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Introduction of a Normal Human Chromosome 8 Corrects Abnormal Phenotypes of Werner Syndrome Cells Immortalized by Expressing an h*TERT* Gene

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Werner syndrome/Chromosome transfer/Functional complementation.

Werner syndrome (WS) is an autosomal recessive disease characterized by premature aging and caused by mutations of the *WRN* gene mapped at 8p12. To examine functional complementation of WS phenotypes, we introduced a normal human chromosome 8 into a strain of WS fibroblasts (WS3RGB) immortalized by expressing a human telomerase reverse transcriptase subunit (h*TERT*) gene. Here, we demonstrate that the abnormal WS phenotypes including cellular sensitivities to 4-nitroquinoline-1-oxide (4NQO) and hydroxy urea (HU), and chromosomal radiosensitivity at G_2 phase are corrected by expression of the *WRN* gene mediated by introducing a chromosome 8. This indicates that those multiple abnormal WS phenotypes are derived from a primary, but not secondary, defect in the *WRN* gene.

INTRODUCTION

Werner syndrome (WS) is a rare autosomal recessive disorder characterized by the premature onset of a number of processes associated with aging.[1,2] The *WRN* gene, defected in WS, is mapped at 8p12 and encodes a WRN protein that is consisted of 1432-amino acids.[3] The WRN protein has both 3'→5' helicase and 3'→5' exonuclease activities[4,5] and has been suggested to function in DNA replication, repair and telomere processing.[6] The precise physiological roles of the WRN protein, however, remain to be obscure. Cells derived from WS patients show the hypersensitivities to selected DNA-damaging agents including 4-nitroquinoline-1-oxide (4NQO),[7,8] topoisomerase inhibitors,[9,10] DNA cross-linking agents[11] and hydroxy urea (HU).[12] In addition, WS cells demonstrate an abnormal radiation response at G_2 phase.[13] These studies suggest that the WRN protein plays an important role in DNA metabolism including DNA repair and replication pathways.

Because WS is caused by a defect of the *WRN* gene, it is highly expected that all abnormal phenotypes observed in WS cells can be rescued by expression of the *WRN* gene. To examine this anticipation, we introduced a normal human chromosome 8 into a SV 40-immortalized WS cell line by microcell fusion and studied functional complementation of the *WRN* gene.[14] However, we failed to complement abnormal phenotypes of the WS cells such as 4NQO hypersensitivity and a high incidence of spontaneous deletion mutation[15,16] in spite of expression of the WRN protein. This result raises the question whether the cellular abnormal phenotypes observed in WS cells are due to a primary effect or a secondary effect. On the other hand, it is also possible that secondary genetic changes due to the intrinsic instability of recipient SV40-immortalized WS cells might hamper a correction of abnormal phenotypes.

In the present study, to make clear these uncertainties concerning the complementation of the WS phenotype by the expression of the *WRN* gene, we introduced a normal human chromosome 8 into a WS cell line immortalized by expressing a human telomere reverse transcriptase subunit (h*TERT*) gene, and studied sensitivities to 4NQO and HU,

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and chromosome aberrations by X-irradiation at G_2 phase in microcell hybrids introduced with a human chromosome 8. In contrast to the previous study,[14] the expression of the *WRN* gene corrected the abnormal phenotypes examined.

MATERIALS AND METHODS

Cells and cell culture

Mouse A9 cells containing a single copy of human chromosome 8, which was tagged with a blasticidin S resistance gene, were used as chromosome donors. WS3RGB/T cells derived from WS3RGB fibroblasts (42-year-old female WS patient) were immortalized by introducing the h*TERT* gene and used as recipients. Normal human fibroblast cells immortalized by the h*TERT* gene, BJ/hTERT, were used as a control. The A9 cells were cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM; Nissui Pharmaceutical, Tokyo) supplemented with 10% fetal bovine serum (FBS; Trace Bioscience, Melbourne), 100 U/ml penicillin, and 100 μg/ml streptomycin. WS3RGB/T cells and BJ/hTERT cells were cultured in α-modified minimum essential medium (α–MEM; Invitrogen, Carlsbad, CA) supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Microcell hybrid (WS3RGB/T-8) cells were cultured in the α–MEM supplemented with 1.5 μg/ml blasticidin S (Funakoshi Co., Tokyo). Cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

