Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.4, No.1, 1-7, 2017

Original Article

Simultaneous depletion of WRNIP1 and RAD52 restores resistance to oxidative stress

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(Received January 19, 2017; Accepted January 21, 2017)

ABSTRACT — WRNIP1 was originally identified as Werner (WRN) helicase interacting protein in a yeast two-hybrid screen. The WRN helicase, which is mutated in the progeroid disorder Werner syndrome, has been implicated in homologous recombination, DNA repair, and other DNA transactions. WRN also interacts with RAD52, which is conserved from yeast to human and may function in homologous recombination under certain conditions. In this study, we addressed the physical and functional relationship between WRNIP1 and RAD52. WRNIP1 and RAD52 formed a complex in cells. *WRNIP1* and *RAD52* double gene knockout cells grew slightly slower than each of the single gene knockout cell lines and wild-type cells, indicating that WRNIP1 and RAD52 are required to maintain the normal rate of cell growth. Interestingly, the sensitivity to hydrogen peroxide of each single gene knockout cell line was suppressed in double gene knockout cells, suggesting that a new pathway dealing with oxidative stress-induced DNA lesions operates in the absence of both WRNIP1 and RAD52.

Key words: WRN, WRNIP1, RAD52, H_2O_2

INTRODUCTION

Werner syndrome (WS) is an autosomal recessive disease characterized by premature aging. At an early age, WS patients exhibit features associated with human physiological aging. The affected gene, *WRN,* encodes a 1432 amino acid protein, which belongs to the RecQ family of helicases (Gray *et al*., 1997). WRN protein possesses both helicase and exonuclease activities, and WS is associated with defects in these activities (Huang *et al*., 2006). WRN plays roles in cellular DNA metabolic pathways, including DNA replication, repair, and recombination, as well as telomere maintenance (Bohr, 2005). WRN interacts with many proteins that are involved in DNA replication, repair, and recombination such as DNA polymerase δ, Ku70, and RAD52 (Kamath-Loeb *et al*., 2000; Li *et al*., 2004; Baynton *et al*., 2003). In addition to these proteins, we identified Werner helicase-interacting protein 1 (WRNIP1) as an interacting partner of WRN (Kawabe *et al*., 2001).

DNA double-strand breaks (DSBs) are DNA lesions induced by exogenous and endogenous sources, such as ultraviolet light, γ rays, reactive oxygen species (ROS), and metabolites, in a DNA replication-dependent or -independent manner. DSBs are serious threats to cell viability and genome stability if they are unrepaired or repaired incorrectly. There are two main mechanisms to repair DSBs: nonhomologous end joining and homologous recombination (HR) (Chapman *et al*., 2012; Ceccaldi *et al*., 2016). WRN interacts *in vitro* and *in vivo* with RAD52, which is thought to be involved in HR (Baynton *et al*., 2003). RAD52 modulates WRN helicase activity in a DNA structure-dependent manner, and WRN stimulates RAD52 strand-annealing activity (Baynton *et al*., 2003). Therefore, WRN and RAD52 might cooperatively facilitate rescue of stalled or blocked DNA replication forks.

Rad52 plays a crucial role in HR and HR-mediated DNA repair in yeast, and consequently a deficiency of Rad52 causes severe recombination and repair defect phenotypes (Symington *et al*., 2002; Krogh and Symington, 2004; Hanamshet *et al*., 2016). By contrast, a deficiency

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of RAD52 only slightly affects HR in vertebrates (Rijkers *et al*., 1998; Yamaguchi-Iwai *et al.*, 1998). The difference in the importance of Rad52 between yeast and vertebrates may be due to the presence of proteins that possess functions similar to those of yeast Rad52 in vertebrates. Yeast Rad52 mediates the formation of Rad51 nucleoprotein filaments involved in homology search and strand pairing in HR. In higher eukaryotic cells, BRCA2 is the prominent mediator of Rad51 nucleoprotein filaments (Prakash *et al*., 2015). However, the conservation of Rad52 from yeast to humans indicates its importance in certain conditions. Recent studies demonstrate that depletion of human RAD52 is synthetically lethal with BRCA2 deficiency (Feng *et al*., 2011; Lok *et al*., 2013), suggesting that RAD52 plays a back-up role when the major RAD51 pathway is disabled.

