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DNA-PK inhibition causes a low level of H2AX phosphorylation and homologous recombination repair in Medaka (*Oryzias latipes*) cells

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ABSTRACT

Nonhomologous end joining (NHEJ) and homologous recombination (HR) are known as DNA double-strand break (DSB) repair pathways. It has been reported that DNA-PK, a member of PI3 kinase family, promotes NHEJ and aberrant DNA-PK causes NHEJ deficiency. However, in this study, we demonstrate that a wild-type cell line treated with DNA-PK inhibitor and a mutant cell line with dysfunctional DNA-PK showed decreased HR efficiency in fish cells (Medaka, *Oryzias latipes*). Previously, we reported that the radiation-sensitive mutant RIC1 strain has a defect in the Histone H2AX phosphorylation after γ-irradiation. Here, we showed that a DNA-PK inhibitor, NU7026, treatment resulted in significant reduction in the number of γH2AX foci after γ-irradiation in wild-type cells, but had no significant effect in RIC1 cells. In addition, RIC1 cells showed significantly lower levels of DNA-PK kinase activity compared with wild-type cells. We investigated NHEJ and HR efficiency after induction of DSBs. Wild-type cells treated with NU7026 and RIC1 cells showed decreased HR efficiency. These results indicated that aberrant DNA-PK causes the reduction in the number of γH2AX foci and HR efficiency in RIC1 cells. We performed phosphorylated DNA-PKcs (Thr2609) and 53BP1 focus assay after γ-irradiation. RIC1 cells showed significant reduction in the number of phosphorylated DNA-PKcs foci and no deference in the number of 53BP1 foci compared with wild-type cells. These results suggest that low level of DNA-PK activity causes aberrant DNA-PKcs autophosphorylation in RIC1 cells. It is known that 53BP1 is involved in both DNA-PK dependent and independent NHEJ. Therefore we suggest that DNA-PK independent NHEJ repair DSBs under the condition of decreased DNA-PK activity, which causes reduction of HR efficiency.

1. Introduction

Double-strand breaks (DSBs) can lead to genomic instability, which causes cancer and promotes aging. Cells maintain genomic stability by activating the DNA damage response after DSBs. As the first step of the cellular response to DSBs, the histone H2A variant H2AX located around DSB sites is phosphorylated on Ser139 (known as γH2AX) by the phosphatidylinositol-3 (PI3) kinase family proteins. Subsequently, γH2AX plays a critical role in DNA damage signaling, which induces cell-cycle checkpoints, apoptosis, and DSB repair [1,2]. The PI3 kinase family includes DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM), and ATM and Rad3-related (ATR). H2AX is phosphorylated by ATM and DNA-PK after DSBs, and by ATR after replication stress and SSB or UV [2].

Nonhomologous end joining (NHEJ) and homologous recombination (HR) are two major repair pathways of DSBs. Each pathway requires a set of repair factors [3]. Ku70 and Ku80 recognize and bind the DSB sites after DNA damage. Subsequently, the Ku70/80–DNA complex stabilizes and stimulates DNA-PK catalytic subunit (DNA-PKcs) to form the DNA-PK complex on the DSB sites. DNA-PK complex phosphorylates various proteins and promotes NHEJ [4]. Therefore, the absence of DNA-PK leads to increased HR efficiency after DSBs, which is consistent with the passive shunting of DSBs from NHEJ to HR [5]. However, several studies reported that DNA-PK inhibition causes a decrease in HR efficiency [6,7]. In addition, recent studies have been reported that NHEJ consists of several separate pathways containing classical and alternative NHEJ [3,8]. The classical NHEJ is well-known as Ku heterodimer and DNA-PKcs dependent pathway. The alternative NHEJ has been detectable in the absence of DNA-PKcs, Ku70 or Ku80, therefore

Abbreviations: DSBs, double-strand breaks; NHEJ, non-homologous end joining; HR, homologous recombination; ENU, N-ethyl-N-nitrosourea; RIC, radiation induced curly tailed; DNA-PKcs, DNA dependent protein kinase catalytic subunit; ATM, ataxia telangiectasia mutated; ATR, ATM and Rad3-related; 53BP1, p53 binding protein 1.

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this pathway is called DNA-PK independent or backup NHEJ [9]. These suggest the complicated regulation of DSB repair by DNA-PK but the molecular mechanism of which is still unknown. In addition, many studies reported the regulation mechanism of DSB repair pathways in mammalian cells, especially humans and mice. Therefore, the common DSB mechanism among vertebrates is still unknown.

