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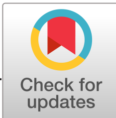

RESEARCH ARTICLE

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The testicular soma of *Tsc22d3* knockout mice supports spermatogenesis and germline transmission from spermatogonial stem cell lines upon transplantation

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Abstract

Spermatogonial stem cells (SSCs) are adult stem cells that are slowly cycling and self-renewing. The pool of SSCs generates very large numbers of male gametes throughout the life of the individual. SSCs can be cultured in vitro for long periods of time, and established SSC lines can be manipulated genetically. Upon transplantation into the testes of infertile mice, long-term cultured mouse SSCs can differentiate into fertile spermatozoa, which can give rise to live offspring. Here, we show that the testicular soma of mice with a conditional knockout (conKO) in the X-linked gene *Tsc22d3* supports spermatogenesis and germline transmission from cultured mouse SSCs upon transplantation. Infertile males were produced by crossing homozygous Tsc22d3 floxed females with homozygous ROSA26-Cre males. We obtained 96 live offspring from six long-term cultured SSC lines with the aid of intracytoplasmic sperm injection. We advocate the further optimization of Tsc22d3-conKO males as recipients for testis transplantation of SSC lines.

KEYWORDS
germline stem cell, GILZ, spermatogenesis, SSC

1 | INTRODUCTION

Spermatogonial stem cells (SSCs) are adult stem cells that continuously undergo self-renewal to maintain the undifferentiated state, and that differentiate to form mature spermatozoa throughout the lifetime of males (Kanatsu-Shinohara & Shinohara, 2013). Theoretically, a single SSC yields ~4,096 haploid gametes by passing through successive mitoses and one meiosis—a process that takes ~35 days in mouse. SSCs reside on the basement membrane of the seminiferous tubules in the testis. A specialized microenvironment that is termed a niche supports the self-renewal and differentiation of SSCs (Oatley & Brinster, 2012). The widely used biological assay to assess SSC activity within a population of cells entails the transplantation of SSCs by microinjection into the testes of infertile mice (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994). Transplanted cells give rise to fertile spermatozoa and then to donor cell-derived offspring either by natural mating or with the aid of an assisted reproductive technology such as intracytoplasmic sperm cell injection (ICSI). Long-term culture of mouse SSCs became possible by adding to the medium self-renewal factors such as glial cell line-derived neurotrophic factor (GDNF; Kanatsu-Shinohara et al., 2003; Kubota, Avarbock, & Brinster, 2004). It is estimated that only a small fraction (<1%) of the cells of an SSC line harbor the stem cell potential (Kanatsu-Shinohara & Shinohara, 2013). Genetic manipulation of the genome of SSC lines has enabled the generation of genetically modified strains of mice (Kanatsu-Shinohara et al., 2006; Sato et al., 2015; Wu et al., 2015) and rats (Chapman et al., 2015), but has not replaced gene targeting in embryonic stem cells.

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There are two commonly used types of infertile recipients for SSC transplantation in mice. The first type is the Kit^{W}/Kit^{Wv} mutant mouse, which has smaller testes that contain almost no germ cells and are devoid of spermatogenesis. But because Kit^{W}/Kit^{W} homozygous mice die after birth, compound heterozygous Kit^{W}/Kit^{Wv} males must be generated from crosses of mice carrying the Kit^{W} or Kit^{Wv} alleles in the heterozygous state. The second type is obtained by intraperitoneal injection of busulfan, an alkylating chemotherapeutic agent that preferentially kills spermatogonial stem cells. But the typical dose of 40 mg busulfan/kg body weight results not only in infertility, but also in substantial morbidity and mortality. In the few studies that report mortality rates, these ranged from 30% (Ma, Wang, Gao, & Jia, 2018), 31.6% (Qin et al., 2016), 60% (Ganguli et al., 2016), to as high as 87% (Wang, Zhou, Yuan, & Zheng, 2010) for 40 mg busulfan/kg body weight. In certain studies, busulfan-treated mice received a bone marrow transplant to relieve hematopoietic suppression (Aoshima, Baba, Makino, & Okada, 2013; Kanatsu-Shinohara et al., 2002, 2003; Ogawa, Dobrinski, Avarbock, & Brinster, 1999). We believe that, in the interest of animal welfare and the 3R principle of humane experimental technique with animals (Russell & Burch, 1959), it is imperative to explore alternative candidates for SSC transplantation recipients as they become available through new discoveries or new technological developments.

A novel candidate recipient that has emerged recently but has not been explored yet is the genetically infertile male mouse that is the centerpiece of the goGermline technology (Koentgen et al., 2016). This technology was originally developed to produce chimeric mice that give 100% germline transmission of the embryonic stem cell-derived genome upon microinjection of embryonic stem cells into blastocysts or eight-cell embryos. Infertile males are produced by crossing two strains that can be maintained in the homozygous and hemizygous state and that are healthy and fertile: females homozygous for a Tsc22d3 conditional (floxed) gene-targeted mutation are crossed with males homozygous for a ROSA26-Cre gene-targeted mutation. There is no mortality at any stage; there is no genotyping of any mouse needed; and 100% of male offspring of the cross are Tsc22d3-conKO and infertile. The goGermline technology appeared to us as promising to fulfill the reduction imperative of the 3R principle as source of an alternative, third-type of recipient for SSC transplantation. Here, we report spermatogenesis and germline transmission of the SSC-derived genome upon transplantation into the testes of infertile Tsc22d3-conKO males.