Microcell Fusion

Microcell fusion was performed by the procedure described previously.[14] Briefly, donor cells (~1×10^6) were inoculated in 25-cm^2 flasks, and micronuclei were induced by treatment with 50 ng/ml Colcemid in the DMEM containing 20% FBS and 3 μg/ml blasticidin S for 48 h. The flasks were filled with serum free medium (SFM) containing 10 μg/ml cytochalasin B (Sigma Chemical Co., St. Louis, MO), and then micronuclei were isolated by centrifugation at 11,000 r.p.m. for 30 min at 34°C. The crude microcells were purified by filtration through a series of polycarbonate filters with pore sizes of 8, 5, and 3 μm. The purified microcells were resuspended in SFM containing 25 μg/ml phytohemagglutinin (Sigma Chemical Co., St. Louis, MO) and attached to the recipient WS3RGB/T cells by incubation at 37°C for 15 min. The cells were treated with 3 ml of polyethylene glycol (PEG; Sigma Chemical Co., St. Louis, MO) mixed with SFM (PEG : SFM, 1 : 1.4) for 30 sec, overlaid with 3 ml of a low-concentration PEG (PEG : SFM, 1 : 3) and treated for another 40 sec. After washing with SFM three times, the cells were filled with the α–MEM containing 10% FBS. After 48 h of incubation at 37°C, the recipient cells were replated for selection in the α–MEM containing 15% FBS and 1.5 μg/ml blasticidin S for 3–4 weeks. Blasticidin S-resistant microcell hybrids were isolated and grown in the α–MEM containing 10% FBS and 1.5 μg/ml blasticidin S.

Whole chromosome painting

Exponentially growing cells were treated with Colcemid (60 ng/ml) for 2 h and harvested. Chromosome samples were prepared as described previously.[14] For whole chromosome painting, the slide was air-dried overnight and immersed in pre-treatment solution (2X SSC / 0.5% NP-40, pH 7.0) for 30 min at 37°C. Then the slide was immersed in denaturing solution (70% formamide in 2X SSC, pH 7.0) for 3 min at 72°C. After dehydration by successive treatments with 70, 85 and 100% ethanol for each 2 min, the slide was dried with an airjet. A DNA probe that was specific for chromosome 8 (Q-Biogene, Montreal) was denatured at 72°C for 10 min and applied to a pre-warmed chromosome slide (45°C). The slide was covered with the probe mixture and glass coverslip and sealed with rubber cement to avoid evaporation. The hybridization was performed at 37°C for 16 h in the humidified atmosphere. After hybridization, the coverslip was removed, and the slide was incubated in wash buffer (0.5X SSC/ 0.1% SDS) for 5 min at 65°C, rinsed in PBD buffer (Q-Biogene, Montreal) at room temperature for 5 min, and stained with 8 μl of 20 ng/ml DAPI (Q-Biogene, Montreal) in antifade. The metaphase chromosomes were observed using a fluorescence microscope (Olympus, Tokyo) and digital images were recorded using a CCD camera (Olympus).

Expression of the WRN gene and protein

Expression of the *WRN* gene and protein in microcell hybrids was examined by reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blotting, respectively, as described previously.[14,17] The relative expression of the *WRN* gene and protein was calculated from the band corresponding to the *WRN* gene and protein. The intensity of the band was adjusted by that corresponding to the *c-myc* gene for RT-PCR. For Western blotting analysis, the equal loading of protein was confirmed by Coomassie blue staining of the gels.

4NQO sensitivity

A 1 mM stock solution of 4NQO in ethanol (Wako Pure Chemical Industries, Osaka) was diluted with SFM prior to use. To examine cellular sensitivity to 4NQO, cells were plated into 100-mm dishes and cultured for 12 h. Then, the cells were washed once with SFM and treated with the SFM containing a graded concentration of 4NQO at 37°C for 1 h. After the treatment, the cells were washed once with complete culture medium, refed with the complete culture medium, and cultured for 14 days. Colonies more than 50 cells were scored as survivors.

