Trivial role for NSMCE2 during in vitro proliferation and differentiation of male germline stem cells

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Abstract

Spermatogenesis, starting with spermatogonial differentiation, is characterized by ongoing and dramatic alterations in composition and function of chromatin. Failure to maintain proper chromatin dynamics during spermatogenesis may lead to mutations, chromosomal aberrations or aneuploidies. When transmitted to the offspring, these can cause infertility or congenital malformations. The structural maintenance of chromosomes (SMC) 5/6 protein complex has recently been described to function in chromatin modeling and genomic integrity maintenance during spermatogonial differentiation and meiosis. Among the subunits of the SMC5/6 complex, non-SMC element 2 (NSMCE2) is an important small ubiquitin-related modifier (SUMO) ligase. NSMCE2 has been reported to be essential for mouse development, prevention of cancer and aging in adult mice and topological stress relief in human somatic cells. By using in vitro cultured primary mouse spermatogonial stem cells (SSCs), referred to as male germline stem (GS) cells, we investigated the function of NSMCE2 during spermatogonial proliferation and differentiation. We first optimized a protocol to generate genetically modified GS cell lines using CRISPR-Cas9 and generated an Nsmce2−/− GS cell line. Using this Nsmce2−/− GS cell line, we found that NSMCE2 was dispensable for proliferation, differentiation and topological stress relief in mouse GS cells. Moreover, RNA sequencing analysis demonstrated that the transcriptome was only minimally affected by the absence of NSMCE2. Only differential expression of Sgsm1 appeared highly significant, but with SGSM1 protein levels being unaffected without NSMCE2. Hence, despite the essential roles of NSMCE2 in somatic cells, chromatin integrity maintenance seems differentially regulated in the germline.


Introduction

The process of spermatogenesis is characterized by ongoing and dramatic alterations in composition and function of chromatin. Chromatin is the supramolecular complex, consisting of DNA and proteins, which packages, shapes and orchestrates the genome and safeguards genomic stability. Incorrect spatiotemporal organization of chromatin can initiate germ cell apoptosis, leading to spermatogenic arrest and male infertility (de Rooij & de Boer 2003). If all spermatogenic arrest mechanisms fail, incorrect chromatin architecture can even cause chromosomal aberrations or aneuploidies, leading to congenital abnormalities in the offspring (Jan et al. 2012).

Numerous chromatin-based processes, such as cell cycle progression, cellular differentiation and genomic integrity maintenance, are to a large extent modulated by the structural maintenance of chromosomes (SMC) protein complexes: SMC1/3 (cohesin), SMC2/4 (condensin) and SMC5/6. Besides SMC5 and SMC6, the SMC5/6 complex contains several non-SMC elements (NSMCEs), including NSMCE2. Together with these NSMCEs, the SMC5/6 complex has a ring-like structure large enough to hold two double-stranded DNA molecules together. This property of the SMC5/6 complex is pivotal for recombination-mediated DNA damage repair (De Piccoli et al. 2006, Potts 2009, Wu & Yu 2012) and resolving replication-induced topological stress in yeast (Carter & Sjogren 2012, Jeppsson et al. 2012, Langston & Weinert 2015). Of the four NSMCEs in mammals, NSMCE2 specifically associates with SMC5, where it exhibits a C-terminal SP-RING domain with an E3 small ubiquitin-related modifier (SUMO)


In mitotically dividing yeast, the SMC5/6 complex is essential for the maintenance of replication fork stability and the prevention of replication-induced topological stress (Carter & Sjögren 2012, Jepppson et al. 2012, Langston & Weinert 2015). Consistently, depletion of SMC5 in mouse embryonic stem (ES) cells leads to the accumulation of cells in G2 and subsequent mitotic failure and apoptosis (Pryzhkova & Jordan 2016). Using a human osteosarcoma cell line (U2OS), we have recently shown that SMC6 interacts with DNA topoisomerase II α (TOP2A) (Verver et al. 2016a). TOP2A is a topoisomerase that prevents supercoiling of replicating DNA (Wang 1996, 2002). Moreover, we have shown that the CRISPR-Cas9-mediated removal of NSMCE2 in these cells led to increased sensitivity to the topoisomerase inhibitor etoposide (Verver et al. 2016a).

In the mouse and human, the SMC6 protein is most highly expressed in the testis where it appears to be involved in spermatogonial differentiation (Verver et al. 2013, 2014) and meiosis (Gomez et al. 2013, Verver et al. 2013, 2014). Likewise, also NSMCE2 has been identified to be expressed in developing mouse male germ cells, from spermatogonia to round spermatids (Jacome et al. 2016). Recently, a study using a conditional knock-out (KO) mouse model showed essential roles for SMC5/6 during meiotic chromosome segregation (Hwang et al. 2017). However, suitable KO models to study the SMC5/6 complex in mitotically dividing spermatogonia are currently not available. In mice, complete ablation of SMC6 or NSMCE2 results in embryonic lethality (Ju et al. 2013, Jacome et al. 2016). Hence, the role of SMC5/6 or NSMCE2 in spermatogonia remains largely unknown.

Male germline stem (GS) cells, initially termed by Shinohara’s group, refer to the cultured spermatogonial stem cells (SSCs) able to propagate in vitro for over 2 years without losing SSC properties (Kanatsu-Shinohara et al. 2005a). In the current study, we first optimized a protocol to generate genetically modified mouse GS cell lines using CRISPR-Cas9. By applying this optimized protocol, we generated an Nsme2<sup>−/−</sup> GS cell line. Using this GS cell line, we studied the role of Nsme2 during in vitro spermatogonial proliferation and differentiation, gene expression and the spermatogonial response to topological stress.

Materials and methods

Animal use and care
Neonatal (4–5 d.p.p.) DBA/2J (Charles River) male mice were used for GS cell isolation. To acquire neonatal testis materials, donor mice were first anesthetized by 4% isoflurane total body anesthesia followed by killing by decapitation and inactivation of the brain. Testes were collected and the tunica albuginea was removed. Testicular tissues were cryopreserved in supplemented MEM (Gibco, Thermo Fisher Scientific) containing 20% fetal bovine serum (FBS) and 8% DMSO in a Coolcell freezing device and stored in liquid nitrogen (−196°C) for future GS cell isolation. All animal procedures were in accordance with and approved by the animal ethical committee of the Academic Medical Center, University of Amsterdam.

