

OGG1 protects mouse spermatogonial stem cells from reactive oxygen species in culture¹

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Abstract

Although reactive oxygen species (ROS) are required for spermatogonial stem cell (SSC) self-renewal, they induce DNA damage and are harmful to SSCs. However, little is known about how SSCs protect their genome during self-renewal. Here we report that *Ogg1* is essential for SSC protection against ROS. While cultured SSCs exhibited homologous recombination-based DNA double-strand break repair at levels comparable to those in pluripotent stem cells, they were significantly more resistant to hydrogen peroxide than pluripotent stem cells or mouse embryonic fibroblasts, suggesting that they exhibit high levels of base excision repair (BER) activity. Consistent with this observation, cultured SSCs showed significantly lower levels of point mutations than somatic cells, and showed strong expression of BER-related genes. Functional screening revealed that *Ogg1* depletion significantly impairs survival of cultured SSCs upon hydrogen peroxide exposure. Thus, our results suggest increased expression of BER-related genes, including *Ogg1*, protects SSCs from ROS-induced damage.

56 **Introduction**

57 DNA repair of self-renewing tissues is an important topic because any defects
58 in stem cells are transmitted to their progenitor cells and influence the tissue turnover and
59 function [1]. When DNA lesions directly impair self-renewal, the tissue will be depleted
60 of stem cells and will fail to function properly due to a lack of mature differentiated cells.
61 Even a subtle difference in the self-renewal rate can be deleterious because stem cells in
62 many tissues compete against each other for their optimal microenvironment. DNA
63 mutations in the germline are particularly critical because they not only lead to infertility
64 but also generate de novo mutations that may give rise to new diseases in the next
65 generation. Five percent of live-born human offspring have a genetic disorder and 20%
66 of these disorders are caused by de novo mutations [2]. Compared to female germline
67 cells, male ones undergo extensive replication before gamete production, which has the
68 potential to generate more mutations in the male-derived genome [3]. To counter this
69 potential dilemma, it is likely that germ cells possess a unique DNA repair system that
70 mitigates the accumulation of de novo point mutations.

71 Studies of male germ cells have been hampered by difficulties in introducing
72 genetic manipulation. However, an exception has been spermatogonial stem cells (SSCs).
73 SSCs divide continuously to sustain spermatogenesis throughout the life of male animals
74 [4,5]. Indeed, they are thought to comprise only 0.02-0.03% of total germ cells in the
75 testis [5,6]. Despite their small number, SSCs can be expanded in vitro by their self-
76 renewal activity. With supplementation of SSC self-renewal factors, such as GDNF and
77 FGF2, grape-like clusters of spermatogonia can proliferate in vitro for > 2 years [7]. These

cells, designated as germline stem (GS) cells, can reinitiate spermatogenesis upon transplantation into the seminiferous tubules of infertile animals. Although the frequency of SSCs in GS cell cultures is low (~1-2%), this culture system allows the in vitro expansion and genetic manipulation of SSCs, facilitating biochemical and molecular analyses of the male germline [8]. Thus, using GS cells, we previously showed that reactive oxygen species (ROS) drive SSC self-renewal [9]. NOX1 is a major producer of ROS and mice deficient in *Nox1* exhibit impaired self-renewal in vivo, whereas supplementation of H₂O₂ enhances GS cell proliferation in vitro. Deletion or depletion of genes involved in ROS generation, such as *Mapk14* or *Mapk7*, impairs GS cell proliferation and induces apoptosis [10].

However, ROS also induce DNA damage. DNA double-strand breaks (DSBs) are the most severe type of damage and are lethal and oncogenic. DSBs are repaired by non-homologous end-joining (NHEJ) or homologous recombination (HR)[11]. HR is an error-free repair process that occurs most efficiently in the late S and G2 phases of the cell cycle because HR demands homologous strands to serve as an original template for correcting damaged DNA. On the other hand, NHEJ occurs throughout the cell cycle but is prone to error. ROS also induce other forms of DNA damage through oxidization of nucleotide bases. 8-oxoguanine (8-OxoG) is one of the most common DNA lesions caused by oxidative stress [12]. The lesion results in G:C to T:A transversions. Oxidized bases are typically repaired by the base excision repair (BER) pathway, but when they occur simultaneously on opposing strands, attempted BER can lead to the generation of

DSBs. To ensure pristine genome preservation, SSCs must correct these mutations as precisely as possible during self-renewal.

Several *in vivo* studies have suggested that male germ cells possess a unique DNA repair machinery. For example, morphological analyses of irradiated testes have shown that ZBTB16⁺ spermatogonia lack histone-associated signaling components of the DNA repair machinery, such as γ H2AX/MDC1, which are recruited immediately to DSBs after damage in somatic cells [13]. Male germ cells also undergo dynamic changes in DNA mutation frequency. A series of studies that used *LacI* mutation-reporter mice (“Big Blue mice”) showed that the estimated point mutation frequencies of a mixed population of spermatogenic cells are lower than those in somatic cells [14]. Closer examination revealed a decline in mutation frequency during spermatogenesis, such that the mutation frequencies in type B spermatogonia and all subsequent stages of spermatogenesis were lower than the frequency in primitive type A spermatogonia [2]. In addition, spermatogenic cells from old mice showed significantly higher mutation frequencies than those from young mice [2]. Therefore, DNA repair and mutation frequency in spermatogenic cells change according to their differentiation status and age. More recent studies have suggested that the numbers of DNA mutation frequencies in embryonic stem (ES) cells, induced pluripotent stem cells and spermatogonia are very low compared to somatic cells [15,16], which suggested high levels of BER activity in these cells. Although these results suggest a similarity between pluripotent stem cells and spermatogonia, GS cells are significantly more sensitive to irradiation than ES cells [17]. While ES cells continue to proliferate after irradiation [18], GS cells undergo apoptosis

at significantly lower radiation doses. These results suggest that SSCs have a unique DNA repair machinery not found in ES cells or somatic cells.

In this study, we sought to understand the mechanism by which GS cells tolerate ROS. We evaluated the DSB repair machinery by transfecting DNA substrates into GS cells and determined the contributions of NHEJ and HR. We found that the expression of genes involved in BER is up-regulated in GS cells relative to other cell types and revealed that *Ogg1* is responsible for protecting GS cells from ROS.

Materials and Methods

Animals

The Big Blue mice were obtained from Taconic Farms, Inc. (Hudson, NY). These mice were maintained in a C57BL/6 (B6) background. The Institutional Animal Care and Use Committee of Kyoto University approved all animal experimentation protocols.