WRNIP1 was originally identified as an interacting partner of WRN, has homology to replication factor C family proteins, and is conserved from yeasts to humans (Kawabe *et al*., 2001). WRNIP1 and the yeast homologue (Mgs1) interact with proteins of the DNA replication machinery, such as PCNA and DNA polymerase δ, to repair or prevent DNA lesions during DNA replication or in post-replication repair (Kanu *et al*., 2015; Vijeh Motlagh *et al*., 2006; Saugar *et al*., 2012; Tsurimoto *et al*., 2005). Recently, it was reported that WRNIP1 works in conjunction with RAD51 in response to replication stress (Leuzzi *et al*., 2016). HR proteins, such as BRCA2 and RAD51, are essential components of a mechanism responsible for defense against replication stress, and WRNIP1 protects replication forks from degradation by promoting RAD51 stabilization on single stranded DNA (Leuzzi *et al*., 2016).

Taking into account the interactions of WRN with WRNIP1 and RAD52, we supposed that WRNIP1 and RAD52 form a complex. In the present study, we confirmed the physical interaction of WRNIP1 with RAD52. In addition, to clarify the functional relationship between WRNIP1 and RAD52 in higher eukaryotic cells, we constructed *WRNIP1/RAD52* double gene knockout cells (*WRNIP1*$^{-/-/-}$*/RAD52*$^{-/-}$ + c*WRNIP1* cells) in which *WRNIP1* gene expression could be switched off by doxycycline (Dox) treatment. RAD52-depleted cells reportedly show increased sensitivity to oxidative stress (de Souza-Pinto *et al*., 2009); therefore, we examined sensitivity to hydrogen peroxide (H_2O_2) of the double gene knockout cells as well as that of each single gene knockout cell line. *RAD52*$^{-/-}$ and *WRNIP1*$^{-/-/-}$ cells were more sensitive to H_2O_2 than wild-type (WT) cells. Unexpectedly, the H_2O_2 sensitivity of double gene knockout cells was similar to that of WT cells, suggesting that a new pathway to deal with oxidative stress-induced DNA lesions operates in the absence of both WRNIP1 and RAD52.

MATERIAL AND METHODS

Immunoprecipitation

Human 293 cells were cultured in DMEM supplemented with 10% fetal bovine serum. Cells were transfected with a construct encoding FLAG-tagged human WRNIP1 using Lipofectamine 3000 Reagent (ThermoFisher Scientific, MA, USA). Cell lysates were prepared with lysis buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, and a protease inhibitor cocktail (EDTA-free) (Roche Diagnostics, Basel, Switzerland). The lysate was collected by centrifugation and incubated with IgG-Sepharose (GE healthcare UK Ltd., Bucks, England) at 4°C for 30 min. After centrifugation, the supernatant was incubated with anti-FLAG agarose beads (Sigma Aldrich, MO, USA) at 4°C for 1 hr. The beads were washed five times with lysis buffer, resuspended in SDS sample buffer (50 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 5% β-mercaptoethanol), and boiled for 3 min. Proteins were separated by SDS-PAGE and detected by western blotting using an anti-FLAG antibody (Sigma Aldrich, MO, USA) or an anti-RAD52 antibody (Santa Cruz Biotechnology, Texas, USA).

Gene knockout DT40 cells and cell culture

WRNIP1$^{-/-/-}$ and *RAD52*$^{-/-}$ cells were generated from chicken DT40 cells as described previously (Yamaguchi-Iwai *et al*., 1998; Yoshimura *et al*., 2014). Cells were cultured in RPMI 1640 supplemented with 100 μg/mL kanamycin, 10% fetal bovine serum, and 1% chicken serum at 39°C in the presence or absence of 1 μg/mL Doxycycline (Dox).