2 | MATERIALS AND METHODS

2.1 | Mice

The CAG::mRFP1 strain is Tg(CAG-mRFP1)1F1Hadj/J (Long, Lackan, & Hadjantonakis, 2005; The Jackson Laboratory, #5645). The UBI-GFP strain is C57BL/6-Tg(UBC-GFP)30Scha/J (Schaefer, Schaefer, Kappler, Marrack, & Kedl, 2001; The Jackson Laboratory, #4353). The Tg(act-EGFP) strain is C57BL/6-Tg(CAG-EGFP)131Osb/LeySopJ (Okabe, Ikawa, Kominami, Nakanishi, & Nishimune, 1997; The Jackson Laboratory, #6567). The D4/XEGFP strain is Tg(CAG-EGFP)D4Nagy/J (Hadjantonakis, Gertsenstein, Ikawa, Okabe, & Nagy, 1998; The Jackson Laboratory, #3116). The B6-iDTR strain is C57BL/6-Gt(ROSA)26Sortm1(HBEGF)Awai/J (Buch et al., 2005; The Jackson Laboratory, #7900). The ROSA26-EGFP strain is B6.Cg-Tg(Gt(ROSA)26Sor-EGFP)I1Able/J (Giel-Moloney, Krause, Chen, Van Etten, & Leiter, 2007; The Jackson Laboratory, #7897). The B6-GFP strain is C57BL/6-Tg(CAG-EGFP)1Osb/J (Okabe et al., 1997; The Jackson Laboratory, #3291). Mouse embryonic fibroblasts were prepared from strain Tg(DR4)1Jae/J (The Jackson Laboratory, #3208). The Tsc22d3-flox strain (Koentgen et al., 2016) was established in embryonic stem cell line Bruce4; chimeras were crossed with ROSA26-Flp in a C57BL/6 background; and the strain was then maintained in a C57BL/6 background with the *Tyr*^{c} allele and the wild-type *Tyr* allele segregating at the albino locus, and the *A* and *a* alleles segregating at the agouti locus. The ROSA26-Cre strain was established in Bruce4, and mice were intercrossed for more than 20 generations with several backcrosses to C57BL/6 along the way. The Tsc22d3-flox and ROSA26-Cre strains are available to the research community. Mouse experiments were performed in accordance with the German Animal Welfare Act, the European Directive 2010/63/EU, and the institutional ethical and animal welfare guideline of the Max Planck Research Unit for Neurogenetics. Approval came from the *Regierungspräsidium* Darmstadt and the *Veterinäramt* of the City of Frankfurt.

2.2 | SSC culture

The culture medium consisted of StemPro-34 SFM with Stem Pro supplement (Kanatsu-Shinohara, Ogonuki, et al., 2003; Wu et al., 2015; Zhang et al., 2014; Gibco, #10639–011), 25 μg/mL insulin (Sigma, #I1882), 100 μg/mL transferrin (Sigma, #T1428), 60 μM putrescine (Sigma, #P5780), 30 nM sodium selenite (Sigma, #S5261), 6 mg/mL D-(+)-glucose (Sigma, #G7021), 30 μg/mL pyruvic acid (Sigma, #P4562), 1 μL/mL DL-lactic acid (Sigma, #L7900), 5 mg/mL bovine serum albumin (Calbiochem, #126609), 2 mM L-glutamine (Millipore, #TMS-002-C), 100x β-mercaptoethanol (Specialty Media, #ES-007-E), minimal essential medium vitamin solution (Gibco, #11120–052), 100x nonessential amino acid solution (Gibco, #11140–035), 1% penicillin/streptomycin (Specialty Media, #TMS-AB2-C), 0.1 mM ascorbic acid (Sigma, #A4034), 10 μg/mL d-biotin (Sigma, #B4639), 20 ng/mL recombinant human epidermal growth factor (Gibco, #PMG8041), 10 ng/mL recombinant human basic fibroblast growth factor (PeproTech, #AF-100-18B-250), 10 ng/mL recombinant human GDNF (PeproTech, #450–10-250), 1 IU/mL Leukemia Inhibitory Factor (Millipore, #ESG1107), and 1% fetal bovine serum (HyClone, #SH30071.03). Cells were maintained at 37°C with 5% CO_2.

2.3 | Transplantation of SSCs

Tsc22d3-conKO males at 4–12 weeks were used as recipient mice. Cell suspensions of 10–15 μL, which contained $2–3 \times 10^{5}$ cells, were injected into seminiferous tubules (Ogawa, Aréchaga, Avarbock, & Brinster, 1997) using a micropipette (40–80 μm diameter tips). A Nikon SMZ25 stereofluorescence microscope was used to visualize green

fluorescence in the testes. Mice were sacrificed by cervical dislocation, and testes collected and cryopreserved for immunofluorescence.

