Original Article

Persistence of red blood cells with *Pig-a* mutation in *p53* knockout mice exposed to X-irradiation

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ABSTRACT — The *Pig-a* mutation assay is becoming one of the major experimental procedures used to assess *in vivo* genotoxicity. The assay allows simple *in vivo* analysis and enables gene mutations in the hematopoietic system to be measured using high throughput flow cytometry. Previously, we demonstrated that X-irradiation increased the *Pig-a* mutant frequencies in red blood cells (RBCs) of mice in a radiation dose-dependent manner. In this study, to understand how RBCs with *Pig-a* mutation induced by X-irradiation persist, we compared *Pig-a* mutant frequencies between irradiated C57BL/6J (*p53*^{+/+}) mice and irradiated *p53* homozygous knockout (*p53*^{-/-}) mice by using the RBC *Pig-a* assay. After the peak in radiation-induced *Pig-a* mutant frequencies, a gradual decrease in mutant frequencies in irradiated *p53*^{-/-} mice was observed, while irradiated *p53*^{+/+} mice had a rapid decrease, which suggests that RBCs with *Pig-a* mutation are eliminated normally in irradiated *p53*^{+/+} mice but not in irradiated *p53*^{-/-} mice due to lack of p53 function. In addition, we also found that the p53 function affected the regulation of *Pig-a* mutagenesis in aging mice. Our results suggest that p53 function, distinct types of mutation, and the life span of RBCs play key roles in the persistence of *Pig-a* mutation in the hematopoietic system of RBCs after irradiation.

Key words: *Pig-a* mutation, Flow cytometry, Red blood cells, X-irradiation, *p53* knockout mice

INTRODUCTION

Recently, the assay used to examine mutation in the phosphatidylinositol glycan-class A gene (*Pig-a*) has emerged as one of the major assays to evaluate *in vivo* genotoxicity of chemical exposures. The *Pig-a* gene-mutation assay is used to screen for mutagenicity *in vivo* and allows simple temporal analyses. *Pig-a* mutation can be measured rapidly using small samples of peripheral blood and flow cytometry, which accounts for the popularity of this assay (Dobrovolsky *et al.*, 2010). *Pig-a* encodes a protein involved in the synthesis of glycosylphosphatidylinositol (GPI) anchors that link a specific subset of proteins to the cell surface, including the antigens CD59, CD55, CD24, and CD48 (Kawagoe *et al.*, 1994; Hernandez-Campo *et al.*, 2007).

The *Pig-a* mutation assay has mainly been used to assess the mutagenicity of chemicals known to be powerful point mutagens. In a previous study, we developed our *Pig-a* assay based on Kimoto's *Pig-a* gene-mutation assay

(Kimoto *et al.*, 2011, 2012) using mouse red blood cells (RBCs) stained with 3 markers, including a fluorescently labeled anti-CD24 antibody, an anti-TER-119 antibody, and an anti-CD71 antibody (Ohtani *et al.*, 2012). It has become a useful method for detecting the *in vivo* genotoxic effects of ionizing radiation on impaired hematopoietic stem cells. Interestingly, we found that *Pig-a* mutant frequencies in irradiated mice showed less persistence than in mice treated with N-ethyl-N-nitrosourea (ENU) (Ohtani *et al.*, 2012). In addition, the *Pig-a* mutant frequency in rats treated with ENU remain higher levels (Miura *et al.*, 2009; Phonethepswath *et al.*, 2010), whereas the frequency in irradiated mice returned to background levels during 6-7-week after irradiation (Ohtani *et al.*, 2012).

p53 homozygous knockout (*p53*^{-/-}) mice are potentially more sensitive to genotoxic effects produced by strong clastogens and mutagens. The p53 protein, a tumor suppressor, plays crucial roles in activating the cell cycle checkpoint (Agarwal *et al.*, 1995) and DNA repair (Kastan *et al.*, 1991), and in inducing apoptosis (Lowe

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et al., 1993) in multicellular organisms. Therefore, we hypothesized that the frequency of *Pig-a* mutations in RBCs derived from irradiated $p53^{-/-}$ mice would be higher than in RBCs from irradiated $p53^{+/+}$ mice, and we also expected that there should be some disparities in the persistence of *Pig-a* mutant frequencies between irradiated $p53^{-/-}$ and $p53^{+/+}$ mice because of abnormalities of elimination in hematopoietic stem cells, erythroid progenitor cells, and/or erythroblast caused by the lack of p53 protein. Previously, we observed that *Pig-a* mutant frequencies in irradiated $p53^{+/+}$ mice showed radiation dose-dependent responses from Day 14 to Day 21 after irradiation (Ohtani *et al.*, 2012), and that mutant frequencies of *T cell receptor* (*TCR*), which belongs to the hematopoietic cell system, appeared a slight decline after peaking following γ -irradiation of $p53^{-/-}$ mice (Kato *et al.*, 2002). Therefore, in irradiated $p53^{-/-}$ mice, the persistence of RBCs with *Pig-a* mutation would decline gradually, much like with the persistence of *TCR* mutant frequencies in the study by Kato *et al.* (2002).