Generation of *WRNIP1*$^{-/-/-}$*/RAD52*$^{-/-}$ + c*WRNIP1* cells

RAD52$^{-/-}$ cells were transfected with linearized *WRNIP1*-Bleo and then with *WRNIP1*-Puro to disrupt the first and second alleles of *WRNIP1* by electroporation using a Gene Pulser apparatus (Bio-Rad, CA, USA) at 550 V and 25 mF. Subsequently, pUHG10-3 with tet-repressible promoter carrying HA-tagged chicken *WRNIP1* (c*WRNIP1*) was transfected into isolated *WRNIP1*$^{-/-/+}$*/RAD52*$^{-/-}$ cells to generate *WRNIP1*$^{-/-/+}$*/RAD52*$^{-/-}$ + c*WRNIP1* cells. Finally, the third allele of *WRNIP1* was disrupted by transfection of linearized *WRNIP1*-Ecogpt. Colonies were selected in 96-well plates with medium containing the corresponding drug. Gene disruption was verified by genomic PCR and RT-PCR. RNA were pre-

pared from *WRNIP1*$^{-/-/+}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells cultured in the presence or absence of Dox for 24 hr. cDNA were prepared by RT-PCR and electrophoresed in a 2% agarose gel.

Western blot analysis

Cells were cultured in the presence or absence of Dox for the indicated durations. Cells (1×10^6) were suspended in SDS sample buffer. Samples were separated by SDS-PAGE and transferred to PVDF membrane (Bio-Rad, CA, USA). HA-tagged WRNIP1 was detected using an anti-HA primary antibody (Roche Diagnostics, Basel, Switzerland). Bands were visualized using ECL Prime detection reagents (GE healthcare UK Ltd., Bucks, England).

Growth curve

Cells (1×10^5) were inoculated and cultured at 39°C for the specified durations. They were enumerated and the growth rates were estimated.

Measurement of sensitivities to DNA-damaging agents

Cells were cultured in the presence or absence of Dox for 48 hr, suspended in medium containing 100 μM H_2O_2 or 2 μM cisplatin in the presence or absence of Dox, and cultured for 48 hr. In the case of ultraviolet (UV) irradiation, cells treated with or without Dox were suspended in 2 mL of phosphate-buffered saline, inoculated in 6-well plates, and irradiated with 4 J/m^2 UV. UV-irradiated cells were suspended in medium containing or lacking Dox and cultured for 48 hr. To determine sensitivities to the DNA-damaging agents, cells were treated with propidium iodide for 5 min and the ratio of dead cells to live cells was determined using a Tali image-based cytometer (ThermoFisher Scientific, MA, USA).

RESULTS AND DISCUSSION

WRNIP1 interacts with RAD52 in cells

The interactions of WRN with RAD52 and WRNIP1 have been confirmed previously; therefore, we examined whether WRNIP1 interacts with RAD52 in cells. FLAG-tagged WRNIP1 was expressed in 293 cells and immunoprecipitation experiments were performed with an anti-FLAG antibody. RAD52 was co-precipitated with WRNIP1 (Fig. 1). This result shows that a portion of WRNIP1 and RAD52 form a complex in cells.

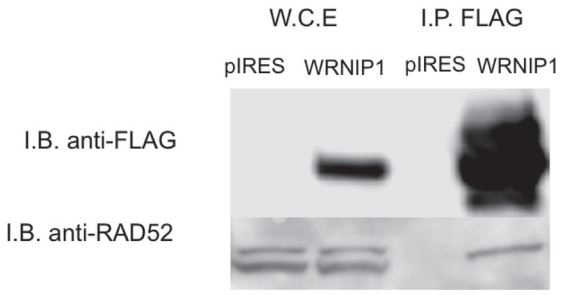

Fig. 1. Physical interaction of WRNIP1 with RAD52. Western blot analysis of immunoprecipitates using an anti-FLAG antibody. Lysates of 293 cells transfected with the FLAG-tagged WRNIP1/pIRES neo3 or pIRES neo3 vector were immunoprecipitated with an anti-FLAG antibody, followed by immunoblotting with anti-FLAG and anti-RAD52 antibodies. I.B., immunoblotting; I.P., immunoprecipitation; W.C.E., whole cell extract.

Generation of *WRNIP1/RAD52* double gene knockout DT40 cells

To investigate the functional relationship between WRNIP1 and RAD52 in cells, we attempted to generate *WRNIP1/RAD52* double gene knockout chicken DT40 cells. Because the *WRNIP1* gene resides on chromosome 2, which is trisomic in chicken DT40 cells, we disrupted each of the three *WRNIP1* alleles in *RAD52*$^{-/-}$ cells. This attempt to generate *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ cells failed. We therefore suspected that *WRNIP1/RAD52* double gene knockout causes synthetic lethality and subsequently generated *WRNIP1/RAD52* double gene conditional knockout cells. After disruption of two of the three *WRNIP1* genes, a construct containing the sequence of HA-tagged c*WRNIP1*, whose expression was under the control of a tetracycline-repressible promoter, was transfected into cells and then the third allele was disrupted to generate *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells. Upon addition of Dox to the culture of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells, disruption of the *WRNIP1* genes was confirmed by RT-PCR (Fig. 2A). cWRNIP1-HA protein in *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells disappeared within 2 days after addition of Dox (Fig. 2B). Thus, we refer to *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells cultured in the presence of Dox for 2 days as *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ cells.