Cell culture

For GS cell cultures, wild-type (WT) and *Trp53* knockout (KO) GS cells in an ICR background were previously described [19]. We also derived GS cells from Big Blue mice (B6 × DBA/2 F1; BDF1). These cells were derived from 2-8 days old pups. For experiments using multipotent germline stem (mGS) cells from Big Blue mice, mGS cells were induced by suppressing *Dmrt1* and *Trp53* in GS cells, as previously described [20]. ES cells (R1) were a generous gift from Dr. M. Ikawa (Osaka University, Osaka, Japan).

Mouse embryonic fibroblasts (MEFs) were prepared from 13.5 days post-coitum ICR or Big Blue mouse BDF1 embryos. Tail tip fibroblasts (TTFs) were collected from adult Big Blue mice. GS cells were maintained on MEFs using Iscove's modified Dulbecco's medium supplemented with GDNF and FGF2, as described previously [21]. ES cells and mGS cells were maintained on MEFs using Dulbecco's modified Eagles's medium (DMEM) supplemented with 15% fetal bovine serum (FBS), 1000 U/ml leukemia inhibitory factor (ESGRO; Merck Millipore, Darmstadt, Germany), nonessential amino acid mixture (Invitrogen, Carlsbad, CA), 0.1 mM 2-mercaptoethanol (2-ME), 2 μ M PD0325901 (Selleck Chemicals, Houston, TX), and 3 μ M CHIR99021 (Biovision, Milpitas, CA). MEFs and TTFs were cultured in DMEM supplemented with 10% FBS. Where indicated, culture medium was supplemented with H₂O₂ (Wako, Kyoto, Japan).

Virus transfection

The lentiviral knockdown (KD) vectors used in the present study were purchased from Open Biosystems (Huntsville, AL), and pLKO1-Scramble shRNA was used as a control (Addgene, Cambridge, MA). For overexpression of cDNAs, mouse *Ogg1* was cloned into CSII-EF-IRES-puro (RIKEN Bioresource Center, Tsukuba, Japan). Lentivirus transfection was performed as described previously [9]. Multiplicity of infection (MOI) was adjusted to 10.0 and 1.0 for lentivirus infection. All KD vectors are listed in Supplemental Table S1.

Immunostaining

Testes were fixed in 4% paraformaldehyde for 2 h at 4°C before embedding in Tissue-Tek OCT compound (Sakura Finetek, Tokyo, Japan) for cryosectioning. Then sections of 4 µm thickness were prepared and slides were incubated in 0.1% Triton X-100. For staining of GS cells, cells were dissociated using trypsin, and single cell suspensions were concentrated on glass slides by centrifugation using a Cytospin 4 unit (Thermo Elecron Corp, Cheshire, UK). The slides were incubated in 0.01% Triton-X for 15 min for permeabilization. After immersion of slides in the blocking buffer [0.1% Tween 20, 1% bovine serum albumin (BSA) and 10% normal donkey serum in phosphate-buffered saline (PBS)], samples were incubated with indicated antibodies or rhodamine-labeled PNA (Vector Laboratories, Burlingame, CA). OGG1 levels were quantified using the MetaMorph software (Molecular Devices, Sunnyvale, CA). Cells were counterstained with Hoechst 33342 (Sigma, St. Louis, MO). The antibodies used are listed in Supplemental Table S2.

Alkaline comet assay

The alkaline comet assay was performed as described previously [22]. In brief, cells were incubated with H₂O₂ for 30 min on ice followed by the incubation in their respective culture medium at 37°C for the indicated periods of time. After centrifugation, cells were embedded in 0.75% low-gelling temperature agarose (A4018; Sigma) on poly-L-lysine-coated glass slides (Matsunami Glass, Osaka, Japan). Then the cells were lysed by incubating the slides at 4°C in lysis buffer [1% Triton X-100, 0.5% N-Lauroryl sarcosine sodium salt, 2.5 M NaCl, 100 mM EDTA (pH 8.0), and 10 mM Tris-HCl (pH

10.0)]. After washing twice in PBS, the slides were immersed in pre-chilled electrophoresis buffer (0.3 M NaOH and 1 mM EDTA in water) for 40 min and electrophoresis was performed at 25 V for 50 min at 4°C. Then the samples were washed twice in 400 mM Tris-HCl (pH 7.0) at room temperature and soaked in ethanol for 5 min, followed by overnight drying at 37°C. DNA was detected by ethidium bromide staining for 30 min at room temperature. After rinsing in PBS, slides were observed via confocal microscopy and quantified using the Metamorph software.

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining

Apoptotic cells were detected using the in situ cell death detection kit: TMR red (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Cells were counterstained using Hoechst 33342.

Gene expression analyses

Total RNA was isolated using TRIzol (Invitrogen). For real-time polymerase chain reaction (PCR), first-strand cDNA was produced using a Verso cDNA synthesis kit. Real-time PCR was performed using StepOnePlus™ real-time PCR system (Applied Biosystems, Cheshire, UK) and the Power SYBR Green PCR Master Mix (Applied Biosystems). Transcript levels were normalized against *Hprt* expression. The PCR conditions were as follows: 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each PCR experiment was performed in triplicates. PCR primers are listed in Supplemental Table S3.

209

210 *Analyses of mutation frequency by Big Blue mice*

211 The Big Blue mouse assay was performed as described previously [16]. In brief,
212 genomic DNA collected from experimental samples was used to recover the lambda
213 shuttle vector containing the *LacI* mutation-reporter gene according to the manufacturer's
214 instructions (Stratagene; La Jolla, CA). SCS-8 *E coli* strain cells were mixed with the
215 packaged phage and plated at < 17,500 plaque-forming units per 25 × 25 cm NZY agar
216 assay tray (Stratagene). Plates were incubated at 37°C for 16-18 h, and stained by
217 incubating with X-gal to detect mutant plaques. The *LacI* gene was amplified by PCR
218 from DNA samples recovered from the mutant phages. The gene sequence of the mutant
219 *LacI* gene was compared with that of the WT *LacI* gene to a) confirm the presence of a
220 mutation, b) determine the spectrum of mutations detected in each sample, and c) identify
221 and adjust for any clonal mutants (= the same mutation at the same location in the *LacI*
222 gene in more than one mutant phage plaque). Numbers of mutations were analyzed by a
223 Poisson model with parameter estimates obtained by the method of maximum likelihood
224 [23]. Because of the low expected frequencies, exact p-values were calculated by the
225 exact conditional test for Poisson variables to compare differences between mutation
226 frequencies, using the Exactci package implemented in R [24,25].