It has been reported that *TCR* mutant frequencies were re-elevated earlier in heterozygous $p53$ knockout ($p53^{+/+}$) mice at about 44 weeks when compared to wild-type mice, resulting in a late effect (Igari *et al.*, 2006). The differences between $p53^{-/-}$ and wild type mice were caused by presence or absence of apoptotic activity. These findings on the late effect raised the possibility that radiation exposure may also increase the accumulation of natural *Pig-a* mutation in the hematopoietic cell system. Therefore, long-term changes in *Pig-a* mutant frequency should be measured, in order to identify age-related effects of ionizing radiation on *Pig-a* mutation *in vivo*.

In the present study, to understand how RBCs with *Pig-a* mutations are processed in the hematopoietic cell system in response to ionizing radiation, we analyzed the persistence of RBCs in irradiated $p53^{+/+}$ (control) and $p53^{-/-}$ mice by using our established RBC *Pig-a* assay. We also investigated the late-effects of ionizing radiation on *Pig-a* mutations in the hematopoietic system of $p53^{-/-}$ and control mice to clarify the link between aging and *Pig-a* gene-mutagenesis.

MATERIALS AND METHODS

Animals

All experimental procedures involving animals were approved by the committee for animal experimentation at the National Institute of Public Health (Wako, Saitama, Japan). The $p53^{-/-}$ mice, which were obtained from the University of Occupational and Environmental Health (Kitakyushu, Fukuoka, Japan), had a genetic background

almost identical to that of C57BL/6 mice. The knockout mice were mated and 7-week-old male $p53^{-/-}$ mice were used in this study. The 6-week-old male C57BL/6J ($p53^{+/+}$) mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were housed in an air-conditioned animal room with free of mouse-specific pathogens on a 12-hr light/12-hr dark cycle. Temperature and relative humidity were maintained at $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$ and approximately 55%, respectively.

X-ray irradiation

Mice were placed in an acrylic exposure chamber and exposed without anesthesia to X-rays generated by a Faxitron CP-160 X-ray apparatus (Faxitron Bioptics LLC; Tucson, AZ, USA). X-irradiation was conducted at 160 kVp with 0.5-mm Cu and 0.5-mm Al filters. The dose rates were set at 0.52 Gy/min. The $p53^{+/+}$ and $p53^{-/-}$ mice were divided into two groups as follows: eight each $p53^{+/+}$ and $p53^{-/-}$ mice received whole-body irradiation of 1 Gy; and seven $p53^{+/+}$ and eight $p53^{-/-}$ mice were not irradiated and were used as controls for each exposure group. One of the eight $p53^{+/+}$ control mice showed abnormal growth and was excluded from analysis.

Blood sample collection

Blood was collected from each experimental group ($n = 7$ or 8 mice/group) on Days 0, 2, 7, 14, 21, 28, 35, 42, 49, 56, 63, 77, 91, 112, 133, 225, 323, 413, and 713 after radiation exposure (with Day 0 being the exposure day). For the last 4 blood collections (on Days 225, 323, 413, and 713), only $p53^{+/+}$ mice were used because almost all $p53^{-/-}$ mice died before Day 225. Blood (14 μl) was withdrawn from the tail vein of mice and mixed with 10 μl of 50 mM ethylenediaminetetraacetic acid (EDTA).