Medaka (*Oryzias latipes*) is used as a useful model in radiation biology [10,11]. RIC1 strain was isolated as a radiation-sensitive mutant strain of Medaka via N-ethyl-N-nitrosourea ENU-mutagenesis screening [12]. Previously, we established four RIC1 cell lines from each single embryo and reported that RIC1 embryonic cells have a defect in γH2AX foci formation after γ-irradiation [13]. The result suggested that the *ric1* gene is involved in the regulation of ATM or DNA-PK. In this study, we performed γH2AX focus assay and NHEJ and HR reporter assays after ATM or DNA-PK inhibitor treatment, and examined phosphorylated DNA-PKcs (Thr2609) and tumor suppressor p53 binding protein 1 (53BP1) focus after γ-irradiation. The results suggest a model that 53BP1 dependent NHEJ is an alternative mechanism to repair DSBs under the condition that decreases DNA-PK activity, which causes reduced HR efficiency.

2. Materials and methods

2.1. Cells and plasmid

The Medaka cell lines CAB (wild type) and RIC1 were established from a single embryo of each strain [13]. Cells were cultured in L-15 medium (Irvine, CA, USA) supplemented with 20% fetal bovine serum (Biological Industries, Kibbutz Beit-Haemek, Israel), 50 μg/mL streptomycin, and 10 mM 2-[4-(-hydroxyethyl)-1-piperazinyl]-ethanesulfonic acid (HEPES) (pH 7.5) and incubated at 33 °C.

The medaka 53BP1 fragment, except for the start codon, was amplified by PCR from a HdrR (wild-type inbred strain) embryo cDNA using PCR primers with a BglII site and a four-glycine linker at the 5' terminus and a SalI site at the 3' terminus. TagFP635c-tagged 53BP1 was generated by an in-flame ligation of a 53BP1 fragment into pTagFP635-c. The sequence of the cloned gene was confirmed by sequencing. CAB and RIC1 cells were transfected with the TagFP635-c-53BP1 plasmid and clones with stable expression were established.

2.2. Irradiation and chemicals

Cells grown in culture dishes or glass-bottom dishes were exposed to various doses of γ-rays from a ^{137}Cs Source at a dose rate of 10 Gy/min (Elan 3000; MDS Nordion, Ottawa, Canada). The ATM inhibitor KU-55933 (Calbiochem, CA, USA) was dissolved in DMSO to prepare a 10 mM stock solution. The DNA-PK inhibitor NU7026 (Calbiochem) was dissolved in DMSO to prepare a 2 mM stock solution. All chemicals are stored at −30 °C.

2.3. Immunocytochemistry

Cells were irradiated and fixed with several antibodies as previously described [13]. The cells were incubated with a mouse monoclonal anti-phospho-H2AX (Ser139) antibody (Upstate, NY, USA) at

