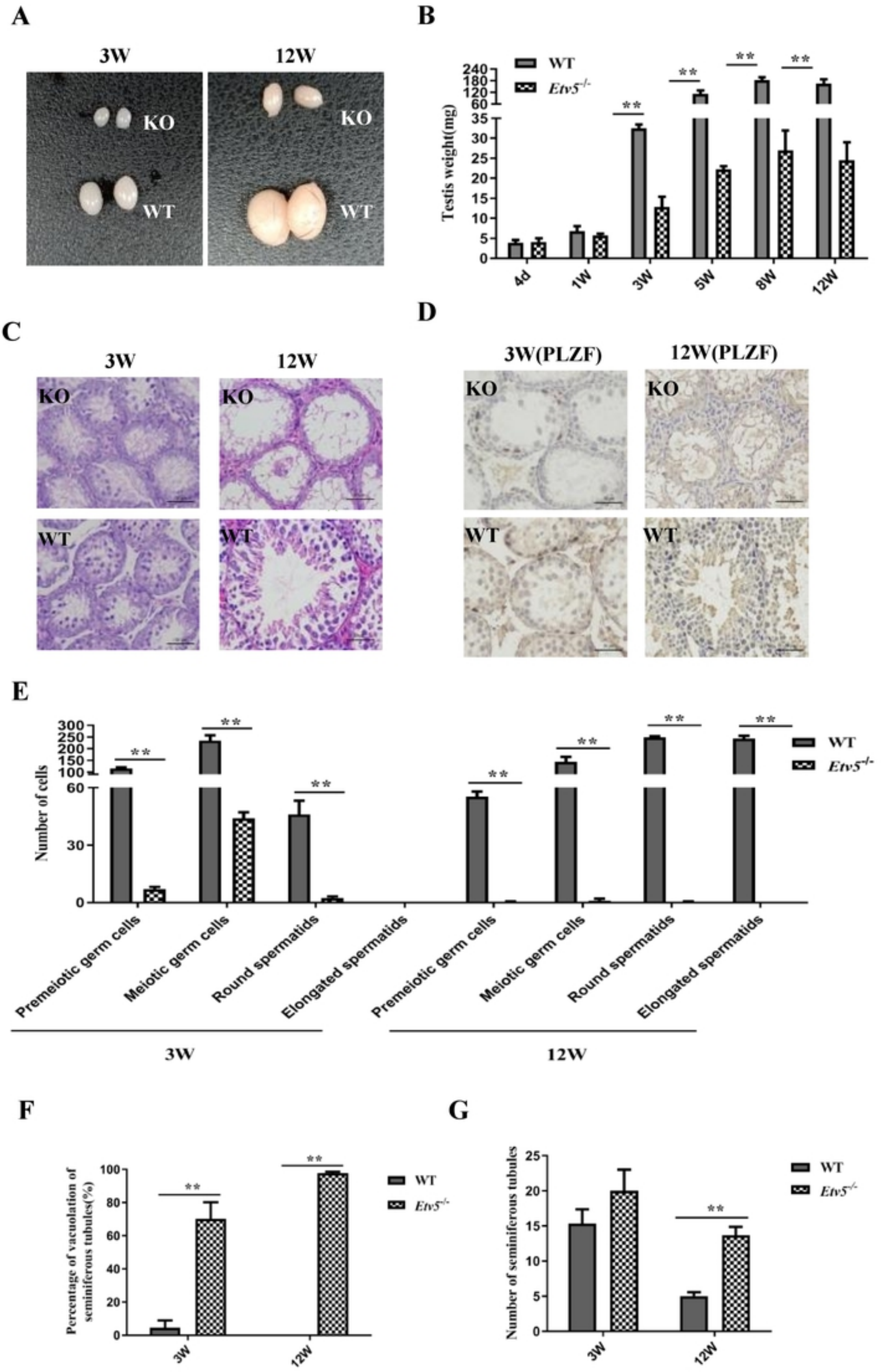
A
3W
12W
KO
WT
B
WT
Etv5-/-
Testis weight(mg)
4d
1W
3W
5W
8W
12W
C
3W
12W
KO
WT
D
3W(PLZF)
12W(PLZF)
KO
WT
E
Number of cells
Premeiotic germ cells
Meiotic germ cells
Round spermatids
Elongated spermatids
3W
12W
F
Percentage of vacuolation of seminiferous tubules(%)
G
Number of seminiferous tubules