Flow cytometric analysis

The *Pig-a* mutation assay was based on our previous procedure (Ohtani *et al.*, 2012). Briefly, the following antibodies were added to 9 μl of the blood-EDTA mixtures from each mouse: 2 μg of fluorescein isothiocyanate-conjugated anti-mouse CD24 antibody (anti-CD24-FITC); 0.5 μg phycoerythrin-conjugated anti-mouse CD71 antibody (anti-CD71-PE); and 1 μg of phycoerythrin-cyanine-5-conjugated anti-mouse TER-119 antibody (anti-TER-119-PE-Cy5) (eBioscience; San Diego, CA, USA). The blood solutions were diluted to 50 μl with phosphate-buffered saline (PBS) and incubated at 4°C for 30 min. After washing with PBS and centrifuging, the stained cells were resuspended in 700 μl of PBS and then analyzed for scoring *Pig-a* mutant frequencies in RBCs using a Cytomics FC500 flow cytometer (Beckman

Persistence of radiation-induced *Pig-a* mutant RBCs(A) $p53^{+/+}$ (C57BL/6J) (B) $p53^{-/-}$

Fig. 1. Accumulation and persistence of *Pig-a* mutant frequencies in X-irradiated (A) C57BL/6J ($p53^{+/+}$) RBCs and (B) $p53^{-/-}$ RBCs. The horizontal axis shows the time (d) after radiation and the vertical axis shows the average number of CD24/TER-119⁺ events [RBCs $\times 10^6$ cells]. (A) *Pig-a* mutant frequencies are compared between 1 Gy irradiated $p53^{+/+}$ and unirradiated $p53^{+/+}$ mice from 0 to 713 d after radiation. (B) *Pig-a* mutant frequencies are compared between 1 Gy irradiated $p53^{-/-}$ mice and unirradiated $p53^{-/-}$ mice from 0 to 133 d after radiation. Peripheral blood was withdrawn from the tail vein and analyzed for *Pig-a* mutant frequencies by using flow cytometry (mean $\pm$ S.E., $n = 7$ or 8 mice/dose). The *Pig-a* mutant frequencies in irradiated $p53^{-/-}$ mice decreased gradually. Student's *t* test was used to analyze the differences between the unirradiated (control) group and the irradiated groups at each time point from Day 0 to Day 713 or 133 (* $P < 0.05$, ** $P < 0.01$). Closed circles: 1 Gy irradiation in $p53^{+/+}$, closed squares: no irradiation in $p53^{+/+}$, closed triangles: 1 Gy irradiation in $p53^{-/-}$, crisscrosses: no irradiation in $p53^{-/-}$.

Coulter; Miami, FL, USA) with CXP software for data acquisition. The compensation parameters were based on the values of our previous assay (Ohtani *et al.*, 2012). In total, approximately 4,500,000 erythrocytes were counted (3 repetitions with 1,500,000 events collected for each specimen). Erythroid cells were first gated on a dot-plot cytogram with forward light scattering on the vertical axis and side scattering on the horizontal axis. Gated RBCs were plotted on a cytogram comparing the fluorescence of the GPI-anchored marker that was identified by anti-CD24-FITC versus erythroid cells labeled by anti-TER-119-PC5 in the present study. The parameter of CD71, which was reticulocyte marker, was not used in this study

because of showing a decrease of reticulocytes by bone marrow suppression after irradiation. Therefore, to analyze more precisely, we selected just two markers, anti-CD24-FITC and anti-TER-119-PC5.

Statistical analysis

For RBC *Pig-a* mutant frequencies in both $p53^{+/+}$ and $p53^{-/-}$ mice, two-tailed Student's *t* tests were used to compare irradiated groups and unirradiated control groups at each time point from Day 0 to Day 713 ($p53^{+/+}$) or Day 133 ($p53^{-/-}$) after irradiation. However, when unequal variances existed between groups, the Welch *t* test was performed instead of the Student's *t* test. Moreover, for *Pig-a*

Table 1. *Pig-a* mutant frequencies in Red Blood Cells (RBCs) of *p53*^{+/+} and *p53*^{-/-} mice exposed to 1 Gy of X-rays.