227

228 *Magnetic cell sorting (MACS)*

229 Testis cells from Big Blue mice were dissociated using 1 mg/ml collagenase
230 type II for 10 min (Sigma) and dissociated into single cells by repeated pipetting, as

described previously [26]. The dissociated cells were incubated with anti-CDH1 antibody for 10 min (ECCD2; a gift from Dr. M. Takeichi, RIKEN CDB, Kobe, Japan). The cells were washed twice with PBS supplemented with 1% FBS, and anti-rat IgG magnetic beads were added and incubated for 15 min on ice (Miltenyi, Auburn, CA). Magnetic cell sorting was carried out as using Miltenyi large cell column.

NHEJ assay

The NHEJ assay was performed as described previously [27]. In brief, GS cells were electroporated with pJH200, *Rag1*, and *Rag2*-expression plasmids using the Neon Transfection System (Thermo Fisher Scientific, Waltham). The survival rate was measured by trypan blue staining. The extrachromosomal plasmids were collected from the transfected cells after 48 h using a modified Hirt extraction method [28], which was used to transform competent DH5 α *E. Coli* cells. Transformed bacteria were plated on LB-agar containing 100 μ g/ml ampicillin (Amp) and/or 5 μ g/ml chloramphenicol (Cm; both from Wako), and incubated for 24 h at 37°C.

HR assay

The HR assay was performed as described previously [29,30]. In brief, GS or ES cells were electroporated with pHPRT-DRGFP (gift from Dr. M. Jasin; Sloan Kettering Institute, New York, NY) using the Neon Transfection System (Thermo Fisher Scientific) and stable clones were obtained by puromycin selection and confirmed by Southern blotting. To assay green fluorescent protein (GFP) expression, cells were

transfected with pSce-I endonuclease (Addgene), and the pCAG-DsRed reporter by lipofectamine 3000 (Thermo Fisher Scientific). Transfection efficiency was estimated by quantifying DsRed fluorescence by FACSCalibur (BD Bioscience, Franklin Lakes, CA).

Statistical analyses

Results are presented as the means $\pm$ SEM. Data were analyzed using the Student's *t*-tests. Multiple comparison analyses were performed using ANOVA followed by Tukey's HSD test.

Results

Increased survival of GS cells against H₂O₂

We previously showed that ROS are required for GS cell proliferation [9]. To compare the sensitivity of GS cells with other cell types, we used ES cells, mGS cells, a pluripotent cell type derived from GS cells [19], and MEFs. These cells were cultured in the presence of H₂O₂ (10 μ M to 1 mM), which is permeable through the cell membrane. Although exposure to high concentrations of H₂O₂ killed GS cells (> 500 μ M), low concentrations of H₂O₂ promoted GS cell proliferation without apparent damage (Fig. 1A, B). This enhancement of proliferation is consistent with a previous study [9]. However, H₂O₂ induced extensive apoptosis of other cell types even at 10 μ M. The number of apoptotic cells increased in a dose-dependent manner. When TUNEL staining was carried out at 50 μ M, significantly enhanced apoptosis was evident (Fig. 1C). In particular, MEFs showed the most severe response. Because apoptosis from irradiation can be attenuated

by *Trp53* deficiency in GS cells [17], we investigated the impact of *Trp53* on H₂O₂ - induced apoptosis at 500 μ M. As expected, loss of *Trp53* increased cell recovery, and significantly reduced the number of TUNEL⁺ cells (Supplemental Fig. S1A, B), which suggests that *Trp53* is involved in ROS-induced GS cell apoptosis.

To quantify DNA damage, we performed an alkaline comet assay [31], which detects both single-strand breaks (SSBs) and DSBs, and measured the length of the DNA tail. Although GS cells showed enhanced proliferation at 50 μ M H₂O₂ (Fig. 1B), they also showed DNA damage at this concentration, which was quickly recovered after 10 min. MEFs showed a more significant increase in the DNA fraction that migrated into the comet tail even at 10 μ M (Fig. 1D). However, all cell types showed significant recovery after 30 min. GS cells also showed extensive damage at 500 μ M H₂O₂, but also recovered efficiently. These results suggest that GS cells are more resistant to ROS than other cell types.

Analyses of DSB repair machinery in GS cells

DSBs represent major damage caused by high concentrations of H₂O₂. Previous studies have shown that H₂O₂ (10-100 μ M) can induce chromosome damage in primary skin cells of mice [32]. To understand the mechanism of H₂O₂ resistance, we first analyzed the expression of genes involved in DSB repair. DSBs are repaired by NHEJ or HR pathways. Using real-time PCR, we examined the expression of *Xlf* (NHEJ), *Xrcc6* (NHEJ), *Brca1* (HR), and *Rad51* (HR). We found stronger expression of all of these genes

in GS cells than in other cell types (Fig. 2A). Some, but not all of these genes were influenced by supplementation of H₂O₂ (Supplemental Fig. S2).

To directly examine the type of DSB repair in a functional manner, we next carried out a series of transfection experiments to test NHEJ activity using pJH200 (Fig. 2B). This plasmid contains two heptamer-nonamer immunoglobulin-joining signal sequences flanking a prokaryotic transcription terminator, which prevents expression of the Cm-resistant genes [27]. Signal sequence removal by *Rag1* and *Rag2* recombinase gene transfection allowed Cm-resistant gene expression. Based on the previous experiments using lymphocytes [27], we transfected pJH200 substrate vector into GS cells. The viability of cells after transfection, as estimated by trypan blue staining, was $31.0 \pm 4.2\%$, $34.4 \pm 4.1\%$, $32.8 \pm 3.8\%$, and $82.8 \pm 2.5\%$, respectively, for GS cells, ES cells, mGS cells, and MEFs, respectively ($n = 4$). The substrate plasmid was recovered 2 days after transfection and introduced into *E. coli* before plating on LB agar plates containing either Amp or Amp/Cm. When the ratio of successful recombination was quantified by measuring the number of Cm-resistant colonies and Amp-resistant colonies, we found that MEFs had significantly elevated NHEJ activity compared to other cell types (Fig. 2C). This is consistent with previous studies that have found that somatic cells, but not ES cells, use NHEJ predominantly to repair DSBs [33]. We did not find statistical significance in levels of NHEJ among the other cell types.