To examine the effect of WRNIP1 and RAD52 depletion on cell growth, we monitored the growth of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells in the presence or absence of Dox. Although the growth rate of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells was not significantly different from those of WT and *RAD52*$^{-/-}$ cells in the absence of Dox, *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells grew slight-

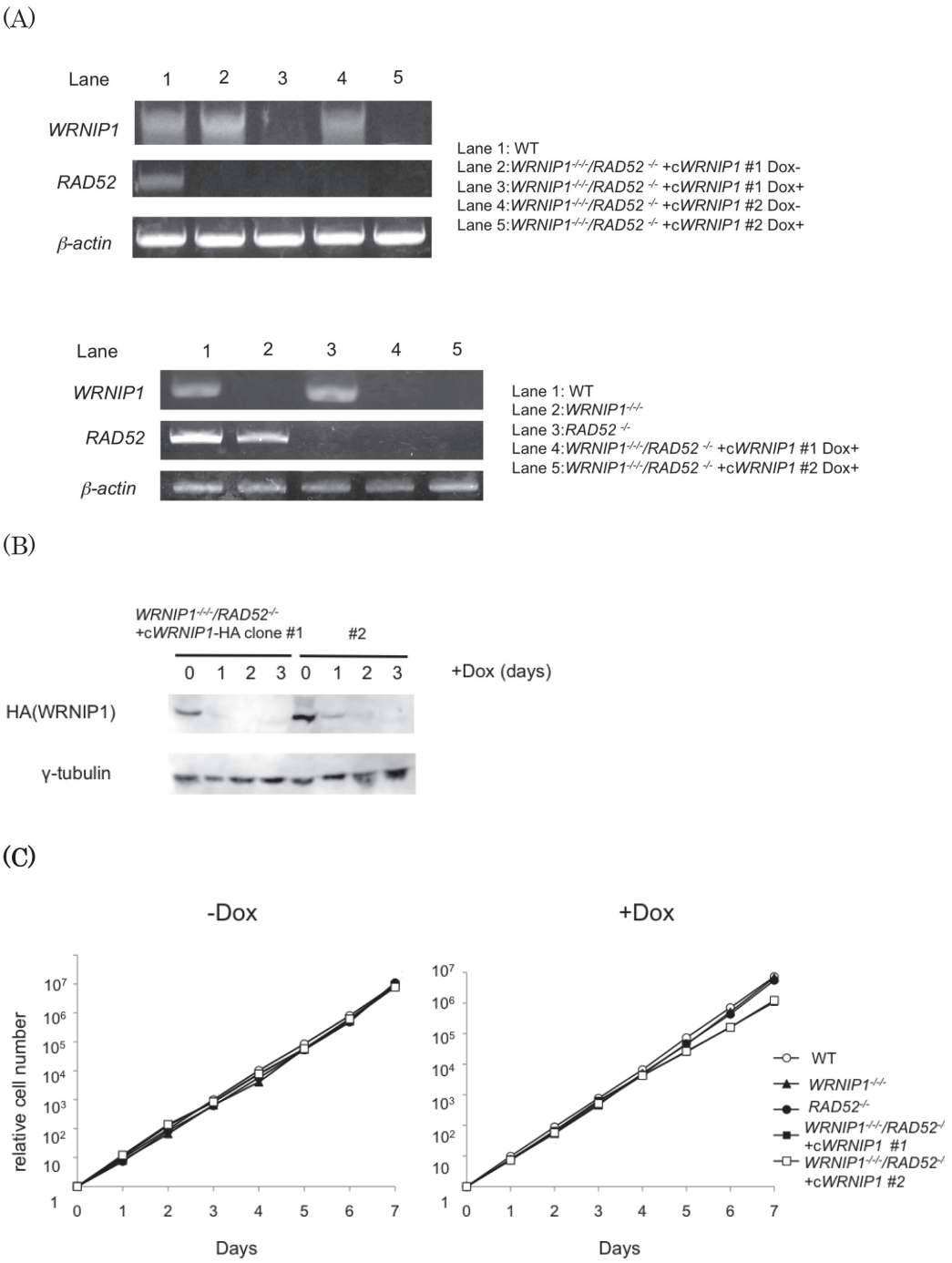

Fig. 2. Generation of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells. (A) Confirmation of gene disruption in *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells by RT-PCR. Upper panel, RNA was prepared from WT and *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells cultured in the presence or absence of Dox for 48 hr. *cWRNIP1* cDNA was prepared by RT-PCR and electrophoresed. β-actin was used as the loading control. Lower panel, RNA was prepared from WT, *WRNIP1*$^{-/-/-}$, *RAD52*$^{-/-}$, and *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells cultured in the presence of Dox and processed as described above. (B) Disappearance of cWRNIP1 protein upon addition of Dox. *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells were cultured in the presence of Dox for the indicated durations and then whole cell lysates were prepared. cWRNIP1 and γ-tubulin (loading control) were detected by western blotting. (C) Growth curves. Cells (1×10^5) were inoculated in 1 ml of medium and cultured for the indicated durations in the presence or absence of Dox.

ly slower than either of the single mutant cell lines and WT cells in the presence of Dox (Fig. 2C). The independent clones (*WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* #1 and #2) showed the same growth rate. These results indicate that the double gene knockout does not cause synthetic lethality, but that both gene products are required to support the normal rate of cell growth.