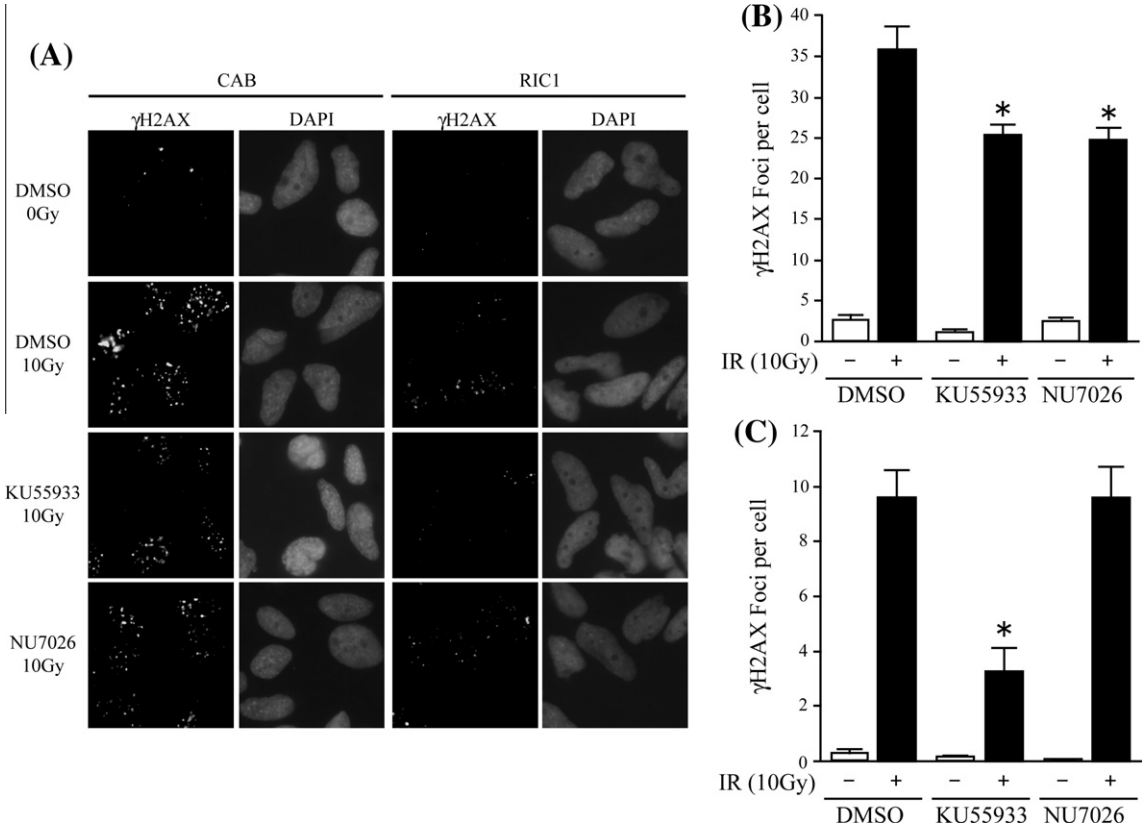

Fig. 1. DNA-PK inhibition causes a decrease in the number of γH2AX foci after γ-irradiation in CAB cells, but not in RIC1 cells. (A) Effects of ATM and DNA-PK inhibitors on the formation of γH2AX foci. CAB and RIC1 cells were treated with 10 M KU55933 or 10 M NU7026 for 1 h before γ-irradiation (10 Gy). Cells were then incubated for 15 min and fixed. DMSO is the control of the inhibitor treatment. (B, C) Average of the number of γH2AX foci in CAB cells (B) or RIC1 cells (C) treated with KU55933 or NU7026. The open bars denote the number of γH2AX foci in not irradiated cells, and the closed bars denote that in irradiated cells ($n > 50$). Data were obtained from one representative experiment among three experiments and error bars represent standard errors of the means (SEM). Statistical analysis was performed using Student's t test (*significantly different from "DMSO + ", $^*P < 0.001$).

a 1:5000 dilution or a mouse monoclonal anti-phospho-DNA-PKcs (Thr2609) antibody (abcam, MA, USA) at a 1:1000 dilution in blocking buffer. Primary antibodies were detected with an Alexa Fluor 488 goat anti-mouse IgG secondary antibody (Molecular Probes). The cells were counterstained with 1 μg/mL 4',6-diamino-2-phenylindole (DAPI). Images were captured using a fluorescence microscope (IX-81; Olympus, Tokyo, Japan) equipped with a digital camera (DP70; Olympus).

2.4. DNA-PK assay

DNA-PK activity was measured through DNA-pull-down assay with filter-binding analysis [14]. The peptide substrate, medaka p53-S15 (sequence: DLPESQGAFQELWEAKK) was synthesized by Hokkaido System Science Co., Ltd. (Hokkaido, Japan). The assay procedures were described previously [14].

2.5. HR and NHEJ reporter assays

The pDR-GFP reporter construct was used to monitor DSB repair via HR [15]. The pEJ was also used as a reporter construct to monitor DSB repair via NHEJ [16]. To measure the repair of I-SceI-generated DSBs, cell lines transfected with pEJ or pDR-GFP stably were electroporated with the I-SceI expression vector (pCBASce). The electroporation was performed using a MicroPorator-mini device (MP100; Digital Bio Technology, Seoul, Korea) according to the Invitrogen Neon™ Transfection System protocol (Neon™ Transfection System 10 μL Kit, catalog number MPK1096). To calculate the amount of NHEJ repair, the percentage of GFP-positive cells was quantified 4 days after electroporation using a flow cytometer (BD Biosciences, CA, USA).