Design of Nsme2—single-guide (sgRNA) and construction of CRISPR-Cas9 plasmids
The online Optimized CRISPR Design Platform (http://crispr.mit.edu/) was utilized to design Nsme2-sgRNA. 5’-ACCGGTTACATATCCTTCAG-3’, followed by the protospacer-adjacent motif (PAM) TGG (in exon 2) was selected as the target site. The corresponding forward and reverse strand oligonucleotide was synthesized by Sigma-Aldrich, and then annealed and cloned into the commercial linearized vector GeneArt CRISPR Nuclease Vector with OFP Reporter (Thermo Fisher Scientific), following the protocol provided by the manufacturer. The correct double-strand oligonucleotide insertion was confirmed by Sanger sequencing after transformation and plasmid extraction.

GS cell culture and differentiation
A mouse GS cell line was established following a previously published protocol (Kanatsu-Shinohara et al. 2003). Briefly,
germ cells were isolated from the cryopreserved testes of 4–5 d.p.p DBA/2J male mice by a two-step enzymatic dissociation. After overnight incubation on gelatin-coated wells, the floating and loosely attached cells were collected and cultured in the complete GS cell medium composed of StemPro-34 SFM medium (Thermo Fisher Scientific), StemPro-34 Supplement (Thermo Fisher Scientific), 1% FBS, 10 ng/mL recombinant human GDNF (Peprotech), 10 ng/mL recombinant human bFGF (Peprotech), as well as other 17 components as previously reported (Kanatsu-Shinohara et al. 2003, Kanatsu-Shinohara & Shinohara 2010). The cells were refreshed every 2–3 days and passed every 5–7 days at a ratio of 1:4–6. From the third passage, the cells were transferred to inactivated primary mouse embryonic fibroblast (MEF) feeder cells that had been treated with 10 µg/mL mitomycin-C (Sigma-Aldrich) for 2–3 h at 37°C. The cells were maintained at 37°C in an atmosphere of 5% CO₂ in air. After ~1 month, the growth of GS cells became stable. GS cells were cultured on MEFs unless otherwise stated. For feeder-free culture, GS cells were seeded on wells pre-coated with laminin (20 µg/mL, Sigma-Aldrich). For retinoic acid (RA)-induced differentiation of GS cells, the feeder-free culture was adopted, and GS cells were exposed to medium containing 2 µM all-trans-RA (Sigma-Aldrich) for 3 days. In control groups, vehicle (0.1% ethanol) was added to the medium.

**GS cell electroproportion**

The constructed CRISPR plasmids targeting Nsmce2 were delivered to low-passage GS cells (<P10) by Neon electroprotator (Thermo Fisher Scientific), following the manufacturer’s guidance. The program used for electroproportion was voltage 1100, width 20 ms and pulse 2. Two days after electroproportion, OFP⁺ cells were sorted by fluorescence-activated cell sorter (FACS, BD Biosciences) and cultured on MEFs for recovery.

**Surveyor assay**

One week after FACS sorting, the genomic DNA of GS cells was extracted, and the genomic region around the target site was amplified by PCR with the forward primer 5'-GATGATGGCACAGTGCTTGG-3' and the reverse primer 5'-GGCAGTTCTGAGTGGAGGATTAC-3'. Hercule II fusion polymerase (Agilent Technologies) was used for high-fidelity PCR amplification. PCR products were purified, denatured and re-annealed to generate DNA heteroduplexes, followed by the Surveyor nuclease digestion (Integrated DNA Technologies) digestion, according to the protocol provided by the manufacturer. The Surveyor nuclease digestion products were run and visualized on agarose gels. The incidence of insertion/deletion (indel) was calculated using a previously described formula (Ran et al. 2013).

**GS cell clonal isolation and expansion**

One week after FACS sorting, the recovered GS cells were dissociated by accutase (Thermo Fisher Scientific) and filtered through a 50 µm mesh to remove cell aggregates. The single GS cells were plated on 6-well plates pre-coated with laminin, at a density of 2000–4000 cells/well. One week after plating, single GS cell-derived patches were detached with 0.5 mM EDTA, manually picked under the microscope, and each cell patch was transferred to one well of a 96-well plate pre-coated with MEFs. After ~1 month, the expanded colonies were dissociated by accutase and transferred to individual wells of a 48-well plate with MEFs and to larger wells thereafter. Since the clonal expansion proceeded to 6-well plates, the cell culture was carried out routinely.

**Genotyping monoclonal GS cell lines**

Before genotyping, a subpopulation of monoclonal GS cell lines was cultured on laminin for several passages to thoroughly eliminate the mixed MEFs. Then, the genomic DNA was extracted from each monoclonal GS cell line, and the region around the target site was amplified by PCR with the uniform primers for the Surveyor assay, but with a different high-fidelity polymerase (Easy-A high-fidelity PCR cloning enzyme, Agilent Technologies). The purified PCR products were cloned into T-Vector pMD19 (TaKaRa) following the manufacturer’s guidance. After transformation and overnight incubation, twenty colonies for each reaction were picked at random and sequenced.

**Off-target analysis**

The 10 top-ranking potential off-target loci, provided by the online Optimized CRISPR Design Platform (http://crispr.mit.edu/), were analyzed. In brief, the genomic DNA was first extracted from monoclonal GS cell lines, and the regions flanking each potential off-target site were PCR-amplified with the Hercule II fusion polymerase (Agilent Technologies) and the primers shown in Table 1. The purified PCR products were then sequenced for off-target analyses.