HU sensitivity

To examine cellular sensitivity to HU, growing cells were washed once with SFM and treated with the complete culture medium containing 2 mM HU (Wako Pure Chemical Industries, Osaka) for 24 h. Then the cells were replated into 100-mm dishes and cultured with the complete culture medium for 14 days. Colonies more than 50 cells were scored as survivors.

Chromosome aberrations induced with X-rays at G_2 phase

To examine chromosome aberrations induced with X-rays at G_2 phase, exponentially growing cells were irradiated with 1 Gy of X-rays using an X-ray generator (M-150 WE; Softex, Osaka) operating at 150 kVp and 5 mA with a 0.1 mm Cu filter at a dose rate of 0.425 Gy/min, treated with Colcemid (60 ng/ml) for 2 h, and harvested. Chromosome samples were prepared as described above and stained with 5% Giemsa solution for 10 min. One hundred metaphases for each cell strain were scored for chromatid gaps and breaks.

RESULTS

Presence of an extra chromosome 8 in the microcell hybrid cells

Microcell fusion experiments were tried six times to introduce a human chromosome 8 containing a normal *WRN* gene into h*TERT*-immortalized WS3RGB/T cells. Average number of recipient cells assayed for one trial of the chromosome transfer was 2.0×10^6. We finally obtained one microcell hybrid that acquired blasticidin S resistance and designated it as WS3RGB/T-8, of which frequency for chromosome transfer was 8.3×10^{-8}. To confirm the presence of an extra copy of chromosome 8 in the microcell hybrid, we examined the copy number of chromosome 8 using whole chromosome painting by scoring 100 metaphases each in parental WS3RGB/T cells and WS3RGB/T-8 cells (Table 1 and Fig. 1). In the parental WS3RGB/T cells, all metaphases (100%) had two copies of chromosome 8; one was intact and the other was translocated to another chromosome (Fig. 1A). In contrast to this, all metaphases (100%) retained an extra copy of intact chromosome 8 in WS3RGB/T-8 cells (Fig. 1B), confirming a successful transfer of a chromosome 8.

Expression of the WRN gene and protein

To examine the expression of the *WRN* gene in WS3RGB/T-8 cells, we used RT-PCR to amplify cDNAs for the *WRN* gene and the *c-myc* gene in BJ/hTERT cells, WS3RGB/T cells and WS3RGB/T-8 cells. We adopted *c-myc* gene as a control for gene expression because its expression was relatively constant through cell cycle[18] and its gene dosage was the same as that of the *WRN* gene based on the fact that both

Table 1. Chromosome analyses in WS3RGB/T cells and WS3RGB/T-8 cells

Cell	Introduced chromosome	No. of metaphases scored	Percentage of cells with the extra chromosome 8	Morphology of the extra chromosome 8
WS3RGB/T	–	100	–	–
WS3RGB/T-8	8	100	100	Intact