3. Results

3.1. H2AX phosphorylation is not affected by NU7026 treatment in RIC1 cells

We have shown that RIC1 cells have a defect of H2AX phosphorylation after γ-irradiation [13]. It is known that both ATM and DNA-PK mainly phosphorylate H2AX after DSBs. Therefore, we predicted that the *ric1* gene is involved in ATM or DNA-PK function. To investigate whether the defective H2AX phosphorylation is caused by aberrant function of ATM or DNA-PK, cells were treated with ATM inhibitor (KU55933) or DNA-PK inhibitor (NU7026) and γH2AX was visualized using immunofluorescence staining with an anti-γH2AX antibody (Fig. 1A). The treatment of 20 mM inhibitors caused severe toxicity, but the treatment with 10 μM KU55933 or 10 μM NU7026 did not cause any inhibition of cell proliferation and any toxicity in both CAB and RIC1 cells (data not shown). The size of γH2AX foci was much smaller in RIC1 cells than that in CAB cells before and after γ-irradiation (Fig. 1A). KU55933 treatment led to a significant decrease in the number of γH2AX foci 15 min after γ-irradiation in both CAB and RIC1 cells (Fig. 1A and B). Although CAB cells treated with NU7026 showed a significant decrease in the number of γH2AX foci 15 min after γ-irradiation, RIC1 cells showed no significant effect in the number of γH2AX foci (Fig. 1A, B and C). These results suggest that aberrant DNA-PK function causes the unstable induction of γH2AX foci in RIC1 cells.

3.2. DNA-PK activity in RIC1 cells is lower than that in CAB cells

We examined the level of DNA-PK activity in RIC1 cells using DNA-pull-down assay with filter-binding analysis [14]. RIC1 cells showed a significant lower level of DNA-PK activity than that of CAB cells (Fig. 2B). In addition, we examine whether NU7026 inhibits medaka DNA-PK. Our results demonstrate that DNA-PK activity was decreased after treatment with NU7026 in a dose-dependent manner (Fig. 2C). These results suggest that low level of DNA-PK activity causes decreased H2AX phosphorylation in RIC1 cells.

3.3. CAB cells treated with NU7026 and RIC1 cells show a significant decrease in HR efficiency compared with untreated CAB cells

Our previous studies revealed that RIC1 cells and embryos showed a delayed DSB repair [12,13]. To evaluate HR and NHEJ efficiency independently, we performed HR and NHEJ reporter assays.