**Western blot analysis**

Before Western blot analysis, a subpopulation of Nsmce2⁺⁺ and Nsmce2⁻⁻ GS cells were cultured on laminin for several passages to thoroughly eliminate the mixed MEFs. Then, the protein was isolated and Western blot analysis was conducted as previously reported (Verver et al. 2013, 2016a), with the LI-COR Odyssey imaging system (Biosciences). The primary antibodies used were rabbit anti-NSMCE2 (1:200; provided by Oscar Fernandez-Capetillo), rabbit anti-SMC5 (1:500; A300-236A, Bethyl Laboratories), guinea pig anti-SMC6 (1:200; custom made, peptide: KRPRQEELEDFDKDGDEDE), mouse anti-PLZF (1:100; D-9, Santa Cruz Biotechnology), mouse anti-OCT4 (1:100; C-10, Santa Cruz Biotechnology), rabbit anti-STRA8 (1:1000; ab49602, Abcam), rabbit anti-SGSM1 (1:1000; ab171943, Abcam), mouse anti-β-actin (1:5000; A1978, Sigma-Aldrich) and rabbit anti-GAPDH (1:400; FL-335, Santa Cruz Biotechnology).

**Cell cycle analysis**

Cell cycle analysis based on DNA content was performed as previously described (Verver et al. 2016a). DNA content
was analyzed with the FACS analyzer (BD Biosciences) and the figures were constructed via the FlowJo software. Data were presented as the mean ± standard error of mean (S.E.M.). Differences between groups were assessed using the Student’s t-test. A difference was considered significant when P < 0.05.

**EdU assay**

The cell proliferation assay was performed using a Click-iT EdU Alexa Fluor 488 imaging kit (Thermo Fisher Scientific), following the protocol provided by the manufacturer. In brief, GS cells were grown on laminin-coated glass coverslips in 24-well plates. On the day of the treatment, cells were incubated with 10 µM EdU diluted in complete medium for 2 h at 37°C, followed by fixation in 4% paraformaldehyde (PFA) for 10 min. After permeabilization, cells were incubated with the reaction cocktail for 30 min at room temperature (RT), and then counterstained with DAPI for 5 min. Cells were mounted on glass slides with the Prolong Gold anti-fade mountant (Thermo Fisher Scientific) and later subjected to visualization under the microscope. For quantification of EdU+ cells, at least 300 cells were analyzed in each group. Data were presented as the mean ± S.E.M. Differences between groups were assessed using the Student’s t-test. A difference was considered significant when P < 0.05.

**Immunocytochemistry**

GS cells were grown on laminin-coated glass coverslips in 24-well plates for all immunocytochemical experiments. In case of etoposide treatment, GS cells were incubated with 10 µM etoposide for 3 h at 37°C, and then fixed at different time points, i.e. 0 h (immediately post treatment), 1, 3 and 5 h after treatment respectively, in 4% PFA for 10 min. Cells were permeabilized in phosphate-buffered saline (PBS) with 0.1% triton-X for 15 min, followed by 1 h of blocking in PBS with 1% bovine serum albumin (BSA) and 0.25% Tween20. Primary antibodies were applied to cells at 4°C overnight. The primary antibodies used were mouse anti-PLZF (1:50; D-9, Santa Cruz Biotechnology), mouse anti-OCT4 (1:50; C-10, Santa Cruz Biotechnology), rabbit anti-LIN28A (1:100; ab46020, Abcam), rabbit anti-ID4 (1:100; M106, CalBioreagents), mouse anti-OCT4 (1:20,000; 05-636, Merck Millipore) and guinea pig anti-SMC6 (1:400; custom made). Omission of the primary antibodies and replacement with mouse, rabbit and guinea pig IgGs were used as negative controls. After washing with PBS on the next day, the cells were incubated with the corresponding secondary antibodies (Alexa Fluor 488 or 555, 1:1000; Thermo Fisher Scientific) for 1 h at RT. Primary antibodies were applied to cells at 4°C overnight. The primary antibodies used were mouse anti-PLZF (1:50; D-9, Santa Cruz Biotechnology), mouse anti-OCT4 (1:50; C-10, Santa Cruz Biotechnology), rabbit anti-LIN28A (1:100; ab46020, Abcam), rabbit anti-ID4 (1:100; M106, CalBioreagents), mouse anti-γ-H2AX (1:20,000; 05-636, Merck Millipore) and guinea pig anti-SMC6 (1:400; custom made). Omission of the primary antibodies and replacement with mouse, rabbit and guinea pig isotype IgGs were used as negative controls. After washing with PBS on the next day, the cells were incubated with the corresponding secondary antibodies (Alexa Fluor 488 or 555, 1:1000; Thermo Fisher Scientific) for 1 h at RT. After counterstaining with DAPI for 5 min at RT, the cells were mounted on glass slides with the Prolong Gold anti-fade mountant (Thermo Fisher Scientific) and later subjected to visualization under the microscope. For quantification of γ-H2AX+ cells (>5 γ-H2AX foci/cell), at least 50 cells were analyzed in each group. Data were presented as the mean ± S.E.M. Differences between groups were assessed using the Student’s t-test. A difference was considered significant when P < 0.05.

**Microscopy**

Fluorescence microscopy images were acquired at RT using a Leica DM5000B microscope equipped with a Leica DFC365 FX CCD camera. Images were analyzed using Leica Application Suite Advanced Fluorescence (LAS AF) software. The presented figures were constructed using Adobe Photoshop CS6.

**RNA sequencing (RNA-seq)**

Total RNA was extracted from Nsmce2−/− and Nsmce2+/- GS cells, respectively, using PureLink RNA Micro Kit (Thermo Fisher Scientific). Biological triplicates from different passages were prepared. Total RNA was sent to BGI Tech Solutions for RNA sequencing (RNA-seq) analysis. The selected potential off-target sites and the corresponding PCR primers for sequencing are listed in Table 1.