2.4 | Immunofluorescence and imaging

SSC lines were cultured in 12- or 24-well dishes. Cells were fixed in 4% paraformaldehyde at room temperature for 15 min, treated for 1 hr at room temperature with 0.5% Triton X-100 and 5% normal donkey serum (Jackson ImmunoResearch Laboratories, #017–000-121) diluted in phosphate buffered saline with Triton X-100 (PBST), which consists of 1 g bovine serum albumin (Calbiochem, #126609), 5 mL 20% Triton X-100 (Sigma, #P9416) and 100 mL 10 × PBS (Sigma, #P5493) to 900 mL ddH$_2$O. Testes were fixed in 4% paraformaldehyde overnight, followed by cryoprotection in 30% sucrose, frozen in Optimal Cutting Temperature compound, and sectioned at 12 μm. Primary antibodies were diluted at 1:50–1:500 in 0.1% Triton X-100 and 1% normal donkey serum, and samples incubated at 4 °C overnight or at 37 °C for 1 hr. After three 15-min washes in PBST, samples were incubated for 1 hr at 37 °C in a 1:500 dilution of secondary antibody in PBST, then washed and covered with H$_2$O containing nuclear stain 4′,6-diamidino-2-phenylindole (DAPI, Thermo Fisher scientific, #D1306) for 5 min. Primary antibodies were MVH (abcam, #Ab13840) at 1:500 dilution, GILZ (Santa Cruz FL-134, #sc-33780) at 1:50, TRA98 (abcam, #ab82527) at 1:500, TRIM36 (abcam, #ab116212) at 1:200, TNP1 (abcam, #ab73135) at 1:150, SCP3 (abcam, #ab15093) at 1:150, WT1 (abcam, #ab89901) at 1:150, SOX9 (Merck Millipore, #AB5535) at 1:500, and CD146 (BioLegend, #134701) at 1:200. Secondary antibodies from Jackson ImmunoResearch Laboratories were Cy5 AffiniPure Donkey anti-goat IgG (H + L; #705–175-147), and Cy5 AffiniPure Donkey anti-rabbit IgG (H + L; #711–175-152). Secondary antibodies from Invitrogen were Donkey anti-rabbit IgG (H + L) with Alexa Fluor 546 (#A10040), Donkey anti-mouse IgG with Alexa Fluor 546 (#A10036), Donkey anti-mouse IgG with Alexa Fluor 488 (#A21202), and Goat anti-chicken IgY (H + L) with Alexa Fluor 488 (#A11039). Images were taken with an AMG EVOS (Life Technologies) and with a Zeiss LSM 710 confocal microscope.

2.5 | Intracytoplasmic sperm injection (ICSI)

Metaphase II-arrested oocytes were collected from superovulated BDF1 females (3–4 weeks) or C57BL/6J females (3–4 weeks). Cumulus cells were removed using hyaluronidase (Sigma, #H3884) at 37 °C for 5 min. The cauda epididymis was washed twice with Dulbecco's phosphate buffered saline (DPBS, Gibco, #14190094), then directly put in 1 mL of EmbryoMax Human Tubal Fluid (Merck Millipore, #MR-070-D). Cell suspensions were exposed to ultrasound for 1–2 min. Spermatozoa without tail were picked up into a blunt piezo-driven pipette with a tip of 10–15 μm diameter. A single sperm head was injected into a single oocyte in a droplet of M2 medium (Sigma, #M7167) containing 5 μg/mL cytochalasin B (Sigma, #C6762) using a pipette with a tip of 10–15 μm diameter, and a piezo micromanipulator controller (Japan Prime Tech, #PMAS-CT150). Injected oocytes were maintained in KSOM medium (Merck Millipore, #MR-106-D) at 37 °C with 5% CO$_2$ in air. Two-cell embryos were transferred into the oviducts of pseudopregnant ICR females. Offspring were born on day 19.5 of gestation.

3 | RESULTS

3.1 | Derivation of SSC lines

We derived a dozen SSC lines from a variety of wild-type, gene-targeted, and transgenic mouse strains. HZ-1 was derived from strain CAG::mRFP1 (Long et al., 2005), and displays the intrinsic red fluorescence from RFP (Figure 1a). HZ-2 was derived from strain UBI-EGFP (Schaefer et al., 2001). HZ-3 was derived from strain Tg(act-EGFP)

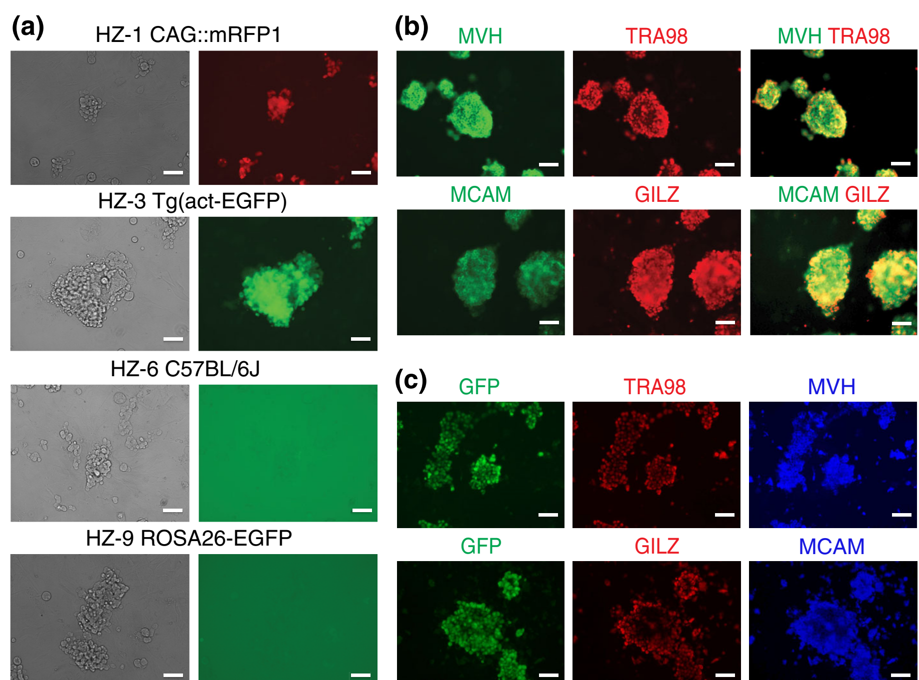

FIGURE 1 SSC lines in culture. (a) Left, brightfield images, and right, fluorescence images. Strain from which the SSC line was derived is indicated. HZ-1 displays the red intrinsic red fluorescence of RFP. HZ-3 displays the intrinsic green fluorescence of GFP. HZ-6 and HZ-9 do not display the intrinsic green fluorescence of GFP. (b) Immunofluorescence of HZ-6 (derived from C57BL/6J) with antibodies for MVH, TRA98, MCAM, and GILZ. (c) Immunofluorescence of HZ-11 (derived from B6-GFP) with antibodies for GFP, TRA98, MVH, GILZ, and MCAM. Scale bars, 50 μm