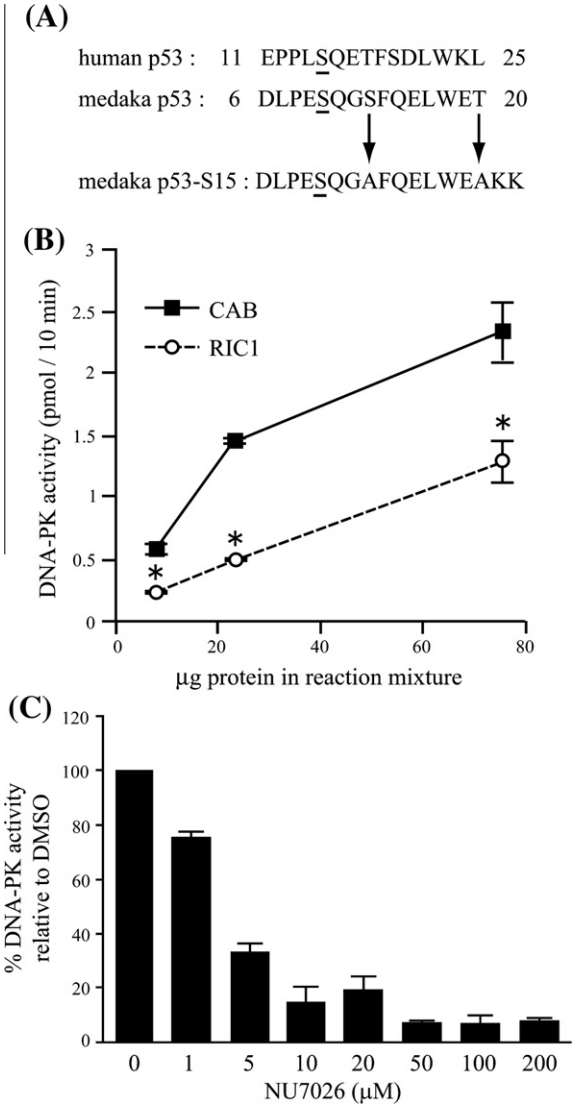

Fig. 2. Low level of DNA-PK activity in RIC1 cells. (A) The amino acid sequence of synthetic peptide medaka p53-S15. The DNA-PK phosphorylation site, serine 10, is underlined. Serine 13 and Threonine 20 in medaka p53 have been changed to alanine and two extra lysines have been appended to the C terminus. (B) DNA-PK activity of CAB and RIC1 cells. Cell lysates of CAB and RIC1 were diluted to 0.5 mg/ml, 1.5 mg/ml and 5 mg/ml, respectively, and assayed by the DNA-pull-down/filter-binding procedure. Closed squares denote the DNA-PK activity in CAB cells and open circles denote that in RIC1 cells. Statistical analysis was performed using Student's *t* test (*significantly different from the DNA-PK activity of the same concentration of cell lysate from CAB cells, $^{*}P < 0.05$). (C) Phosphorylation inhibitory effects of NU7026. Cell lysates was mixed with the indicated concentrations of NU7026 and DNA-PK activity was measured. The *Y*-axis indicates the percentage of DNA-PK activity relative to the DMSO (used as vehicle control).

We introduced the pDRGFP substrate to monitor HR or the pEJ substrate to monitor NHEJ in both CAB and RIC1 cells and measured the percentage of GFP-positive cells after I-SceI-induced DSBs using flow cytometry (Fig. 3A and B). Although CAB cells showed ~3.4% GFP-positive cells, the GFP-positive cells were significantly decreased (1.1%) in RIC1 cells, as assessed using the HR reporter assay (Fig. 3C). CAB cells included 2.3% of GFP-positive cells and RIC1 cells included 3.2% of GFP-positive cells, as assessed using the NHEJ reporter assay (Fig. 3D). To investigate whether the decreased HR efficiency was caused by aberrant DNA-PK function in RIC1 cells, we performed HR reporter assays after NU7026 treatment. NU7026 treatment caused a significant decrease in HR efficiency in CAB cells, but not in RIC1 cells (Fig. 3E). These results suggest that aberrant DNA-PK function causes a decrease in HR efficiency.

3.4. RIC1. cells showed a decrease in the number of phosphorylated DNA-PKcs foci and no deference in the number of 53BP1 foci

DNA-PKcs and Ku heterodimer rapidly form DNA-PK complex on DSB sites and DNA-PKcs autophosphorylation causes DNA-PK dissociation from DSB sites. It is known that DNA-PKcs is autophosphorylated at Thr2609. To investigate the effect of aberrant DNA-PK activity on DNA-PKcs autophosphorylation, CAB and RIC1 cells were stained with phosphorylated DNA-PKcs (Thr2609) antibody after γ-irradiation (Fig. 4A). RIC1 cells showed significant reduction in the number of phosphorylated DNA-PKcs foci each time point after irradiation compared with CAB cells (Fig. 4B). In spite of low level of DNA-PK activity, RIC1 cells showed no significant differences in NHEJ efficiency compared with CAB cells (Fig. 3D). 53BP1 was identified as a protein that binds to the DNA-binding domain of tumor suppressor protein p53 [17]. Several studies reported that 53BP1 is involved in both classical and alternative NHEJ [3,18,19]. To evaluate NHEJ, we performed 53BP1 foci assay by using TagFP635 (Red fluorescent protein) - tagged 53BP1 (Fig. 4A). RIC1 cells showed no significant differences in the number of 53BP1 foci after DSB compared with CAB cells (Fig. 4C). These results suggest that the low level of DNA-PK activity causes aberrant DNA-PKcs autophosphorylation, which does not affect 53BP1 foci formation after DSBs in RIC1 cells.

4. Discussion

DNA-PKcs and the Ku heterodimer (Ku70 and Ku80) constitute the DNA-PK complex, which is a serine/threonine kinase. DNA-PK