<table>
<thead>
<tr>
<th>Potential off-target sites</th>
<th>Genomic loci (GRCm38/mm10)</th>
<th>Mis-matches</th>
<th>PCR primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACCTGGTGACATCCATCGG AGG</td>
<td>60636826–60636848, chromosome 12, +</td>
<td>4, 19</td>
<td>R: AGTACGGTACGTTAGAAAAACG</td>
</tr>
<tr>
<td>TCCTGGTAACATCCATCGG CAG</td>
<td>4770918–4770940, chromosome 1, +</td>
<td>1, 4, 10</td>
<td>F: GCCTCCTGGTTTCATTCTCTCA</td>
</tr>
<tr>
<td>ACCTGTACATACATCCAG CAG</td>
<td>24277599–24277621, chromosome 18, +</td>
<td>3, 5, 6</td>
<td>R: CCTGGAGGTCAAGGAACAA</td>
</tr>
<tr>
<td>GGGCGCTCAGATCCAGGG TGG</td>
<td>107576559–107576581, chromosome 6, +</td>
<td>2, 3, 5, 8</td>
<td>F: CTTGAGAACTAATCTAGGGAGT</td>
</tr>
<tr>
<td>AGCCGTCATCATCCAGGG TGG</td>
<td>148326544–148326566, chromosome 2, +</td>
<td>2, 3, 7, 8</td>
<td>R: ATCCAGAGTGCTCCACT</td>
</tr>
<tr>
<td>TCCATGCCATATCCAGGG TGG</td>
<td>91837662–91837684, chromosome 11, +</td>
<td>1, 2, 4, 9</td>
<td>F: CCAAGGTGCTGCTCAG</td>
</tr>
<tr>
<td>GCCATGCTCACATCCAGGG CAG</td>
<td>34570031–34570053, chromosome 2, +</td>
<td>1, 4, 8, 11</td>
<td>R: CATGACATCTGTGCTG</td>
</tr>
<tr>
<td>ACATGTTCCAGATCCAGGG AAG</td>
<td>75040008–75040030, chromosome 3, +</td>
<td>3, 4, 8, 11</td>
<td>F: GTGTCGATTCTTGAGTGGGTA</td>
</tr>
<tr>
<td>ACTTCTAACAATCCCTAGG TGG</td>
<td>16346304–16346326, chromosome 14, +</td>
<td>3, 4, 5, 11</td>
<td>R: TCTGAGGGTCTGGTGGAGG</td>
</tr>
<tr>
<td>GCCATGCCATATCCATCGG TAG</td>
<td>123031365–123031387, chromosome 10, +</td>
<td>1, 4, 8, 12</td>
<td>R: AGCTGAGGCAGCTCATAGCTCAAT</td>
</tr>
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</table>

Table 1: The selected potential off-target sites and the corresponding PCR primers for sequencing.
(Hong Kong, China) for library construction and sequencing (Illumina HiSeq 4000, paired end 100 bp). Reads were subjected to quality control and aligned to the UCSC mm10 (GRCm38. p4) genome using HISAT2 (v2.0.4) (Kim et al. 2015). Counts were obtained using HTSeq (v0.6.1) (Anders et al. 2015) using the UCSC mm10 GTF. Statistical analyses were performed using the edgeR (Robinson et al. 2010) and limma (Ritchie et al. 2015) R (v3.2.2)/Bioconductor (v3.0) packages. All genes with no counts in any of the samples were removed (15,633 genes), while genes with more than 1 count-per-million reads (CPM) in at least 2 of the samples were kept (31,435 genes). Count data were transformed to log$_2$-counts per million (logCPM), normalized by applying the trimmed mean of M-values method (Robinson et al. 2010) and precision weighted using voom (Law et al. 2014). Differential expression was assessed using an empirical Bayes moderated t-test within limma’s linear model framework including the precision weights estimated by voom. Resulting P values were corrected for multiple testing using the Benjamini–Hochberg false discovery rate. Corrected P values of <0.05 were considered as statistically significant. Genes were re-annotated using biomaRt (v2.26.1) using the Ensembl genome databases (v85). The resulting DEGs (adj. P < 0.05) and entire RNA-seq data are shown in Table 2 and Supplementary Table 1 (see section on supplementary data given at the end of this article) respectively. Gene Set Enrichment Analysis (GSEA) software (Mootha et al. 2003, Subramanian et al. 2005) was used to analyze differentially expressed gene sets, and the GSEA results are shown in Supplementary Table 2.

Accession numbers

All sequence data have been submitted to NCBI (SRA) and will be available under the accession number: ID PRJNA379902.

Results

Construction of CRISPR-Cas9 plasmids and gene targeting of Nsmce2 in GS cells

To perform loss of function study for Nsmce2, we first designed a sgRNA to target exon 2 of the mouse Nsmce2 locus (Fig. 1A). The target site is located in an early and conserved coding sequence of the two Nsmce2 transcript variants. Frameshift mutations at this site will eliminate NSMCE2 function, including its only known activity, GTPase. To overcome this hurdle, we determined to pick single cell-derived patches. Specifically, one week after FACS sorting, we conducted a Surveyor assay (Ran et al. 2013) based on the sorted cells. The target site-specific PCR amplicons were digested by Surveyor nuclease, yielding fragments with expected sizes indicative of indel mutations, demonstrating the occurrence of gene editing (Fig. 1F).

Table 2 The DEGs obtained by analysis of RNA-seq data from Nsmce2$^{-/-}$ and Nsmce2$^{+/+}$ GS cells.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Description</th>
<th>log FC</th>
<th>Adj. P val (&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mx1</td>
<td>MX dynamin-like GTPase 1</td>
<td>2.647288067</td>
<td>0.046661415</td>
</tr>
<tr>
<td>Skor1</td>
<td>SKI family transcriptional corepressor 1</td>
<td>0.715634301</td>
<td>0.046661415</td>
</tr>
<tr>
<td>Myog</td>
<td>Myogenin</td>
<td>4.244660863</td>
<td>0.044188003</td>
</tr>
<tr>
<td>Gbgt1</td>
<td>Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1</td>
<td>1.234137519</td>
<td>0.046661415</td>
</tr>
<tr>
<td>Sgsm1</td>
<td>Small G protein signaling modulator 1</td>
<td>-1.795957187</td>
<td>0.003750701</td>
</tr>
<tr>
<td>Zip358</td>
<td>Zinc finger protein 358</td>
<td>-1.292106048</td>
<td>0.046661415</td>
</tr>
<tr>
<td>Smim22</td>
<td>Small integral membrane protein 22</td>
<td>2.450729769</td>
<td>0.046661415</td>
</tr>
<tr>
<td>Cm9</td>
<td>Predicted gene 9</td>
<td>6.259824968</td>
<td>0.046661415</td>
</tr>
<tr>
<td>Cm30332</td>
<td>Predicted gene 30332</td>
<td>-6.010552517</td>
<td>0.003750701</td>
</tr>
</tbody>
</table>

To establish a GS cell line, we isolated germ cells from the testes of 4–5 d.p.p. DBA/2J male mice and cultured the isolated germ cells following an established protocol published by Shinohara’s group (Kanatsu-Shinohara et al. 2003). After 3–4 weeks, the GS cells were transferred to inactivated MEFs for subculture and they formed the distinctive grape-like colonies (Fig. 1C), consistent with former reports (Kanatsu-Shinohara et al. 2003, Kubota et al. 2004).

