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# Implication of E3 ligase RAD18 in UV-induced mutagenesis in human induced pluripotent stem cells and neuronal progenitor cells

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## ABSTRACT

Pluripotent stem cells (PSCs) have the potential to differentiate to any of the other organs. The genome DNA integrity of PSCs is maintained by a high level of transcription for a number of genes involved in DNA repair, cell cycle and apoptosis. However, it remains unclear how high the frequency of genetic mutation is and how these DNA repair factors function in PSCs. In this study, we employed Sup F assay for the measurement of mutation frequency after UV-C irradiation in induced pluripotent stem cells (iPSCs) as PSC models and neural progenitor cells (NPCs) were derived from iPSCs as differentiated cells. iPSCs and NPCs exhibited a lower mutation frequency compared with the original skin fibroblasts. In RNA-seq analysis, iPSCs and NPCs showed a high expression of RAD18, which is involved in trans-lesion synthesis (TLS) for the emergency tolerance system during the replication process of DNA. Although RAD18 is involved in both error free and error prone TLS in somatic cells, it still remains unknown the function of RAD18 in PSCs. In this study we depleted of the RAD18 by siRNA knockdown resulted in decreased frequency of mutation in iPSCs and NPCs. Our results will provide information on the genome maintenance machinery in PSCs.

**Keywords:** induced pluripotent stem cells (iPSCs); mutation frequency; RAD18; UV exposure

## INTRODUCTION

Pluripotent stem cells (PSCs), including embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have the potential to differentiate to all other organs and are promising for use in regenerative medicine. PSCs exhibit high metabolic activity, which leads to the production of a high amount of reactive oxygen species (ROS), which is the major source of spontaneous DNA damage. ROS cause several types of DNA damage, including base damage and DNA single and double strand breaks which result in genetic mutation and aneuploidy [1]. Since accumulation of genetic mutation causes tumorigenesis, there is a concern on PSCs for regarding their application in medicine [2, 3]. To prevent genome instability, PSCs upregulate the expression of a number of genes involved in genome maintenance, such as DNA repair, DNA replication, the cell cycle checkpoint and apoptosis [4–9]. In PSCs, a highly activated DNA repair pathway can efficiently remove DNA damage, and cells with potential carcinogenic DNA damage are removed *via* apoptosis [1, 10, 11].

PSCs also exhibit high proliferative activity suggesting promotion of DNA replication [12], which is orchestrated by DNA polymerases and processing factors. Since damaged base causes a stack of progressive replication, the trans-lesion synthesis (TLS) pathway bypasses the stacked strand. Although the TLS pathway is important for bypassing the deleterious replication stack, it precedes improper DNA repair leading to genetic mutation. Because of the highly proliferative

activity in PSCs, it is confronted with the risk of TLS-dependent genetic mutation. However, it is largely unknown how PSCs prevent TLS-dependent mutation from occurring.

RAD18 is E3 ubiquitin ligase that mono-ubiquitinates PCNA to promote the TLS pathway [13, 14]. RAD18 expression levels fluctuate through the cell cycle and peaking at S phase [15]. RAD18 is conserved from bacteria through humans [16, 17] and interacts with several DNA repair factors such as NBS1 to promote TLS-associated DNA repair [18, 19]. The mutation of RAD18 in yeasts resulted in hypersensitivity to the genotoxic stress. RAD18 is involved in both error free and error prone TLS in somatic cells. The gene knockout of RAD18 in mouse ESCs resulted in the enhancement of sensitivity to the genotoxic agents and a decreased mutation frequency [13]. These results indicate that the RAD18-dependent TLS pathway is involved in genome stability and regulates the frequency of the occurrence of mutation. However, the role of RAD18 in human PSCs still remains unknown. Previously, we analyzed the gene expression change during reprogramming and differentiation in human skin fibroblasts, iPSCs and neural progenitor cells (NPCs) with the RNA seq analysis [11]. Our data showed higher expression of DNA repair factors including RAD18 in iPSCs and NPCs compared with the original skin fibroblasts suggesting a specific role for RAD18 in iPSCs and NPCs.

