Genome Editing in Mouse Spermatogonial Stem Cell Lines Using TALEN and Double-Nicking CRISPR/Cas9

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SUMMARY

Mouse spermatogonial stem cells (SSCs) can be cultured for multiplication and maintained for long periods while preserving their spermatogenic ability. Although the cultured SSCs, named germline stem (GS) cells, are targets of genome modification, this process remains technically difficult. In the present study, we tested TALEN and double-nicking CRISPR/Cas9 on GS cells, targeting Rosa26 and Stra8 loci as representative genes dispensable and indispensable in spermatogenesis, respectively. Harvested GS cell colonies showed a high targeting efficiency with both TALEN and CRISPR/Cas9. The Rosa26-targeted GS cells differentiated into fertility-competent sperm following transplantation. On the other hand, Stra8-targeted GS cells showed defective spermatogenesis following transplantation, confirming its prime role in the initiation of meiosis. TALEN and CRISPR/Cas9, when applied in GS cells, will be valuable tools in the study of spermatogenesis and for revealing the genetic mechanism of spermatogenic failure.

INTRODUCTION

Spermatogonial stem cells (SSCs) are pivotal for maintaining the lifelong sperm production of mammalian males. They maintain a delicate balance between self-renewal and commitment to differentiation for sperm production. Mouse SSCs can be maintained under culture conditions, continuing to proliferate for up to 2 years without losing their spermatogenic ability as well as maintaining genetic, epigenetic, and karyotypic stability. The cultured SSCs, named germline stem (GS) cells, are now useful in research on various aspects of spermatogenesis. (Kanatsu-Shinohara et al., 2003; Kubota et al., 2004). In order to make GS cells more widely applicable for the study of spermatogenesis at the genetic and molecular levels, it is desirable to be able to modify their genome without disturbing their spermatogenic ability. In fact, this was shown to be possible by the transfection of genes into GS cells (Kanatsu-Shinohara et al., 2005) or by targeting particular genes in GS cells by homologous recombination (Kanatsu-Shinohara et al., 2006a). Successfully treated GS cells were selected during the subsequent cultivation and transplanted into the seminiferous tubules of host mice to produce genome-modified sperm. These results proved that GS cells, like embryonic stem cells (ESCs), can be used as genetically modified cells for the production of animals with modified genomes. Thus, GS cells appear to be a useful tool not only for the study of spermatogenesis but also for many other areas of biological research. However, it is rather difficult to manipulate the genome of GS cells compared to ESCs because of their low transfection efficiency, slow cycling, and difficulty in cloning (Kanatsu-Shinohara et al., 2005, 2006b; Tamm et al., 2013). Thus, targeted genome-modifying experiments using GS cells are not common, and, to the best of our knowledge, only three reports have been published (Iwamori et al., 2012; Kanatsu-Shinohara et al., 2006a, 2008a).

Recently, new genome-modifying methods using sequence-specific endonucleases, such as zinc-finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), and clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (CRISPR/Cas9) systems, were developed one after another (Gaj et al., 2013). In addition, gene-targeting experiments with these nuclease systems were very recently reported to be successful in SSC lines (Fanslow et al., 2014; Wu et al., 2015, Chapman et al., 2015). In the present study, we adopted the double-nicking system of CRISPR/Cas9 and tested it, along with TALEN, on GS cells. Our results demonstrated that these artificial endonucleases are powerful tools for genetic-modification experiments in GS cells, providing an effective way to study the genetic mechanism of spermatogenic failure in particular.