Sample No.	<i>Pig-a</i> mutant frequency (/1,000,000)																		
	d0	d2	d7	d14	d21	d28	d35	d42	d49	d56	d63	d77	d91	d112	d133	d225	d323	d413	d713
<i>p53</i> ^{+/+} Expose	#1	2.64	1.19	0.38	0.37	0.39	0.69	1.17	0.00	1.23	1.77	0.99	0.00	4.19	1.56	0.70	0.35	0.72	0.00
	#2	1.10	0.42	0.97	0.39	2.05	<u>48.68</u>	11.28	3.33	2.41	0.00	0.65	0.64	3.65	0.36	0.36	2.13	0.00	1.54
	#3	0.90	0.00	4.53	3.87	6.12	3.77	1.66	0.31	0.37	0.00	0.71	0.32	0.00	0.33	0.31	0.00	0.35	2.74
	#4	1.53	1.43	7.61	<u>19.30</u>	6.09	7.45	3.33	2.44	0.75	0.34	0.00	0.00	0.00	0.63	0.00	0.97	0.00	1.49
	#5	1.70	0.67	3.19	0.38	0.00	0.42	0.00	3.90	1.03	3.21	2.05	<u>26.79</u>	8.73	2.97	1.07	1.45	3.33	3.61
	#6	1.61	0.78	4.83	3.91	2.26	0.37	0.43	0.45	0.98	0.00	2.69	1.03	3.95	4.41	1.03	1.70	0.00	5.22
	#7	0.00	0.88	5.38	<u>21.98</u>	2.67	5.27	1.30	0.00	0.00	1.75	0.53	0.55	2.64	2.02	1.17	1.70	2.73	3.39
	#8	4.62	2.69	2.54	1.86	<u>39.64</u>	<u>20.94</u>	9.59	7.42	1.98	0.73	0.36	3.57	2.36	1.09	0.65	0.40	0.30	0.00
Sum ave.	1.77	0.99	3.58	6.45	7.45	10.37	3.72	2.04	1.15	0.97	1.03	3.53	3.13	1.85	0.66	1.02	0.82	1.13	2.11
<i>p53</i> ^{+/+} Control	#11	1.56	0.39	0.69	0.00	0.00	0.73	0.34	0.71	0.35	1.25	4.24	1.00	3.17	2.76	4.30	2.05	3.87	0.46
	#12	1.33	0.73	0.37	0.34	0.00	0.40	0.00	0.70	0.38	0.96	0.33	1.68	0.00	1.64	1.99	0.36	0.67	1.13
	#13	2.19	0.00	0.63	3.17	11.78	8.19	3.51	0.69	0.32	0.34	0.33	1.62	2.35	2.85	2.13	<u>15.28</u>	12.69	1.97
	#14	7.20	3.67	3.43	0.98	0.36	0.34	0.63	0.00	0.61	0.00	0.00	0.31	1.02	7.47	13.99	2.81	4.08	0.00
	#15	1.60	0.34	0.37	0.68	0.32	1.96	1.36	1.60	1.06	1.08	0.83	0.69	2.07	0.83	0.00	1.56	1.49	0.00
	#16	2.82	0.69	0.65	1.04	0.00	0.47	8.36	4.54	1.73	3.62	2.10	0.34	1.66	7.92	1.34	2.72	0.72	0.00
	#17	0.00	0.65	1.52	1.13	1.57	3.48	1.36	7.98	3.10	2.47	2.32	0.30	0.93	2.27	0.40	1.46	2.42	4.13
	Sum ave.	2.30	0.95	1.09	1.05	1.89	2.26	2.21	2.14	1.05	1.28	1.39	0.79	1.59	3.71	3.46	2.73	4.02	1.07
<i>p53</i> ^{-/-} Expose	#21	0.00	0.00	0.00	2.97	13.68	8.61	<u>14.15</u>	10.73	6.87	7.86	<u>27.86</u>	<u>13.63</u>	<u>16.73</u>	<u>3.48</u>	<u>25.27</u>			
	#22	0.31	0.61	0.61	2.04	13.33	12.29	<u>25.47</u>	<u>14.79</u>	<u>15.75</u>	3.69	1.45	2.39	10.37					
	#23	1.23	2.73	1.60	3.78	8.55	<u>49.17</u>	<u>19.90</u>	9.39	1.68	3.11	1.77	0.98	7.14	0.37				
	#24	1.46	1.98	1.55	4.67	8.76	<u>31.48</u>	11.04	10.88	<u>15.91</u>	11.40	<u>48.91</u>							
	#25	1.31	1.00	4.50	6.02	<u>14.30</u>	<u>19.99</u>	9.83	11.57	13.50	<u>20.89</u>	<u>19.67</u>	3.20	5.10	7.17				
	#26	2.24	1.29	7.64	7.69	5.37	<u>41.90</u>	<u>35.78</u>	<u>48.10</u>	13.51	<u>15.19</u>	2.05	4.98	9.96	<u>53.57</u>				
	#27	0.31	1.57	0.33	2.10	7.86	4.05	5.17	4.83	4.07	2.72	5.95	2.32	7.45					
	#28	0.30	0.00	0.95	5.02	7.85	10.97	10.29	<u>19.54</u>	12.34	3.13	8.91	<u>17.08</u>	<u>16.37</u>	6.19	8.27			
Sum ave.	0.88	1.13	2.02	4.22	10.12	<u>21.59</u>	<u>16.30</u>	<u>15.09</u>	10.35	8.44	<u>15.17</u>	6.49	10.43	<u>14.25</u>	<u>17.16</u>				
<i>p53</i> ^{-/-} Control	#31	<u>16.46</u>	<u>33.50</u>	<u>22.70</u>	<u>15.18</u>	8.36	7.23	2.44	n.d.	4.19	5.39	5.70	0.76	1.07	1.92				
	#32	5.60	9.80	3.10	0.97	0.99	1.01	2.06	n.d.	0.69	4.40	3.19	0.72	7.35	6.81	<u>17.27</u>			
	#33	<u>20.36</u>	<u>30.39</u>	10.66	4.16	4.27	1.58	4.61	n.d.	3.86	3.06	4.46	4.35	4.53	3.45	7.97			
	#34	0.62	0.96	0.92	8.65	5.11	5.53	11.52	n.d.	3.60	<u>19.25</u>	5.44	2.38	3.56	<u>15.15</u>				
	#35	1.01	0.71	4.62	1.87	1.19	2.67	1.91	n.d.	1.43	5.24	6.80	7.04	10.10	9.32	<u>69.41</u>			
	#36	0.74	1.20	1.84	0.77	11.26	1.22	0.41	n.d.	1.39	10.79	12.94	4.13	8.84	2.61	12.81			
	#37	2.38	3.70	3.09	0.31	0.36	2.20	2.81	n.d.	7.19	12.60	10.15	9.64	9.18	3.06	3.17			
	#38	0.35	2.22	0.95	1.51	0.65	1.92	4.95	n.d.	5.96	3.94	2.65	0.34	7.36	<u>26.50</u>				
Sum ave.	5.98	9.72	5.94	4.13	3.87	2.65	3.58	3.27	7.99	6.03	3.39	6.31	9.19	<u>20.14</u>					