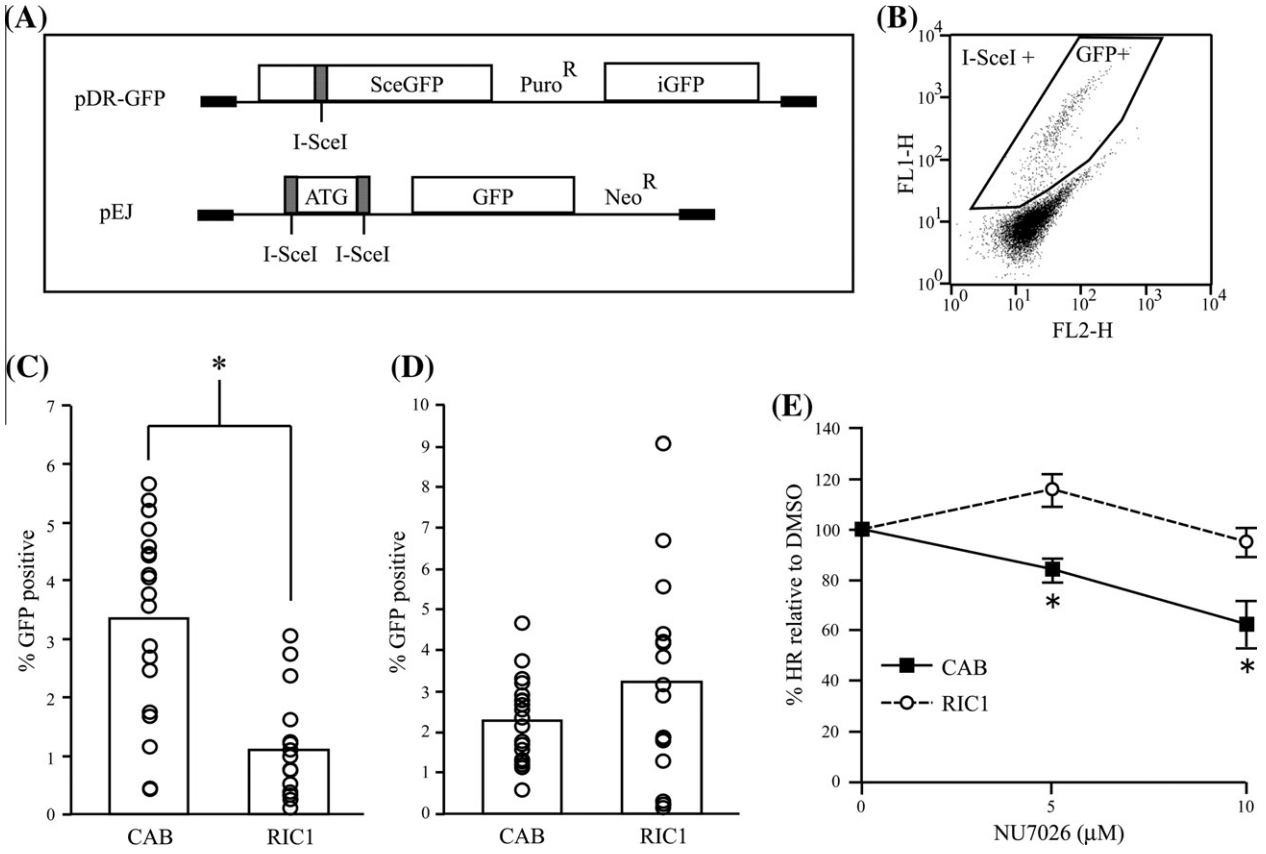

Fig. 3. CAB cells treated with NU7026 and RIC1 cells exhibit a decrease in HR efficiency. (A) Reporter substrates. pDRGFP contains two non-functional GFP genes (SceGFP and iGFP). Repair of the endonuclease I-SceI-induced DSB by HR results in functional GFP. pEJ encodes an initial codon between two I-SceI sites, which prevents GFP translation. Repair of the I-SceI-induced DSB by NHEJ restores GFP translation. (B) Both CAB and RIC1 cells were transfected stably with the pDR-GFP or pEJ plasmid and single cell clones were established. GFP-positive cells were counted using flow cytometry. The dot plot shows the fluorescence intensity of the pDR-GFP stable clone after introduction of I-SceI. (C) I-SceI was introduced into cells transfected with pDR-GFP (19 independent clones from CAB cells and 16 independent clones from RIC1 cells) and cells were analyzed using flow cytometry. Open circles denote the average of the percentage of GFP-positive cells after I-SceI introduction in each single cell clone ($n = 3$ per single cell clone). Open bars denote the average HR efficiency in CAB ($n = 19$) or RIC1 ($n = 16$) clones. Statistical analysis was performed using Student's t test (*$P < 0.0001$). (D) I-SceI was introduced into cells transfected with pEJ (19 independent clones from CAB cells and 16 independent clones from RIC1 cells) and cells were analyzed using flow cytometry. Open circles denote the average of the percentage of GFP-positive cells after I-SceI introduction in each single cell clone ($n = 3$ per single cell clone). Open bars denote the average NHEJ efficiency in CAB ($n = 19$) or RIC1 ($n = 16$) clones. (E) Effect of NU7026 on HR efficiency. I-SceI was introduced into six single cell clones transfected with pDR-GFP in CAB and RIC1 cells and cells were treated with NU7026 simultaneously and incubated for 4 days. Cells were then analyzed using flow cytometry. Closed squares denote the repair efficiency in CAB cells and open circles denote the repair efficiency in RIC1 cells. Each graph shows the average of the repair efficiency in six clones and the Y-axis indicates the percentage of repair efficiency relative to the DMSO control. Error bars represent standard errors of the means (SEM). Statistical analysis was performed using Student's t test (*significantly different from the DMSO control, *$P < 0.05$).