(Okabe et al., 1997), and displays the intrinsic green fluorescence from GFP (Figure 1a). HZ-4 was derived from strain D4/XEGFP (Hadjantonakis et al., 1998). HZ-5 was derived from strain B6-iDTR (Buch et al., 2005). HZ-6, HZ-7, and HZ-10 were derived from wild-type C57BL/6J (Figure 1a). HZ-8 was derived from wild-type CD-1. HZ-9 was derived from strain ROSA26-EGFP (Giel-Moloney et al., 2007), but does not display the intrinsic green fluorescence from GFP (Figure 1a). HZ-11 and HZ-12 were derived from strain B6-GFP (Okabe et al., 1997). These SSC lines were cultured continuously for long periods of time (up to 1 year), and could be frozen and thawed successfully.

To confirm that the newly derived cell lines have the characteristics of SSCs, we performed immunofluorescence with antibodies for markers with various cellular localizations: cytoplasm, nucleus, and cell surface. The mouse vasa homologue (MVH) is expressed in the cytoplasm of germ cells; Vasa (also known as Ddx4) is an ATP-dependent RNA helicase that is highly conserved among vertebrates and invertebrates (Castrillon, Quade, Wang, Quigley, & Crum, 2000; Gustafson & Wessel, 2010). The antigen detected by the TRA98 antibody is the *Gcna1* gene product (Carmell et al., 2016), and resides in the nucleus of testis germ cells (Inoue, Onohara, & Yokota, 2011). CD146, also called melanoma cell adhesion molecule (MCAM), is a transmembrane glycoprotein used as an SSC cell surface marker (Kanatsu-Shinohara, Morimoto, & Shinohara, 2012). We also used antibodies against GILZ, the protein encoded by the gene (Koentgen et al., 2016). Cell lines derived from a C57BL/6J mouse (Figure 1b) and from a GFP-expressing mouse (Figure 1c) were immunoreactive for MVH, TRA98, MCAM, and GILZ, confirming that they express classical markers for SSCs.

3.2 | Testes of Tsc22d3-conKO mice

We performed immunofluorescence on cryosections of testes of Tsc22d3-conKO mice and C57BL/6J mice. Immunoreactivity for GILZ is not detectable in Tsc22d3-conKO testes at 3 weeks (Figure 2a). Immunoreactivity for GILZ colocalizes with cells expressing the germ cell marker that is detected by the TRA98 antibody (Tanaka et al., 1997) in C57BL/6J mice at 4 weeks (Figure 2a). There are TRA98+ germ cells in Tsc22d3-conKO testes at 3 weeks but no longer at 10 weeks (Figure 2a–c). SOX9 and WT1, markers for Sertoli cells (Gao et al., 2006; Hemendinger, Gores, Blacksten, Harley, & Halberstadt, 2002; Kreidberg et al., 1993), are expressed in Tsc22d3-conKO testes at 3 weeks and 10 weeks (Figure 2b,c).

3.3 | Testes of Tsc22d3-conKO mice after SSC transplantation

We transplanted seven SSC lines into the testes of Tsc22d3-conKO mice (Figure 3a, Table 1), 10 mice per SSC line, for a total of 70 transplanted mice. Five SSC lines were derived from GFP+ mice:

