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Article

A novel undifferentiated spermatogonia-specific surface protein 1 (USSP1) in neonatal mice

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ABSTRACT

Mammalian spermatogenesis is maintained by a rare population of spermatogonial stem cells (SSCs), which are important for male fertility. SSCs remain a subset of undifferentiated spermatogonia, which can be isolated by a combination of surface markers. Specific markers to identify and isolate undifferentiated spermatogonia are lacking. *Ussp1*, a transcript previously annotated as long noncoding RNA (RIKEN cDNA 4933427D06, Gene ID: 232217), virtually encodes a membrane protein, USSP1, in a highly testis-specific manner in mouse. We demonstrate its expression on the membrane of undifferentiated spermatogonia by a homemade polyclonal rabbit antibody against the protein. *In vivo*, USSP1$^+$ clusters consist mainly of As, Apr (GFRα1$^+$) and Aal (PLZF$^+$) cells. USSP1$^+$ cells exhibit enrichment of undifferentiated spermatogonia, as shown by increased expression of SSC self-renewal molecular markers and the potential to form SSC clones *in vitro* and *in vivo*. However, *Ussp1* knockout did not affect the number of SSCs or spermatogenesis in mice. Thy1$^+$ cells from *Ussp1* null mice did not show any defect in the SSC colony formation capacity, indicating that USSP1 is not essential for SSC self-renewal. Our data demonstrate that *Ussp1* is specifically expressed in undifferentiated murine spermatogonia, indicating the potential to sort undifferentiated spermatogonia with USSP1 antibodies. *Ussp1* might be a good maker for SSC enrichment in neonatal mice.

1. Introduction

Spermatogonial stem cells (SSCs) were first demonstrated by Brinster and his colleagues [1] based on the observation of donor-derived spermatogenesis in the seminiferous tubules of infertile recipient mice after cell transplantation. SSCs maintain the ability to re-establish complete spermatogenesis and derive fertile offspring following transplantation, even after long-term culture or cryopreservation [2,3]. Combining these techniques, the transplantation of germ cells has shown tremendous potential for clinical application, such as (1) treating male infertility, and (2) fertility preservation for young boys undergoing chemotherapy for cancer [4]. SSCs are derived from primordial germ cells (PGCs), which differentiate into prospermatogonia when they become enclosed in seminiferous cords [4]. During the first postnatal week, prospermatogonia are specified into A_{single} (A_s) spermatogonia and further proliferate. A_s spermatogonia progress through multiple rounds of incomplete division to produce A_{paired} (A_{pr}) and chains of 4, 8, and 16 cells, known as $A_{aligned}$ (A_{al}) spermatogonia [5]. Remaining on the basement membrane of seminiferous tubulars, A_s, A_{pr} and A_{al} are collectively described as undifferentiated spermatogonia (USPG), while the identity of truly SSCs remains unclear [6]. Recently, single-cell RNA sequencing showed SSCs could not be distinguished via whole transcriptome data [7], supporting a plastic spermatogonial model rather than a hierarchal model. Upon differentiation, A_{al} convert to A1 spermatogonia and sequentially give

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rise to A2-A3-A4-In-B spermatogonia and spermatocytes, which further migrate toward the tubular lumen and enter meiosis [8].

To investigate the molecular mechanism underlying the behavior of undifferentiated spermatogonia and dissect its hierarchy, a series of markers has been identified to label these cells. GFRα1 (GDNF family receptor–α1), which mediates the response to GDNF signaling for spermatogonia self-renewal [9,10], was shown to be expressed on the surface of early $A_{s,\ pr,\ al}$ spermatogonia [11,12]. Furthermore, downstream transcription factors of GDNF/GFRα1 signaling, BCL6b [13] and ETV5 [14], were demonstrated to be critical for SSC self-renewal. Inhibitor of differentiation 4 (ID4), was showed to be exclusively expressed in A_s spermatogonia [15]. Lineage tracing and transplantation experiments demonstrated the stem cell capacity of $ID4^+$ cells [16,17]. Similar to ID4, Pax7 was found to be expressed in As spermatogonia, and Pax7+ cells can form long-term spermatogenic patches after transplantation [18]. PLZF (promyelocytic leukemia zinc-finger), another important transcription repressor involved in SSC maintenance, is expressed in all stages of undifferentiated spermatogonia [19,20]. A target of PLZF, c-KIT [21], was a negative-selection marker of SSCs [22–24].

We have identified several hundred candidate genes, which are specifically expressed in undifferentiated $Thy1^+$ spermatogonia by RNA sequencing [25]. While analyzing the transmembrane domain-containing genes, we discovered a protein encoded by a transcript annotated as long noncoding RNA (lncRNA), *RIKEN cDNA 4933427D06Rik*. An open reading frame (ORF) was predicted to encode a polypeptide of more than 200 amino acids. To test whether the transcript encodes a protein, we made a polyclonal rabbit antibody against the predicted protein. We demonstrated that this protein, which was predicted to have a transmembrane domain in its C-terminal, was specifically expressed on the membrane of undifferentiated spermatogonia, as determined by antibody-based methods. Furthermore, the protein sequence was verified by IP-MS/MS. We named it undifferentiated spermatogonia-specific surface protein 1 (USSP1) because it could be utilized to isolate spermatogonia with enriched SSCs. To further explore the function of USSP1 in mouse spermatogonia, the *Ussp1* gene was knocked out with the CRISPR/Cas9 system in mice as previously described [26]. *Ussp1* knockout (KO) did not affect SSC self-renewal or spermatogenesis. But our study sheds light on a novel testis-specific marker, which may prove useful for isolation of undifferentiated spermatogonia in neonatal mice.

2. Materials and methods

2.1. Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University, China. C57BL/6, C57BL/6-Tg(ACTB-EGFP)1Osb/J and CD1 mice were maintained with free access to food and water in a specific pathogen-free (SPF) animal facility. Mice were kept under temperature-controlled (22 ± 1 °C) and light-controlled (14:10 h light:dark cycles) conditions. C57BL/6 mice were chosen to generate knockout mice and subsequent experiments. To reduce the off-target effects of CRISPR/Cas9 system, heterozygote mice were serially bred with wild-type mice for at least three generations. Homozygous mice were produced by crossing F4 heterozygotes.