We next compared HR activity using pHPRT-DRGFP, which contains two GFP cassettes: a nonfunctional mutant GFP, SceGFP, engineered to contain an 18-bp restriction site for the endonuclease Sce-I, and an internal GFP, which contains a 5'- and

3'-truncated fragment capable of correcting the mutation in the SceGFP cassette. Transfection of Sce-I into cells that contain pHPRT-DRGFP induces a DSB, which can be corrected with GFP by HR, as described previously (Fig. 2D)[29]. Using this system, we focused on GS cells and ES cells because these cells did not show significant differences in the NHEJ activity. Because this assay depended on analysis of stably transfected cells, we were not able to analyze MEFs, which undergo senescence during repeated passages. We found a significant increase in GFP expression in both GS cells and ES cells after transfection of the Sce-I-expressing plasmid (Fig. 2E). However, no significant differences in GFP fluorescence were seen between the two cell types. These results suggest that GS cells and ES cells have comparable levels of HR-based DNA repair activity in restoring GFP expression.

Quantification of DNA mutations using Big Blue mice

Although our results suggest that GS cells use HR to survive H₂O₂ treatment, the experiment in the preceding section could not explain the difference between ES and GS cells. However, H₂O₂ can cause several types of DNA damage. DSBs constitute the most severe types of damage, but H₂O₂ also induces point mutations, which are corrected by BER [12]. Considering their strong resistance to ROS and previous studies regarding the high BER activity of male germ cells [34], we speculated that GS cells exhibit a strong BER capacity. Indeed, freshly collected spermatogonia are reported to have a low point mutation frequency compared to somatic cells [16]. However, because cell proliferation decreases BER activity in some cell types [35,36], we analyzed whether GS cells maintain

the low mutation frequency phenotype. For this experiment, we used Big Blue mice (Fig. 3A). These mice contained the prokaryotic *LacI* gene as a mutation reporter transgene. This transgene can be recovered from any cell type and cloned into a lambda shuttle vector, which can be packaged into a phage that can infect *E. coli* host cells. If any inactivating mutation arises in the *LacI* transgene, repression of the *Lac* operon will cease, allowing beta galactosidase to be produced from the *E. coli*, which can be detected by adding X-gal to the plating medium.

In this experiment, we derived GS cells from Big Blue mice. We also collected CDH1⁺ undifferentiated spermatogonia from mice 8- to 10-months old by MACS and compared their mutation frequencies. Flow cytometric analysis of CDH1-selected cells showed that CDH1⁺ cells comprise $24.0 \pm 2.0\%$ ($n = 4$)(Supplemental Fig. S3). For analyses of GS cells, cells from two different time points (8 and 31 months after culture initiation) were analyzed. This was because we were interested to study the impact of in vitro aging. We also used mGS cells as a pluripotent cell type. MEFs and TTFs were used as somatic cell controls. Overall, quantification of mutation frequency revealed that GS cells exhibited relatively low mutation frequencies among the different cell types (Fig. 3B). CDH1⁺ spermatogonia or mGS cells also showed a lower frequency than MEFs or TTFs, consistent with the results of a previous study [16]. The difference between 31 months old GS cells (31M-GS cells) and MEFs ($p = 2.74E-05$) or TTFs ($p = 7.06E-09$) were statistically significant. In contrast, we did not find statistical difference between 31M-GS cells and CDH1⁺ spermatogonia ($p = 0.13$), mGS cells ($p = 0.24$) or 10M-GS cells ($p = 0.61$). Sequencing of *LacI* mutants showed both transversions and transitions

in all cell types (Supplemental Table S4). These results suggest that GS cells similarly maintain a low point mutation frequency, similar to that found in freshly prepared spermatogonia.

Expression of BER-related genes in GS cells

To study the mechanism of the low point mutation frequency in GS cells, we performed real-time PCR and examined expression of BER-related genes in GS cells, ES cells, mGS cells, and MEFs. The BER-related genes include *Apex1*, *Ercc6*, *Fen1*, *Mbd4*, *Mth1*, *Mutyh*, *Neil1*, *Ogg1*, *Pnkp*, *Rpa1-3*, *Tdg*, *Ung*, and *Xrcc1*. These genes were selected based on the previous publications that showed their functional involvement in BER [37-40]. The lack of these genes can cause various defects. For example, deletion of *Ercc6*, *Mbd4*, *Ogg1*, *Mutyh*, *Neil1*, *Rpa1-3* or *Ung* increases the risk of neoplasm, while *Apex1* or *Tdg* deficiency results in embryonic lethality [37-40]. Of the 15 genes that were examined, 11 genes were most strongly expressed in GS cells (Fig. 4A). To study the impact of BER-related genes in a functional manner, we transfected lentiviruses that expressed short hairpin RNA (shRNA) against each BER-related gene (Supplemental Fig. S4). Cell recovery showed a significant decrease in GS cell number after depletion of *Ogg1* (Fig. 4B). *Ogg1* is also one of the genes that were influenced by H₂O₂ (Supplemental Fig. S2). OGG1 is responsible for the removal of oxidized base. It recognizes the damaged base and excises it from the DNA strand. *Ogg1* can control transcription factor homing, induce allosteric transition of G-quadruplex structure, or recruit chromatin remodelers [41, 42]. Because ROS are constantly generated during GS

cell proliferation [10], these results raised a possibility that *Ogg1* is responsible for protecting GS cells from ROS damage.

Western blotting showed that OGG1 is expressed more strongly in GS cells than in other cell types (Fig. 4C). However, immunostaining of WT testis showed that OGG1 expression was not specific to spermatogonia (Fig. 4D). It was found not only in the nucleus, but also in the cytoplasm. The cytoplasmic localization of OGG1 may reflect its function in the mitochondrial DNA repair [42]. Compared to GFRA1⁺ undifferentiated spermatogonia, the number of cells expressing OGG1 slightly but significantly increased during differentiation of spermatogonia into spermatocytes, while its expression decreased significantly in peanut agglutinin (PNA)-reactive haploid cells. The increased expression of OGG1 in spermatocytes and round spermatids was previously noted and suggested that OGG1 is involved in progression of meiosis [43]. OGG1 is not specific to germ cells because Sertoli cells are also immunoreactive to the antibody.

We then tested the function of OGG1 on GS cells upon H₂O₂ treatment. We first used a comet assay to examine its function on DNA damage. Our analysis showed that *Ogg1* KD decreased the head/total DNA ratio, while overexpression (OE) of *Ogg1* resulted in the opposite effect, which suggests that *Ogg1* expression levels are closely correlated with the level of DNA damage in GS cells (Fig. 5A). In the second set of experiments, we tested the function of *Ogg1* KD or OE on GS cell proliferation in the presence of H₂O₂ (Fig. 5B). As expected, cell recovery after culture was significantly reduced by *Ogg1* KD (Fig. 5C). However, *Ogg1* OE did not improved survival of transfected cells.

