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RESEARCH ARTICLE

8-Hydroxyguanine levels and repair capacity during mouse embryonic stem cell differentiation

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Abstracts

To evaluate the defence capacities of embryonic stem (ES) cells against gene impairment, this study measured the levels of 8-hydroxyguanine (8-OH-Gua), a well-known marker of oxidative stress in DNA, and its repair capacity during differentiation. Undifferentiated ES cells (EB3) were cultured without leukaemia inhibitory factor (LIF) for 0, 4 and 7 days and are referred to as ES-D0, ES-D4 and ES-D7, respectively. These three cell lines were treated with 300 μM hydrogen peroxide (H_2O_2) for 48 and 72 h. After treatment, the amounts of 8-OH-Gua in the cells were determined by the high-performance liquid chromatography (HPLC)-electrochemical detector (ECD) method. The levels of 8-OH-Gua in ES-D7 treated with H_2O_2 were higher than those in ES-D0 and ES-D4, suggesting that the DNA in the undifferentiated cells was protected against gene impairment, as compared to that in the differentiated cells. To examine the repair capacity for 8-OH-Gua, this study analysed the expression of 8-OH-Gua repair-associated genes, *8-oxoguanine DNA glycosylase 1* (*OGG1*), *MutY homolog* (*MUTYH*) and *Mut T homolog 1* (*MTH1*), in ES-D0, ES-D4 and ES-D7. The mRNA levels of *MUTYH* and *MTH1* showed no significant change, whereas *OGG1* mRNA was significantly decreased in ES-D7 treated with H_2O_2. Moreover, it was observed that ES-D7 treated with H_2O_2 readily underwent apoptosis, in comparison to its undifferentiated counterparts, ES-D0 and ES-D4. Taken together, ES cells are more resistant to DNA oxidative stresses than differentiated cells.

Keywords: *DNA damage, DNA repair, hydrogen peroxide, 8-oxoguanine DNA glycosylase 1, reactive oxygen species*

Introduction

In recent years, extensive progress has been made in regenerative medicine, including the establishment of induced pluripotent stem (iPS) cells [1]. Although they seem to lack some of the negative issues associated with the use of embryonic stem (ES) cells for regenerative medicine, such as allogeneic immune rejection and the ethical problems of using human embryos for establishing ES cell lines, they still have several problems. The most notable problem is the genomic instability leading to cancer stem cell generation [2–4]. In this context, the avoidance of DNA damage must be a significant step for the safe use of stem cells for regenerative medicine.

Reactive oxygen species (ROS) are believed to play a crucial role in carcinogenesis, by generating oxidative DNA damage. Some forms of oxidative DNA damage have been recognized as pre-mutagenic base modifications, because they induce mutations in genomic DNA [5–7]. In the case of the differentiation of ES cells, DNA damage generation should be a critical event, because DNA damage-induced mutations may result in catastrophic changes that would affect many different cell types in the organism.

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Therefore, it is reasonable to predict that ES cells should be equipped with highly efficient defense mechanisms against oxidative stresses and DNA damage.

8-Hydroxyguanine (8-OH-Gua) is the most abundant form of DNA damage generated by ROS and is believed to play an important role in carcinogenesis [8,9]. Since 8-OH-Gua causes GC to TA transversion type point mutations in DNA [10–12], which are often detected in various cancer cells, it is important to determine the levels of 8-OH-Gua in nuclear DNA when exploring the molecular mechanisms of carcinogenesis.

Several genes encoding antioxidant enzymes, such as thioredoxin-glutathione reductase, glutathione peroxidases 2, 3 and 4 and glutathione-S-transferase, reportedly became down-regulated during the differentiation of mouse ES cells into embryoid bodies [13]. As for the 8-OH-Gua generation, the steady state levels of 8-OH-Gua were lower in human ES cells than those in differentiated human primary fibroblasts, WI-38 [14]. These studies suggested that the defense capacities for genomic integrity were higher in ES cells than in differentiated cells. In fact, Cervantes et al. [15] reported that the mutation frequency in ES cells was significantly lower than that in mouse embryonic fibroblasts.