To obtain insight into the slow growth phenotype of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ cells, the distribution of cells according to the cell cycle was monitored. The cell cycle distribution pattern of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ cells did not differ markedly from those of WT, *WRNIP1*$^{-/-/-}$, and *RAD52*$^{-/-}$ cells (data not shown). Moreover, the number of dead cells was not increased with *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ cells (data not shown). Thus, the deficiency of WRNIP1 or RAD52 function does not apparently cause a growth defect, whereas the deficiency of both WRNIP1 and RAD52 causes a subtle cell growth defect that does not change the cell cycle distribution or increase the number of dead cells.

Sensitivity of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ cells to DNA-damaging agents

UV irradiation generates cyclobutane pyrimidine dimers, which are bypassed by translesion DNA polymerase, Polη, during DNA replication, and WRNIP1 functions upstream of Polη upon UV irradiation (Yoshimura *et al*., 2014). *cis*-Diamminedichloroplatinum (II) (cisplatin) forms intrastrand cross-links, interstrand cross-links, DNA-protein cross-links, and monoadducts with DNA. The interstrand cross-links are repaired by a complicated mechanism involving translesion synthesis and HR (Haynes *et al*., 2015). H_2O_2 is a ROS that oxidizes bases in DNA to generate 8-oxoguanine (8-oxoG). 8-OxoG is removed by oxoguanine DNA glycosylase (OGG1), and RAD52 stimulates OGG1 incision activity (de Souza-Pinto *et al*., 2009). In addition, RAD52-depleted cells show increased sensitivity to oxidative stress. Thus, the sensitivities of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ cells to UV radiation, cisplatin, and H_2O_2 were compared with those of each single gene knockout cell line.

The sensitivities of *WRNIP1*$^{-/-/-}$ and *RAD52*$^{-/-}$ cells to UV radiation were the same as that of WT cells. *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells cultured in the presence or absence of Dox were not more sensitive to UV radiation than WT cells (Fig. 3). Next, we examined the sensitivity of these cells to cisplatin. *WRNIP1*$^{-/-/-}$, *RAD52*$^{-/-}$, and *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells cultured in the presence or absence of Dox showed a similar sensitivity to cisplatin as WT cells. These results suggest that neither WRNIP1 nor RAD52 functions in dealing with DNA cross-links.