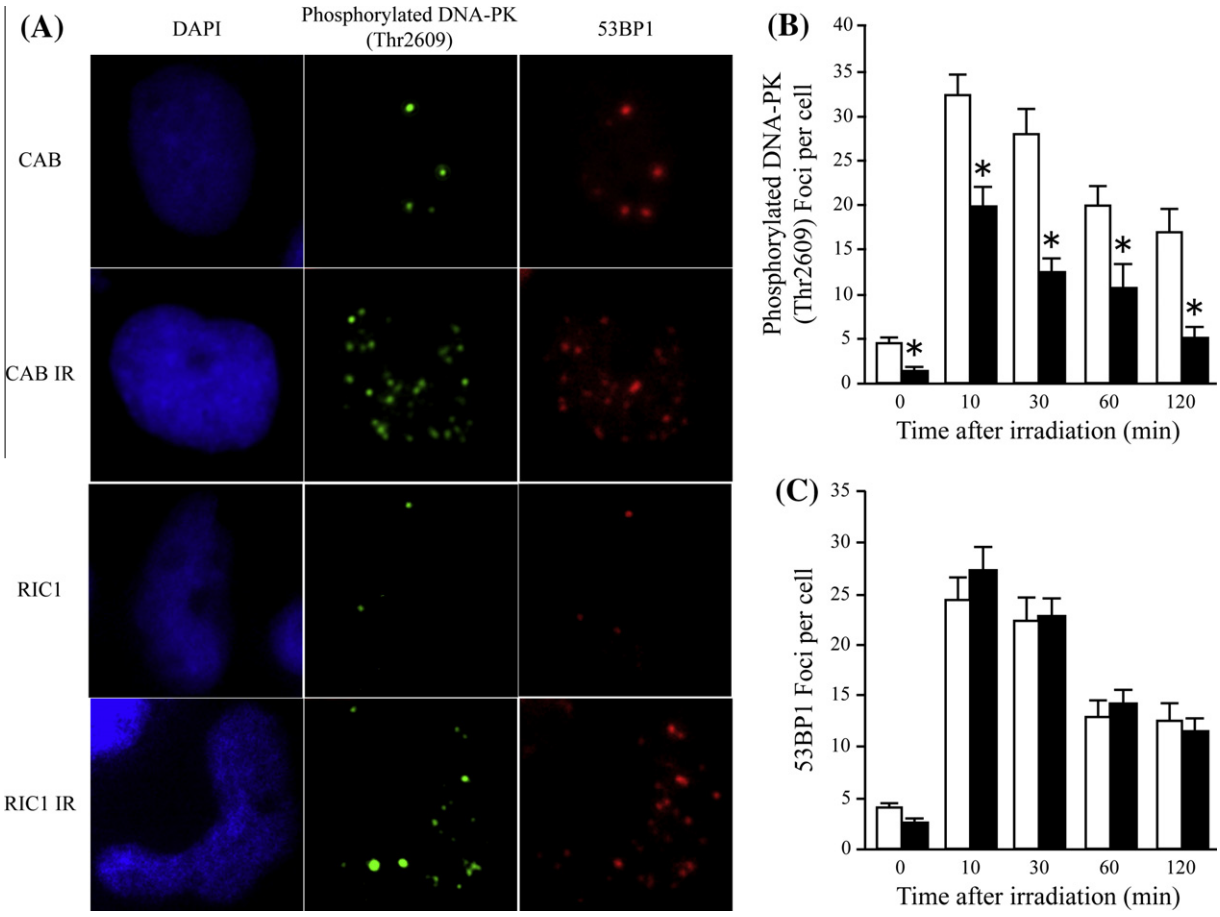

Fig. 4. Decrease in the number of autophosphorylated DNA-PK foci and no defference in the number of 53BP1 foci in RIC1 cells. CAB and RIC1 cells stably transfected with pTagFP635-c-53BP1 were 5 Gy γ-irradiated. Cells were then incubated for the indicated times and fixed. (A) Phosphorylated DNA-PKcs (Thr2609) foci and 53BP1 foci 10 min after γ-irradiation. (B, C) Average of the number of phosphorylated DNA-PK (Thr2609) foci (B) or 53BP1 foci (C) after γ-irradiation in CAB (open bars) or RIC1 cells (closed bars) ($n > 20$). Data were obtained from one representative experiment among three experiments and error bars represent standard errors of the means (SEM). Statistical analysis was performed using Student's t test (*significantly different from the number of foci in CAB cells irradiated and incubated for same time, $^*P < 0.01$).