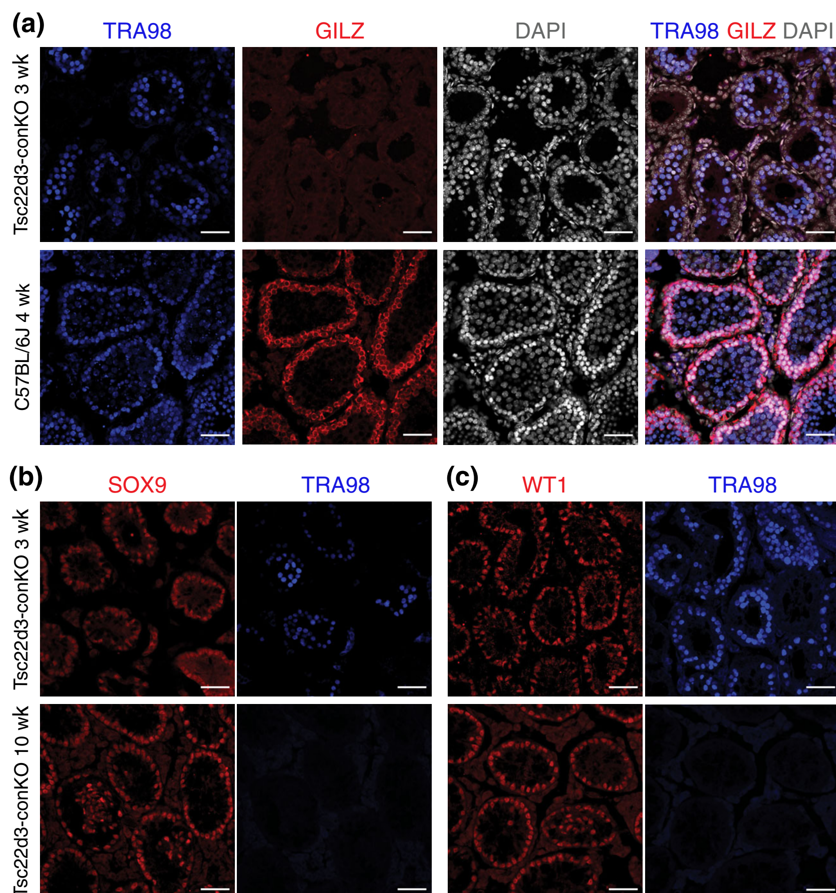

FIGURE 2 Testes of Tsc22d3-conKO and C57BL/6J mice. (a) Immunofluorescence for TRA98 and GILZ in cryosections of testes of a Tsc22d3-conKO mouse at 3 weeks, and of a C57BL/6J mouse at 4 weeks. DAPI (white) serves as nuclear stain. (b) Immunofluorescence for SOX9 and TRA98 in cryosections of testes of Tsc22d3-conKO mice at 3 weeks and 10 weeks. (c) Immunofluorescence for WT1 and TRA98 in cryosections of testes of Tsc22d3-conKO mice at 3 weeks and 10 weeks. Scale bars, 50 μm

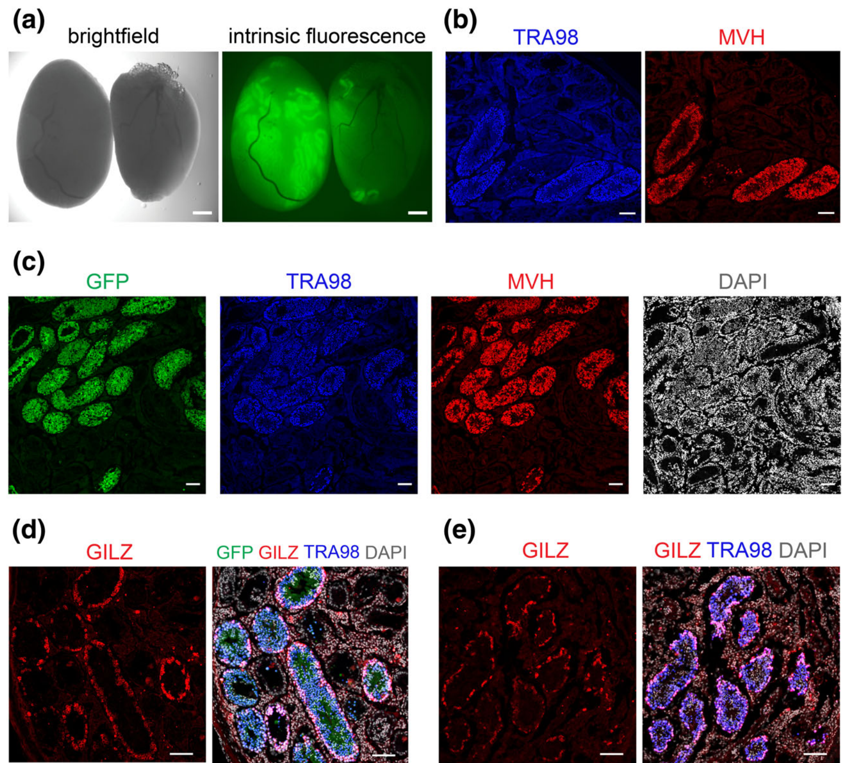

FIGURE 3 Testes of Tsc22d3-conKO mice after SSC transplantation. (a) Macroscopic view of Tsc22d3-conKO testes transplanted with HZ-12 (derived from B6-GFP). (b) Immunofluorescence for TRA98 and MVH in cryosections of a Tsc22d3-conKO testis transplanted with HZ-6 (derived from C57BL/6J). (c) Immunofluorescence for GFP, TRA98, and MVH in a cryosection of a Tsc22d3-conKO testis transplanted with HZ-11 (derived from B6-GFP). DAPI (white) serves as nuclear stain. (d) Immunofluorescence for GILZ, TRA98, and GFP in a cryosection of a Tsc22d3-conKO testis transplanted with HZ-12 (derived from B6-GFP). DAPI (white) serves as nuclear stain. (e) Immunofluorescence for GILZ and TRA98 in a cryosection of a Tsc22d3-conKO testis transplanted with HZ-6 (derived from C57BL/6J). DAPI (white) serves as nuclear stain. Scale bars, 2 mm in (a) and 100 μm in (b–e)

HZ-2 (UBI-EGFP), HZ-3 Tg(act-EGFP), HZ-9 (ROSA26-EGFP), HZ-11, and HZ-12 (B6-GFP). Two SSC lines were derived from wild-type mice: HZ-6 and HZ-7 (C57BL/6J). Two months after transplantation, colonization of the seminiferous tubules of Tsc22d3-conKO testes by the GFP+ cells from HZ-12 (derived from B6-GFP) could be visualized readily by exposure to UV light in a macroscopic view of a testis (Figure 3a). Immunoreactivity for TRA98 and MVH was present in cryosections of a Tsc22d3-conKO testis transplanted with HZ-6 (derived from C57BL/6J; Figure 3b). Cryosections of a Tsc22d3-conKO testis transplanted with HZ-11 (derived from B6-GFP) showed colocalization of GFP, TRA98, and MVH immunoreactivity (Figure 3c). In cryosections of a Tsc22d3-conKO testis transplanted with HZ-12 (derived from B6-GFP), GILZ immunoreactivity colocalized with the GFP and TRA98 signals in seminiferous tubules (Figure 3d). Immunofluorescence in cryosections of a Tsc22d3-conKO testis transplanted with HZ-6 (derived from C57BL/6J) revealed numerous TRA98+ and GILZ+ cells in seminiferous tubules (Figure 3e).