RESULTS

Preparation of TALEN and CRISPR/Cas9 Plasmids Targeting the Rosa26 Locus
We designed TALEN and CRISPR/Cas9 constructs targeting a site in the first intron of the Rosa26 locus that was
previously targeted successfully with the ZFN system (Perez-Pinera et al., 2012) (Figure S1A). The Rosa26 locus was chosen because inserted genes will be expressed ubiquitously without any additional effects on the characteristics of target cells (Zambrowicz et al., 1997). Thus, the targeted GS cells were expected to maintain spermato- genic competency. We used the Platinum TALEN plasmid, which has enhanced cleavage activity compared to conventional TALEN (Sakuma et al., 2013). As for CRISPR/Cas9, we chose the pX335 CRISPR/Cas9 expression vector, which uses a double-nicking strategy, by which the occurrence of a double-strand break (DSB) becomes more specific and accurate (Mali et al., 2013; Ran et al., 2013). To confirm the target-site-specific cutting efficiency of the TALEN and CRISPR/Cas9-expressing vectors, we performed a Surveyor nuclease assay with the cell line 15P-1, which confirmed their accurate and sufficient cutting efficiency (Figure S1B).

Gene Targeting of the Rosa26 Locus Using TALEN and CRISPR/Cas9

The construct of the targeting vector was composed of a splice acceptor, an EGFP, internal ribosome entry sites, and puromycin-resistant gene sequences, sequentially connected in this order and flanked with short homologous sequences on both the 5′ and 3′ sides (Figure 1A). When gene targeting is successful, the targeted GS cells express GFP and become resistant to puromycin.

We performed transfection with the targeting vector together with the TALEN or CRISPR/Cas9-expressing vector by electroporation of GS cells derived from the wild-type mouse. Approximately 2 weeks after electroporation,
the selection of GS cells with puromycin was initiated. Puromycin-resistant colonies became apparent in ~1 week, and some of them were picked up for cloning without checking GFP expression. We obtained 12 and 8 clones of transfected GS cells with TALEN and CRISPR/Cas9, respectively. Excluding one clone produced by TALEN, every GS cell clone expressed GFP (Figure 1B). We used PCR genotyping to examine whether the Rosa26-targeting construct was integrated into the target site. All of the 11 and 8 GFP-expressing clones induced by TALEN and CRISPR/Cas9, respectively, showed the correct insertion of the target sequence into the Rosa26 locus (Figure 1C). Among them, 8 out of the 11 clones and 5 out of the 8 clones showed homozygous insertion (Figure 1C). Southern blotting analysis demonstrated that there was no integration of the donor construct other than at the target site (Figure 1D). Collectively, targeting efficiencies mediated by TALEN and CRISPR/Cas9 were quite high: 11 clones out of 12 and all 8 clones, respectively (Table 1). Thus, these results indicate that genome editing mediated by the TALEN or CRISPR/Cas9 double-nicking CRISPR/Cas9 double-nicking system was highly effective in introducing a transgene at target site in the genome of GS cells.

Spermatogenic Ability of Targeted GS Cells

We tested whether those GS cells with a target construct at the Rosa26 site (Rosa26-GS cell) remain as functional SSCs and differentiate into competent sperm. We transplanted lines of Rosa26-GS cells into the testes of W/Wv (W) mice whose testes contain very few germ cells because of genetic defects in the c-kit gene, making them an ideal host testis. The transplanted Rosa26-GS cells colonized the seminiferous tubules and formed stretches of colonies that expressed GFP (Figure 2A). By histological observation, we confirmed that those colonies supported spermatogenesis up to sperm formation. The immunohistologic features, as well as regular histological findings on periodic acid-Schiff (PAS) stain, demonstrated normal spermatogenesis (Figures 2B, 2D, and 2E). When testis tissue was dissociated, sperm were obtained (Figure 2C). In summary, we transplanted 11 and 4 clones of Rosa26-GS cells of TALEN and CRISPR/Cas9, respectively, resulting in 9 and 4 clones giving rise to spermatogenesis, respectively. The two clones of Rosa26-GS cells with the TALEN procedure showed neither colonization nor spermatogenesis in the W mouse testes, for unknown reasons.