2.2. RT-PCR and real-time quantitative PCR

Total RNA from tissues at different developmental stages were extracted using TRIzol reagent (15596–018, Invitrogen). Reverse transcription (RT), with oligo (dT) primer, was performed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, #K1621) according to the manufacturer's instructions. Primers were designed based on sequences in the National Center for Biotechnique Information (NCBI) database.

For RT-PCR, rTaq polymerase (R001A, TAKARA) was used under the following cycling conditions: 95 °C for 3 min and 30 cycles of 95 °C for 15 s, 60 °C for 15 s, 72 °C for 45 s, with a final extension at 72 °C for 5 min.

For real-time PCR, the StepOnePlus Real-Time PCR system and SYBR green master mix (4367659, ABI) were used according to the manufacturer's instructions (Applied Biosystems).

All primers used for RT-PCR and real-time PCR are listed in Table S1 (online).

2.3. Generation of rabbit anti-USSP1 polyclonal antibody

The predicted coding region of mouse *Ussp1* (612 bp) was cloned into pENTR/D-TOPO vector (Invitrogen). The full-length CDS was LR recombined into pDEST17 (Invitrogen). The 6 × His fusion protein was expressed in *E. coli* strain BL21 and purified using a Ni Sepharose High Performance column (17-5268-01, GE Healthcare), according to the manufacturer's instructions. Purified protein was used to immunize two healthy rabbits twice (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences). Antibody was purified from the serum of the rabbits by affinity purification using an antigen-coupled column generated with AminoLink Plus Coupling Resin (20501, Thermo). For fluorescence-activated cell sorting (FACS), purified antibody was labeled with Alexa Fluor™ 555 NHS Ester (A20009, Invitrogen) according to the manufacturer's instructions.

2.4. Western blot analysis

Tissues or cells were homogenized in cold NETN buffer with protein inhibitor cocktail (P8340, Sigma). Lysates were then centrifuged at 15,000 r/min for 15 min at 4 °C, and the supernatants were collected. Proteins were fractionated by SDS-PAGE and electrotransferred to a nitrocellulose membrane (HATF00010, Millipore). The membrane was blocked with milk and incubated with the following diluted primary antibodies overnight at 4 °C: rabbit anti-USSP1 antibody (1:5,000) and mouse anti-GAPDH antibody (1:5,000; 60004-1-Ig, Proteintech group). Goat anti-mouse IgG (1:10,000; 926–32220, LI-COR) and goat anti-rabbit IgG (1:10,000; 926–32211, LI-COR) were used as secondary antibodies. The images were captured by scanning membrane with the Licor Odyssey system.

2.5. Immunoprecipitation-Mass spectrometry

Nine dpp (days postpartum) pup testes were collected and homogenized in cold NETN buffer with protein inhibitor cocktail. Lysates were then centrifuged at 15,000 r/min for 15 min at 4 °C, and the supernatants were collected. Protein A/G beads (20422, Thermo) coupled with anti-USSP1 antibody were added into the lysates and kept rotated for 3 h with at 4 °C. The beads were then centrifuged and washed three times in NETN buffer, followed by heating in Protein Loading Buffer (FD002, Fudebio-tech). Proteins were fractionated by SDS-PAGE, and followed by sliver staining. A single band at approximately 28 kD was cut and submitted to LC-MS/MS (Liquid Chromatography with tandem mass spectrometry, Fitgene Biotech, Guangzhou) analysis.

2.6. Whole-mount immunofluorescence

Seminiferous tubules were fixed in 4% paraformaldehyde solution on ice for 20 min. After washing with PBS, samples were

incubated with blocking solution (PBS with 10% FBS and 0.1% Tween-20) for 1 h at room temperature. Antibodies were diluted in blocking buffer; the following primary antibodies were incubated overnight at 4 °C: rabbit anti-USSP1 antibody (0.5 μg/mL), goat anti-PLZF antibody (1:500; AF2944, R&D systems), goat anti-GFRα1 antibody (1:500; AF560, BD Biosciences), goat anti-GATA4 antibody (1:200; sc-1237, Santa Cruz Biotechnology). The following secondary antibodies were incubated at room temperature for 1 h: Alexa Fluor 647-conjugated donkey anti-goat IgG (1:500; A21447, life technologies) and Alexa Fluor 555-conjugated donkey anti-rabbit IgG (1:500; A21206, life technologies). Tissues were then incubated in Hoechst33342 (B2261, Sigma), observed and photographed using a Nikon microscope.

2.7. Magnetic-activated cell sorting

Thy1$^+$ germ cells were isolated through magnetic-activated cell sorting, as previously described [27]. Briefly, 9 dpp pup testes were collected and digested into single cell suspension by the combination of 0.25% Trypsin-EDTA and DNase I (10 mg/mL). A single cell suspension was then separated with a 30% Percoll solution, and the pellet was collected and incubated with magnetic microbeads conjugated with anti-Thy1 antibody (130–049–101, Miltenyi Biotec) for 20 min. Thy1$^+$ cells labeled with magnetic beads were isolated by the separation column and MACS Separation Unit (130-142-102, Miltenyi Biotec).

2.8. Fluorescence-activated cell sorting

Single cell suspension from 9 dpp pup testes was prepared for magnetic-activated cell sorting. The cells were incubated in antibodies diluted in PBS-S (PBS with 1% FBS, 10 mmol/L HEPES, 1 mg/mL glucose, 1 mmol/L Soduim pyruvate, 50 units/mL penicillin and 50 μg/mL streptomycin) on ice for 1 h and washed twice in PBS-S. Then, 5 μg/mL Propidium Iodide (P4170, Sigma) was added to the cell suspension 5 min before loading onto a BD FACSAria II to eliminate dead cells. The antibodies included Alexa Fluor 488-conjugated rabbit anti-USSP1 antibody (1 μg/mL) and rat anti-Thy1 antibody (12-0903-81, eBioscience). Rabbit IgG were used as isotype control (1:500; 31235, life technologies).

2.9. Cell culture

Thy1$^+$ germ cells were cultured as previously described [27] with slight modifications. Briefly, Thy1$^+$ germ cells were maintained on MEF feeder cells in serum-free medium with GDNF (212-GD-010, R&D Systems), bFGF (354060, BD Biosciences), and GFRα1 (560-GR-100, R&D Systems) at final concentrations of 20, 150, and 10 ng/mL, respectively.

