Restore natural fertility of Kit\textsuperscript{W}/Kit\textsuperscript{WV} mouse with nonobstructive azoospermia through gene editing on SSCs mediated by CRISPR-Cas9

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Abstract

Background: Male infertility is a serious social problem in modern society. Nonobstructive azoospermia (NOA) caused by germ cell gene defects is an important reason for male infertility, but effective clinical treatment for this disease has not been established.

Methods: We choose Kit\textsuperscript{W}/Kit\textsuperscript{WV} mouse as a research model and try to develop a new treatment strategy and “cure” its infertility. Mutant spermatogonial stem cells (SSCs) were isolated from one single unilateral testis of a 14-day-old Kit\textsuperscript{W}/Kit\textsuperscript{WV} mouse and propagated in vitro. The C to T point mutation on Kit\textsuperscript{WV} site of these SSCs was corrected through CRISPR-Cas9-mediated homology-directed repair (HDR) in vitro. Then, the repaired SSCs were screened out, proliferated, and transplanted into the remaining testis, and complete spermatogenesis was established in the recipient testis.

Results: Healthy offsprings with wild type Kit gene or Kit\textsuperscript{W} mutation were obtained through natural mating 4 months after SSC transplantation.

Conclusion: In this study, we established an effective new treatment strategy for NOA caused by germ cell gene defects through a combination of SSC isolation, CRISPR-Cas9-mediated gene editing, and SSC transplantation, which brought hope for these NOA patients to restore their natural fertility.

Keywords: CRISPR-Cas9, Nonobstructive azoospermia, Spermatogonia stem cells

Background

Male infertility has become an increasingly serious social problem in modern society, and nonobstructive azoospermia (NOA), which accounts for 10% of all infertile men, is an important cause for this [1–3]. Genetic causes, including chromosomal aberrations and spermatogenesis gene mutations, are often detected on severe NOA patients [4]. Gamete-deficient NOA due to germ cells’ genetic mutations, such as RBMY [5], KLHL10 [6], and SYCP3 [7], has been incurable by assisted reproductive technologies and difficult to bear for patients [8]. Cell therapies are considered to be one of the most promising strategies to rescue this type of infertility [9].

c-Kit is expressed in germ cells and controls germ cell differentiation in mammalian testis [10]. Heterozygous mutant mice with W and WV mutation of this gene, the Kit\textsuperscript{W}/Kit\textsuperscript{WV} mice, whose spermatogonia are considerably reduced or exhausted, have been used as an excellent animal model to develop therapeutic strategies for gamete-deficient patients due to genetic mutations by Yuan et al [9]. They isolated tail-tip fibroblasts from adult Kit\textsuperscript{W}/Kit\textsuperscript{WV} mice and then derived embryonic stem cells from these cloned blastocysts obtained by somatic cell nuclear transfer. The produced mutant ESCs’ W site was corrected using TALEN-mediated gene editing and further differentiated into primordial germ cell-like cells in vitro and then transplanted into busulfan-treated mouse
testes for spermatogenesis re-establishment. This is an encouraging strategy which can produce functional haploid cells for intracytoplasmic sperm injection (ICSI). However, since ICSI is still needed to get offsprings, the azoospermia has not been completely cured in fact. Besides, the whole treatment process is so cumbersome that it is difficult to be applied in clinical treatment for patients.

The feasibility of CRISPR-Cas9-mediated gene editing in spermatogonial stem cells (SSCs) has been reported [11, 12]. Transplanted SSCs can generate complete spermatogenesis in recipient testis [13]. If we can carry out CRISPR-Cas9-mediated gene correction on SSCs taken from NOA patients, the treatment process will be greatly simplified, the risk of tumor formation will be reduced, and even the patients are able to restore natural fertility.

Here, to explore the feasibility of this strategy, we improved our lab’s established method for isolating SSCs [14, 15]; so that we can isolate SSCs from single unilateral juvenile KitW/KitWv mouse testis, CRISPR-Cas9-mediated homology-directed repair (HDR) was conducted on isolated mutant SSCs. The repaired SSCs without WV mutation were screened out and propagated, then transplanted into another testis of the donor mouse. Healthy offsprings were obtained through natural mating 4 months after repaired SSC were transplanted. This work provides a more convenient and more humane therapeutic strategy for NOA.