Next, we tested the fertility of those sperm by intracytoplasmic sperm injection (ICSI). We chose the clones of TALENs #8 and #9 (Figures 1C and 1D). All (n = 113) oocytes fertilized by ICSI developed into the two-cell stage on the next day (Table S1). Following embryo transfer, 8 and 23 offspring were delivered from clones #8 and #9, respectively, and all grew healthily (Figure 2F; Table S1). As both clones were targeted homozygously (Figure 1C), all offspring carried the inserted GFP heterozygously (Figure S2A) and expressed GFP throughout the body (Figure 2F). When their reproductive potential was examined by brother-sister mating, they gave birth to offspring, proving its reproductive competency. The second-generation progeny also grew normal and were healthy (Figures S2B–S2D). These results demonstrate that gene modification of GS cells using a TALEN and CRISPR/Cas9 double-nicking system did not affect the characteristics of GS cells, both as stem and spermatogenic cells.

Gene Targeting of Stra8 Gene Using CRISPR/Cas9

The genetic modification techniques when applied to GS cells could be a useful means to examine the function of particular genes during spermatogenesis. Using the double-nicking CRISPR/Cas9, we tried to disrupt the gene of Stimulated by retinoic acid gene 8 (Stra8) by inserting the targeting vector sequence. Stra8 expression is induced by retinoic acid (RA) in spermatogonia and is responsible for entry into meiosis (Baltus et al., 2006; Zhou et al., 2008). We constructed a CRISPR/Cas9-expressing vector, which includes sequence of guide RNA specific to a site in the fourth exon of the Stra8 gene (Table S2), and a targeting vector, which contained tdTomato fluorescence, PGK promoter, and Puro sequences (Figure 3A). We used these three vectors to transfect GFP-GS cells that express GFP constitutively. Amounts of vector DNA transfected were 2 and 6 μg of CRISPR/Cas9 and targeting vectors, respectively, or double doses of them. Nine colonies resistant to puromycin

| Table 1. Summary of Targeting Experiments Using TALEN and CRISPR/Cas9 |
|-----------------------------------------------|
| Targeting Vector | No. of Clones Analyzed | No. of Non-targeted Clones | No. of Targeted Clones | No. of Random Integration | Homozygous Insertion |
|-------------------|------------------------|----------------------------|------------------------|--------------------------|----------------------|
| TALEN             | Rosa-SA-GFPiPuro        | 12                         | 1                      | 11                       | 0                    | 8/11                 |
| CRISPR            | Rosa-SA-GFPiPuro        | 8                          | 0                      | 8                        | 0                    | 5/8                  |
| CRISPR            | Stra8-tdTomato-PGK-Puro | 18                         | 0                      | 18                       | 0                    | 3/9                  |

*Double doses of CRISPR/Cas9 and targeting vectors were used for transfection.*
were picked up from each plasmid dosage group, resulting in 18 clones in total (termed Stra8-GS cells). Each clone was examined by PCR genotyping and Southern blotting to show that the Stra8-targeting construct had been integrated into the target site correctly, without random integration (Figures 3B and 3C). The homologous insertion was noted in three out of nine clones and six out of nine clones, respectively, for each dose set of vectors (Figure 3B).

When Stra8-GS cells were cultured in a medium with RA added for 2 days, almost all Stra8-GS cells expressed tdTomato, confirming that the expression of the inserted construct was induced by RA (Figure 3D). We then chose a line of homozygously knocked in Stra8-GS cells and transplanted them into the testes of W mice. Eighty-four days after, on analysis, the recipient testis showed extensive colonization by GS cells, appearing as tubules colored with GFP (Figure 3E). The colonized cells, however, appeared to be restricted to the periphery of the seminiferous tubules, indicating that they were located on the basement membrane without differentiation to the inside of the RPSIRCNELAT.