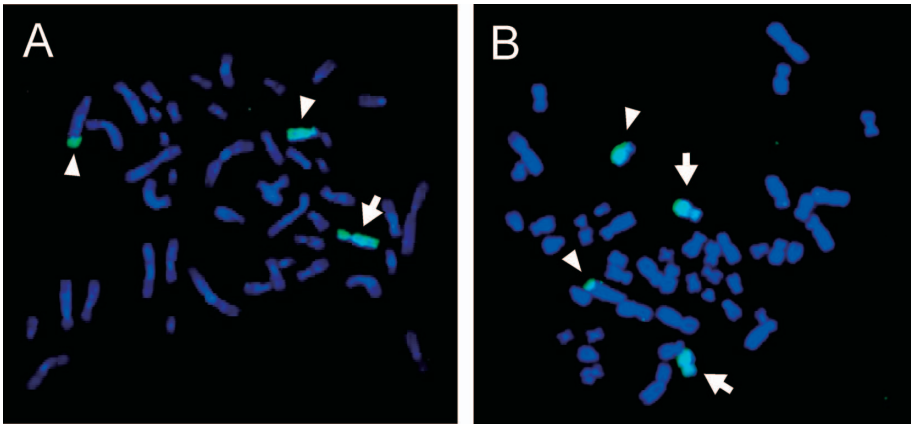

Fig. 1. The presence of an extra copy of chromosome 8 in microcell hybrid WS3RGB/T-8 cells. Metaphase spreads of (A) parental WS3RGB/T cells and (B) WS3RGB/T-8 cells are represented. The presence of an extra copy of chromosome 8 in WS3RGB/T-8 cells was confirmed by whole chromosome painting using a probe specific for chromosome 8. Arrows, an intact chromosome 8; Arrowheads, a rearranged chromosome 8.

genes located on chromosome 8. As shown in Fig. 2, the expression of the *WRN* gene in WS3RGB/T cells was very low, showing 4% level of that in the control BJ/hTERT cells (Fig. 2B), although those two types of cells expressed the *c-myc* gene at similar levels. In contrast, the expression of the *WRN* gene in WS3RGB/T-8 cells shows 48% level of that in the control cells (Fig. 2B). These results were consistent with those obtained by Western blotting, where the expression of the WRN protein was 6% and 52% level of that in the control cells in WS3RGB/T cells and in WS3RGB/T-8 cells, respectively (Fig. 3A and 3B).

Sensitivities to 4NQO and HU

To examine functional complementation of WS phenotypes by the expression of the *WRN* gene, we determined the sensitivities to 4NQO and HU in BJ/hTERT cells, WS3RGB/T cells, and WS3RGB/T-8 cells. As shown in Fig. 4, WS3RGB/T cells were more sensitive to 4NQO than the control (BJ/hTERT) cells. In contrast, the expression of the *WRN* gene conferred resistance to 4NQO in WS3RGB/T-8 (Fig. 4). Similarly, although WS3RGB/T cells were hypersensitive to 2mM HU as compared with the control cells ($p < 0.01$, Student's t-test), the expression of the *WRN* gene recovered resistant to HU ($p < 0.05$, Student's t-test) in WS3RGB/T-8 cells as shown in Fig. 5. These results indicate that the expression of *WRN* gene in the immortalized WS cell line (WS3RGB/T-8) can complement the WS phenotypes such as the hypersensitivities to 4NQO and HU.

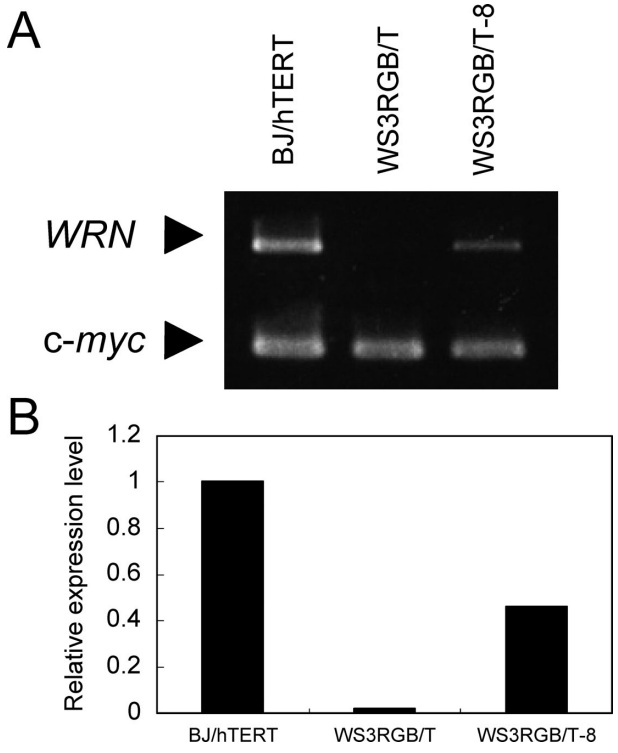

Fig. 2. Expression of the *WRN* gene in WS3RGB/T cells and WS3RGB/T-8 cells. (A) The expression levels of the *WRN* gene and the c-*myc* gene were measured by RT-PCR. Cells used were BJ/hTERT (a human control cell line), WS3RGB/T, and WS3RGB/T-8. (B) The relative expression of the *WRN* gene was determined as described in Material and Methods.