Fig.2

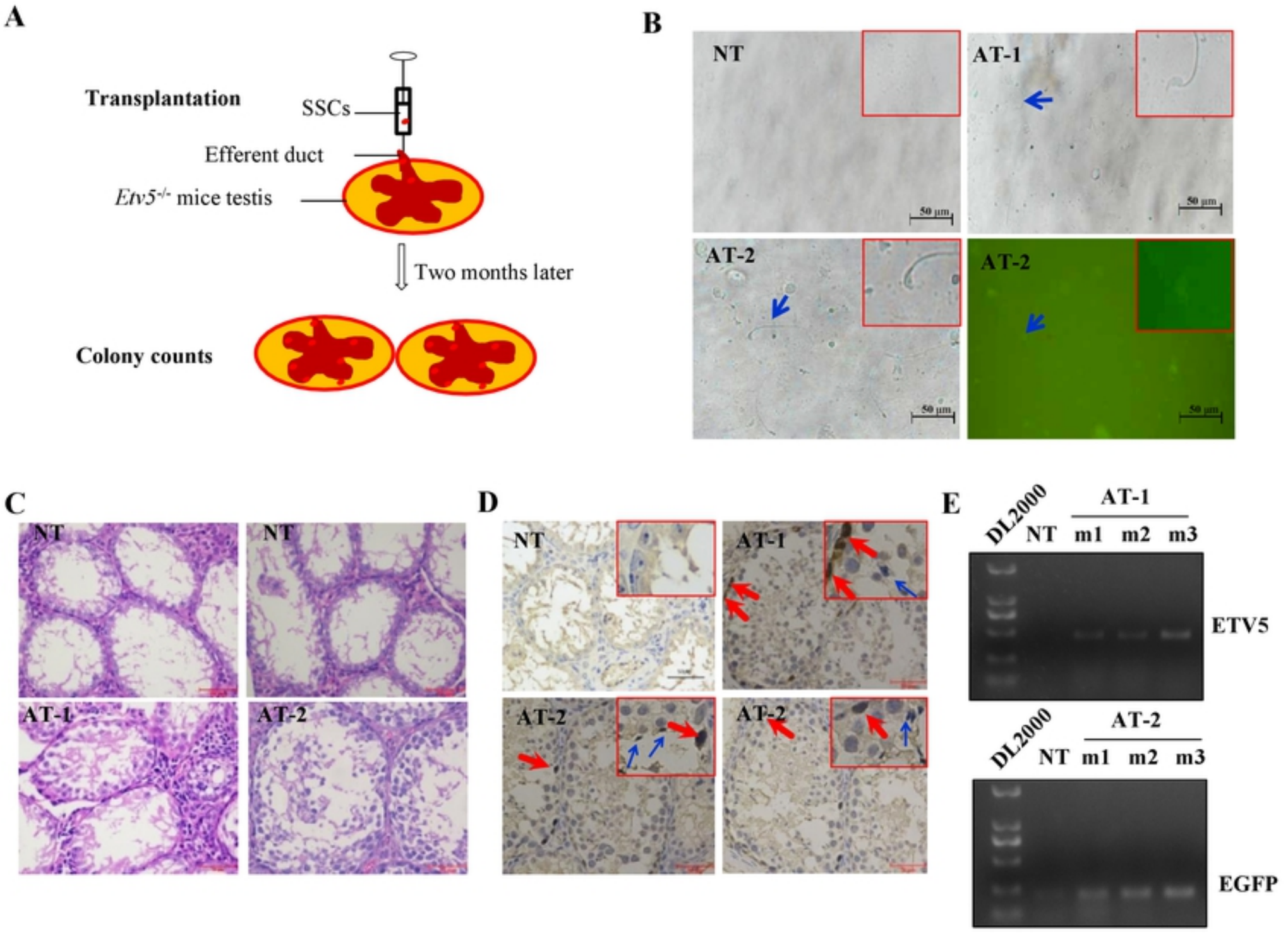
A
Transplantation
SSCs
Efferent duct
Etv5-/- mice testis
Two months later
Colony counts
B
NT
AT-1
AT-2
AT-2
50 μm
C
NT
NT
AT-1
AT-2
D
NT
AT-1
AT-2
AT-2
E
DL2000
AT-1
NT
m1
m2
m3
ETV5
DL2000
AT-2
NT
m1
m2
m3
EGFP

Fig.3

Table 1. Number and motility of sperm in one side epididymis between WT and *Etv5*$^{-/-}$ mice

Age (w)	Sperm		Motility	
	WT	*Etv5*$^{-/-}$	WT	*Etv5*$^{-/-}$
6	$3.73 \pm 0.49 \times 10^{6}$ (c)	$6.25 \pm 0.40 \times 10^{3}$ (e)	68.92%±2.51 (c)	20.01%±2.08 (e)
8	$6.13 \pm 0.33 \times 10^{6}$ (b)	$2.88 \pm 0.25 \times 10^{4}$ (d)	69.33%±0.47 (b)	47.63%±0.75 (d)
12	$7.50 \pm 0.32 \times 10^{6}$ (a)	ND	88.01%±1.50 (a)	ND

ND: No sperm can be detected; WT: wild type

Different letters (a, b, c, d and e) within a column represent significance ($P < 0.01$).

Table 1

Table 2. Development of allogeneic spermatogenesis in *Etv5*$^{-/-}$ recipient mice

Recipient mice		Spermatogenesis after 2 months transplantation							
		No transplantation			After transplantation				
Age (w)	number	Testes	Tubles	Epididymides	Testes	Tubles	epididymides	Sperm	Motility
3	9	ND	ND	ND	9/9(100%)	29.51 ±0.08	7/9(77.78%)	9.58±5.05×10^4	0
7	9	ND	ND	ND	ND	ND	ND	ND	ND

ND: No sperm can be detected.

Table 2