Accumulation of genetic mutation causes genome instability that leads to tumorigenesis. It is thought that the upregulation of the genome maintenance system suppresses genetic mutation in PSCs, however there is currently poor evidence for this. In this study, we first analyzed the mutation frequency using the Sup F reporter system in iPSCs with the model of PSCs and NPCs as differentiated cells. Furthermore, we analyzed the mutation frequency in RAD18-depleted iPSCs and NPCs. RAD18-depleted cells exhibited a decrease in the frequency of mutation, suggesting that the appropriate expression of RAD18 is important for the genome stability and the suppression of mutation in iPSCs and NPCs.

## MATERIALS AND METHODS

### Cell culture

Human skin fibroblasts NB1RGB [20–22] and human iPSCs 201B7 [23] were obtained from RIKEN Bio Resource Center. NB1RGB was maintained with Dulbecco's Modified Eagle Medium and supplemented with 10% FBS and penicillin/streptomycin (Nacalai Tesque, Japan). iPSCs C2 were derived from NB1RGB by messenger RNA (mRNA) integration-free methods using the Stemgent® StemRNATM-NM Reprogramming Kit for Reprogramming Adult and Neonatal Human Fibroblasts (Stemgent, USA) as previously reported [11, 24]. iPSCs were maintained as feeder-free culture with the NutriStem™ XF/FF medium (ReproCELL, Japan) and iMatrix511 silk (Nippi, Japan). NPCs were derived from iPSCs C2 using the PSC neural induction medium (Gibco, Thermo Fisher Scientific, USA) and maintained with the neural basal medium (Gibco, Thermo Fisher Scientific) and iMatrix 511 silk according to the manufacturer's protocols with small modifications. iPSCs and NPCs culture media were supplemented with the Y27632 ROCK inhibitor (WAKO Pure Chemical Industries, Japan) during passage; on the subsequent day, the media were replaced with fresh culture media without the ROCK inhibitor.

### Transfection and siRNA knockdown

pSP189 plasmid vector was introduced into mammalian cells using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, USA) following the manufacture's instruction. siRNA Luciferase (5′- UAAGG CUAUGAAGAGAUACdTdT-3′, 5′- GUAUCUCUUCAUAGCCUU AdTdT-3′) was used for negative control and siRNA RAD18 (5'-GCC AAGGAAAGAUGCUAATT-3′, 5′- UUAGCAUCUUUCCUUGGC TT-3′) were introduced into mammalian cells using Lipofectamine RNAi-MAX (Invitrogen, Thermo Fisher Scientific) following the manufacture's instruction. All siRNAs were used at a final concentration of 50 nM.

### SDS-PAGE and Western blot analysis

To analyze the RAD18 expression level in RAD18 knocked down cells, we performed a Western blot analysis. Cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris HCl, pH 8.0, 250 mM NaCl, 25 mM ethylenediaminetetraacetic acid (EDTA), 0.5% v/v Triton X-100, 0.5% w/v sodium dodecyl sulfate (SDS), 0.5% w/v sodium deoxycholate) containing protease inhibitor cocktail (Nacalai Tesque, cat# 25955-11). The protein concentration was measured by a Bicinchoninic Acid (BCA) Assay Kit (Takara Bio, Japan) using bovine serum albumin as the standard. In all the experiment, 20 $\mu$g of protein was loaded onto SDS polyacrylamide gel electrophoresis (SDS-PAGE) plates. The proteins were electrophoresed at 30 mA/gel plate for 1-1.5 h, and transferred onto a polyvinylidene difluoride (PVDF) membrane at 100 V for 1.5 h. Next, the PVDF membrane was blocked with 1% w/v BSA/TBS-T (tris-buffered saline and Tween 20) for 1 h at room temperature on a shaker. For primary antibody reactions, the following primary antibodies were used for 1- 4 h at room temperature: RAD18 (rabbit, 1:1000, abcam, UK, cat# ab79763), $\beta$-actin (rabbit, 1:2000, Gene-Tex, USA, cat#GTX110564) and GAPDH (mouse, 1:2000, Chemicon, Merck Millipore, Germany, cat#MAB374) for loading control. Next, the PVDF membrane was washed by TBS-T for three times. For secondary antibody reactions, horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse antibodies (DAKO, Agilent Technologies, USA, cat# P0399 or P0477, respectively) were used for 1 h at room temperature. Subsequently, the PVDF membrane was washed with TBS-T for five times. The PVDF membrane was developed by the enhancement of chemiluminescence (LI-COR Biosciences, USA) and was detected by C-digit (LI-COR Biosciences). Western blot band intensity was measured by Image J software.