Figure 2. Spermatogenic Ability of Targeted GS Cells
(A) Recipient testes transplanted with GS cells targeted by TALEN and CRISPR/Cas9 contained seminiferous tubules expressing GFP, which indicates GS cell colonization. A non-transplanted recipient testis (W mouse) did not show GFP expression.
(B) Immunostaining of recipient testis tissue cryosections, transplanted with Rosa26-GS cells targeted by TALEN. Peanut agglutinin (PNA), which reacts with the acrosome, appears as red. Anti-GFP (green) and counterstain with Hoechst dye (blue) were merged.
(C) Flagellated sperm derived from mechanically dissociated host testis transplanted with Rosa26-GS cells targeted by TALEN.
(D) Immunostaining of recipient testis tissue transplanted with Rosa26-GS cells targeted by CRISPR. PNA (red), anti-GFP (green), and counterstain with Hoechst dye (blue) were merged.
(E) A recipient testis transplanted with GS cells targeted by CRISPR/Cas9 shows extensive colonization on day 159 after transplantation (left). This testis processed for histological examination showed normal spermatogenesis with PAS stain (right).
(F) Offspring obtained using the ICSI procedure with sperm derived from two Rosa26-GS cell clones (#8 and #9) targeted with TALEN. GFP expression throughout the bodies of the offspring was confirmed under UV light.

Scale bars represent 1 mm (A and E, left), 200 μm (E, right), 100 μm (B), 50 μm (D), or 20 μm (C).
Figure 3. Targeting of the Stra8 Gene in GS Cells Using CRISPR/Cas9
(A) Schematic overview of the targeting strategy for the Stra8 gene. Arrows (P1, P2, and P3) show primer sites for the genotyping PCR. P1 and P2 primers are located outside the region of the homology arm. The P3 primer is located in the transgene. Expected sizes of the PCR products are 1,687 bp in wild-type and 1,542 bp in targeted alleles. The probe used for Southern blot analysis is shown as a green bar.
(B) PCR genotyping of Stra8-GS cells targeted with CRISPR/Cas9. The leftmost lane shows a 200-bp ladder marker. The W lane shows wild-type controls. Asterisks indicate homozygously targeted clones.
(C) Southern blot analysis of Stra8-GS cells. SpeI-digested genomic DNA was hybridized with the tdTomato probe. The expected fragment size is 6.6 kb.
(D) Expression of the tdTomato was induced in Stra8-GS cells by RA addition to the medium in 2 days. A merged image of tdTomato (red), GFP (green), and DIC is shown.
(E) A recipient testis transplanted with Stra8-GS cells shows extensive colonization of the GS cells. The inset shows a magnified view of the same testis.
(F) H&E staining of a cryosection of the recipient testis, showing no meiotic differentiation.

(legend continued on next page)
tubules. Histological analysis showed that there were tubules containing spermatogonia lining the basement membrane with no progression of spermatogenesis (Figure 3F), which was exactly the same as the phenotype of *Stra8*-deficient mice (Baltus et al., 2006). Furthermore, we performed an immunohistological examination with antibodies to tdTomato and GFP and found that the expression of tdTomato in spermatogonia was different from tubule to tubule (Figure 3G). This observation is consistent with a previous report that the expression of *Stra8* was periodic in spermatogonia and spermatocytes, depending on the wave of the seminiferous epithelium (Zhou et al., 2008). Together, these results demonstrate that CRISPR/Cas9-mediated gene disruption is effective in GS cells, the effects of which could appear as a particular phenotype in spermatogenesis following transplantation into the host testis.

**DISCUSSION**

In the present study, we succeeded in genome modification of the *Rosa26* locus and *Stra8* gene using TALEN or CRISPR/Cas9 systems, with extremely high-level efficiency and accuracy. The accuracy of CRISPR/Cas9 was comparable to that of TALEN and was actually perfect, probably owing to the double-nicking system we used.