GS cells have been shown to be extremely refractory to commonly used transfection methods such as calcium phosphate precipitation and lipofection (Kanatsu-Shinohara et al. 2005c). Nevertheless, novel electroporation devices can be harnessed to transfect spermatogonia with moderate efficiency (Zeng et al. 2012, Fanslow et al. 2014, Chapman et al. 2015, Sato et al. 2015, Wu et al. 2015). To this end, we utilized a Neon electroporator to deliver CRISPR vectors into early passage GS cells. Two days after electroporation, GFP$^+$ cells (Fig. 1D) were enriched by FACS (Fig. 1E) and transferred to MEF feeder cells for recovery. One week after FACS sorting, we conducted a Surveyor assay (Ran et al. 2013) based on the sorted cells. The target site-specific PCR amplicons were digested by Surveyor nuclease, yielding fragments with expected sizes indicative of indel mutations, demonstrating the occurrence of gene editing (Fig. 1F).

Generation of Nsmce2$^{-/-}$ GS cell lines

Next, we derived monoclonal Nsmce2$^{-/-}$ GS cell lines. Initially, we exploited FACS to deposit single GS cells into individual wells of one 96-well plate (1 cell/well) pre-coated with MEF feeder cells. However, after 2 weeks of culture, we did not observe any GS cell clones. To overcome this hurdle, we determined to pick single cell-derived patches. Specifically, one week after FACS sorting, the sorted and recovered GS cells were plated on 6-well plates pre-coated with laminin, at a low density (2000–4000 cells/well) (Fig. 2A). The wells were pre-coated with laminin instead of MEFs because GS cells...
can attach rapidly to laminin, thereby circumventing the problem of polyclonal formation when single GS cells are plated at a low density. By contrast, we observed that GS cells attached slowly to MEFs and that many single cells readily aggregated before attachment. Previous reports have shown that MEFs as feeders are dispensable and GS cells can also be cultured on laminin for a long time (Kanatsu-Shinohara et al. 2005a, 2011, 2014). One week after plating, approximately 1–10% of single GS cells formed cell patches comprising 4–6 cells (Fig. 2B). They were then detached with EDTA, manually picked under the microscope and each cell patch was transferred to one well of a 96-well plate pre-coated with MEFs for further clonal expansion. After ~1 month, the expanded colonies (Fig. 2C) were dissociated and transferred to individual wells of a 48-well plate coated with MEFs and to larger wells thereafter. Eventually, the clonal expansion proceeded to 6-well plates. The whole process of the clonal expansion took ~2.5 months. In total, we derived 7 GS cell lines from 48 picked single-cell patches. PCR amplification of the genomic DNA region around the target site followed by TA cloning and Sanger sequencing revealed that 2 of them carried gene modifications at the Nsmce2 locus. Unfortunately, one Nsmce2−/− GS cell line failed to expand, probably due to premature passage when cells remained at a small number. Fortunately, the other Nsmce2−/− GS cell line, harboring bi-allelic frameshift mutations (Fig. 2D), was successfully expanded. Western blot analysis, using a transfected and single cell-derived Nsmce2+/+ GS cell line as a positive control, confirmed the eradication of the corresponding NSMCE2 protein (Fig. 2E). Protein levels of SMC5 and SMC6 were not influenced by removal of NSMCE2 (Fig. 2E). We validated these results using lysates from Nsmce2+/+ and Nsmce2−/− MEFs (described in Jacome et al. 2016) (Fig. 2E). Finally, we sequenced the 10 top-ranking potential off-target sites (Table 1) in the established Nsmce2−/− cell line and detected no off-target mutations. This Nsmce2−/− GS cell line, together with the control Nsmce2+/+ GS cell line used in Fig. 2E, were used for all further experiments.

**Removal of NSMCE2 does not influence the spermatogonial cell cycle or proliferation**

Morphologically, Nsmce2−/− GS cells were indistinguishable from their Nsmce2+/+ counterparts, and both formed the characteristic grape-like colonies on MEFs (Fig. 3A), distinct from the ES-like multipotent GS (mGS) cell colonies. Because we have previously demonstrated a prolonged cell cycle of Nsmce2-null U2OS cells (Verver et al. 2016a), we investigated whether the removal of NSMCE2 also alters the proliferation/cell cycle time of GS cells. The two single cell-derived GS cell lines, Nsmce2+/+ and Nsmce2−/− respectively, did not have significantly different cell doubling time (Fig. 3B). Consistently, cell cycle analysis by DNA histogram showed no difference between the two cell populations with respect to the ratios of cells in different cell cycle phases (Fig. 3C and E). To further investigate the role of NSMCE2 in controlling GS cell proliferation, we performed an EdU incorporation assay. The proportion of cells that incorporate the thymidine analog EdU (a measurement for DNA synthesis) did not differ...
between Nsmce2+/+ and Nsmce2−/− GS cells (Fig. 3D and F). Furthermore, both cell lines could normally propagate in vitro for more than 35 passages, without significant changes in cellular morphology or cell doubling time. Overall, the results suggest that the removal of NSMCE2 does not influence in vitro spermatogonial proliferation.