Serum-free medium was MEMα (12571, Gibco) supplemented with 0.2% BSA (A9418, Sigma), 50 units/mL penicillin, 50 μg/mL streptomycin, 2 mmol/L L-glutamine (25030, Gibco), 10 mmol/L HEPES (H0887, Sigma), 50 μmol/L 2-ME (M7522, Sigma), 10 μg/mL transferrin (T1283, Sigma), 60 μmol/L putrescine (P5780, Sigma), 5 μg/mL insulin (I5500, Sigma), 30 nmol/L Na_2SeO_3, and 7.6 μeq/L FFA mix (described in ref. [27]).

For colony formation assay, 2×10^5 sorted cells were resuspended in serum-free medium with growth factors, and placed in wells of 12-well plates containing MEF monolayers. Clumps were counted at Day 7 and submitted for statistical analysis.

2.10. Germ cell transplantation

Pregnant mice were treated with a single dose of busulfan (50 mg/kg; Sigma-Aldrich) at E13.5d. Male pups were used as recipients at 10–12 dpp. Approximately 2–3 μL of donor-cell suspension at a concentration of 1×10^7 cells/mL was introduced into each testis of recipients. Seventy days after transplantation, recipients were sacrificed and the testes were observed and photographed using a Stereo Lumar.V12 fluorescent stereoscope. A donor spermatogonial stem cell was defined by its ability to produce an EGFP-positive spermatogenic colony.

2.11. gRNA design and vector construction

We designed four gRNAs targeting exon 3 of *Ussp1* based on http://crispr.mit.edu. All gRNAs were cloned into pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid #42230) and pDR274 (Addgene plasmid #42250), respectively. The gRNA ligated pX330 vectors were transfected into V6.5 embryonic stem cells, which was followed by the T7EI assay to confirm the activity of each gRNA (data not shown).

2.12. In vitro transcription of Cas9 mRNA and gRNA

The Cas9 expression vector MLM3613 was a gift from Keith Joung [28] (Addgene plasmid #42251), and the Cas9 sequence in MLM3613 was then replaced with a mammalian expression optimized version from pX330. The vector was linearized and recovered by gel extraction, which was followed by *in vitro* transcription using mMESSAGE mMACHINE T7 Ultra Kit (AMB1345, Ambion). The gRNA ligated pDR274 was linearized and *in vitro* transcribed into RNA using a MEGAshortscript T7 Transcription Kit (AM1354, Ambion).

2.13. Single-cell embryo injection

Female mice (C57BL/6J, 4–6 weeks old) were used as embryo donors following superovulation. After mating, resultant fertilized embryos were collected from the oviducts and cultured in KSOM medium. Then, 100 ng/μL Cas9 mRNA and 50 ng/μL gRNA were mixed, and 10 pL of the mixture was injected into the cytoplasm of each zygote. Surviving injected embryos were transferred into oviducts of pseudopregnant ICR females.

2.14. Mouse genotyping

Tails of 3-week-old pups were collected for genotyping using the KAPA Mouse Genotyping Kit (KAPA biosystems, KK7302) according to the manufacturer's protocol. PCR was performed using the following primers:

Ussp1-T7E1-F: 5′- CAGTCTCCCTATTGAGTTTCAGA -3′,

Ussp1-T7E1-R: 5′- AAACAAACATAGCCAGGGAG -3′.

Target region amplicons were cloned into a T-cloning vector (B522211, Sangon Biotech.), transformed into *E. coli* DH5α competent cells and plated onto on LB plate containing 100 μg/mL ampicillin. Single clones were chosen, and plasmid DNA was isolated and sequenced by a commercial sequencing company (IGE Biotech.).

2.15. Histological analysis

Testis tissue were fixed in Bouin's solution overnight, embedded in paraffin and sectioned. After rehydration in an ethyl alcohol series, the samples were stained with hematoxylin and eosin (HE) and subsequently observed and photographed using a Carl Zeiss microscope.

2.16. Statistical analysis

In histograms, all data were represented by the mean ± SEM of at least three replicates for each experiment. Where indicated, the

results were compared in different groups using the chi-square and student's *t*-test analyses. Differences were considered significant with $P < 0.05$ or 0.01.

3. Results

3.1. Ussp1 is specifically expressed in mouse testis

Our previous RNA-seq data showed that *Ussp1* (*4933427D06*, Gene ID: 232217) is expressed in mouse testis cells [25]. This result was confirmed by RT-PCR of different somatic tissues, and only testes expressed *Ussp1* mRNAs (Fig. 1a). *Ussp1* was annotated as long noncoding RNA in the NCBI database and showed high tissue specificity and low conservation across species. Meanwhile, its ORF was predicted to encode a 203-amino-acid polypeptide. To test whether the transcript encodes a protein, a polyclonal rabbit antibody was generated against the full length of the predicted protein. To confirm the specificity of the homemade USSP1 antibody, we fist transiently transfected HEK293T cells with pCMV-USSP1-SFB or pCMV-GFP-SFB expression vector and then performed Western blotting with Flag antibody and USSP1 antibody. Results showed that our antibody could distinguish USSP1 protein specifically (Fig. S1 online). Using various mouse tissues, a single band at approximately 28 kD, which is slightly larger than the expected molecular mass at 22.9 kD, was detected in the testes, but not in other tissues by western blot analysis (Fig. 1b). And then, the approximately 28 kD endogenous proteins from pup testes that immunoprecipitated by the homemade antibody were submitted to LC-MS analysis. The mass spectrum of the polypeptide R.SEPTEPTEPAGAAGR was match to the predicted sequence (Fig.S2, S3a, b online), certainly confirming the translation of this lncRNA. Further, the expression pattern of USSP1 transcript across different developmental stages was investigated. The mRNA expression level of *Ussp1* peaked at 9 days post-partum (Fig. 1c), which is the time at which undifferentiated spermatogonia amplify to a maximum relative number and begin to differentiate. Intriguingly, its expression pattern was similar to a well-known SSC marker gene, *Plzf* (Fig. 1d). These data imply that *Ussp1* is a testis-specific gene and encodes a protein.