To understand the mechanism of OGG1-mediated protection from ROS, we first analyzed whether *Ogg1* OE or KD might change the expression levels of genes involved in DNA repair. However, real-time PCR analysis did not show strong impact on their expression levels (Supplemental Fig. S5). We then performed immunostaining. Although OGG1 was predominantly found in the cytoplasm of GS cells under normal culture conditions, it moved to the nucleus upon H₂O₂ exposure (Fig. 5D), consistent with the previous observation that relocalization of OGG1 initiates DNA repair [37]. Taken together, these results suggest that *Ogg1* plays a critical role in preventing H₂O₂-induced DNA damage in GS cells.

Discussion

We investigated how SSCs protect their genome from ROS, which are essential for self-renewal division. ROS-mediated self-renewal in SSCs is counterintuitive because ROS induce DNA mutations and genetic instability. Our initial analysis revealed that GS cells are significantly more resistant to H₂O₂ than pluripotent stem cells or MEFs. We did not expect this result because ES and mGS cells are significantly resistant to irradiation than GS cells [17]. In that experiment, GS cells were arrested at the G1 phase of the cell cycle and many cells underwent rapid apoptosis, while ES and mGS cells did not show apparent changes. Indeed, γ H2AX staining persisted longer in GS cells, which suggested that DSB repair is relatively slower in GS cells [17]. This slow DSB repair was in sharp contrast to rapid recovery of DNA damage after H₂O₂ exposure.

We initially thought that GS cells and ES cells are similar in their DNA repair machinery because both cell types allow gene targeting by HR [44-46]. DSBs are predominantly repaired by the NHEJ pathway in higher eukaryotes [13], including MEFs and tissue-specific stem cells, such as hematopoietic stem cells (HSCs) and neural stem cells (NSCs) [1,47,48]. In this sense, ES or GS cells are unique that they can use HR for DSB repair. This may be why gene targeting is feasible in ES or GS cells. Because germ cells transmit genetic information to the next generation, it is reasonable that they preferentially use more precise HR to repair DNA damage to minimize replicative errors. However, because the HR efficiency was comparable between ES and GS cells, this activity alone probably does not explain the high survival rate of GS cells after H₂O₂ exposure.

We next checked BER activity because ROS also induce point mutations. Although male germline cells show modest nucleotide excision repair and DSB repair activities [49,50], they exhibit elevated levels of BER activity [51]. However, it has not been easy to test the BER activity of SSCs or undifferentiated spermatogonia because they comprise a small population in total testis germ cells. We overcame this problem using GS cells because it is possible to collect a large number of SSCs. However, because in vitro culture might change their property, we examined the mutation frequency of GS cells from Big Blue mouse, which were previously used to demonstrate low mutation frequency in freshly isolated spermatogonia [16]. This analysis revealed that GS cells from Big Blue mice still maintained a low mutation frequency. When compared to TTFs or MEFs, mutation frequencies were lower in fully established GS cells ($0.41\text{-}0.69 \times 10^{-6}$

⁵). These values were comparable to those found in THY1⁺ spermatogonia (0.59×10^{-5}), but were lower than those found in spermatogonia of 6-day-old pup testes (2.34×10^{-5}) or prospermatogonia in 15-day-old embryos (0.99×10^{-5}) in a previous study [16]. Although CDH1⁺ spermatogonia (1.93×10^{-5}) showed relatively high mutation frequencies, this was probably due to contamination by somatic cells, which is inevitable during MACS-mediated cell recovery. These results confirm previous observations of high BER activity in male germ cells and suggest that GS cells can be used to analyze factors involved in high BER activities.

Because ES cells also have a lower point mutation frequency than somatic cells [33], it was possible that difference in the BER activity explains the ROS resistance. To test this hypothesis directly, we examined the expression of BER-related genes in various cell types. As we expected, many BER-related genes are expressed in GS cells compared with other cell types. Through functional screening of BER-related genes, we eventually found that *Ogg1* depletion significantly impaired the survival of GS cells. *Ogg1* is a DNA glycosylase enzyme that is responsible for the excision of 8-OxoG [52]. We further confirmed the involvement of *Ogg1* in ROS protection against H₂O₂ (Fig. 5A). Although OGG1 was relatively widely expressed in the testis, its strong expression in GS cells and our functional analysis strongly suggest that OGG1 is responsible for ROS protection in GS cells.

While our results showed the importance of *Ogg1* in GS cells, *Ogg1* KO mice do not show apparent spermatogenic defects despite increased 8-OxoG formation in somatic tissues [53]. We think that culturing spermatogonia probably increases the

exposure to ROS because ROS are required for GS cell proliferation [9]. Because GS cells proliferate more actively than undifferentiated spermatogonia in vivo [4], GS cells probably depend more heavily on ROS than SSCs in vivo, which likely have less mutations. In this sense, it is probably not surprising that reproductive defects do occur in the descendants of triple compound *Ogg1/Mth1/Mutyh* mutants [54]. Because GS cells are exposed to high levels of ROS and they also divide faster than SSCs in vivo, it is likely that such conditions induce more mutations in the genome of GS cells. However, SSCs in vivo probably take longer periods to accumulate mutations. MTH1 degraded 8-OxoG in the nucleotide pool to prevent its incorporation into DNA, while MUTYH removed adenine misincorporated by replicative polymerases opposite the oxidized purine 8-OxoG. Interestingly, the triple KO mice were fertile but failed to produce progeny after eight generations. They exhibit an increased incidence of hydrocephaly and cancers, suggesting that these genes influence the frequency of de novo mutations in germ cells. Although the target cell type was not examined in that study, SSCs are most likely the best candidate cell because newly generated mutations in committed progenitors will disappear as they undergo only a limited number of cell divisions and complete spermatogenesis. SSCs are the only cells that can accumulate these mutations by self-renewal. Therefore, these results support our observation that BER enzymes, including OGG1, play critical roles in the protection of SSCs against ROS.

One of the important next questions is how OGG1 protects GS cells from ROS-induced damage. OGG1 translocated into the nucleus when high concentration of H₂O₂ was added to culture medium, which suggested that GS cells have a mechanism to

monitor and modulate ROS levels upon acute ROS-induced damages. Several anti-oxidant molecules reportedly protect spermatogonia from ROS. For example, spermatogonia express significantly high levels of NRF2 or SOD1 [55,56]. Because animals lacking these molecules are extremely sensitive to ROS, it is likely that they share a close relationship with OGG1. In fact, OGG1 is reportedly induced by NRF2 [57], and *Sod1* OE decreases mutation frequency in Big Blue mice [58]. Therefore, these molecules likely create a network of ROS defenses. The next important step is to clarify the relationships among these molecules, which will lead to a better understanding of the mechanism by which ROS allow self-renewal without damaging the genome of SSCs.