### Mutation frequency

The Sup F assay was performed as previously reported [25–27]. The pSP189 plasmid vector was exposed to the UV-C irradiation at indicated dose (200, 500 and 1000 J/m$^2$). And then, pSP189 was transfected into mammalian cells with Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific) according to the manufactural procedure. After incubation for 24 - 48 h, pSP189 were extracted and purified by FastGene Plasmid Mini Prep Kit (Nippon Genetics, Japan). To remove the non-replicated plasmid vector, pSP189 was treated with Dpn1 restriction enzyme. Purified pSP189 was introduced into MBM7070 *Escherichia coli* by electroporation (BioRad, USA). And then, super optimal broth with catabolite repression

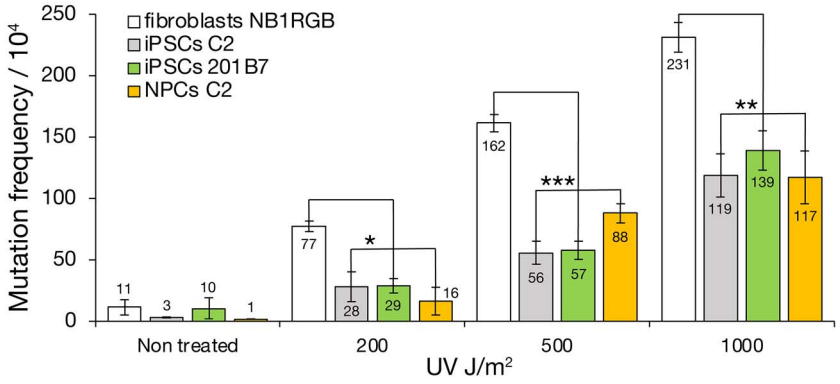


**Fig. 1. Mutation frequency in fibroblasts, iPSCs and NPCs. Mutation frequency was measured using the Sup F assay in fibroblasts (NB1RGB), iPSCs (C2 and 201B7) and NPCs C2. UV exposure was used as a mutagen at the indicated dose (200, 500 and 1000 J/m²). Each experiment was conducted at least three times. The error bars represent SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.005$, Welch's t-test.**

(SOC) was added immediately after and incubated for 1 h at 37°C. condition. MBM7070 *E. coli* were plated on the X-gal Ampicillin LB plate and incubated for 24 h at 37°C condition and bacteria colonies were counted.

### Micronucleus assay

After transfection of siRNA RAD18 or Luciferase, cells were incubated for 24 h and exposed to the UV-C irradiation at 20 J/m². After 24 h incubation, cells were fixed with 4% paraformaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were observed by fluorescence microscopy (ZEISS, Axio Observer 7) and counted at least 200 cells.

### Statistical analysis

A statistical analysis was performed using Microsoft Excel. Welch's t-test was employed to analyze the statistical significance of the difference between the two experimental groups. All experiments were independently performed at least three times.