3.2. USSP1 specifically marks a subset of spermatogonia in vivo

To explore the USSP1$^+$ cell subset, whole-mount immunostaining was then performed using seminiferous tubular fragments at different development stages of testis. We chose PLZF to label undifferentiated spermatogonia, GFRα1 to label A_s and A_{pr} spermatogonia, and GATA4 to label Sertoli cells [29]. As shown by the immunofluorescent pictures, USSP1 was located on the surface of cells. Most USSP1$^+$ cells are PLZF$^+$ across all development stages and vice versa (Fig. 2a, b). Almost all the GFRα1$^+$ cells are USSP1$^+$, however, only a subset of USSP1$^+$ cells is GFRα1$^+$, and the percentage of this subset decreased with the development of testes after 9 dpp (Fig. 2c, d). In contrast, very few USSP1$^+$ cells are GATA4$^+$ (Fig. 2e, f). Taken together, these data demonstrate the high specificity of USSP1 for undifferentiated spermatogonia.

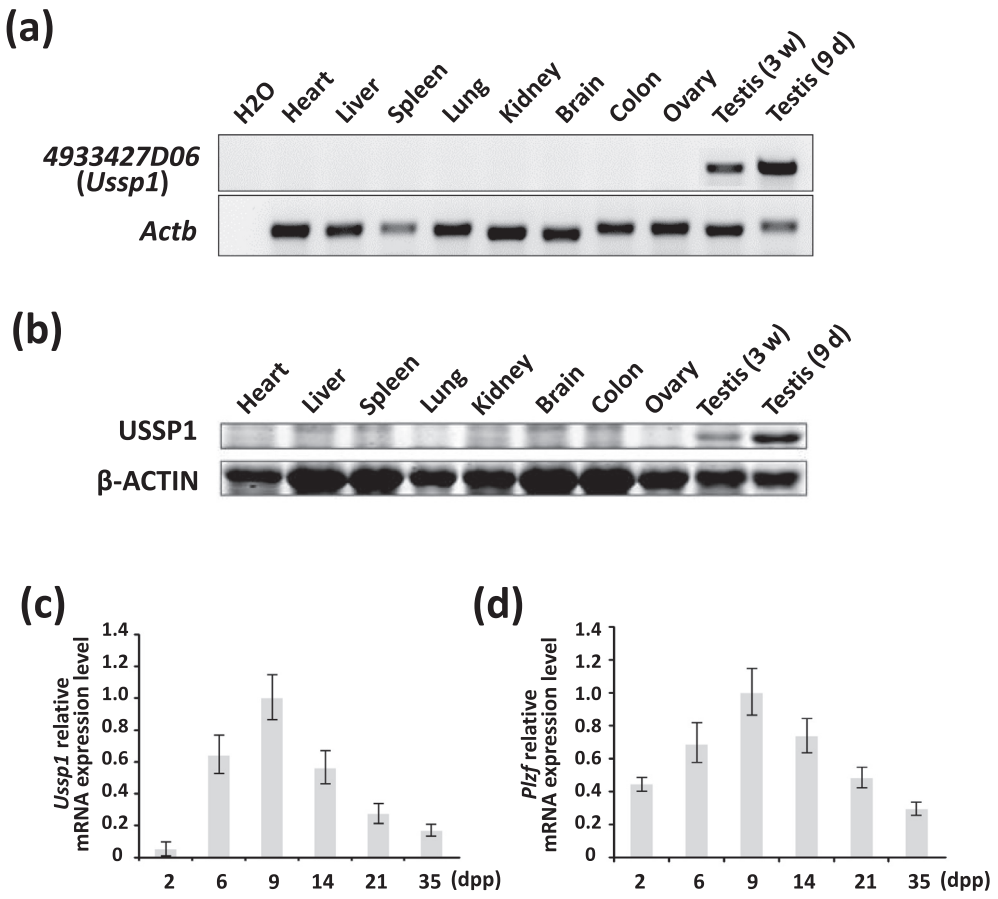

Fig. 1. *4933427D06* (*Ussp1*) is specifically expressed in mouse testis. (a) RT-PCR of *4933427D06* (Gene ID: 232217, hereafter referred to as *Ussp1*) in different tissues from 3-week-old mice and 9 dpp mouse testis. (b) Western blot analysis of USSP1 in different tissues from 3-week-old mice and 9 dpp mouse testis. (c) mRNA expression pattern of *Ussp1* in different ages of male mice by quantitative real-time PCR of *Ussp1*. (d) mRNA expression pattern of *Plzf* in different ages of male mice by quantitative real-time PCR. dpp, days postpartum.