3.4 | Differentiation and spermatogenesis after SSC transplantation

To determine if the transplanted SSCs are committed towards meiosis, we performed immunofluorescence for synaptonemal complex protein 3 (SCP3), a meiosis-specific component of the axial/lateral element of the synaptonemal complex (Yuan et al., 2000). Immunofluorescence for SCP3 of Tsc22d3-conKO testes transplanted with HZ-6 (derived from C57BL/6J) showed numerous immunoreactive cells (Figure 4a). We detected costaining with SCP3, GFP, and TRA98 antibodies in a Tsc22d3-conKO testis transplanted with HZ-9 (derived from ROSA26-EGFP; Figure 4b). Thus, the transplanted donor SSCs can proceed to meiosis after transplantation into Tsc22d3-conKO testes. Next, we performed immunofluorescence for transition nuclear protein 1 (TNP1), a marker for elongating and condensing spermatids (Dadoune, 1995; Han et al., 2016; Yassine et al., 2015), and for RNF98 (also known as Trim36 and Haprin), a marker for elongated

TABLE 1 Transplantation of long-term cultured SSCs

SSC line	Strain	Age of male from which SSC line was derived	Genotype	Genetic background	No. of recipients	No. of recipients analyzed	No. (%) of recipients with germline colonization
HZ-2	UBI-EGFP	2 weeks	Homozygous	C56BL/6 J	10	7	2 (29%)
HZ-3	Tg(act-EGFP)	1 week	Homozygous	C56BL/6 J	10	6	5 (83%)
HZ-6	C57BL/6J	3 weeks	Wild-type	C56BL/6 J	10	7	3 (43%)
HZ-7	C57BL/6J	1 week	Wild-type	C56BL/6 J	10	9	4 (44%)
HZ-9	ROSA26-EGFP	5 weeks	Heterozygous	C56BL/6 J	10	10	5 (50%)
HZ-11	B6-GFP	12 weeks	Hemizygous	C56BL/6 J	10	5	3 (60%)
HZ-12	B6-GFP	4 weeks	Hemizygous	C56BL/6 J	10	8	3 (38%)

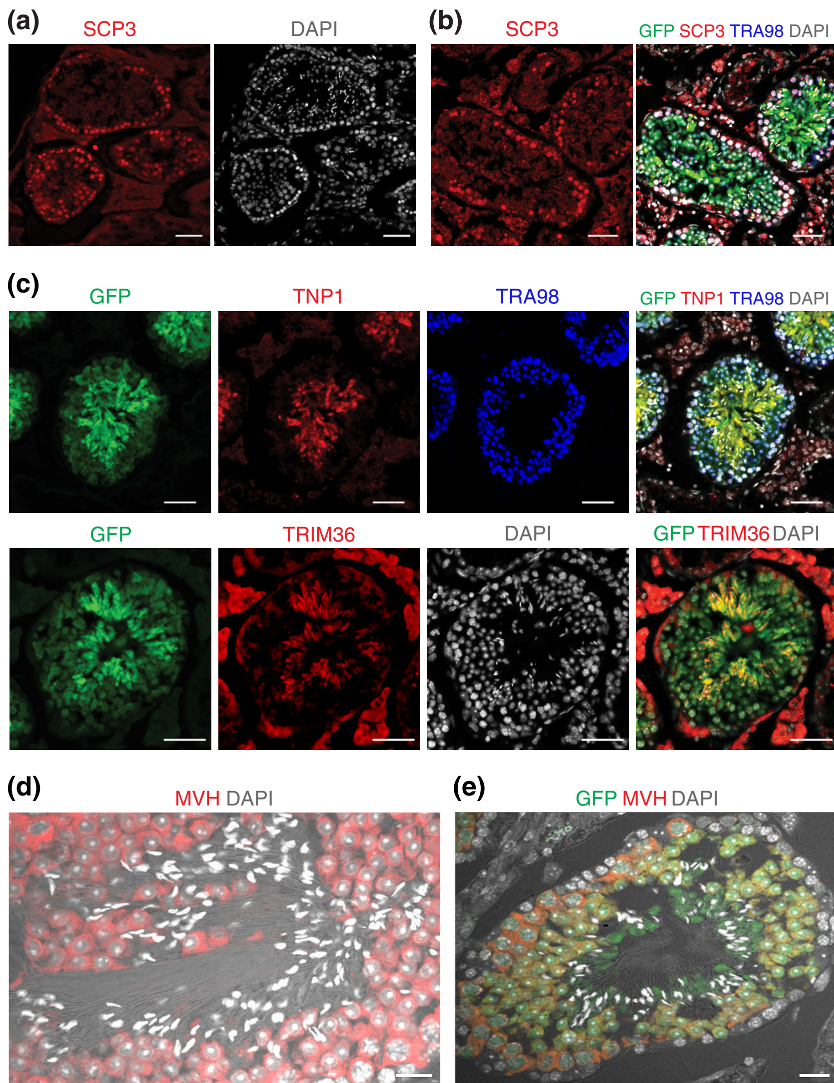

FIGURE 4 Spermatogenesis in testes of Tsc22d3-conKO mice after SSC transplantation. (a) Immunofluorescence for SCP3 in a cryosection of a Tsc22d3-conKO testis after transplantation of HZ-6 (derived from C57BL/6J). DAPI (white) serves as nuclear stain. (b) Immunofluorescence for SCP3, GFP, and TRA98 in a cryosection of a Tsc22d3-conKO testis after transplantation of HZ-9 (derived from ROSA26-EGFP). DAPI (white) serves as nuclear stain. (c) Immunofluorescence for GFP, TNP1, TRA98, and TRIM36 in cryosections of a Tsc22d3-conKO testis after transplantation of HZ-9 (derived from ROSA26-EGFP). DAPI (white) serves as nuclear stain (d) Immunofluorescence for MVH in a cryosection of a Tsc22d3-conKO testis after transplantation of HZ-7 (derived from C57BL/6J). DAPI (white) serves as nuclear stain. (e) Immunofluorescence for GFP and MVH in a cryosection of a Tsc22d3-conKO testis after transplantation of HZ-11 (derived from B6-GFP). DAPI (white) serves as nuclear stain. Scale bars, 50 μm in (a–c) and 20 μm in (d, e)