Our study shows that GS cells depend on OGG1 to protect their genome from ROS. Although we still do not know the long-term impact and phenotype of abnormal *Ogg1* expression, this can be examined using transplantation assay of Big Blue GS cells after overexpression or KD. Understanding the degree of DNA damage in SSCs is important for future clinical applications of SSCs for male infertility treatment [59], because increased mitosis during culture may give rise to undesired de novo mutations, which has been a concern for regenerative medicine [60]. Thus, the analysis of DNA mutation patterns and understanding the mechanism of BER activity in SSCs have important implications in not only understanding the mechanism of the self-renewal process, but also for understanding disease etiology and for developing future applications in medicine.

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Figure legends

Figure 1. Enhanced resistance of GS cells to ROS.

(A) Appearance of different types of cells cultured with H₂O₂. Cells were maintained for 6 days. Scale bar, 50 μ m.

(B) Survival rate of H₂O₂-treated cells. GS cells were cultured for 6 days, while the other cell types were recovered 3 days after culture initiation because they proliferate more quickly (mean $\pm$ SEM, n = 6). Results of three experiments. Significantly more GS cells survived compared to other cell types. *P < 0.05.

(C) TUNEL staining. GS cells were analyzed 6 days after culture, whereas the rest of the cells were analyzed at 3 days. At least 150 cells were counted. Results of three experiments. Scale bar, 50 μ m. Counterstained by Hoechst 33342. *P < 0.05.

(D) Comet assay. Each cell type was exposed to different doses of H₂O₂ for 30 min and then incubated in normal media for indicated periods. The cells were then electroporated and stained with ethidium bromide. The head (round shape) corresponds to undamaged DNA while the tail (smear) corresponds to damaged DNA. At least 40 cells were picked up and the intensity ratio of head over total DNA was quantified. Results of two experiments. This experiment was replicated three times. *P < 0.05.

Figure 2. Repair of DSBs in GS cells.

(A) Real-time PCR analyses of genes involved in DSB repair (mean $\pm$ SEM, n = 4).

(B) Experimental scheme of NHEJ assay using pJH200 substrate vector and *Rag1/2* expressing plasmids. pJH200 and *Rag1/2* were cotransfected (i). RAG1/2 cut both sides of the stop codon (ii). NHEJ machinery facilitates joining of the blunt ends and the

construct was introduced into *E.Coli* (iii). Colonies were counted whose number represents the efficacy of successful NHEJ (iv).

(C) Quantification of colonies (mean $\pm$ SEM, n = 5). NHEJ ratio was measured by dividing the colony numbers on Amp⁺/Cm⁺ plates by those on Amp⁺ plates. The number on Amp⁺ plates reflects transfection efficiency. Results of five experiments. *P < 0.05.

(D) Experimental scheme of the HR assay using the pHPRT-DRGFP plasmid. DRGFP contains two incomplete GFP sequences (i). DRGFP, *DsRed*, and *Sce-I* were transfected into GS and ES cells. *SCE-I* cut the target sequence in the GFP sequence and DsRed was used as a control for measuring transfection efficiency (ii). Recombination occurred between the two incomplete GFP sequences (iii). The resultant GFP had a complete sequence for emitting fluorescence (iv).

(E) Flow cytometric analyses of transfected cells (mean $\pm$ SEM, n = 5). Results of two experiments. Y-axis indicates the ratio of GFP⁺ DsRed⁺ population over DsRed⁺ population. Successful recombination of pHPRT-DRGFP yields green fluorescence and DsRed is used to estimate transfection efficiency.

Figure 3. Big Blue assay of GS cells.

(A) Experimental scheme of Big Blue assay. Each cell type was collected from Big Blue mice carrying the *LacI* transgene (i). Genomic DNA was extracted from Big Blue cells (ii), and packaged into a lambda phage (iii), which infected *E. coli* (iv). The ratio of blue and white plaque numbers was determined (v).

(B) Mutation frequency. Plaque forming unit (Pfu) stands for the total number of plaques counted. The *LacI* transgene from blue plaque was sequenced to confirm the independency of each mutation. For 2.5M-GS cells, one of the 8 mutant plaques showed two different mutations, and 9 independent mutations were found via sequencing. Since the sequence change was different for each of these mutations, we counted them as 2 unique, independent mutations in this case. NA, not applicable because cells were not cultured.

Figure 4. Impaired GS cell proliferation by *Ogg1* depletion.

(A) Real-time PCR analyses of BER-related genes (mean $\pm$ SEM, n = 4). Results of two experiments. *P < 0.05.

(B) Cell recovery after transfection of shRNA against indicated genes 6 days after transfection (mean $\pm$ SEM, n = 6). Results of two experiments. *P < 0.05.

(C) Western blotting analyses of OGG1 in GS cells (mean $\pm$ SEM, n = 6). Results of three experiments. *P < 0.05.

(D) Immunostaining of OGG1 and spermatogenic markers. Cells in 35 tubules were counted. Scale bar, 20 μ m. Counterstained by Hoechst 33342. *P < 0.05.

Figure 5. Impact of *Ogg1* on H₂O₂ resistance of GS cells.

(A) Comet assay. GS cells were transfected with shRNA or cDNA and analyzed 2 days after transfection. Cells were then exposed to H₂O₂ (500 μ M) for 30 min followed by

753 recovery at indicated time points. At least 30 cells were counted for each cell type (mean
754 $\pm$ SEM). Results of two experiments. *P < 0.05.

755 (B) Experimental scheme of H₂O₂ resistance analyses. GS cells were transfected with
756 shRNA, and half of the transfected cells were exposed to H₂O₂ (200 μ M). The ratio of
757 cells that survived [cells with H₂O₂ (a)/cells without H₂O₂ (b)] was determined 6 days
758 after transfection.

759 (C) Survival of GS cells after *Ogg1* OE (n = 7)/KD (n = 6)(mean $\pm$ SEM). The ratio of
760 cells that survived [cells with H₂O₂ (a)/cells without H₂O₂ (b)] in (B) was determined in
761 at least 2 independent experiments 6 days after transfection.

762 (D) Immunostaining of OGG1 in GS cells 1 day after H₂O₂ exposure (mean $\pm$ SEM, n =
763 24). Results of two experiments. BF, bright field. Scale bar, 10 μ m. Counterstained by
764 Hoechst 33342. *P < 0.05.