In a previous report, the gene-targeting efficiency in GS cells without genome-editing technology was as low as 1.7%, as 2 out of 120 clones were selected (Kanatsu-Shinohara et al., 2004). In order to make GS cells useful for the study of spermatogenesis, this characteristic of GS cells must be kept in mind. In the present study, we showed that both TALEN and double-nicking CRISPR/Cas9 were effective for genome editing in GS cells. In fact, we demonstrated that 2.8–4.7 kbp of transgenes could be successfully introduced accurately into the target site in the genome of GS cells. This result suggests that genome-editing technology in GS cells allows us to repair more extensive mutations than those involving single nucleotides. More importantly, our study demonstrated that the *Rosa26*-GS cells retained full capacity for complete spermatogenesis up to the formation of competent sperm, which was not shown in either of the two previous studies. It is noteworthy that GS cells are prone to lose spermatogenic potential, possibly after being cultured under stressful conditions such as overgrowth, repeated freezing and thawing, or high passage numbers. In an extreme case, GS cells not only lose spermatogenic ability but also gain multipotency, turning into ESC-like cells (Kanatsu-Shinohara et al., 2004). In order to make GS cells useful for the study of spermatogenesis, this characteristic of GS cells must be kept in mind. In the present study, we successfully showed that the spermatogenic ability of GS cells was disturbed when the *Stra8* gene was disrupted. This result demonstrated that GS cells can be used to test whether or not a particular gene or genes are functioning in spermatogenesis when cultured under the appropriate conditions.

As was shown in this study, along with many other previous studies, TALEN and double-nicking CRISPR/Cas9 genome-editing techniques are significantly accurate, with only a negligible risk of random gene disruption, in the drug-selected clones. This result indicates that disrupting a particular gene without disturbing any other genes in GS cells could replace gene-manipulating...
technologies with ESCs in the study of spermatogenesis. When GS cells of species other than mice become available for gene targeting as well, it may become possible to study the role of particular genes in their spermatogenesis, which would be rather difficult or practically impossible with ESC technology. In fact, culturing of the SSCs of the rat, hamster, and rabbit was reported to be feasible (Hamra et al., 2005; Kanatsu-Shinohara et al., 2008b; Kubota et al., 2011; Ryu et al., 2005), making this research strategy realistic. In addition, whole-genome sequencing is now available, owing to the many technological innovations in that field. Based on such a huge volume of genome information along with sophisticated analysis methods, genetic analysis of infertile patients (azoospermia in particular) could reveal genes responsible for spermatogenic failure. The genome-modifying technologies shown in this study will be extremely useful to examine such candidate genes responsible for spermatogenic defects in the future.

**EXPERIMENTAL PROCEDURES**

**Animals and Culture of GS Cells**

To establish wild-type GS and GFP-GS cells, male C57BL/6 (CLEA Japan) and C57BL/6-Tg (CAG/Act-GFP) (Okabe et al., 1997) transgenic mice, respectively, were mated with wild-type ICR females (CLEA Japan) to produce F1 pups. The testes of F1 pups were dissected out and used as a source of GS cells. The culture medium used for GS cells was StemPro-34 based (Kanatsu-Shinohara et al., 2003), with minor modifications (Sato et al., 2013). Wild-type GS and GFP-GS cells were used for targeting experiments of Rosa26 and Stra8, respectively. WβB6F1-W/+ (Japan SLC), at 6–15 weeks old, were used as recipients for spermatogenic transplantation. W/W+ allows colonization of GS cells of ICR/B6 background without apparent rejection (Araki et al., 2010). The spermatogenic ability of GS cell lines was checked by transplantation into the testes of W/W+ before using for targeting experiments (Figures S3A and S3B). All animal experiments conformed to the *Guide for the Care and Use of Laboratory Animals* and were approved by the Institutional Committee of Laboratory Animal Experimentation (Animal Research Center of Yokohama City University, Yokohama, Japan).

**Gene Targeting of GS Cells**

GS cells used for targeting to Rosa26 and Stra8 were at passages 6 and 8, respectively. They were harvested using 0.25% trypsin, and 1.5–4 × 10^6 cells resuspended in T solution of Cell Line Nucleofector Kit were electroporated with 6 μg of targeting vector plasmids and 2 μg of each TALEN or CRISPR/Cas9-expressing plasmid using Nucleofector 2b with program A-023. Selection with puromycin (0.15 μg/ml) was performed 10–14 days after electroporation. When puromycin-resistant colonies expanded, usually at ~4 weeks after electroporation, we picked up individual colonies using a micropipette (P200 pipetman) under an inverted microscope for successive culturing.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Supplemental Experimental Procedures, three figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.05.011.

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