## RESULTS

### Mutation frequency is suppressed in iPSCs and NPCs

To determine the frequency of mutation for PSCs, we used iPSC C2, which was derived from human skin fibroblasts NB1RGB, and iPSCs 201B7 at Kyoto university were generally used a model for iPSCs. NPCs C2 derived from iPSC C2 were used as differentiated cells and NB1RGB was used as a control for somatic cells. The pSP189 plasmid for encoding a suppressor tRNA (Sup F) reporter system was used to measure mutation [25, 28]. pSP189 plasmids were exposed to the indicated dose of UV-C irradiation (200, 500 and 1000 J/m²) and transfected into cells. After incubating for 48 h, the replicated plasmid was then isolated and transformed into an MBM7070 *E. coli* host containing a lacZ gene with a premature stop codon. The mutation frequency was assessed using blue-white colony selection. In each sample, 1000-30 000 colonies were counted. All types of cells exhibited an increased mutation frequency in dose dependent manner (Fig. 1). Both iPSCs C2 and 201B7 showed low mutation frequency in the high dose exposure (at 500 J/m², C2: $56 \times 10^{-4}$, 201B7: $57 \times 10^{-4}$, at 1000 J/m², C2: $119 \times 10^{-4}$, 201B7: $139 \times 10^{-4}$) compared with skin fibroblasts (at 500 J/m², $162 \times 10^{-4}$, at 1000 J/m², $231 \times 10^{-4}$). NPCs showed similar level of mutation frequency (at 500 J/m², $88 \times 10^{-4}$, at 1000 J/m², $117 \times 10^{-4}$) with iPSCs. These results suggest that PSCs and NPCs have genetic mutation suppressing ability.

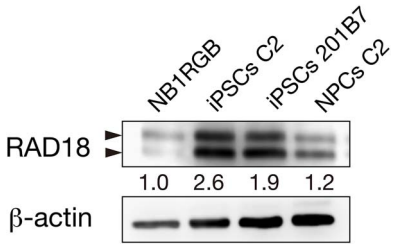


**Fig. 2. RAD18 expression in fibroblasts NB1RGB, iPSCs and NPCs. The RAD18 protein expression level via a Western blot analysis is in the indicated cells. Both band indicates RAD18. $\beta$-actin was used for loading control. Each band intensity was measured by Image J software and normalized by $\beta$-actin intensity.**

### Knockdown of RAD18 suppresses genetic mutation in iPSCs and NPCs

We addressed how iPSCs and NPCs suppress the frequency of mutation. In a previous study, we conducted the RNA-seq analysis in fibroblasts, iPSCs and NPCs and found that the TLS pathway factor RAD18 expression was upregulated in iPSCs and NPCs compared with fibroblasts [11]. We checked the protein expression using Western blot analysis (Fig. 2). Although iPSCs showed a high expression of RAD18 compared with fibroblasts, NPCs showed relatively lower RAD18 expression than iPSCs. This result might reflect the differentiation state of NPCs, because gene expression alternation depends on differentiation state [11]. Based on these findings, we hypothesized the RAD18 plays a suppressive role in iPSC mutation [29].To address our hypothesis, the RAD18 expression was depleted by siRNA knockdown in skin