Figure 1

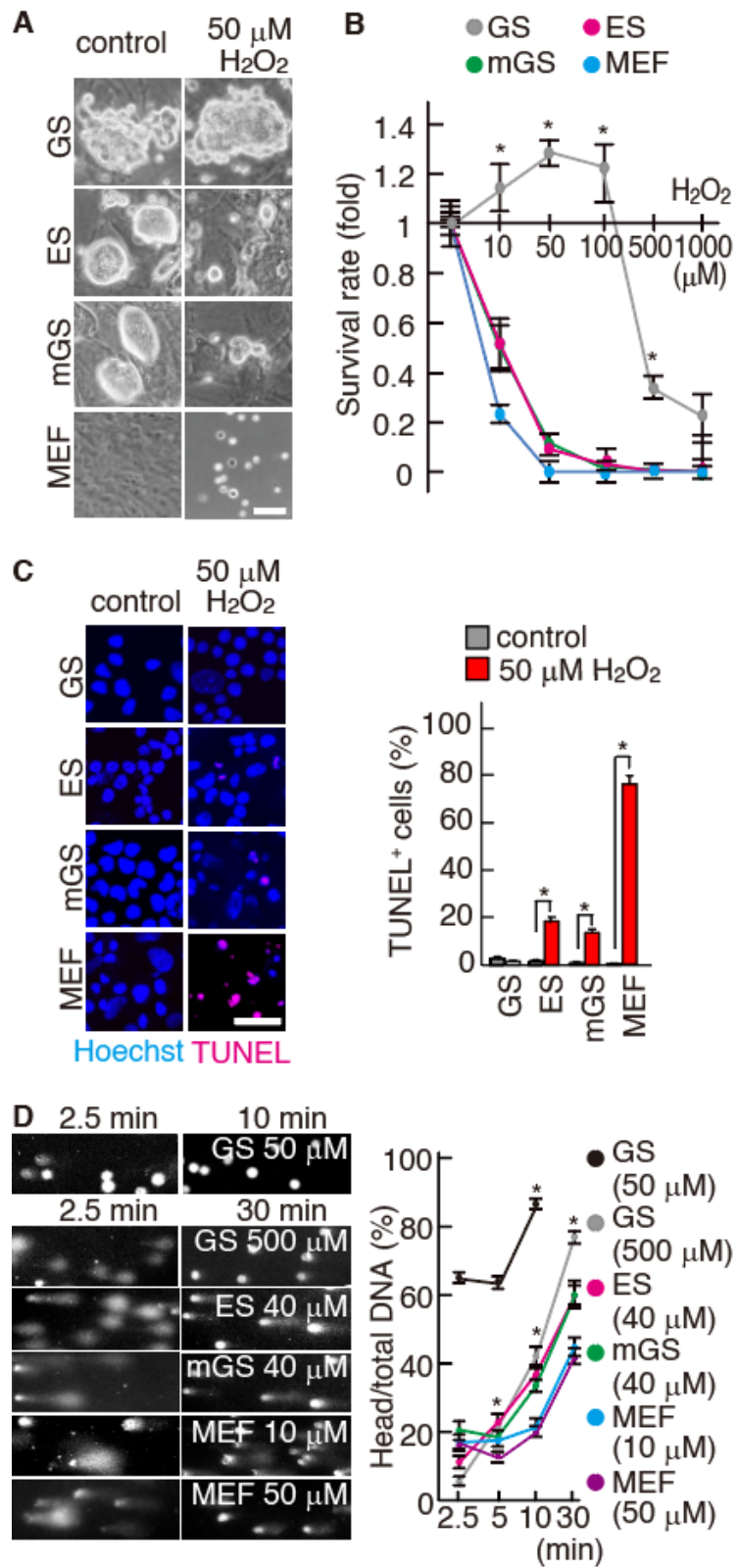


Figure 2

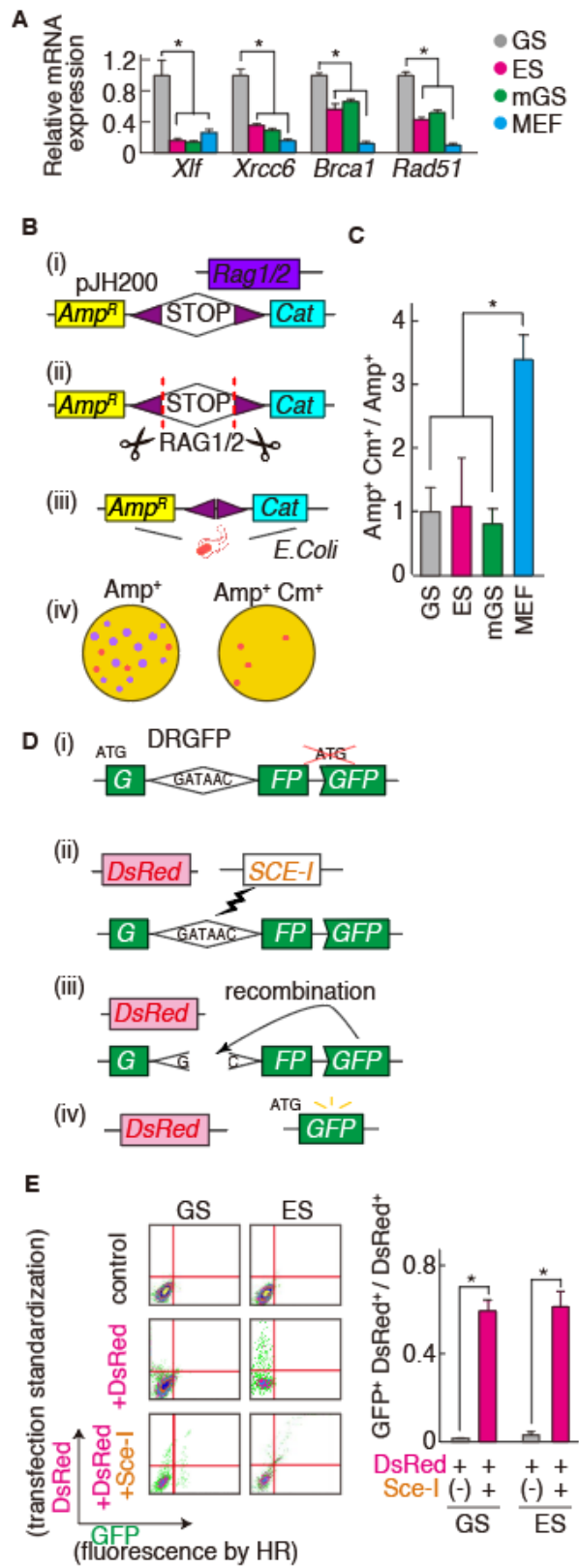
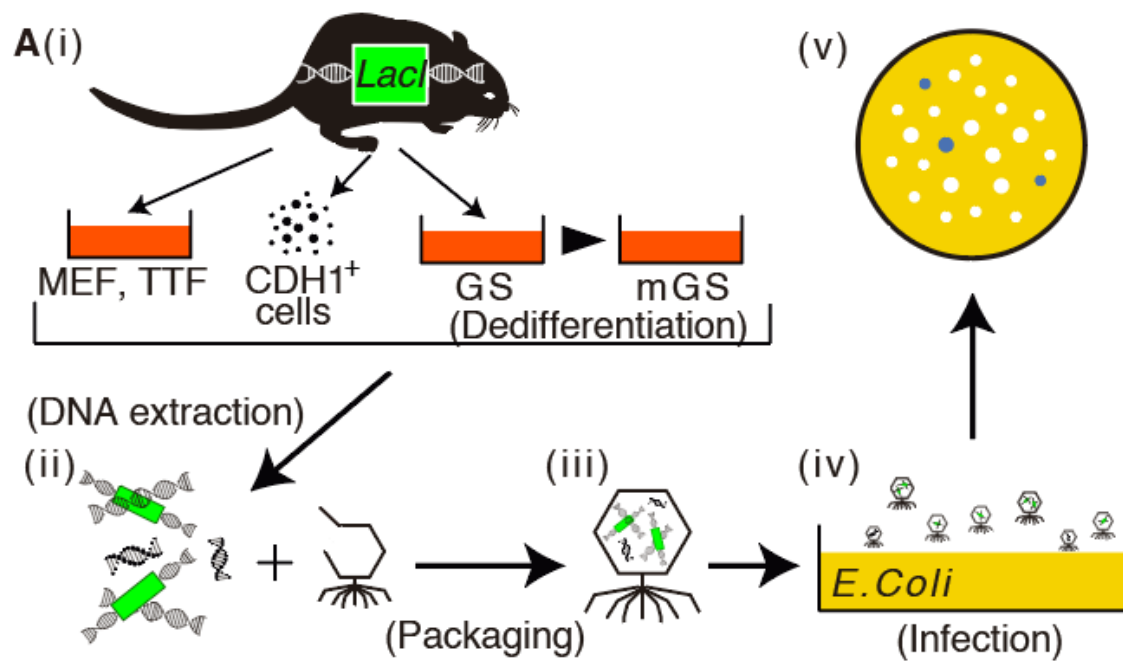