Method
Animals
KitW/KitWv and C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). The use of mice and all pertinent surgical procedures were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

SSC cultures, gene editing, and transplantation
One-step enzymatic digestion with collagenase I (GIBCO) and DNase I (AppliChem) was used for isolation of testicular cells from ~ 14 days postpartum (dpp) mice to small seminiferous tubule fragments. The fragments were plated on 100-mm dishes in mouse embryonic fibroblast (MEF) medium, one dish for one testis. Then dishes were observed under a microscope after 18 h of culture. Dishes containing germ cells were screened out, loosely attached germ cells were collected with repetitive pipetting, and these germ cells were found to migrate into the culture dish. Thereafter, cells were transferred to new freshly prepared mouse embryonic fibroblast (MEF) dish to enrich SSC cultures. SSCs were cultured in the serum-free MEMa (Invitrogen) supplemented with 25 μg/ml insulin (Sigma), 100 μg/ml transferrin (Sigma), 20 μg/ml putrescine (Sigma), 3 mg/ml BSA (ICN), 20 μg/ml ascorbic acid (Sigma), 2 mM L-glutamine (GIBCO), 55 μM 2-mercaptoethanol (Sigma), 10 mM HEPES (Sigma), 50 units/ml penicillin (Sigma), 50 μg/ml streptomycin (Sigma), 20 ng/ml human GDNF (R&D), and 5 ng/ml human bFGF (Peprotech). The enriched SSC cultures were passaged every 4 to 5 days at a dilution of 1:2 to 1:4 depending on the size of the cell clumps and the growth of the somatic cells to remove testicular somatic cells in each passage. As such, relatively pure mutant SSC clumps, which have 93% GFRα-1-positive cells and 3% c-Kit-positive cells as reported earlier (14), were routinely obtained after a total of ~ 4–5 passages.

The WV-sgRNA was designed and selected from http://crispor.tefor.net/, then cloned into Bbs I sites of pX458 plasmids (Addgene plasmid 48138). This plasmid and oligo DNA were transfected into SSCs with Amaxa Cell Line Nucleofector Kit L (Lonza) using Amaxa Nucleofector according to the manufacturer’s instruction.

For transplantation, SSC clumps were trypsinized into single cells and prepared in concentrations of 5 × 10⁷ cells/ml for SSC transplantation. Ten microliters cell suspension were injected into each testis of KitW/KitWv mice via the efferent duct.

Off-target effect analysis
We identified a total of 41 potential “off-target” sites by the website used in gRNA design. We examined the five highest scoring sites. DNA sequencing of PCR products amplified from these genomic sites was performed on three different passages of the successfully repaired SSC line. The five highest scoring sites and sequences of the primers for the amplification of these five off-target sites were as follows: intergenic of Lca5 and Sh3bglf2 (Forward-CAGAGG CACCTGCATTT, Reverse-TGGAAACCATCCTAACC); intergenic of Gm1815 and Zfp353 (Forward-GTCCAC ATCACATCTCA, Reverse-ACATGGGACTTTACCTT); intergenic of Kif2b and Gm11498 (Forward-CGCTGG CTCTATGCTTA, Reverse-CCCAAACGTGGTGGTGA); intergenic of Eph4a and mmu-mir-6352 (Forward- GTCCATCAAAATCCACAA, Reverse-GGCTATTAA TCAACCA); intron of Dagla (Forward-AGCTTCTCAG ATTTCCC, Reverse-TCCAGACTCTATCCCC).

Immunofluorescence microscopy
Testes collected from KitW/KitWv mice 2 months after SSC transplantation were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Tissue sections (5 μm in thickness) were obtained in a microtome and mounted on glass slides. Sections were dewaxed and rehydrated, followed by antigen retrieval in 10 mM
sodium citrate buffer. After blocking with 5% BSA in PBS (wt/vol) for 1 h, the sections were incubated with primary antibody at 4 °C overnight (see Table 1). Secondary antibody conjugated with either FITC or TRITC (Jackson ImmunoResearch) at 1:200 dilution was used and incubated for an hour at room temperature. Slides were then stained with DAPI (blue) to visualize cell nuclei and mounted in prolonged anti-fade mounting medium. Fluorescence images were captured using a Zeiss LSM780 confocal microscope.

Genotyping
We used phenotypes to determine genotypes for the Kit+/Kitw mouse and related strains according to the “Genotyping Protocol” provided by The Jackson Laboratory. The hybrid Kit+/Kitw mice are the offspring of a cross between Kit+/Kitw (Stock No. 000692) females and Kitw+/Kitw (Stock No. 000049) males. The Kitw+/Kitw mouse is black with a white belly spot, occasionally has a white head blaze, and tail has a white tip. The Kitw+/Kitw mouse is grey with a light belly and white spot and a light tail. However, the Kitw+/Kitw mouse has a white coat and black eyes. Researchers can easily distinguish these three strains of mice by their different coat color characteristics.