On the other hand, to prevent such mutations, several repair systems are ubiquitously present, from bacteria to human [16,17]. To prevent point mutations due to the generation of 8-OH-Gua, many organisms possess a three-component enzymatic system (termed the 'GO system'), which reduces mutagenesis by 8-OH-Gua and possibly other oxidized purines [18,19]. The human GO system consists of three enzymes, 8-oxoguanine DNA glycosylase 1 (OGG1), MutY homolog (MUTYH) and Mut T homolog 1 (MTH1). OGG1 is a major repair enzyme that removes 8-OH-Gua from DNA [20]. MUTYH removes adenine from Adenine:8-OH-Gua pairs, which only occur in DNA after misincorporation events. MTH1 prevents the incorporation of 8-OH-Gua into DNA from the pool of oxidized dGTP.

In this study, we assessed the defense capacities of mouse ES cells against oxidative DNA damage during differentiation, by measuring the levels of 8-OH-Gua and its repair capacity. Our study provides valuable information for the use of stem cells for regenerative medicine in the future.

Materials and methods

Culture of mouse ES cells and hydrogen peroxide treatment

The EB3 cells (undifferentiated ES cells established in RIKEN, Kobe, Japan) were a kind gift from Dr Hitoshi Niwa, CDB of RIKEN, and were maintained on gelatin-coated dishes in Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) supplemented with 10% foetal bovine serum (FBS, GIBCO-BRL, Grand Island, NY), 0.1 mM 2-mercaptoethanol (Sigma), 0.1 mM non-essential amino acids (GIBCO-BRL), 1 mM sodium pyruvate (Sigma) and 1,000 U/ml of leukaemia inhibitory factor (LIF, GIBCO-BRL). We used gelatin-coated dishes without feeder cells for all cell cultures in this study. After 24 h of sub-culture, 5×10^3 cells were cultured for 4 and 7 days without LIF. These cells are referred to as ES-D4 and ES-D7, respectively, and the undifferentiated ES cells are referred to as ES-D0. The differentiation stages of these cells were examined by analysing *Oct 3/4* mRNA expression by the RT-PCR method. These three cell lines (ES-D0, ES-D4 and ES-D7) were inoculated at the beginning of a 24 h period at 5×10^5 cell/mL, as a sub-culture. After the 24 h sub-culture, 300 μM hydrogen peroxide (H_2O_2, Nacalai Tesque, Inc., Kyoto, Japan) or the same volume of sterile water as a control were added to the medium. The H_2O_2 solutions were freshly prepared from a 30% stock solution. LIF (final conc: 1000 units/mL) was added to only ES-D0. After the indicated periods of cultivation, the cells were harvested, washed with sterile PBS and subjected to analyses.

Determination of 8-hydroxyguanine in DNA

The assay for measuring the 8-OH-Gua levels was described previously [21]. Briefly, the DNA from the cells was isolated by the sodium iodide method, using a DNA Extraction WB Kit (Wako, Japan). For homogenization, a lysis solution containing 1 mM desferal (deferoxamine mesylate, Sigma) was used. The isolated DNA was digested with nuclease P1 (Yamasa, Choshi, Japan) to obtain a deoxynucleoside mixture. The solution was clarified with an Ultrafree-Probind filter (Millipore, Bedford, MA) and was injected into a high-performance liquid chromatography (HPLC) column (Capcell Pak C18 MG, Shiseido, Tokyo, Japan) equipped with an electrochemical detector (ECD) (Coulochem II, ESA, Chelmsford, MA). The 8-OH-Gua value in the DNA was calculated as the number of 8-OH-dG per 10^5 deoxyguanosine (dG).

Conventional RT-PCR

To examine the expression of cell differentiation associated genes, we performed PCR with reverse transcription (RT-PCR) assays specific for the mouse *Oct 3/4* mRNA. mRNA was isolated using an mRNA isolation kit (GE Healthcare) and the first strand of cDNA was synthesized from the isolated mRNA. Furthermore, we examined the expression of mouse *OGG1*, mouse *MUTYH* and mouse *MTH1* mRNA to study the 8-OH-Gua repair capacity of the cells.

Table I. Primers for RT-PCR.

Gene name	Sequence	Product size (bps)
Oct 3/4	5′-AGCTGCTGAAGCAGAAGAGG-3′(F)	468
	5′-CCTGGGAAAGGTGTCCTGTA-3′(R)	
OGG1	5′-ATCTGTTCCTCCAACAACAAC-3′(F)	504
	5′-GCCAGCATAAGGTCCCCACAG-3′(R)	
MUTYH	5′-GGCCTCTGTCTCCCCATATCAT-3′(F)	397
	5’-CTGCTGTAGGGTCTCTGCTGTA-3′(R)	
MTH1	5′-GTCTGAGCGTGGATACACTGCA-3′(F)	341
	5’-TGTGTAGCATCCTGAGTGGCCA-3′(R)	
GAPDH	5′-AACGGGAAGCTCACTGGCATG-3′(F)	304
	5′-TCCACCACCCTGTTGCTGTAG-3′(R)	

(F): Forward; (R): Reverse.