Figure 3



B

Cell-type	Doubling time (h)	Pfu number	Mutation number (before sequencing)	Independent mutation	Mutation frequency (x10 ⁻⁵)
GS (8 mo)	75	1,213,368	5	5	0.41
GS (31 mo)	60	726,680	5	5	0.69
CDH1 ⁺ (9±1 mo)	NA	363,052	7	7	1.93
mGS	23	362,943	20	6	1.65
MEF	30	136,682	11	10	7.32
TTF	132	51,471	10	10	19.43

Figure 4

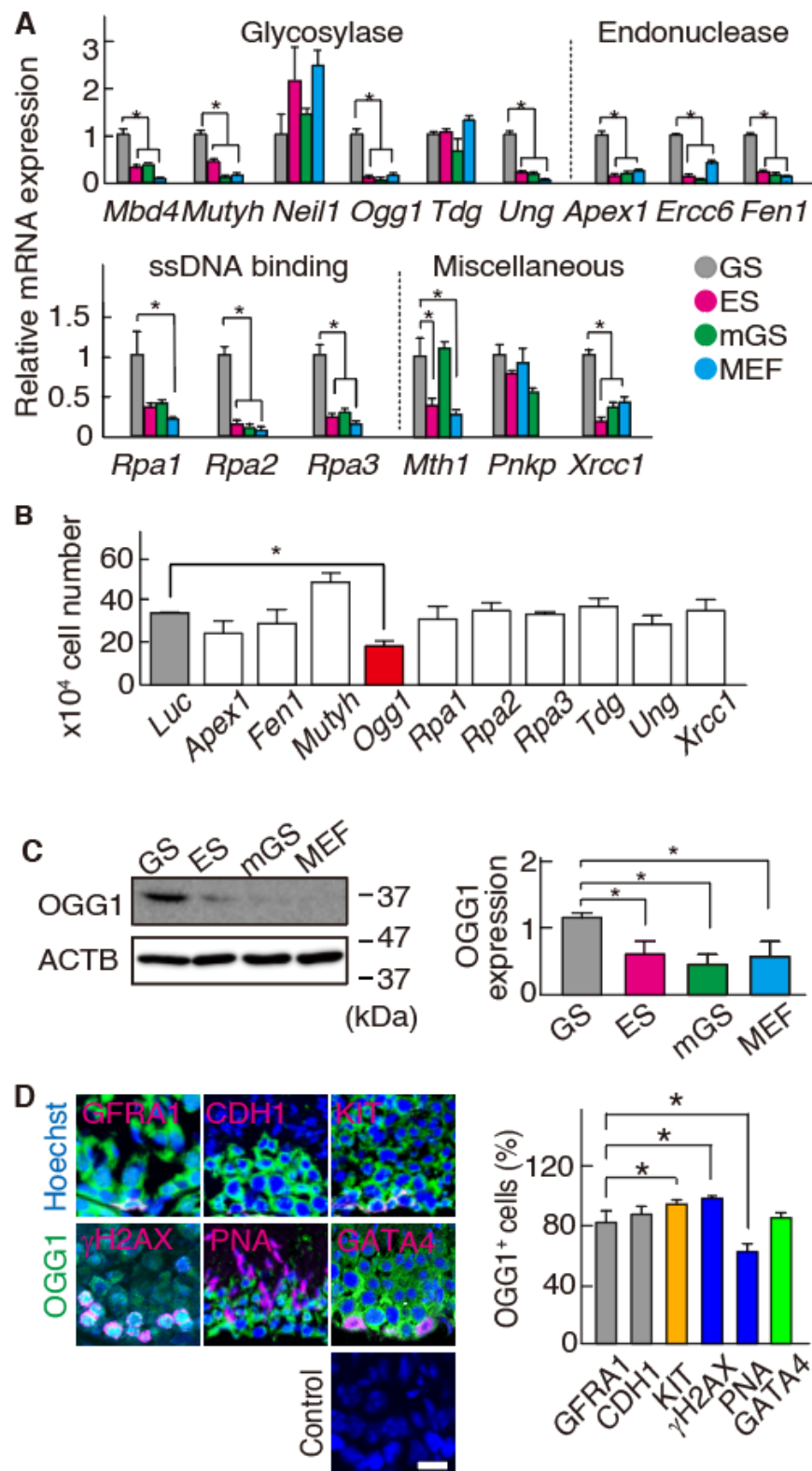


Figure 5