Bisulfite sequencing
To confirm the DNA methylation state, bisulfite sequencing was performed using the EZ DNA methylation kit (Zymo Research) following the manufacturer's manual. Primers for bisulfite sequencing used in this study were as follows: H19 (Forward-TATGAGTATT TAGGAGGTAT AAGAATT, Reverse-ATTTTATCAAA TRITC (Jackson ImmunoResearch) at 1:200 dilution was used and incubated for an hour at room temperature. Slides were then stained with DAPI (blue) to visualize cell nuclei and mounted in prolonged anti-fade mounting medium. Fluorescence images were captured using a Zeiss LSM780 confocal microscope.

Results
Isolation of SSCs from one Kitw+/Kitw mice
The overall experiment process of this study is shown in Fig. 1. Gene-defective SSC isolation was the first step. Some of the Kitw+/Kitw mutant testes completely lack spermatogonia, and the remaining testes contained only a few spermatogonia [16], which leads us unable to isolate SSCs as usual [15], so a donor testis screening procedure was added to the SSC isolation process. Besides, in order to ensure mouse survival after removal of unilateral testis and convenient to surgical operation, 14-dpp (day postpartum)-old mice were used. Unilateral testes taken from donor mice were digested into small seminiferous tubule fragments and respectively plated on dishes for 18 h. Most empty tubules are shown as the lower left panel of Fig. 2a, except for tubules containing spermatogonia shown as the upper right panel of Fig. 2a. Only the testis in which spermatogonia could be observed under an inverted microscope was selected for subsequent SSC separation and enrichment experiments. The obtained Kitw+/Kitw mutant SSCs clone had normal cell morphology (Fig. 2b), and their SSC identity was confirmed by positive immunostaining of germ cell's marker Tra98 and stem cell marker c-Kit; all these SSC identities were consistent with wild type SSCs (Fig. 2c). The presence of W and WV point mutations on isolated SSCs were confirmed by DNA sequencing analysis (Fig. 3a).

Table 1 Antibodies used in this study

| Antibody | Vendor | Catalog no. | Host | Working dilution |
|----------|--------|-------------|------|-----------------|
| Tra98    | Abcam  | Ab82527     | Rat  | 1:200           |
| GFRA-1   | R&D    | AF560       | Goat | 1:200           |
| C-kit    | Abcam  | Ab5506      | Rabbit | 1:200 |
| SCP3     | Abcam  | Ab97672     | Mouse | 1:200 |
| N-cadherin | Santa Cruz | sc-7939 | Rabbit | 1:200 |
| JAM-A    | Thermo | 36-1700     | Rabbit | 1:200 |
| Lin28a   | R&D    | AF3757      | Goat  | 1:200           |
| SOX9     | Abcam  | ab76997     | Mouse | 1:200 |

Abcam, Cambridge, UK; Santa Cruz Biotechnology, Dallas, TX, USA; Thermo Fisher Scientific, Waltham, MA, USA; R&D Systems, Minneapolis, MN, USA.
Fig. 1 Diagram for the cure strategy of the NOA in the Kitw/Kitw mouse via CRISPR-Cas9-mediated gene editing in SSCs. Kitw/Kitw SSC cell line was established from one testis of a 14-dpp Kitw/Kitw mouse. Pxs48 plasmid with WV-sgRNA and exogenously supplied donor DNA were electroporated into Kitw/Kitw SSCs. GFP-positive cells, which are transfected SSCs, are enriched for further expansion and identification. SSC cell line carrying the corrected WV site without off-target mutations is selected for transplantation into the other testis of the donor mouse. The cure of the NOA was evidenced by the birth of a healthy offspring 4 months after SSC transplantation.