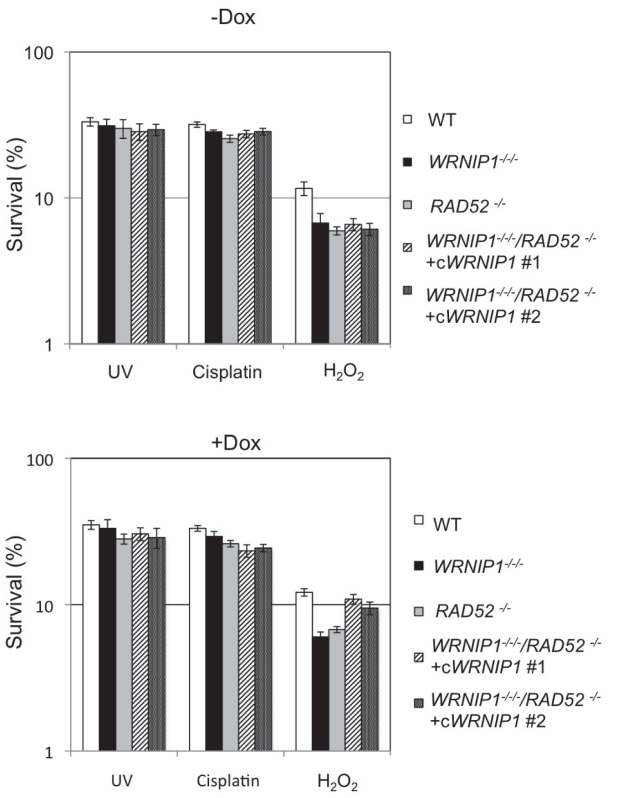

Fig. 3. Sensitivities to DNA-damaging agents. Cells were cultured in the presence or absence of Dox for 48 hr and then treated with UV radiation, cisplatin, or H_2O_2 as described in the Materials and Methods. Cells were further cultured in the presence or absence of Dox for 48 hr. The number of living cells was estimated.

In contrast with the sensitivities to UV radiation and cisplatin, *WRNIP1*$^{-/-/-}$, *RAD52*$^{-/-}$, and *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ + c*WRNIP1* cells were slightly more sensitive to H_2O_2 than WT cells (Fig. 3; upper panel). Unexpectedly, the sensitivity of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ cells to H_2O_2 was reduced to the level of WT cells (Fig. 3; lower panel). The higher sensitivity of *RAD52*$^{-/-}$ cells is consistent with the finding that depletion of RAD52 sensitizes cells to H_2O_2 (de Souza-Pinto *et al*., 2009). The H_2O_2 sensitivity of *WRNIP1*$^{-/-/-}$ cells indicates that WRNIP1 helps to deal with oxidative DNA damage. If WRNIP1 functions in a RAD52-independent pathway to deal with oxidative DNA damage induced by H_2O_2, the sensitivity of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ cells to H_2O_2 should be synergistic. However, the sensitivity of *WRNIP1*$^{-/-/-}$/*RAD52*$^{-/-}$ cells was reduced to the level of WT cells, suggesting that a new pathway operates in the absence of both WRNIP1 and RAD52.

Our previous study demonstrated that the UV sensitivity of Polη-deficient cells is suppressed by depletion of WRNIP1, suggesting that UV-induced DNA damage is processed by a Polη-independent pathway in the absence of WRNIP1 (Yoshimura *et al*., 2014). Thus, we proposed that Polδ and PrimPol might help to process UV-induced DNA damage in the absence WRNIP1 and Polη. In addition, we found that depletion of WRNIP1 increases the expression of PrimPol in cells (these data will be published elsewhere). PrimPol is a novel enzyme with dual DNA primase and DNA polymerase activities (García-Gómez *et al*., 2013; Rudd *et al*., 2013; Bianchi *et al*., 2013). PrimPol has emerged as a new type of DNA damage tolerance enzyme that possesses the flexibility to bypass replication-pausing DNA lesions such as UV-induced DNA adducts (García-Gómez *et al*., 2013; Bianchi *et al*., 2013; Mourón *et al*., 2013) and 8-oxoG via translesion synthesis (Zafar *et al*., 2014; Guilliam *et al*., 2016) and to initiate DNA synthesis downstream of the lesion. Considering the above-mentioned results together, it seems likely that PrimPol helps to deal with oxidative DNA damage in the absence of both WRNIP1 and RAD52. If this is the case, why does this pathway not function in *WRNIP1*$^{-/-}$ cells? This issue must be addressed in a future study.

ACKNOWLEDGMENTS

This study was supported by JSPS KAKENHI Grant Numbers JP26440065.

Conflict of interest---- The authors declare that there is no conflict of interest.

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