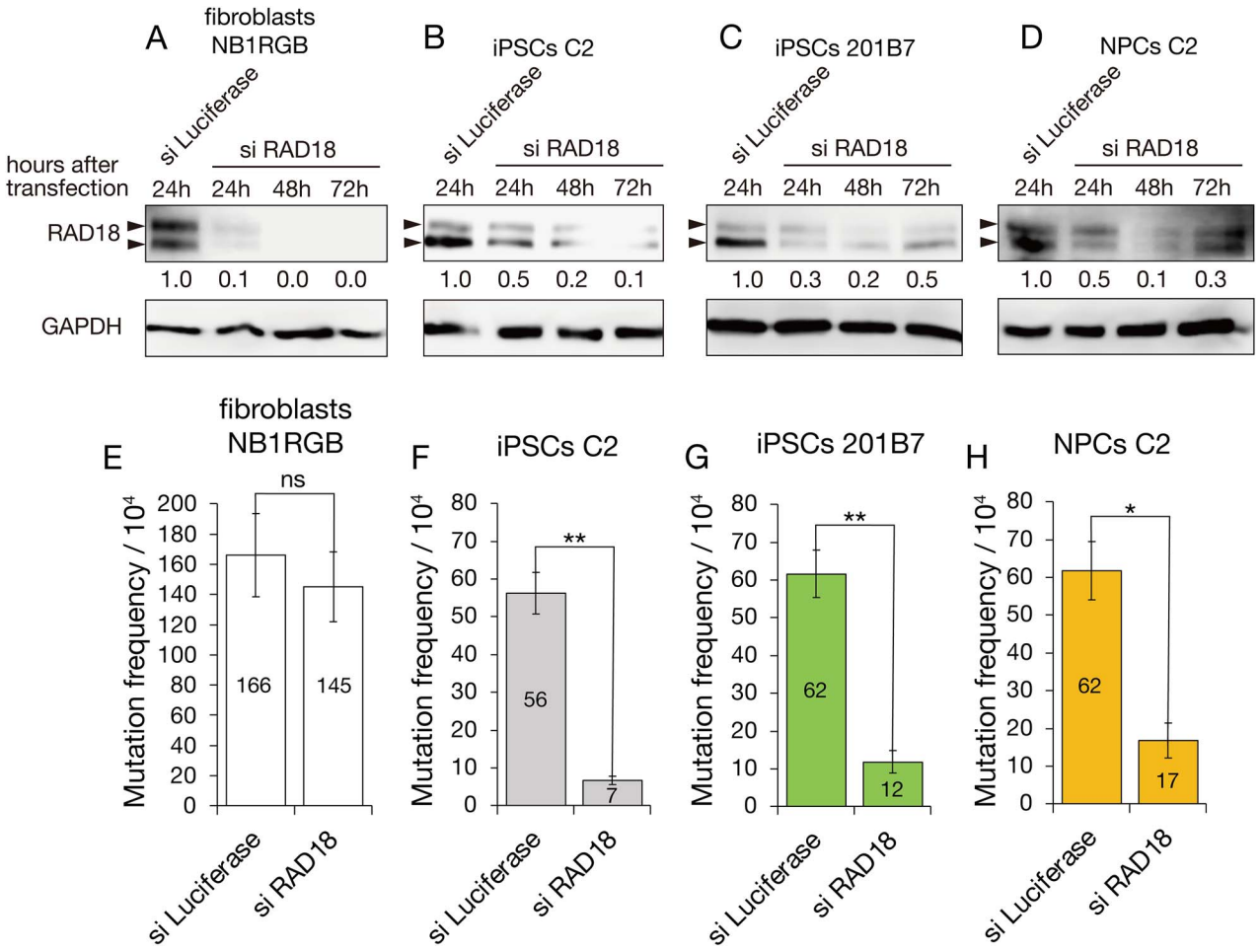


**Fig. 3. Mutation frequency in RAD18 knockdown fibroblasts, iPSCs and NPCs. siRNA knockdown experiment in fibroblasts NB1RGB (A), iPSCs C2 (B), 201B7 (C) and NPCs C2 (D). After transfection of siRNA RAD18 oligo, protein was extracted from cells at indicated time. The lower band indicates the Rad18 expression. The GAPDH antibody was used for loading control. Each band intensity was measured by Image J software and normalized by GAPDH intensity. The mutation frequency was measured using the Sup F assay in fibroblasts NB1RGB (E), iPSCs C2 (F) and 201B7 (G) and NPCs C2 (H) after the siLuciferase or siRAD18 knockdown treatment. UV exposure at 500 J/m² was used as a mutagen. Each experiment was performed at least three times. The error bars represent SEM. n.s. no significant, $^{*}P < 0.01$, $^{**}P < 0.005$, Welch's t-test.**