GAPDH mRNA was employed as an internal standard. The primers used in this experiment are listed in Table I. The RT-PCR products were separated on a 2.0% agarose gel and were visualized by ethidium bromide staining.

Apoptosis analyses: Annexin V-Cy3.18 (AnnCy3) immunofluorescent method

ES-D0 and ES-D7 cells were seeded at 5×10^4 cells/mL and allowed to adhere overnight. After a treatment with 300 μM H_2O_2 for 72 h, the cells were harvested. To determine whether apoptosis was induced in the collected cells, the annexin V-Cy3.18 (AnnCy3) immunofluorescent method was performed. To label the cells with AnnCy3, an AnnCy3 apoptosis detection kit (Sigma) was used. The cells were examined by fluorescence microscopy (BX50; OLYMPUS, Tokyo, Japan). The images facilitated the differentiation between the early apoptotic cells (AnnCy3 positive + 6-carboxyfluorescein diacetate (6-CFDA) positive), necrotic cells (unstained or AnnCy3 positive + 6-CFDA negative) and viable cells (AnnCy3 negative + 6-CFDA positive). These methods were performed according to the manufacturers' instructions.

Statistical analysis

The values in the graphs represent the means ± SD. Statistical analysis was performed using the unpaired Student's *t*-test and an analysis of variance (ANOVA) followed by the Tukey test. All data were analysed by using the SPSS software, version 11.0 (SPSS Japan Inc., Tokyo, Japan). Probability values less than 0.05 were considered to indicate significant differences.

Results

Cell differentiation

Our cell morphology study confirmed that ES-D0, but not ES-D4 or ES-D7, maintains the morphological characteristics of an ES cell, such as a colony formation (Figure 1A). To monitor the cell differentiation at a molecular level, the expression of *Oct 3/4* mRNA was analysed. We observed that ES-D0 and

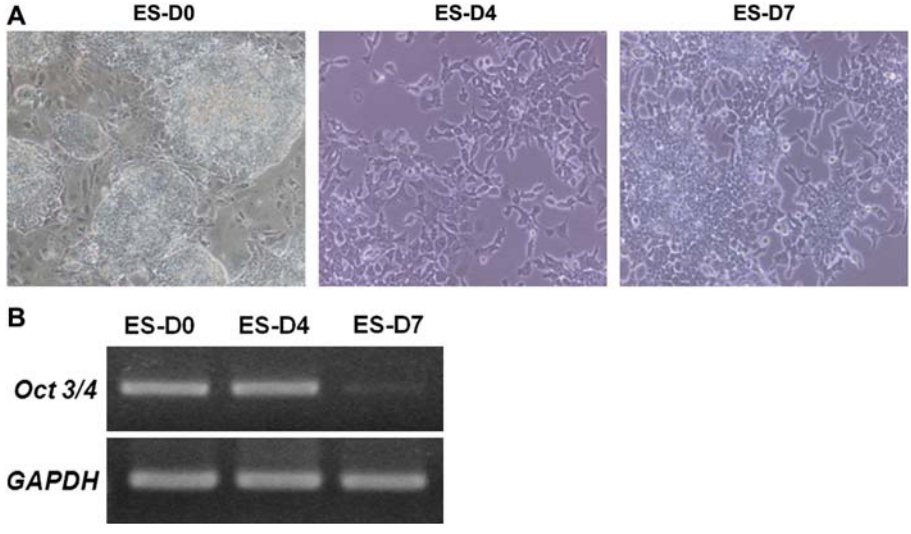

Figure 1. (A) Cell morphology was examined by microscopy with a photography system. ES-D0 cells were observed to form colonies (left), while the ES-D4 (middle) and ES-D7 (right) did not form colonies. (B) *Oct 3/4* mRNA expression. *Oct 3/4* mRNA was expressed in ES-D0 and ES-D4, but not in ES-D7.

ES-D4 expressed *Oct 3/4* mRNA, while ES-D7 did not (Figure 1B). Since Oct *3/4* is a transcriptional regulator required to maintain the totipotentiality of ES cells [22–25], ES-D0 and ES-D4 may maintain the characteristics of the stem cell, while ES-D7 was differentiated and lost some of the characteristics of the stem cell.