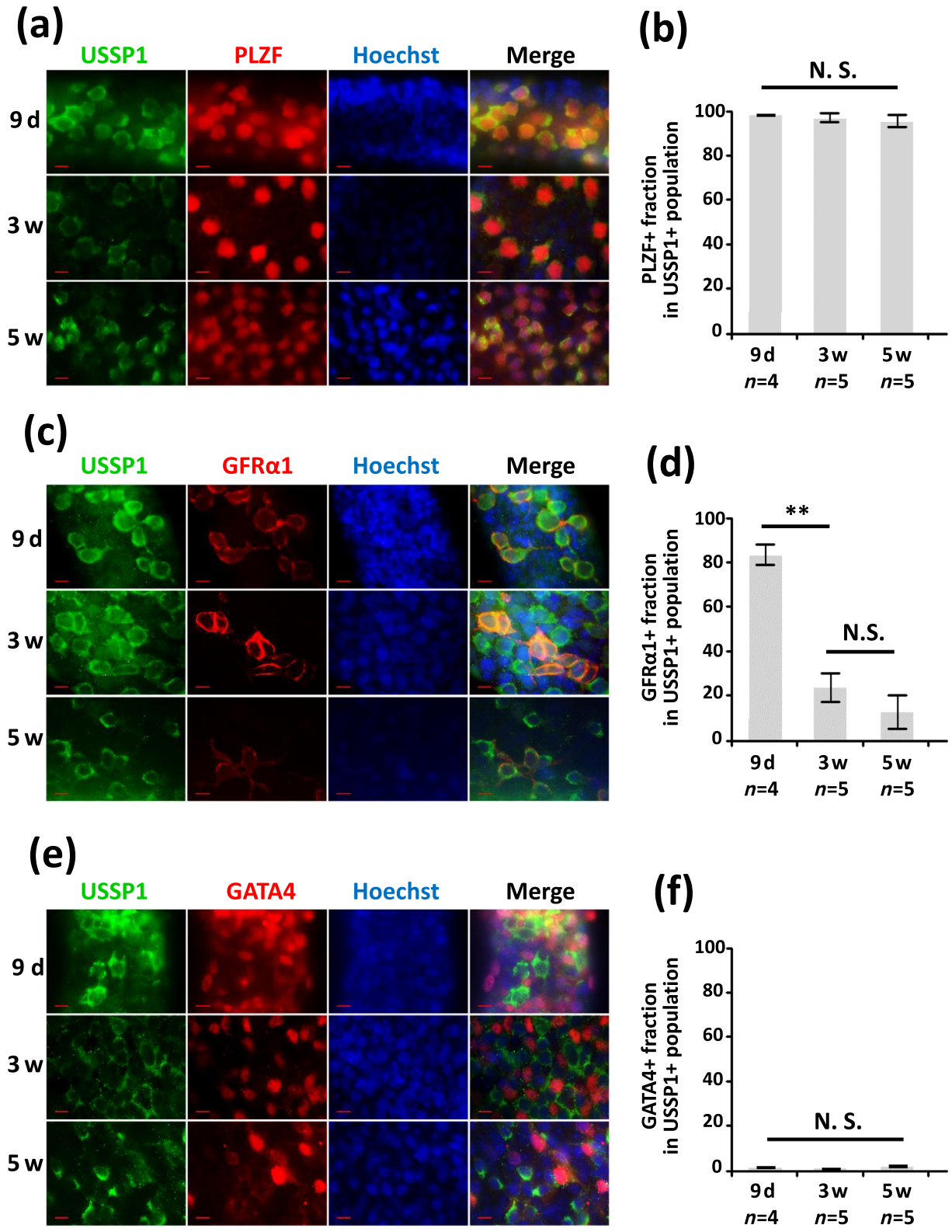

Fig. 2. USSP1 specifically marks a subset of spermatogonia *in vivo*. Distribution patterns and statistical results of USSP1 with PLZF (a, b), GFRα1 (c, d), and GATA4 (e, f) at different spermatogenesis stages. *n* represents the number of individuals. N.S., no significant difference was found between the groups; ** significant difference compared with the control group ($P < 0.01$). *n*, number of mice.

3.3. USSP1$^+$ population enriches undifferentiated spermatogonia

USSP1 was specifically expressed in a subset of cells that are PLZF and GFRα1 positive. We next tested whether USSP1$^+$ cells could enrich undifferentiated spermatogonia. First, we isolated the USSP1$^+$ population through fluorescence-activated cell sorting (FACS) from 9 dpp male mice (Fig. 3a). The recovery rate of USSP1$^+$ cells is 7.26%, which is consistent with the ratio of the PLZF$^+$ pop-

ulation (8.3% from 10 to 14 days postnatal testis) in a previous study [30]. Real-time PCR was then performed using the mRNA from both the sorted USSP1$^+$ population and unsorted population. Several markers that are involved in SSC self-renewal, including *Plzf*, *Bcl6b*, and *Etv5*, were evaluated. Interestingly, all SSC markers showed a significant increase in the USSP1$^+$ population compared to unsorted ones (Fig. 3b). Moreover, differentiating markers of spermatogonia cells, such as c-kit, sohlh1, and sohlh2, significantly