fibroblasts, iPSCs and NPCs and the mutation frequency was measured. The RAD18 expression level was confirmed by Western blot analysis (Fig. 3). Since iPSCs were vulnerable, the combined transfection of siRNA and pSP189 plasmids resulted in a massive induction of cell death in iPSCs. Thus, we selected daily transfection of these factors. After 24 h of incubation following cell seeding, siRNA RAD18 was transfected. In addition, after 24 h incubation, pSP189 plasmids, which had been exposed to UV 500 J/m², were transfected. After another 24 h of incubation, the replicated plasmids were extracted and used for mutation assay. Although the RAD18 depleted fibroblast showed no significant change (siLuciferase, $166 \times 10^{-4}$, siRAD18, $145 \times 10^{-4}$), the RAD18 depleted iPSCs and NPCs cells significantly reduced the frequency of mutation (siLuciferase, $165 \times 10^{-4}$, 201B7: $62 \times 10^{-4}$, NPCs: $62 \times 10^{-4}$, siRAD18, C2: $7 \times 10^{-4}$, 201B7: $12 \times 10^{-4}$, NPCs: $17 \times 10^{-4}$) (Fig. 3). Contrary to our hypothesis, this result indicates that RAD18 has a promoting role of in the mutation frequency in iPSCs and NPCs. Furthermore, to investigate whether RAD18 knockdown leads to the genome instability, we performed micronucleus assay after UV exposure in RAD18 knockdown cells (Fig. 4). Although RAD18 depleted fibroblasts showed no significant change, iPSCs and NPCs showed dramatically increasing of the number of micronuclei after RAD18 knockdown. Furthermore, UV exposure additively increased the number of micronuclei in these cells. These results suggest that RAD18 is required for genome stability independently of occurrence of mutation in iPSCs and NPCs.

## DISCUSSION

In this study, we first measured the frequency of mutation in iPSCs using a plasmid-based reporter assay, which is useful owing to the

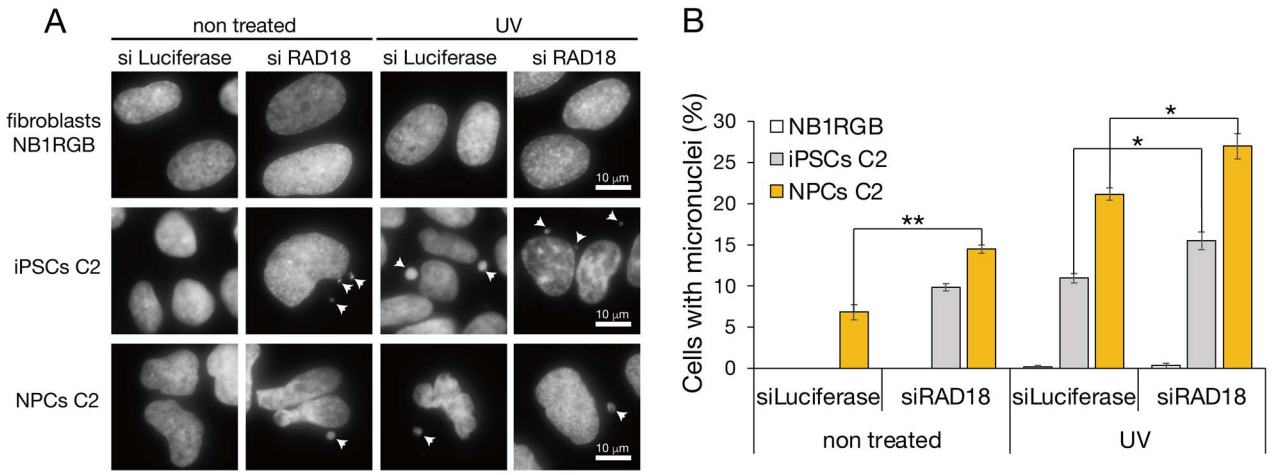


**Fig. 4. Measurement of the number of micronuclei in RAD18 depleted fibroblast, iPSCs and NPCs after UV exposure. After knockdown of RAD18 in fibroblast, iPSCs and NPCs, cells were exposed to UV exposure at 20 J/m$^2$ and incubated 24 h. And then, cells were fixed and stained with DAPI. Cells were observed by fluorescence microscopy (A). Cells were counted at 200 cells and graphed (B). The error bars represent SEM, $^{*}P < 0.05$, $^{**}P < 0.005$, Welch's t-test.**

convenience it offers as well as its applicability to all cells. Meanwhile, recent advances of the next-generation sequencing (NGS) allow the whole genome DNA sequencing to be read, revealing genetic mutation signatures in iPSCs treated with 79 environmental carcinogens [30]. These data showed that the DNA damage induced by diverse environmental mutagen were repaired by disparate DNA repair and the replicative pathways. We believe that the combined analysis with NGS and the Sup F assay could be useful in verifying each DNA repair pathway in iPSCs.