The level of 8-OH-Gua generation and the expression of mouse 8-OH-Gua repair associated genes

The 8-OH-Gua level was analysed by using HPLC coupled with ECD. The 8-OH-Gua levels in the DNA of ES-D7 treated with 300 μM H_2O_2 for 72 h were significantly higher than those of ES-D0 and ES-D4 treated with 300 μM H_2O_2 for 72 h (Figure 2A, ANOVA followed by the Tukey test). These results suggested that oxidative DNA damage was generated more easily in ES-D7 than in undifferentiated cells. On the other hand, no significant differences were detected among any groups in the expression of *mOGG1* mRNA (Figure 2B, ANOVA followed by the Tukey test). However, the level of *mOGG1* mRNA expression was lower in ES-D7 treated with 300 μM H_2O_2 for 72 h than that in ES-D7 without H_2O_2 treatment (Student's *t*-test). As for the other 8-OH-Gua repair-associated genes, there were no significant differences between any cell lines (data not shown).

Apoptosis

The AnnCy3 immunofluorescent method was performed to analyse the type of cell death. More than half of the ES-D7 cells treated with 300 μM H_2O_2 for 72 h were stained with both AnnCy3 (red) and 6-CF (green), which appeared as yellow fluorescence (Figure 3). On the other hand, almost all of the other cells with/without H_2O_2 were stained with 6-CF (green) alone. These results suggested that the 300 μM H_2O_2-treated ES-D7 cells were in the early stage of apoptosis.

Discussion

To examine the defence capacity of stem cells against genomic impairment, we analysed the generation of 8-OH-Gua and the expression of *OGG1* mRNA, *MUTYH* mRNA and *MTH1* mRNA of mouse ES cells at three stages (0, 4th and 7th day) of differentiation. Before starting the experiment, we predicted that the defence systems for DNA would be more effective in ES cells than in differentiated cells, because mutations in ES cells could result in catastrophic changes that could cause disorder in many differentiated cells in the organism. In this study, we employed 300 μM H_2O_2 as an oxidative stressor.

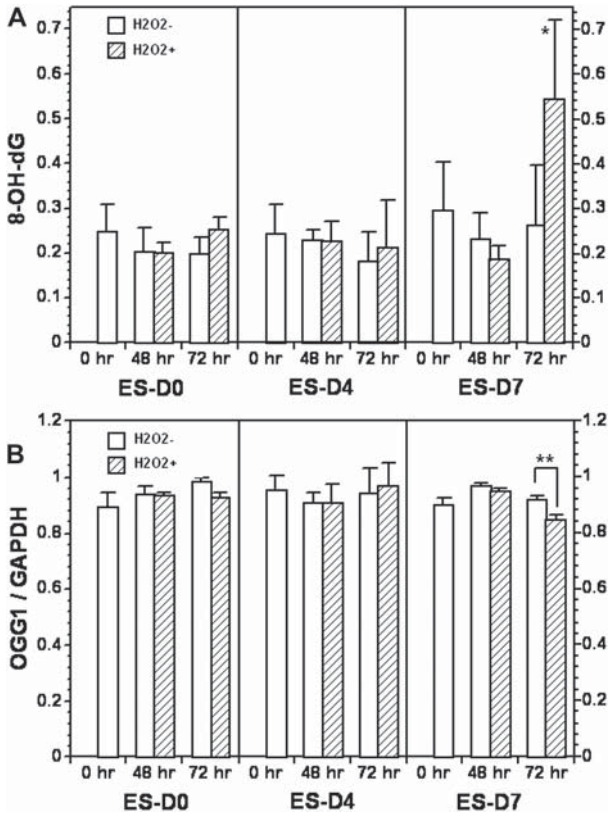

Figure 2. (A) The levels of 8-OH-Gua generated in the DNA of ES cells. The 8-OH-Gua levels are expressed as the number of 8-hydroxydeoxyguanosine (8-OH-dG) per 10^5 deoxyguanosine (dG). (B) *Mouse OGG1* mRNA expression. The expression of *mOGG1* mRNA was lower in ES-D7 treated with 300 μM H_2O_2 for 72 h, as compared to other cells with/without H_2O_2 treatment. The *mOGG1* expression levels were standardized with the *GAPDH* expression levels. Statistical analysis was performed using analyses of variance (ANOVA) followed by the Tukey test and the unpaired Student's *t*-test. $^*p < 0.001$ vs ES-D0 and ES-D4 treated with 300 μM H_2O_2 for 72 h (ANOVA), $p = 0.005$ vs ES-D0 treated with 300 μM H_2O_2 for 72 h and $p = 0.02$ vs ES-D4 treated with 300 μM H_2O_2 for 72 h (Tukey test), $^{**}p < 0.05$: ES-D7 (72 h with H_2O_2) vs ES-D7 (72 h without H_2O_2) (Student's *t*-test).