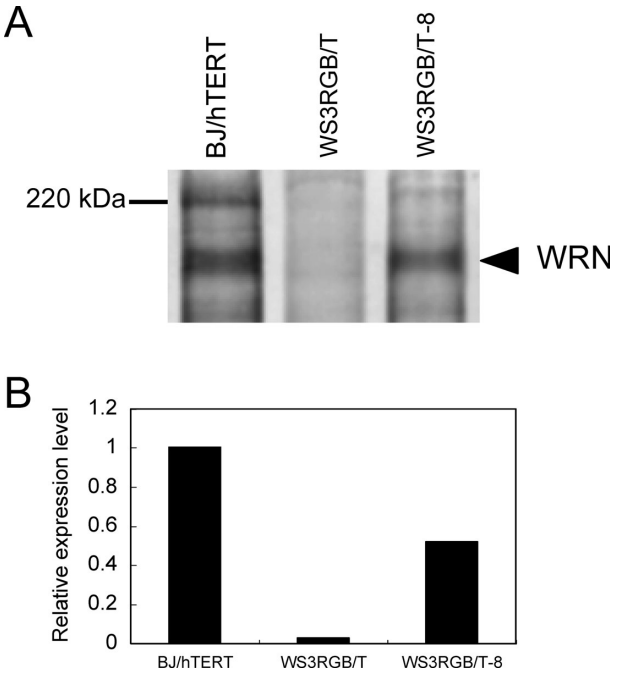

Fig. 3. Western blot analysis of the WRN protein. (A) The expression level of the WRN protein was measured by Western blotting. Cells used were BJ/hTERT, WS3RGB/T, and WS3RGB/T-8. (B) The relative expression of the WRN protein was determined as described in Material and Methods.

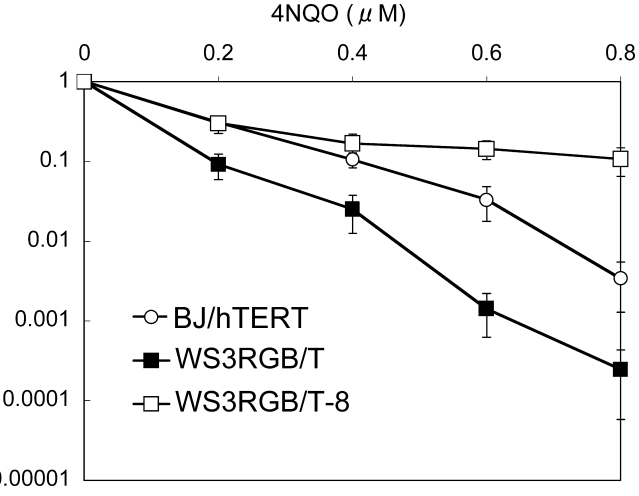

Fig. 4. Sensitivity to 4NQO assayed by colony-forming ability. Cells examined were BJ/hTERT (○), WS3RGB/T (■), and WS3RGB/T-8 (□). The cells were treated with 4NQO for 1 h and incubated for 2 weeks for colony formation.

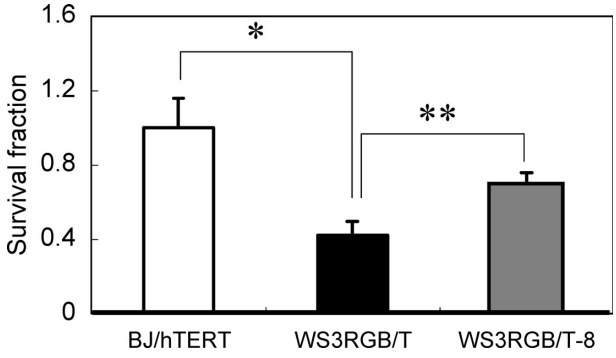

Fig. 5. Sensitivity to HU assayed by colony-forming ability. Cells examined were BJ/hTERT, WS3RGB/T, and WS3RGB/T-8. The cells were treated with 2 mM HU for 24 h and incubated for 2 weeks for colony formation. The differences are significant by Student's t-test (*$p < 0.01$, **$p < 0.05$).