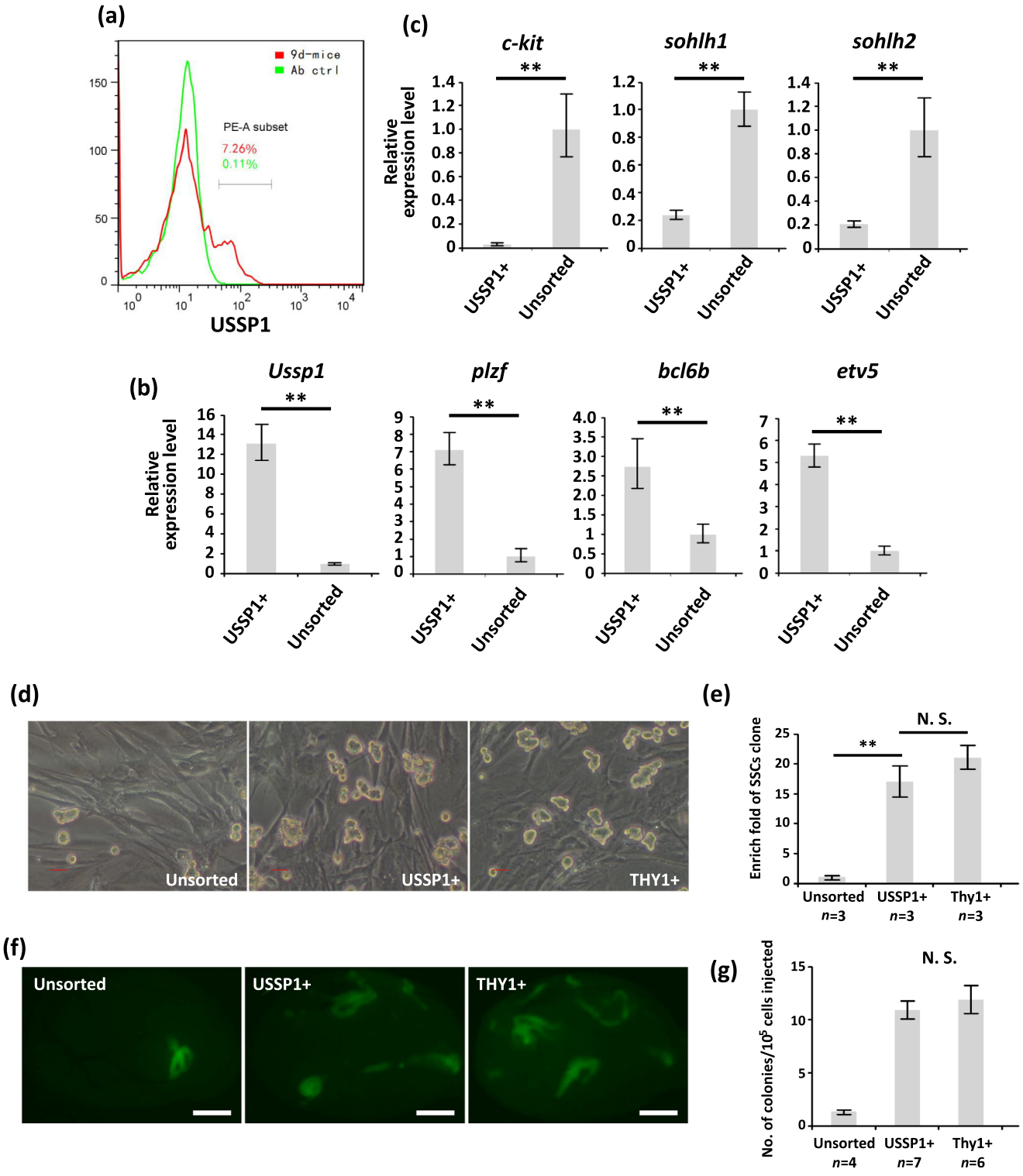

Fig. 3. USSP1 population enriches spermatogonial stem cells. (a) Isolation of the USSP1$^+$ population by fluorescence-activated cell sorting. (b-c) The USSP1$^+$ population showed a higher expression level of non-differentiation spermatogonia markers (*plzf, bcl6b* and *etv5*) (b) and lower expression level of differentiating spermatogonia markers (*c-kit, sohlh1* and *sohlh2*) (c) compared with the unsorted population. (d) Colony formation assays of unsorted, USSP1$^+$ and Thy1$^+$ populations. Scale bar = 50 μm. (e) Statistical result of (d). (f) Donor-derived spermatogenesis in recipient mice of unsorted, USSP1$^+$ and Thy1$^+$ groups. Scale bar = 1 mm. (g) Statistical result of (f). *n* represents experimental triplicate. N.S., no significant difference was found between the compared groups; * significant difference compared with the control group (**$P < 0.01$, ***$P < 0.001$).

decreased (Fig. 3c). These data suggests that the USSP1$^+$ cell population could enrich undifferentiated spermatogonia that contain SSCs. To further validate this hypothesis, unsorted testis cells, were individually cultured in serum-free conditions (Fig. 3d, Fig. S3 online). A cluster of germ cells was defined as a SSC colony. The colony formation efficiency of USSP1$^+$ cells was significant higher than the unsorted population, but comparable to that of Thy1$^+$ cells (Fig. 3d, e). Furthermore, we isolated USSP1$^+$ cells and Thy1$^+$ cells from ACTB-EGFP C57BL/6 testes to perform transplantation. Donor-derived spermatogenesis in recipient testes could be directly visualized under a fluorescent dissecting microscope due to constitutive expression of EGFP in donor cells. Seventy days after transplantation, approximately twice the period necessary for spermatogenesis in mouse, the recipients were sacrificed and the testes were observed (Fig. 3f). The number of donor-derived colonies in the USSP1$^+$ group did not differ significantly from the Thy1$^+$ group, but was about 8-fold compared to the unsorted group (Fig. 3f, g). These data suggested that USSP1$^+$ population enriches real SSCs.

3.4. One-step Ussp1 knockout mice generation with the CRISPR/Cas9 system

To test the functional role of the *Ussp1* gene in SSCs, we generated *Ussp1* knockout mice using the well-established CRISPR/Cas9 system [26]. The *Ussp1* gene is located at chromosome 6 and contains 4 exons with the start codon in exon 3. Additionally, we designed two gRNAs targeting sites in Exon 4 (Fig. 4a). Cas9 mRNA and gRNAs were coinjected into C57BL/6 murine zygotes. Then, surviving zygotes were transplanted into the oviduct of pseudo-pregnant CD1 female mice. Founder mice were screened by Sanger sequencing of the target region (Fig. 4b), and then bred with wild type C57BL/6 mice to generate F1 mice. We obtained six lines of F1 mice with different genotypes (Table S2 online) containing out-frame mutation, deletion and inversion from 3 founder mice. Then, we chose two lines for further functional analysis (Fig. 4c). Line 1 carried a 2-bp depletion in the gRNA target site, which led to pre-mature termination at amino acid 57 with two additional missense amino acid residues. Therefore, it might have a shortest