spermatids and mature sperm (Kitamura, Nishimura, Nishimune, & Tanaka, 2005; Kitamura, Tanaka, & Nishimune, 2003). We found that HZ-9 reinitiated spermatogenesis in a Tsc22d3-conKO testis on transplantation, and that cells developed into haploid cells (Figure 4c). We observed sperm with tail in seminiferous tubules of a Tsc22d3-conKO testis after transplantation with HZ-7 (derived from C57BL/6J; Figure 4d) and with HZ-11 (derived from B6-GFP; Figure 4e).

3.5 | Germline transmission from transplanted SSCs

Males were mated with C57BL/6J females 2 months after transplantation, but no offspring was obtained after 5–14 months. We proceeded to apply the assisted reproduction technique ICSI, injecting sperm without tail into BDF1 (C57BL/6J x DBA/2) oocytes or C57BL/6J oocytes (Figure 5a). Zygotes developed into 2-cell embryos after 12–24 hr culture in KSOM medium (Figure 5b). Following transplantation of 2-cell embryos into oviducts of pseudopregnant ICR females, we obtained a total of 96 live pups: 79 pups using BDF1 oocytes and 17 pups using C57BL/6J oocytes (Table 2). The birth rate for ICSI using C57BL/6J oocytes is known to be very low (Sakamoto, Kaneko, & Nakagata, 2005). Pups exposed to UV light displayed the intrinsic green fluorescence of GFP in a manner that is consistent with the provenance of the SSC line from a heterozygous mouse (Figure 5c, left panel), a homozygous mouse (Figure 5c, middle panel), or a C57BL/6J mouse (Figure 5c, right panel). We confirmed the presence or absence of GFP in genomic DNA from tail biopsies with PCR (data not shown). Thus, the testicular soma of Tsc22d3-conKO mice supports spermatogenesis and germline transmission of long-term cultured SSC lines on transplantation.

4 | DISCUSSION

Spermatogonial stem cells (SSCs) continuously undergo self-renewal to maintain the undifferentiated state, and differentiate to produce eventually spermatozoa, which transmit genetic information to the next generation (Oatley & Brinster, 2012). Recently gene expression signatures have been identified for 11 successive cell types or subtypes within the spermatogenic lineage of the mouse, starting with SSCs (Hermann et al., 2018). Transplantation of SSCs into testes can

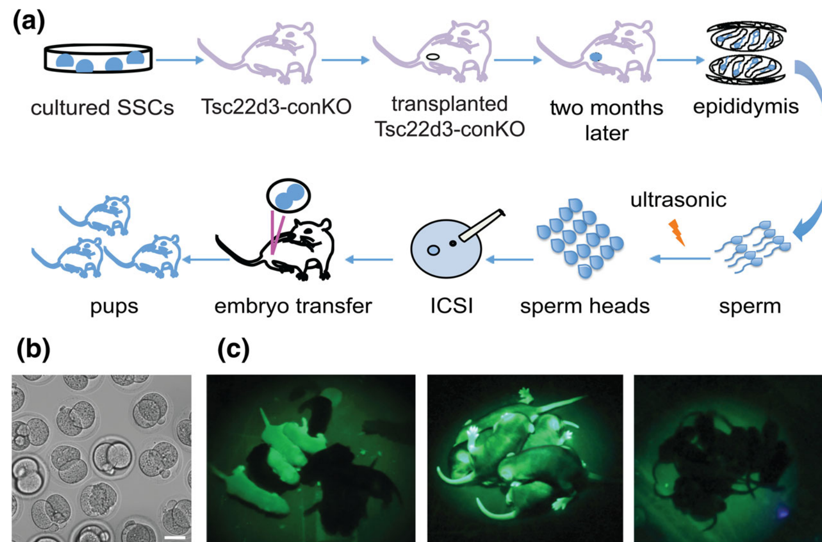

FIGURE 5 Offspring generated by ICSI with sperm from testes of Tsc22d3-conKO mice after SSC transplantation. (a) Experimental strategy. Cultured SSCs were transplanted into the testes of Tsc22d3-conKO mice. Two months later, epididymis spermatozoa were isolated and exposed to ultrasound, and sperm heads were injected into oocytes by ICSI. Following 2-cell embryo transfer, live offspring were obtained. (b) Brightfield image of 2-cell embryos after ICSI into BDF1 oocytes with sperm from a Tsc22d3-conKO male transplanted with HZ-9 (derived from ROSA26-EGFP). Scale bar, 50 μm. (c) Pups exposed to UV light. Left panel, pups obtained with HZ-9, which is hemizygous for the targeted mutation in the ROSA26 locus. Four out of 10 pups display the intrinsic green fluorescence of GFP. Middle panel, pups obtained with HZ-3 (derived from Tg(act-EGFP)), which is homozygous for the transgene. All pups express the intrinsic green fluorescence of GFP. Right panel, pups obtained with HZ-6 (derived from C57BL/6J). No pups express the intrinsic green fluorescence of GFP

be applied to treat male infertility (Ogawa, Dobrinski, Avarbock, & Brinster, 2000); can be used to produce transgenic animals (Chapman et al., 2015; Sato et al., 2015; Wu et al., 2015); and is the most stringent functional assay to assess SSC activity. After transplantation into the seminiferous tubules, SSCs pass through the blood testis barrier that is formed by tight, adherens and gap junctions between adjacent Sertoli cells; a fraction of the transplanted SSCs migrate to the basement membrane of the seminiferous tubules; and some cells complete the process of spermatogenesis (Nagano, Avarbock, & Brinster, 1999).