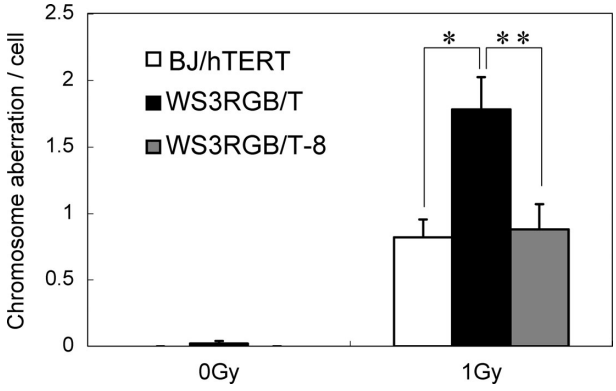

Fig. 6. Chromosome aberrations induced by X-irradiation at G_2 phase. Cells examined were BJ/hTERT (white bar), WS3RGB/T (black bar), and WS3RGB/T-8 (gray bar). The cells were irradiated with 1 Gy of X-rays and one hundred metaphases for each cell strain were scored for chromatid gaps and breaks. The differences are significant by Welch's t-test (*$p < 0.01$, **$p < 0.05$).

Chromosomal sensitivity to ionizing radiation at G_2 phase

We selected chromosomal sensitivity to ionizing radiation at G_2 phase as a WS phenotype[13] to be examined the complementation by the WRN expression. As shown in Fig. 6, WS3RGB/T cells showed a higher frequency of chromosome aberrations than the control cells after exposure to 1 Gy of X-rays at G_2 phase ($p < 0.01$, Welch's t-test). In contrast to this, the frequency of chromosome aberrations in WS3RGB/T-8 cells was reduced to the level as low as that of the control cells ($p < 0.05$, Welch's t-test). The result indicates that the expression of the *WRN* gene can correct the abnormal chromosomal sensitivity to ionizing radiation at G_2 phase observed in WS cells.

DISCUSSION

We demonstrated that the expression of the WRN protein corrects the abnormal WS phenotypes in an immortalized WS cell line. The corrected WS phenotypes are cellular sensitivities to 4NQO[7,8] and HU,[12] and chromosomal radiosensitivity at G_2 phase. This is contrast to the results reported in our previous study,[14] where we failed to complement the abnormal WS phenotypes such as the hypersensitivity to 4NQO and high proportion of deletion mutations at *HPRT* locus by the expression of the *WRN* gene. However, in the present study, we could not determine the mutation types at *HPRT* locus for confirming that spontaneous deletion mutations occur in a high proportion in WS cells, which was well characterized and one of the most interesting cellular phenotype of WS cells.[14–16] Because WS3RGB/T cells used in the present study were female origin in contrast to WS780 cells, which were male origin and used in the previous study, the detection of mutation at *HPRT* locus was not possible in WS3RGB/T cells.

In both previous and present studies, we adopted the same chromosome transfer method to complement the defect of WS phenotypes, but the recipient WS cells were different between two studies. Whereas we used a SV40-immortalized WS cell line (WS780) in the previous study, we adopted an h*TERT*-immortalized WS cell line (WS3RGB/T) in the present study. We do not have a clear answer to explain the reason why we could not correct the WS phenotypes in the SV40-immortalized WS cells in the previous study. A possible answer is that the SV40-immortalized cells are genetically unstable as compared with those immortalized by the h*TERT* gene so that multiple mutations are accumulated during the establishment of cell line. These secondary mutations might be involved in the failure of complementing the WS phenotypes. Therefore, it is not recommendable to use SV40-immortalized human cells to examine the complementation of mutant phenotypes because secondary mutations that might be accumulated in those cells may hamper the function of gene product that is asked. In contrast, as demonstrated in the present study, h*TERT*-immortalized human cells are more stable than SV40-immortalized human cells and useful for examining the complementation test.