Fig. 2 The isolating strategy and characterization of Kitw/Kitw SSCs. a All tested testes were digested separately. With the lack of germ cells, most digested testes samples were tiled in the bottom of the dish like the lower left panel after being digested into small fragments and cultured for 18 h. A small number of testes have rare tubules containing germ cells presented as the upper right panel. Testes samples containing germ cell stacks were selected for SSC enrichment. Scale bar = 100 μm in original size panels and 50 μm in magnified panels. b Morphology of isolated Kitw/Kitw SSC clump. Scale bar = 20 μm. c Identification of SSCs. Kitw/Kitw mutant SSC clumps (DAPI, for cell nuclei) were positive for germ cell marker Tra98 (red) and stem cell GFRα-1 (green), but negative for differentiated germ cell C-kit (gray), which were consistent with the wild type control SSCs shown in the bottom panel of c, indicating that they were undifferentiated spermatogonial stem cells. Scale bar = 20 μm.
characteristics and plated at low density on plates. Then single SSC colonies were picked up, and each colony was cultured in one well of the 48-well culture plate for further expansion and screened to determine the successfully repaired SSC lines when each clone expanded to $2 \times 10^3$ cells during passage. Twenty-one successfully expanded single SSC colonies were detected when the repaired SSC clone was screened out. Both HDR-mediated repair (one SSC clone) and nonhomologous end joining (NHEJ)-mediated deletion gene-editing events (three SSC clones) were detected after the CRISPR-Cas9 induction on the $WV$ mutation site (Fig. 3c, d). Then, the SSC clone with repaired $WV$ locus was screened out for further research (Fig. 3c). This SSC clone maintained paternal genomic imprints (Fig. 3e). Besides, we examined the five highest scoring potential “off-target” sites by DNA sequencing of the PCR products amplified from these genomic sites; no obvious off-target mutations were detected (Fig. 4).

Assembly of normal blood–testis barrier (BTB) after repaired SSC transplantation and differentiation

The BTB is one of the essential prerequisites for spermatogenesis to proceed smoothly [18]. Kit$^w$/Kit$^{ww}$ mice cannot establish a functional BTB to support meiosis due to $c$-Kit mutation usually, but the transplantation of normal SSCs can induce its assembly [15]. In order to examine if the SSCs with repaired $WV$ locus could induce the assembly of functional BTB in Kit$^w$/Kit$^{ww}$ mice, the repaired SSCs were harvested and transplanted into adult Kit$^w$/Kit$^{ww}$ mice after being cultured for several passages. Then, the recipient mice were euthanized for examination by immunofluorescence analysis of typical BTB constituent proteins N-cadherin and JAM-A in the next 8 weeks so that the status of BTB assembly in cross sections of seminiferous tubules was carefully monitored. As anticipated, the functional BTB assembly was conditionality induced as normal SSCs are transplanted [15], which was directly related to the differentiation stage of germ cells within specific domains of the
semiferous epithelium. In tubules without germ cells, the distribution of N-cadherin and JAM-A was diffusely localized and extended all the way to the tubule lumen. However, they were restrictively expressed to the site in the basal region of the seminiferous epithelium where SSCs had differentiated into spermatocytes and round spermatids (Fig. 5), consistent with their location at the BTB in normal wild type control testes.

Establishment of complete spermatogenesis and generation of healthy offsprings after repaired SSC transplantation

After confirming that the SSCs with repaired WV locus could successfully induce the assembly of functional BTB and differentiate in Kit+/Kit+v mice, we examined if the recipient mice could establish complete spermatogenesis, and restoring fertility, the repaired SSCs were transplanted into another testis of the donor mouse and other 14 adult Kit+/Kit+v mice. Unrepaired Kit+/Kit+v SSCs were also transplanted as control. Two months later, three recipient mice of the repaired SSCs were sacrificed for spermato genesis status detection. We observed that both kinds of SSCs recolonized to the basement membrane and proliferated. However, unrepaired Kit+/Kit+v SSCs were still positive for spermatogonia marker Lin28a and could not differentiate to establish complete spermatogenesis (Fig. 6A, a). But the repaired SSCs could differentiate further and establish complete spermatogenesis in seminiferous cords (Fig. 6A, b). The distribution pattern of germ cells in the repaired SSC recipient testes was consistent with that in
wild type testes (Fig. 6A, d), but significantly different from
that in Kitw/Kitw\textsuperscript{v} testes lacking germ cells (Fig. 6A, c).
Then, wild type and Kitw/Kit+ offsprings were obtained
from the donor mouse and other 9 of 11 adult Kitw/Kitw\textsuperscript{v}
recipient mice after mating with wild type C57BL/6 mice
(Fig. 6B), which means the NOA of the Kitw/Kitw\textsuperscript{v} mouse
was cured.