**Nsmce2−/− GS cells express typical markers of undifferentiated spermatogonia and can be induced to differentiation**

To validate their undifferentiated spermatogonial identity, we performed immunocytochemistry on Nsmce2−/− and Nsmce2+/+ GS cells. Both cell populations showed staining for SSC/progenitor cell markers PLZF, LIN28A, OCT4 and ID4 (Fig. 4A), indicating their undifferentiated state. Negative controls, i.e. omission of primary antibodies or replacement with isotype IgGs, did not yield any staining (Fig. 4A). Because our previous studies have shown that SMC6 marks spermatogonial differentiation (Verver et al. 2013), we next investigated whether NSMCE2 plays a role during spermatogonial differentiation. To this end, we induced spermatogonial differentiation by adding RA to the culture medium according to previous papers (Dann et al. 2008, Wang et al. 2016). In line with previous papers (Kanatsu-Shinohara et al. 2005a, Dann et al. 2008), when cultured on laminin, both Nsmce2+/+ and Nsmce2−/− GS cells started to exhibit the typical rhomboid morphology with long pseudopod-like extensions that is characteristic for undifferentiated spermatogonia. Treatment with RA for 3 days made them, as expected for differentiating spermatogonia (Dann et al. 2008), gradually lose this phenotype and become more round (Fig. 4B), indicative of their differentiation. Moreover, Western blot analysis showed that exposure to RA considerably reduced the protein levels of PLZF and OCT4, whereas that of differentiation marker STRA8 (Zhou et al. 2008a,b) was markedly increased in both cell lines (Fig. 4C). Overall, these results demonstrate that Nsmce2−/− GS cells have normal undifferentiated spermatogonial characteristics and can be induced to differentiation normally.
Etoposide-induced DNA damage repair occurs independently of NSMCE2 in GS cells

Because we have recently shown that SMC6 physically interacts with TOP2A and that NSMCE2 is implicated in the response to etoposide-induced topological stress and in subsequent DNA damage repair in U2OS cells (Verver et al. 2016a), we investigated whether NSMCE2 functions similarly in GS cells. To this end, we exposed Nsmce2<sup>+/+</sup> and Nsmce2<sup>−/−</sup> GS cells to etoposide and quantified DNA damage formation and repair marked by γ-H2AX (Fig. 5A and B). Albeit at a low number, γ-H2AX staining could be discerned in a small fraction of cells prior to etoposide treatment. Exposure to etoposide for 3 h triggered an increase of cells displaying γ-H2AX staining, indicative of increased DNA damage. However, the absence of NSMCE2 did not lead to more γ-H2AX+ cells after etoposide treatment (Fig. 5A and B). Also the decrease of γ-H2AX+ cells, indicative of DNA repair, followed similar dynamics between Nsmce2<sup>+/+</sup> and Nsmce2<sup>−/−</sup> GS cells. Moreover, in contrast to U2OS cells (Verver et al. 2016a), γ-H2AX did not co-localize with SMC6 in GS cells, regardless of etoposide treatment (Fig. 5C). Negative controls, i.e. omission of primary antibodies or replacement with isotype IgGs, did not yield any staining (Fig. 5A and C). The above data suggest that NSMCE2 is not involved in the response to etoposide-induced topological stress in GS cells.

Deprivation of NSMCE2 results in significant downregulation of Sgsm1 at the RNA but not protein level

To gain a broader perspective on the overall molecular effects of NSMCE2 removal, we conducted a RNA-seq experiment to compare the transcriptomes of Nsmce2<sup>−/−</sup> and Nsmce2<sup>+/+</sup> GS cells. According to the RNA-seq data of biological triplicates from different passages (Fig. 6A), inactivation of NSMCE2 generated only 9 differentially expressed genes (DEGs, adj. P < 0.05). Of these 9 DEGs, 6 genes showed upregulation and 3 genes showed downregulation (Table 2 and Supplementary Table 1). These RNA-seq data thus suggest that removal of NSMCE2 has only very minimal effects on the spermatogonial transcriptome. Notably, one known gene, Sgsm1, showed a very significant downregulation without NSMCE2 (Table 2) and appeared to be highly expressed in human spermatogonia (Human Protein Atlas, v15 (Uhlen et al. 2015)). Nevertheless, Western blot analysis showed comparable protein expression of SGSM1 in Nsmce2<sup>−/−</sup> and Nsmce2<sup>+/+</sup> GS cells (Fig. 6B).

Subsequent GSEA indicated alterations of gene sets such as H3K27Me3 or H3K4Me2 related to histone methylation and cancer development (Supplementary Table 2). Nonetheless, none of the genes within these gene sets were differentially expressed (adj. P < 0.05).

Discussion

SSCs, a subpopulation of undifferentiated spermatogonia, are best characterized by their capability of self-renewal to maintain sufficient numbers as well as differentiation to mature spermatozoa, thereby maintaining life-long male fertility. SSCs hold great value in reproductive medicine. They can reestablish spermatogenesis following transplantation into recipient testes, and thus they can be harnessed to restore fertility of, for instance, childhood cancer survivors who lose their germ cells.
due to chemotherapy or radiotherapy (Mulder et al. 2016). Moreover, in combination with genomic modification, SSCs could theoretically be employed to cure spermatogenic failure with known genetic causes or prevent inheritance of genomic diseases (Mulder et al. 2016). For rodent SSCs, CRISPR-Cas9 has been applied successfully for precise genomic modification and even to correct a genetic disease in the offspring (Chapman et al. 2015, Sato et al. 2015, Wu et al. 2015). Apart from clinical applications, SSCs are also of substantial utility in biomedical research, e.g. manipulation of SSCs provides an advantageous

Figure 4 Nsmce2−/− GS cells express typical markers of undifferentiated spermatogonia and can be induced to differentiation. (A) The staining of SSC/progenitor cell markers in GS cells on laminin. Bar = 10 µm. (B) Representative images of GS cells (on laminin) with/without RA treatment. Asterisks (*) indicate the long pseudopod-like extension. Bar = 25 µm. (C) Western blot analysis of PLZF, OCT4 and STRA8 proteins in GS cells with/without RA-induced differentiation. GAPDH and β-actin are used as loading controls.
avenue to generate various animal models with specific genotypes and phenotypes (Zheng et al. 2014).