This concentration of H_2O_2 is relatively high, but no significant differences in the viabilities between cells with and without 300 μM H_2O_2 treatment were observed during 72 h (data not shown). The assay of the differentiation marker revealed that the 4-day-differentiated cells (ES-D4) maintained the features of undifferentiated ES cells (ES-D0) because they expressed *Oct 3/4* mRNA, a pluripotency marker, while ES-D4 lost the morphological features of ES cells and could not form colonies (Figure 1). In contrast, ES-D7 did not express *Oct 3/4* mRNA and also lost the morphological features of the ES cells. These results suggested that ES-D4 kept some of the features of the ES cells, but ES-D7 had lost most of them.

Since the generation of 8-OH-Gua is considered to be involved in carcinogenesis, by the induction of point mutations [10–12], the measurement of 8-OH-Gua levels would be useful to evaluate the defence capacities of ES cells. In this study, our observations

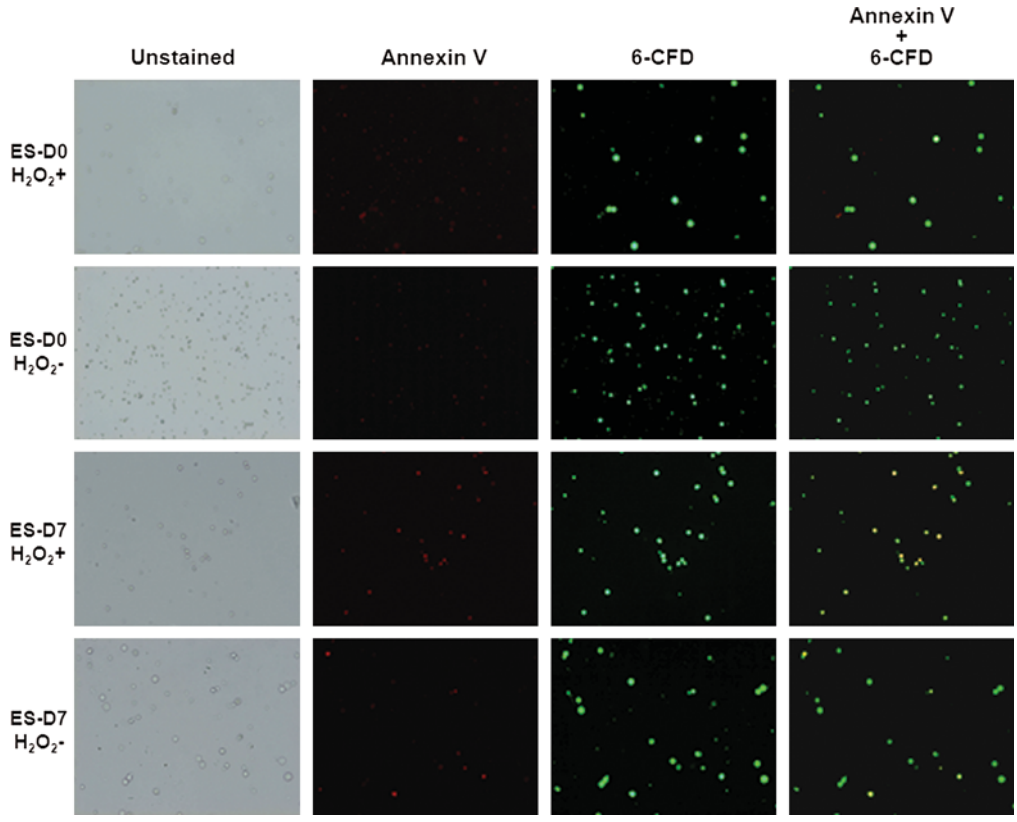

Figure 3. Cells treated with 300 μM H_2O_2 for 72 h were examined by the AnnCy3 immunofluorescent method. When cells are incubated with both AnnCy3 and 6-CFDA, living cells and necrotic cells are labelled with 6-CF (green) and AnnCy3 (red), respectively. Cells in the early stage of apoptosis will be labelled with both AnnCy3 (red) and 6-CF (green). Staining with both AnnCy3 (red) and 6-CF (green), which appeared as yellow fluorescence, was frequently observed in ES-D7 cells treated with 300 μM H_2O_2. ES-D0 treated with 300 μM H_2O_2 showed only living cells.