In terms of the sensitivities to 4NQO and HU, it turned out in the present study that the WRN function is primarily, but not secondarily, involved in the recovery of resistance to 4NQO and HU. 4NQO induces a spectrum of DNA and chromatin lesions including oxidative DNA damage, single-strand breaks, base intercalations, adducts, and cross-links. There has been no direct evidence to indicate that the WRN protein plays a critical role in the repair process of those lesions. However, evidence has been accumulated to show that WS cells are hypersensitive to DNA cross-linking agents such as mitomycin C (MMC) and *cis*-platinum (II)

diamine dichloride (CDDP),[11] suggesting that the WRN protein might participate in the resolution of DNA cross-link damage induced by 4NQO. Another possibility of the 4NQO hypersensitivity in WS cells is due to a defective response to oxidative damage induced by 4NQO because a recent study[19] indicates that knockdown of the WRN expression using RNA interference (RNAi) in primary human fibroblast cells accelerates cellular senescence and accumulation of DNA double-strand breaks caused by oxidative stress. Furthermore, *in vivo* oxidative stress has been shown to be elevated in both WS patients and mutant mice of the mouse *WRN* gene.[20–22] These studies implicate a significant role of the WRN protein in dealing with DNA damage delivered by oxidative stress.

Several lines of evidence suggest a role of the WRN protein in DNA replication. For example, WS cells show extended S-phase[23] and reduced frequency of replication initiation sites compared with normal cells.[24,25] In addition, the WRN protein interacts with RPA protein that is required for replication,[26] and co-localizes with RPA upon replication fork arrest after HU treatment.[27] These results, together with a report demonstrating the requirement of the WRN protein for correct recovery after replication arrest,[12] suggest a significant role of the WRN protein in DNA replication. The evidence that the HU hypersensitivity in WS cells can be corrected by the expression of the WRN protein (Fig. 5) indicates that this abnormal WS phenotype is also caused primarily by a defect of the WRN function.

Although WS has not been categorized as a DNA repair-deficient genetic disease, there has been some reports to suggest that the WRN protein participates in repairing radiation-induced DNA damage. For example, we previously demonstrated that exogenous expression of exonuclease domain-deleted WRN protein in human 293 cells increased radiosensitivity in terms of cell survival and chromosome aberrations,[17] suggesting that a function of WRN exonuclease activity is essential for the repair of radiation-induced DNA damage. Also, Yannone *et al.* demonstrated that WS cells immortalized by the h*TERT* gene were radiosensitive and that this hypersensitivity was complemented by introduction of the *WRN* gene.[28] In addition to these, WS cells were characterized by an abnormal radiation response at G_2 phase.[13] These findings are consistent with our present result that the frequency of X-ray-induced chromosome aberrations at G_2 phase in WS3RGB/T cells was significantly higher than that of the control cells (Fig. 6), suggesting that DNA damage response induced by ionizing radiation at G_2 phase is defected in WS3RGB/T cells. The evidence that chromosomal radiosensitivity is completely normalized in WS3RGB/T-8 suggests that normal DNA damage response at G_2 phase requires the WRN function.

It should be noticed that the abnormal phenotypes in the 4NQO sensitivity (Fig. 4) and the chromosomal radiosensitivity at G_2 phase (Fig. 6) in WS3RGB/T cells are fully recovered in WS3RGB/T-8 cells even though the expression level of the WRN protein is almost half compared with that of the control cells as shown in Fig. 3. This implies that a half expression level of the WRN protein is enough to recover from the phenotypic defects observed in WS cells. The result is consistent with the fact that WS heterozygotes are phenotipically indistinguishable from normal individuals.

In summary, the present study demonstrated that the expression of the *WRN* gene complements abnormal WS phenotypes, i.e., the hypersensitivities to some types of DNA or chromatin lesion and DNA replication arrest, and also the abnormal response to ionizing radiation at G_2 phase, in h*TERT*-immortalized WS cells. This indicates that those multiple abnormal phenotypes are derived from a primary, but not secondary, defect in the *WRN* gene. In addition, the present study clearly indicates that h*TERT*-immortalized human cells are better than SV40-immortalized human cells to use in the complementation study.

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