In spite of this, the establishment of genetically modified SSC lines has been inefficient so far, primarily

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**Figure 5** Etoposide-induced DNA damage formation. (A) Representative images of γ-H2AX staining in Nsmce2<sup>+/+</sup> and Nsmce2<sup>−/−</sup> GS cells after etoposide treatment. GS cells were seeded on laminin prior to etoposide treatment. Control, without etoposide treatment; 0 h, immediately post treatment. Bar=10 µm. (B) Percentage of γ-H2AX<sup>+</sup> cells after etoposide treatment. Control, without etoposide treatment; 0 h, immediately post treatment. Data are presented as the mean ± s.e.m., n=3. (C) No co-localization of γ-H2AX with SMC6. Bar=5 µm.

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**Figure 6** The overall effects of NSMCE2 removal on the transcriptome of GS cells. (A) The multidimensional scaling (MDS) plot (left panel) and heat map (right panel) illustrating the minimal transcriptome variation between the WT and KO cells. KO, Nsmce2<sup>−/−</sup> GS cells; WT, Nsmce2<sup>+/+</sup> GS cells. (B) Western blot analysis of SGS1 in Nsmce2<sup>+/+</sup> and Nsmce2<sup>−/−</sup> GS cells. β-Actin is used as a loading control.
due to low transfection efficiency, difficulty in monoclonal isolation and expansion of SGCs in vitro. These technical difficulties impede the generation of transgenic animal models and the research on genes underlying spermatogenesis. Similar to other primary stem cells, SSCs are very refractory to prevailing transfection approaches such as calcium phosphate precipitation and lipofection (Kanatsu-Shinohara et al. 2005c). Previous studies have also indicated that adenovirus-based virus (AAV) and integration-deficient lentivirus are ineffective to fulfill gene editing in SSCs (Fanslow et al. 2014). Currently, novel electroporation devices are increasingly used to transfect SSCs (Zeng et al. 2012, Fanslow et al. 2014, Chapman et al. 2015, Sato et al. 2015, Wu et al. 2015). In our study, we employed a Neon electroporator to deliver the large CRISPR-Cas9 vectors into GS cells. The cells harboring CRISPR-Cas9 vectors were then enriched and subjected to clonal isolation. To achieve single cell-derived GS cell clones, we first cultured single GS cells in a full 96-well plate, but failed to get any cell clones. Because the initial cell density has been reported to have a big influence on subsequent GS cell culture (Kanatsu-Shinohara et al. 2005c), we then attempted to pick single cell-derived patches. We found that GS cells attached slowly to MEFs and that many single cells readily aggregated before attachment. We therefore used laminin instead of MEF feeder cells. GS cells rapidly attach to laminin, thereby greatly facilitating monoclonal isolation when single GS cells are plated at a low density. Collectively, the entire optimized protocol (elaborated in ‘Materials and methods’ section) is less laborious and more efficient compared to recent reports (Chapman et al. 2015, Sato et al. 2015, Wu et al. 2015). Using this optimized protocol, we successfully generated an Nsmce2−/− GS cell line lacking the NSMCE2 subunit of the SMC5/6 complex.

The SMC5/6 complex has been shown to play pivotal roles in many important biological processes, in particular, genomic integrity maintenance and DNA damage repair (De Piccoli et al. 2006, Potts 2009, Wu & Yu 2012). In yeast, all subunits of the SMC5/6 complex, characterized by the reduced expression of SMC6 and/or SMC5 (Kliszczak et al. 2012, Payne et al. 2014). Using Western blot analysis, we showed that the protein levels of SMC5 and SMC6 were not affected by NSMCE2 depletion in GS cells. Indeed, the stable presence of SMC5 and SMC6 does not mean that the whole SMC5/6 complex remains stable. However, combined with the fact that we did not detect any other significant phenotype, it does suggest that NSMCE2 is not essential for the SMC5/6 stability and function in GS cells.

We have recently found that the removal of NSMCE2 in U2OS cells generated significant differences in phenotypes, including slower cell growth, accumulation of cells in G1-S of the cell cycle, as well as a decreased plating efficiency (Verver et al. 2016a). In line with these, an earlier study in human MCF-7 breast cancer cells showed that knockdown of Nsmce2 resulted in slower cell growth and impaired G1-S transition. Ectopic expression of the full-length NSMCE2, but not its SUMO ligase-inactive mutant, rescued the reduced cell number, implying that the normal growth of these breast cancer cells requires the NSMCE2 SUMO ligase function (Ni et al. 2012). However, consistent with a study conducted in chicken DT40 cells (Kliszczak et al. 2012), we found that the viability and growth of GS cells was not influenced by the removal of NSMCE2. Nsmce2−/− GS cells showed similar doubling time, EdU incorporation and cell cycle progression as their Nsmce2+/− counterparts. Furthermore, GS cells showed normal cellular morphology without nuclear abnormalities, such as micronuclei or nucleoplasmic bridges that are observed in mouse and human Nsmce2-deficient fibroblasts (Payne et al. 2014, Jacome et al. 2016). These disparities imply cell type-specific roles of NSMCE2.