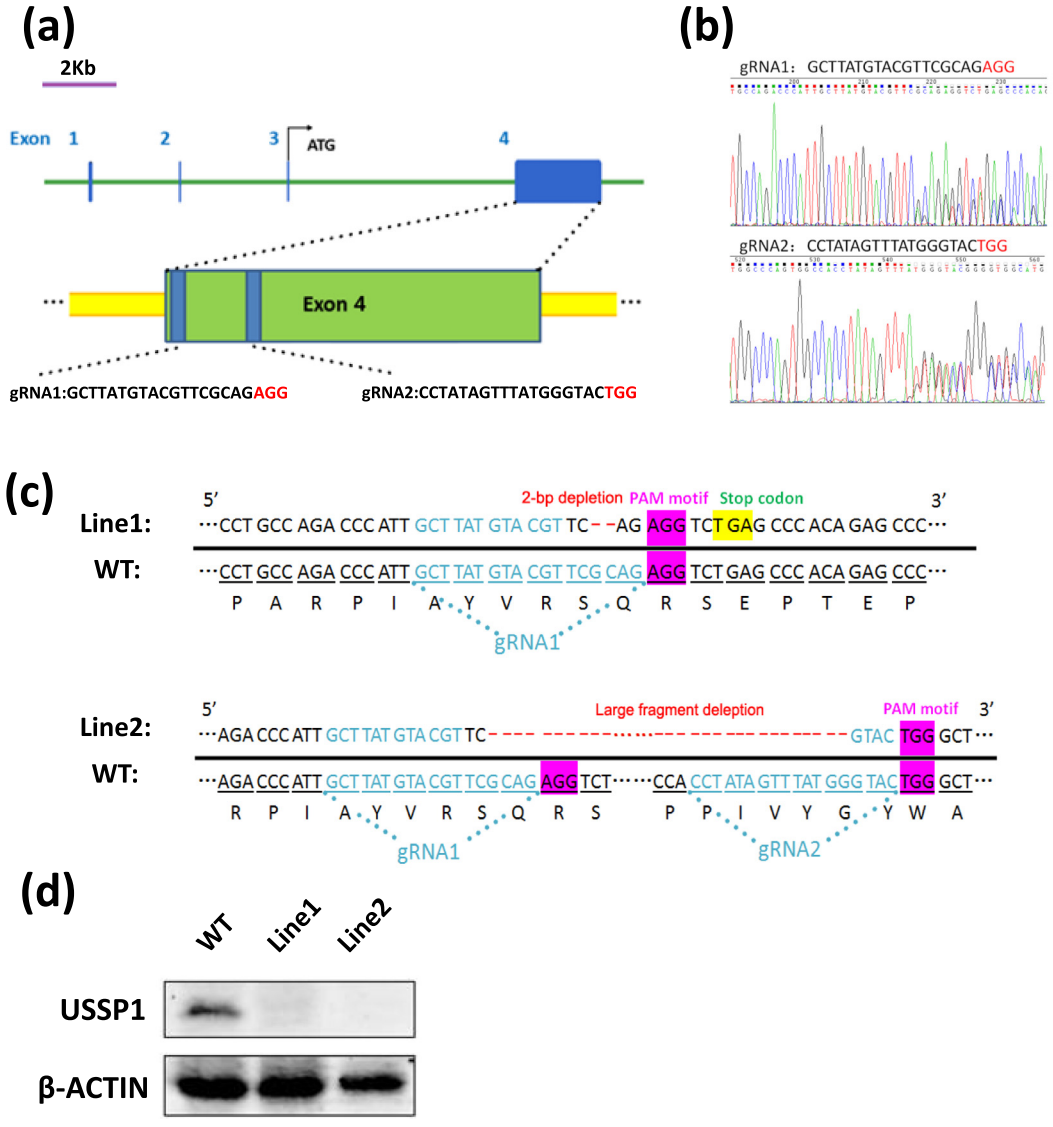

Fig. 4. One-step generation of *Ussp1* knockout mice with CRISPR/Cas9 system. (a) Schematic of the gRNA target sites in the *Ussp1* gene locus. (b) Representative Sanger sequencing results of founder mice are shown. (c) Genotyping results of Line1- and Line2-*Ussp1* null mice. Cas9-mediated indels lead to premature translation stop of both lines. gRNA target sites are shown in blue; Protospacer adjacent motifs (PAM) are labeled with purple; red shows the modified sequence; and the termination codons are labeled in yellow. (d) Western blot analysis of USSP1 expression in testis from WT, Line1- and Line2-*Ussp1* null mice.

truncated protein and fewest missense amino acid residues. Line 2, a 327-bp length fragment in-frame depletion between the gRNA target sites, was chosen for phenotype analysis since *Ussp1* may function as a lncRNA. Both mutations in the *Ussp1* locus depleted the full-length protein, which was confirmed by western blot analysis (Fig. 4d).

3.5. USSP1 is dispensable for spermatogenesis

All *Ussp1* knockout male mice form two selected lines were fertile, and the litter sizes and percentage of male pups were comparable to those of wild-type (WT) male mice (Fig. 5a, b), indicating a dispensable role of *Ussp1* for male fertility. In addition, no significant differences in testes weights were observed between 3-month-old *Ussp1* knockout mice and WT mice (Fig. 5c). Furthermore, there were no anatomical defects in the *Ussp1* null mouse, as evidenced by histological analyses (Fig. 5d). We next investigated the Thy1$^+$ population, which are undifferentiated spermatogonia, in *Ussp1* knockout mice. There was no significant difference in the recovery rate or colony forming efficiency (Fig. 5e, f). Therefore, disruption of *Ussp1* did not affect the maintenance of undifferentiated spermatogonia.