**Discussion**

CRISPR-cas9-mediated gene editing has been applied
on mouse and rat SSCs for the acquisition of healthy
offsprings from parents with somatic cell genetic dis-
ease [11, 12]. This treatment strategy is promising
and relatively easy to implement, because these “pa-
tients” had normal spermatogenesis to provide enough
SSCs. However, it maybe not be suitable for NOA patients
who lack germ cells. In contrast, our study
has brought great hope for NOA patients with germ
cell genetic disease. If their SSCs can be isolated
through testicular biopsy [19] and corrected in vitro,
then transplanted back to the patients’ withered testes
to restart spermatogenesis, the NOA will be cured. In
this study, we verified the feasibility of this treatment
strategy. The establishment of complete spermatogen-
enesis in the recipient testis after repair of SSC
transplantation and generation of healthy offsprings
indicated the feasibility of this strategy.

This strategy only includes SSC isolation, in vitro gene
editing, and transplantation, three main steps which are
greatly simplified compared to a previous report [9].
Besides, autologous transplantation is more ethical and
immunotoxic reactions are removed. SSC cell lines that
carry the desired gene modification without unwanted
mutations can be selected easily in vitro, which brings a
100% efficiency of birth to healthy offsprings [11].
Somatic cell genetic disease also have a chance to be
removed from offsprings in this treatment process.
Furthermore, this strategy is more ethical and easier to
be accepted for NOA patients. If it can be applied in
humans, the NOA will be cured actually, which means
that the patients can give birth according to their own
wishes by the way of natural mating. For NOA patients
without SSCs, the strategy of Yuan et al. is more
applicable.

As mentioned above, this strategy is mainly aimed at
the treatment of NOA caused by germ cell gene defects,
especially these NOA patients whose testes have intact
or partially intact stem cell niche and retained SSCs. It
may not be the most suitable or economical treatment
strategy for other types of NOA patients, such as
azoospermic cancer survivors whose testicular stem cell niche is compromised.

Although we co-transfected a donor DNA to improve the incidence of HDR, the 4.7% (1/21) incidence rate of HDR was still low and lower than the incidence rate of NHEJ 14.2% (3/21). More efforts should be made to improve the incidence of HDR. Satisfactorily, the recipient mice had a high rate (81.8%) of successful reproduction rate after mating with wild type C57BL/6 mice, which means the repaired SSCs had a great chance to successfully restore spermatogenesis.

Researchers have made a lot of effort to reduce the off-target effect in recent years’ application of the CRISPR-cas9 system, and relatively good results have also been achieved [20, 21]. However, unpredictable and unexpected mutations after CRISPR-Cas9 editing still occur. Although no obvious off-target mutations were detected in this study, the detection scheme we used was relatively simple and not comprehensive enough. There may be off-target events that had not been detected. So stricter standards must be used if this strategy is used in a human body. More comprehensive and detailed inspection methods,
such as whole genome sequencing, must be used before transplantation.

The propagation of human SSCs in vitro was reported about 10 years ago [22, 23], but there are still difficulties for other labs to replicate. So, we cannot apply this strategy on human NOA patients immediately, but we believe SSCs will play a key role in getting human offspring without genetic disease in the near future.

**Conclusions**

In this study, we established a new treatment strategy for NOA caused by germ cell gene defects through combination of SSC isolation, CRISPR-Cas9-mediated gene editing, and SSC transplantation, which brought hope for some NOA patients to restore their natural fertility. Not only to cure the NOA patients with germ cell gene defects, but may also for males who can bear normally but with other genetic disorders, be able to produce normal gametes and descendants by combining this strategy and ART technology.

**Abbreviations**

BTB: Blood–tests barrier; dpp: Day postpartum; HDR: Homology-directed repair; ICSI: Intracytoplasmic sperm injection; MEF: Mouse embryonic fibroblast; NHEJ: Nonhomologous end joining; NOA: Nonobstructive azoospermia; sgRNA: Single-guide RNA; SSCs: Spermatogonial stem cells

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**Authors’ contributions**

XL performed the study design, most experiments, and manuscript writing. TS participated in the data analysis and manuscript modification. YL participated in the study design and XL performed the study design, most experiments, and manuscript writing. TS participated in the data analysis and manuscript modification. XW was the main handler of SSC transplantation. YL participated in the study design and TS participated in the data analysis and manuscript modification. All authors read and approved the final manuscript.

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**Availability of data and materials**

All analyzed data are available in the manuscript. Raw data or other materials produced in the conduct of these studies are available from the authors to qualified investigators upon request.

**Ethics approval and consent to participate**

The use of mice and all pertinent surgical procedures were approved by the Animal Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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