TABLE 2 Production of offspring by ICSI

SSC line	Percentage of cultured embryos that developed to the two-cell stage	No. of embryos transferred	No. (%) of pups	No. of GFP+ pups
B6D2F1 oocytes				
HZ-2	77%	61	0 (0%)	0
HZ-3	52%	102	8 (8%)	8
HZ-6	61%	82	21 (26%)	0
HZ-7	43%	100	22 (22%)	0
HZ-9	50%	92	9 (10%)	4
HZ-11	46%	100	19 (19%)	11
HZ-12	60%	100	0 (0%)	0
C57BL/6J oocytes				
HZ-3	80%	92	5 (5%)	5
HZ-6	55%	54	0 (0%)	0
HZ-7	80%	62	2 (3%)	0
HZ-9	61%	84	0 (0%)	0
HZ-11	65%	92	4 (4%)	1
HZ-12	76%	80	6 (8%)	5

Recipients for SSC transplantation have been prepared or bred in several ways over the past decades: by testicular irradiation (Withers, Hunter, Barkley, & Reid, 1974; Zhang, Shao, & Meistrich, 2006), by cooling the testes (Ehmcke, Joshi, Hergenrother, & Schlatt, 2007; Young et al., 1988; Zhang et al., 2004), by heat shock treatment (Ma et al., 2011), by experimental cryptorchidism (Mendis-Handagama, Kerr, & de Kretser, 1990), by injection of the chemotherapeutic drug busulfan (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994; Bucci & Meistrich, 1987), and by breeding Kit^{W}/Kit^{Wv} compound heterozygous mice (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994). A quarter of a century after the first reports of busulfan-treated mice and Kit^{W}/Kit^{Wv} compound heterozygous mice as recipients for spermatogonial transplantation (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994), they remain the most widely used types of recipients. But disadvantages include the morbidity and mortality (busulfan-treated mice), and the inefficient generation of recipients by breeding (only 25% of male offspring from heterozygous parents is Kit^{W}/Kit^{Wv} compound heterozygous). There is thus opportunity for improvement in identifying and optimizing a novel type of recipient that is devoid of morbidity or mortality and that can be bred efficiently in a single cross and without genotyping.

GILZ was originally discovered as an anti-inflammatory protein that is involved in the immunosuppressive effects of glucocorticoids (D'Adamio et al., 1997). The mouse GILZ protein is encoded by the *Tsc22d3* gene, which is located on the X chromosome. Males hemizygous for a Tsc22d3 knockout were unexpectedly found to be infertile

(Bruscoli et al., 2012; Ngo et al., 2013, 2013; Romero et al., 2012; Suarez et al., 2012). It is not possible to generate homozygous Tsc22d3-KO females by breeding (Suarez et al., 2012). Hemizygous Tsc22d3-KO males must be bred by crossing heterozygous females with wild-type males, and 50% of the male offspring of such crosses are hemizygous and infertile. The defect in the germline appears to be intrinsic, as spermatogenesis can be restored in Tsc22d3-KO males by transplantation of freshly prepared wild-type germ cells (Bruscoli et al., 2012). But germline transmission of the donor haplotype, either by natural mating or with the aid of ICSI, remained to be shown for Tsc22d3-KO mice.

Our strategy of the goGermline technology consists of mating homozygous Tsc22d3 floxed females with homozygous ROSA26-Cre males, producing 100% male mice that are Tsc22d3-conKO and infertile (Koentgen et al., 2016). Upon microinjection of embryonic stem cells in blastocysts or eight-cell embryos generated in this cross, chimeras can be generated that yield 100% germline transmission of the embryonic stem-cell derived genome (Koentgen et al., 2016). We have now evaluated Tsc22d3-conKO males as recipients for SSC transplantation. Our newly derived SSC lines and the recipient males are in an inbred C57BL/6J background, and are thus immunologically fully compatible. We were able to generate live offspring carrying the donor haplotype from several SSC lines with the aid of ICSI.

The mechanisms of infertility of Tsc22d3-KO mice have been studied by several groups and in several strains (Bruscoli et al., 2012; La et al., 2018; Ngo, Beaulieu, et al., 2013; Ngo, Cheng, et al., 2013; Romero et al., 2012; Suarez et al., 2012). The phenotype involves an arrest midway through the pachytene of meiosis I, massive apoptosis, SSC exhaustion, resulting in a progressive depletion of the germline, and terminating in a Sertoli cell-only phenotype. Our contribution here is to demonstrate that the testicular soma of adult Tsc22d3-conKO mice supports spermatogenesis and germline transmission upon transplantation of established, long-term cultured SSC lines. The proof of principle that we have delivered paves the way for optimization of Tsc22d3-conKO mice as a third type of recipient for SSC transplantation—a type of recipient that is devoid of morbidity and mortality and that can be bred efficiently and without genotyping. The next step is to obtain offspring by natural mating of transplanted Tsc22d3-conKO males. We speculate that Tsc22d3-conKO pups may be better recipients (Kubota & Brinster, 2018; Shinohara, Orwig, Avarbock, & Brinster, 2001).

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