We have recently found that SMC6 protein expression specifically marks differentiating spermatogonia (Verver et al. 2013), the spermatogonial subpopulation irreversibly committed toward meiosis. However, the exact role of NSMCE2 during spermatogonial differentiation has not been elucidated. We therefore investigated whether absence of NSMCE2 would influence the capacity of GS cells to differentiate. GS cells can be induced to differentiation by adding RA to the culture medium (Dann et al. 2008, Wang et al. 2016). RA-induced differentiation will lead to downregulation of SSC/progenitor cell markers (e.g. PLZF and OCT4), upregulation of differentiation markers (e.g. STRA8), reduced self-renewal and increased apoptosis and eventually to a decline in the total cell number (Dann et al. 2008, Chen et al. 2016, Wang et al. 2016). Before RA-induced differentiation, Nsmce2−/− GS cells exhibited routine expression profiles characteristic for undifferentiated spermatogonia. Here, we found that Nsmce2−/− GS cells could be normally induced to differentiate, exhibiting similar morphology and characteristics as their Nsmce2+/− counterparts. Hence, NSMCE2 does not seem to be involved in in vitro spermatogonial proliferation or differentiation.
NSMCE2 is extensively reported to be crucial for the response to DNA damage, mostly in yeast (McDonald et al. 2003, Andrews et al. 2005, Potts & Yu 2005, Zhao & Blobel 2005, Rai et al. 2011, Kliszczak et al. 2012). However, recent studies performed in multiple mouse and human cell types have indicated that NSMCE2 is redundant for the repair of ionizing radiation (IR)-induced DNA damage in mammalian cells (Fernandez-Capetillo 2016, Jacome et al. 2016, Verver et al. 2016a). Indeed, we have recently shown that also in U2OS cells, NSMCE2 is redundant for the repair of double-strand breaks (DSBs) induced by IR (Verver et al. 2016a). Nonetheless, in the same paper, we showed that CRISPR-Cas9-mediated removal of NSMCE2 led to increased sensitivity to etoposide. Etoposide is a cytotoxic agent that, by forming a complex with DNA and topoisomerase II, causes replication-induced topological stress and DSBs at replication forks (Hande 1998). Surprisingly, here we found that removal of NSMCE2 in GS cells did not cause increased sensitivity to etoposide. Moreover, etoposide-induced DSBs marked by γ-H2AX were repaired efficiently in both Nsmce2+/+ and Nsmce2−/− GS cells. Also in contrast to U2OS cells (Verver et al. 2016a), etoposide-induced γ-H2AX in GS cells did not co-localize with SMC6. It is thus plausible that GS cells hold different mechanisms to resolve topological stress than somatic cells, which might not rely on NSMCE2 or even the SMC5/6 complex.

To gain a broader perspective on the overall molecular effects of NSMCE2 removal, we finally conducted a whole transcriptome RNA-seq analysis for Nsmce2+/+ and Nsmce2−/− GS cells. We previously hypothesized that, by altering chromatin structure, the SMC5/6 complex would influence spermatogonial gene transcription (Verver et al. 2013, 2014). GSEA did indicate the downregulation of gene sets involved in chromatin regulation, such as H3K27Me3 or H3K4Me2 related to histone methylation and cancer development, suggesting that chromatin architecture or function is somehow affected by the absence of NSMCE2. However, removal of NSMCE2 only led to 9 DEGs, of which none were present in these gene sets. Of the 9 DEGs, Sgsm1 showed a very significant downregulation without NSMCE2. SGS1, as well as its two paralogs SGS2 and SGS3, all consist of RUN and TBC motifs and have been reported to orchestrate small G protein-mediated signaling transduction. Unlike SGS2 and SGS3, which show ubiquitous expression in a wide range of tissues, SGS1 is primarily expressed in human brain, heart and testes (Yang et al. 2007). Moreover, in human testes, SGS1 is highly expressed in spermatogonia (Human Protein Atlas, v15 (Uhlen et al. 2015)). In our present study, we also demonstrated the high protein level of SGS1 in GS cells. However, while removal of NSMCE2 significantly downregulated Sgsm1 transcripts, its protein level was not affected. Perhaps the SGS1 protein level was somehow stabilized in the absence of NSMCE2. Alternatively, assuming that SGS1 protein stability is not affected by NSMCE2, the detected lower Sgs1 mRNA level was still sufficient to ensure the wildtype protein level. Nevertheless, the detected Sgs1 downregulation in our study and localization in human spermatogonia indicate an unknown role of this protein in GS cells. To acquire more knowledge about this, loss-of-function studies for SGS1 could be performed in the future.

The absence of evident phenotypes and the minimal transcriptome variation caused by NSMCE2 removal raise the question what the exact role of NSMCE2 in GS cells is. Recently, a case report described 2 female patients with heterozygous frameshift mutations in Nsmce2 that result in decreased expression of NSMCE2. These patients exhibited serious phenotypes like primordial dwarfism, extreme insulin resistance and, notably, primary ovarian failure (Payne et al. 2014). Given that NSMCE2 is required for yeast meiosis (Pebernard et al. 2004), the possibility remains that further downstream steps beyond spermatogonial proliferation and differentiation require the function of NSMCE2. On the other hand, the specific expression of SMC6 in differentiating spermatogonia (Verver et al. 2013), the stable presence of SMC5 and SMC6 without NSMCE2 and the observed lethality of GS cells after transfection with CRISPR-Cas9 plasmids targeting Smc5/6 all suggest that the SMC5/6 complex plays important roles in spermatogonia, but that this spermatogonial function of SMC5/6 is not affected by NSMCE2 removal.

Male germline stem cells are responsible for the lifelong daily production of millions of sperm and the transmission of genetic information to the offspring. Decades of studies have well demonstrated that this unique adult stem cell population is largely distinct from somatic cells in terms of cellular activities, cell fate commitment, developmental plasticity, chromatin architecture and remodeling as well as (epi)genetic features (Guo et al. 2014, Manku & Culty 2015, Tseng et al. 2015). They also hold unique mechanisms to maintain genomic stability (Marjault & Allemand 2016), which can partially explain the divergent roles of NSMCE2 and SMC5/6 in GS cells. It has been known that the SMC5/6 complex, including NSMCE2, is essential for genome integrity maintenance in somatic cells, demonstrated by, e.g. the embryonic lethality of SMC6 or Nsmce2 KO (Ju et al. 2013, Jacome et al. 2016) and the role of NSMCE2 in the prevention of cancer and aging in adult mice (Jacome et al. 2016). Nonetheless, the SMC5/6 complex in male germline stem cells seems to function normally without NSMCE2. Hence, how germline stem cells safeguard genomic integrity, and the role of SMC5/6 herein, remain to be further investigated, especially given the current technical development such as CRISPR-Cas9 and the potential clinical application of...
these cells, for instance, in fertility preservation, curing spermatogenic failure or preventing transmission of genetic diseases (Mulder et al. 2016).

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0173.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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