indicated that the 8-OH-Gua levels in the DNA of ES-D7 treated with 300 μM H_2O_2 for 72 h were significantly higher than those in the other cells with/without H_2O_2 treatment (ANOVA followed by the Tukey test). Maynard et al. [14] previously reported a similar observation, in which the steady state levels of 8-OH-Gua in ES cells were lower than those in differentiated cells. Both our present results and the report by Maynard et al. suggested that ES cells were resistant to the generation of oxidative DNA damage, in comparison to differentiated cells. However, since the measured levels of 8-OH-Gua depend on the balance between generation and repair [19], the repair capacities for 8-OH-Gua must also be assessed to evaluate the defence capacities against gene impairment.

In this context, we analysed the repair capacities for 8-OH-Gua by measuring the *mOGG1* mRNA expression levels of ES-D0, ES-D4 and ES-D7 and could not find any significant differences (ANOVA followed by the Tukey test). However, we found that the *mOGG1* mRNA expression in ES-D7 treated with H_2O_2 was lower than that in cells without H_2O_2 treatment (Student's *t*-test). Therefore, at least under these experimental conditions, the 300 μM H_2O_2 treatment impaired the 8-OH-Gua repair systems. We previously found that various chemical agents, such as cadmium chloride [26], etoposide [27] and arsenic compounds [28], inhibited the 8-OH-Gua repair systems, including the OGG1 activity and *OGG1* mRNA expression. Based on these studies, the finding that 300 μM H_2O_2 also inhibits 8-OH-Gua repair systems in differentiated cells is reasonable. Therefore, the increase in the 8-OH-Gua level in ES-D7 may be partly due to the H_2O_2-induced depletion of the 8-OH-Gua repair capacity. However, Maynard et al. [14] reported different observations, in which H_2O_2 enhanced the OGG1 activity in ES cells over that in differentiated cells within 3 h after 100 μM H_2O_2 exposure. It is difficult to explain why our observations were different from theirs. However, our experiment was performed at 48 and 72 h after exposure to 300 μM H_2O_2. Therefore, the increase in *OGG1* mRNA expression induced by H_2O_2 might be related to the concentration of H_2O_2 or the period of cell culture. We did not observe any significant changes in other 8-OH-Gua repair-associated genes in this experiment. Taken together with the measurements of 8-OH-Gua levels and the *mOGG1* mRNA expression, although other 8-OH-Gua repair associated genes (*MUTYH* and *MTH1*) did not change during cell differentiation, the defence capacities against oxidative DNA damage in differentiated cells could be lower than those in ES cells.

In addition, we analysed the types of cell death of ES-D0 and ES-D7 treated with 300 μM H_2O_2, because the accumulation of DNA damage is known to trigger apoptosis, to eliminate the damaged cells. Our previous observations indicated that the accumulation of 8-OH-Gua might lead to the induction of apoptosis [29]. In this study, more than half of the 300 μM H_2O_2 (72 h)-treated ES-D7 cells were stained with both AnnCy3 (red) and 6-CF (green), which appeared as yellow fluorescence (Figure 3), suggesting that these cells were in the early stage of apoptosis. On the other hand, almost all of the other cells with/without H_2O_2 treatment were stained with 6-CF (green) alone, which indicated that they were alive. Therefore, it is likely that the undifferentiated ES cells are more resistant to apoptosis than the differentiated cells under the conditions with 300 μM H_2O_2. The apoptosis might be induced, at least in part, by the generation of 8-OH-Gua, due to the decrease in the 8-OH-Gua repair capacity. However, the opposite observation was reported by Tichy and Stambrook [30], who suggested that ES cells were hypersensitive to DNA damaging agents and readily undergo apoptosis to eliminate damaged cells from the population. The mechanisms that determine the cell's destiny, alive or dead, should be clarified in the future.

Conclusions

We found that the defence capacities against 8-OH-Gua were higher in ES cells than in differentiated cells, suggesting that as the cells become differentiated, the mutation rates would increase. Therefore, careful attention should be paid to the possibility of oxidative DNA damage when undifferentiated stem cells, such as ES cells and tissue stem cells, are differentiated into target mature cells for use in regenerative medicine.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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