We also used NPCs derived from iPSCs as differentiated cells with stem cell activity for the purpose of measuring mutation frequency. Because NPCs function as neural stem cells and progenitor cells, it states immature than differentiated fibroblasts. The RNA seq analysis revealed that the genome stability related to gene expression in NPCs was similar to that in iPSCs. Thus, a low-frequency mutation in NPCs is reasonable. As it has been reported that the accumulation of damage to DNA in the central nervous system results in neural degeneration [31–33], it is important to identify the relationship between DNA damage-dependent genetic mutation and neural degeneration. Both iPSCs and NPCs showed a lower frequency of mutation compared with skin fibroblasts. This result is consistent with the observation that stem cells have several mechanisms to suppress the instability of genomes [34, 35]. Our data might contribute to the measurement of mutation assays for other tissue cells derived from iPSCs.

To identify the molecular mechanism of suppressing mutation frequency in iPSCs and NPCs, we investigated gene expression using the RNA seq analysis [11] and found high expressions of RAD18 in iPSCs and NPCs compared with fibroblasts. RAD18 is involved in TLS for solving the emergency stalled replication fork. The RAD18-dependent PCNA mono-ubiquitination mechanism is conserved in diverse species, such as yeast, mice and humans. Defective RAD18 results in hypersensitivity to the DNA strand breaks caused by X-rays, suggesting that RAD18 plays a distinct role in repairing DNA damage. In other reports, RAD18 is involved

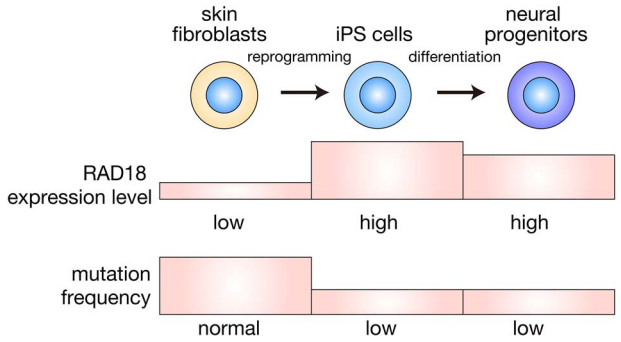


**Fig. 5. Model of RAD18 expression and cell differentiation. RAD18 expression level was increased after reprogramming and mutation frequency rate was decreased after reprogramming.**

in repairing DNA single-strand breaks in the S phase [36], as well as homologous recombination (HR)-mediated DNA double-strand breaks [37].

Although the RAD18-dependent mono-ubiquitination of PCNA is involved in both error-free and error-prone TLS, we hypothesized that a low mutation frequency in iPSCs and NPCs is dependent on the high expression of RAD18. To confirm this hypothesis, we knocked down the RAD18 expression in iPSCs and NPCs and measured the frequency of mutation. Contrary to our hypothesis, the RAD18-depleted iPSCs and NPCs exhibited a lower frequency mutation than the negative control cells. This result suggests that the disruption of an appropriate expression by RAD18 siRNA resulted in the promotion of RAD18-independent, error-free TLS, although a high expression of RAD18 promote error-free TLS, which suppresses the frequency of mutation in the baseline of PSCs (Fig. 5). Considering the complexity of the TLS pathway, the RAD18 knockdown might induce

unscheduled activity in the TLS. In fact, our results are consistent with others' study using ES cells from mice [13]. Meanwhile, RAD18 depletion increased the number of micronuclei in iPSCs and NPCs suggesting RAD18 is required for genome stability independently of mutation suppressing mechanism in iPSCs and NPCs but not in fibroblasts.

In summary, the genome maintenance mechanism of PSCs remains largely unknown. Specifically, a high level of replication activity in DNA, which is associated with damage and genetic mutation in DNA, causes catastrophic damage for cellular homeostasis. Our results might contribute to elucidate the genome maintenance machinery in PSCs.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest associated with this manuscript.

## ACKNOWLEDGMENTS

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