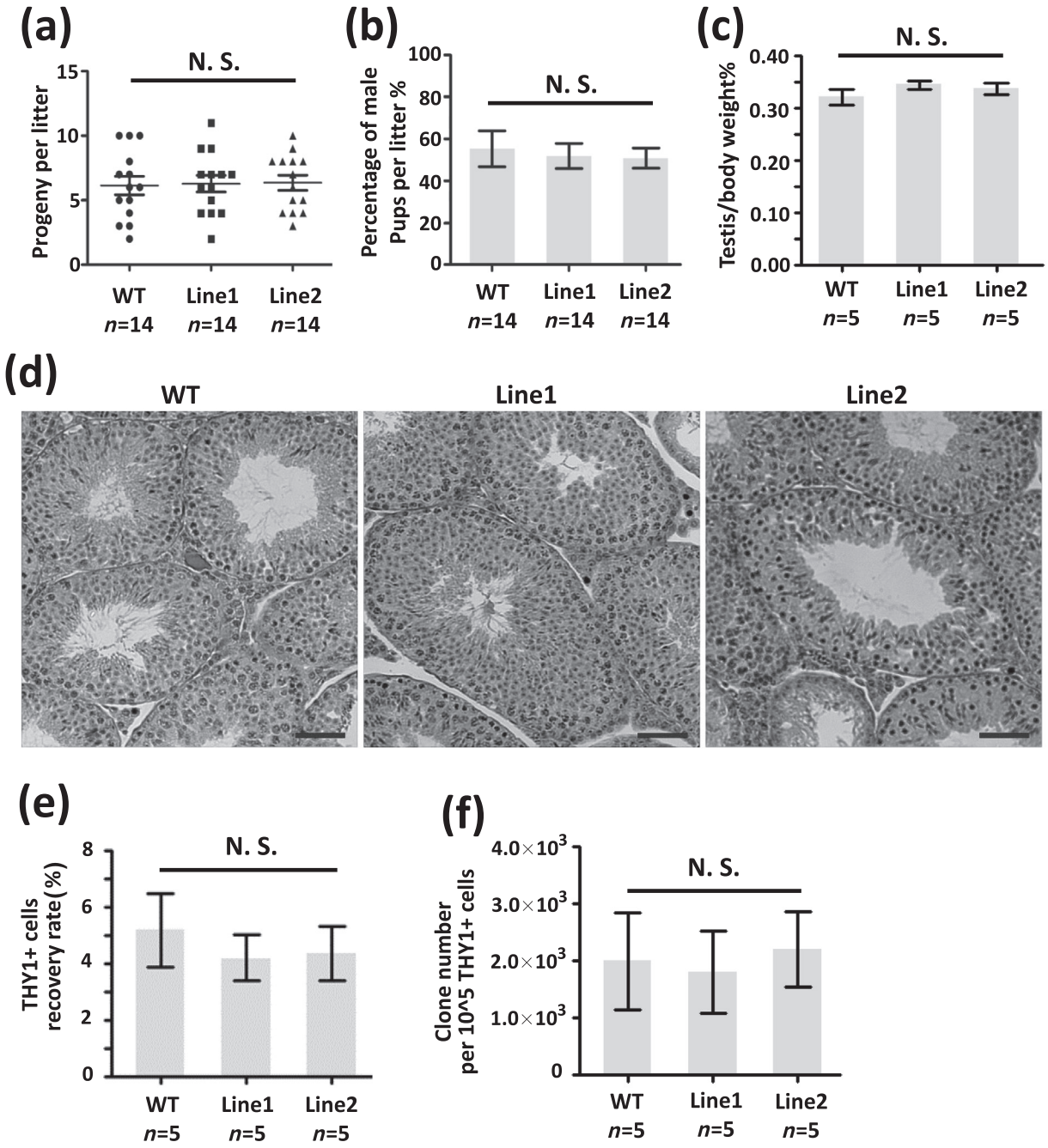

Fig. 5. USSP1 is indispensable for spermatogenesis. (a) Fertility assays of 14-week-old males. (b) Percentage of male pups per litter. (c) Testis/body weight ratio of WT, Line1- and Line2-*Ussp1* null mice at 14 weeks of age. (d) Histological analyses of WT, Line1- and Line2-*Ussp1* null mice at 14 weeks of age. No abnormalities in spermatogenesis or testis morphology were noted. Scale bar = 50 μm. (e) Line1- and Line2-*Ussp1* null mice showed no defect in the THY1$^+$ cell recovery rate. (f) Line1- and Line2-*Ussp1* null SSCs showed no defect in the colony formation rate. N.S., no significant difference was found among the compared groups.

4. Discussion

In this study, we demonstrated a novel protein, USSP1, is encoded by a gene previously annotated as lncRNA, *4933427D06*. We named this gene *Ussp1* due to its specific expression and location on surface of undifferentiated spermatogonia. *Ussp1* is expressed specifically in mouse testis with a pattern similar to *Plzf*. USSP1$^+$ cells form small clusters *in vivo* and comprise GFRα1$^+$ and PLZF$^+$ cells, as a characteristic feature of A_s, A_{pr} and A_{al} spermatogonia. Moreover, USSP1$^+$ cells express high levels of crucial transcriptional factors that are required for SSC maintenance, suggesting the self-renewal ability of this population. Colony formation assays showed that USSP1$^+$ cells could self-renew *in vitro*. As expected, transplantation assay clearly demonstrated SSCs were enriched in the USSP1$^+$ population. Based on these observations, we conclude that USSP1 specifically marks the undifferentiated spermatogonia that are capable of self-renewal. Therefore, to evaluate whether USSP1 plays an important role in this process, we generated *Ussp1*-/- mice with a very simple protocol using the CRIPSR/Cas9 system [31]. We did not observe any significant defects in undifferentiated spermatogonia and spermatogenesis, suggesting that USSP1 is dispensable for male fertility under normal conditions.

LncRNAs, which can be involved in various biological processes, such as gene regulation, X-inactivation and chromatin remodeling, were thought to play important roles in spermatogenesis [32]. LncRNAs were defined as non-protein coding transcripts longer than 200 nucleotides. However, this view was challenged by increasing evidence. For example, antibody-based methods clearly demonstrated the translation of several lncRNAs [33,34]. Moreover, analysis of ribosome profiling data has predicted a portion of lncRNAs to be translated [35]. *Ussp1* was annotated as lncRNA, and it had multiple lncRNA features, such as low conservation across species and high developmental stage specificity. However, signatures of mRNAs were also found, such as a long predicted ORF and abundant expression. We employed an antibody against the predicted protein and demonstrated its translation in undifferentiated spermatogonia, which shows great potential as a selection marker. Nevertheless, we did not observe any significant changes in this population in the pre-mature variant and large fragment deletion variant. Collectively, our result indicates that *Ussp1* is a protein coding gene, but disturbing its function does not affect normal spermatogenesis.

Although *Ussp1* is suggested to be non-functional, USSP1, the surface protein encoded by *Ussp1*, provides an alternative for isolating relatively purified SSCs. Currently, surface markers such as Thy1 and GFRα1 are employed to isolate a highly enriched SSC population [27,36]. However, these markers are not sufficiently specific. Importantly, the antibody-based selection of SSCs may lead to receptor blockade or over-activation of downstream signaling [37], thus restricting the application of some important surface markers, such as GFRα1 [38]. These disadvantages limit the potential agricultural and clinical application of SSCs and highlight the need to identify novel cell surface markers to enrich SSCs without disturbing their function. In our study, our data support that *Ussp1* is specifically expressed in undifferentiated murine spermatogonia, indicating the potential to sort SSCs with USSP1 antibodies. Additionally, *Ussp1* knockout male mice were as fertile as WT mice. These two features suggest that Ussp1 might provide a valuable maker for SSC enrichment and benefit further studies of SSCs.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Author contributions

Junjiu Huang conceived the study. Zhuoheng Lin, Puping Liang, and Junjiu Huang designed the overall experimental scheme. Zhuoheng Lin, Puping Liang, Zhaokai Yao, Yuxi Chen, Xiya Zhang, Rui Huang, Zhen Zhang, Minyan Li, Wenbin Ma, Haiyan Zheng, Shanbo Cao, Guang Shi and Xiaoyang Zhao executed the experiments. Zhou Songyang and Junjiu Huang analyzed the data. Zhuoheng Lin and Junjiu Huang wrote the paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scib.2019.03.022.

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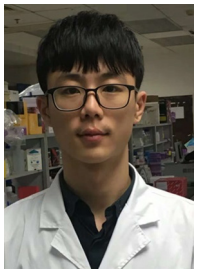

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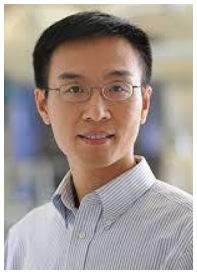

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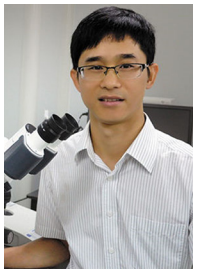

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