phosphorylates H2AX and other substrates, such as main NHEJ factors (Artemis, XRCC4, and DNA ligase IV) [4]. Therefore, DNA-PK deficiency causes a decrease in NHEJ efficiency [5,6]. In this study, however, we showed that aberrant DNA-PK causes a decrease in HR efficiency in Medaka embryonic cell lines (Fig. 3C and E). The core protein components of NHEJ include the Ku heterodimer, DNA-PKcs, XRCC4 and DNA ligase IV [4,20,21]. The DNA-PKcs, Ku heterodimer are recruited immediately to DSB sites and DNA-PK phosphorylates many substrates, including DNA-PKcs itself [22]. DNA-PKcs is trans-autophosphorylated at the ABCDE cluster, which contains six close serine or threonine residues, including Thr2609, Ser2612, Thr2620, Ser2624, Thr2638, and Thr2647 [18,22,23]. In addition, it has been reported that the autophosphorylation of Thr2609 in the ABCDE cluster induces the dissociation of DNA-PK from DSB sites and other repair factors, including both NHEJ and HR repair factors, act on DSB sites [22–24]. XRCC4 and DNA ligase IV bind DNA-PK complex at the DSB sites, which results in the activation of the process for repair by NHEJ. Previous studies reported that Ku heterodimer, XRCC4 and Lig IV can form the complex at the DSB sites independently of DNA-PK activity [20,21], and the complex inhibits end resection of the DSBs which is required for the process for repair by HR [25,26]. These reports proposed a hypothesis that DNA-PK inhibition causes the retention of Ku heterodimer, DNA-PKcs, XRCC4 and DNA ligase IV complex at DSB sites, which prevents HR process by the inhibition of the end resection of DSBs. Therefore, the DNA-PK inhibition that prevents the dissociation of DNA-PK from DSB sites causes defects in both NHEJ and HR process [6,27]. However, the hypothesis has not been tested *in vivo*. RIC1 cells showed low level of DNA-PK activity and decrease in the number of phosphorylated DNA-PKcs (Thr2609) foci after γ-irradiation compared with CAB cells (Fig. 2 and 4B). Furthermore, we previously reported that RIC1 showed delayed DSB repair in pre-early gastrula cells and embryonic cell lines [12,13]. These results suggest that aberrant DNA-PK prevents DNA-PKcs autophosphorylation at Thr2609, which presumably induces the retention of the DNA-PK complex at DSB sites and inhibits both of the NHEJ and HR processes. 53BP1 is involved in classical NHEJ. Therefore, we predicted that the inhibition of NHEJ process suppresses 53BP1 foci formation. However RIC1 cells showed a slight increase in the number of 53BP1 foci after DSB compared with CAB cells (Fig. 4C). In addition, RIC1 cells showed a slight increase in NHEJ efficiency (Fig. 3D). Several studies reported that 53BP1 is involved with alternative NHEJ under the condition of DNA-PK dysfunction [18,19]. Therefore, we suggest that aberrant DNA-PK not inhibit alternative NHEJ which is a complementary mechanism to classical NHEJ and HR.

It is known that DNA-PK binds and activates many DSB repair factors. Therefore, the regulation of DSB repair mechanism by DNA-PK and direct (or indirect) involvement of DNA-PK in HR repair pathway is unclear yet. In this study, we showed that a low level of DNA-PK activity causes decreased H2AX phosphorylation and HR efficiency in fish cells. These results are comparable to previous reports in mammals. Furthermore, our findings suggested that DNA-PK independent NHEJ repairs DSBs under the condition

of decreased DNA-PK activity, which causes reduction of HR efficiency. Therefore, further analysis of the dynamics of DSB repair factors using Medaka mutants may reveal an *in vivo* molecular mechanism for the regulation of classical NHEJ, alternative NHEJ and HR by DNA-PK.

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