The underlined values with colored background are designated as the high values, representing RBC *Pig-a* mutant frequencies of at least 14 mutants/million cells. n.d., no data. Sum ave. is the mean *Pig-a* mutant frequency for the group.

Persistence of radiation-induced *Pig-a* mutant RBCs

Fig. 2. Comparison of *Pig-a* mutant frequencies among the four groups, unirradiated *p53*^{+/+} (control), irradiated *p53*^{+/+}, unirradiated *p53*^{-/-}, and irradiated *p53*^{-/-}, at (A) Day 28 and (B) Day 35 after irradiation. Vertical axis shows the average number of CD24/TER-119⁺ events [RBCs × 10⁶ cells]. The Steel test was used to analyze the differences among wild-type and knockout mice in unirradiated and irradiated groups at Day 28 and Day 35 (* $P < 0.05$, ** $P < 0.01$).

mutant frequencies at Days 28 and 35, a Kruskal–Wallis nonparametric test was used to compare the 4 groups: irradiated *p53*^{-/-}, unirradiated *p53*^{-/-}, irradiated *p53*^{+/+}, and unirradiated *p53*^{+/+} mice. When significant differences were detected using the Kruskal–Wallis test, the Steel test was performed as a post hoc analysis of these comparisons. Levels of significance were set at $P < 0.05$ and $P < 0.01$. The statistical analyses were performed using Excel Statistics 2008 (Social Survey Research Information Co.; Tokyo, Japan).

RESULTS

Transition of *Pig-a* mutant frequencies

Fig. 1 showed the temporal changes in *Pig-a* mutant frequencies in *p53*^{+/+} and *p53*^{-/-} mice after X-irradiation for 2 years, i.e., 0–713 days. In irradiated *p53*^{+/+} mice (Fig. 1A), a significant difference was only observed in the mean of the *Pig-a* mutant frequency at Days 14 after irradiation. The *Pig-a* mutant frequencies were increased from Day 7 to Day 28, followed by a decrease after the peak. In the irradiated *p53*^{-/-} mice, the first peak was showed on Day 28 after irradiation. Thereafter the mutant frequencies were decreased gradually until Day 77, and then increased again from Day 91 after irradiation (Fig. 1B).

All *Pig-a* mutant frequency data was shown in Table 1. We defined a frequency of 14 mutants per million cells as being high, which was approximately 7-times higher than the mean *Pig-a* mutant frequency (1.93×10^{-6}) that

was calculated using measurements from all unirradiated *p53*^{+/+} mice from Day 0 to Day 713. The value of “7” times was defined according to our previous study (Ohtani *et al.*, 2012). By this definition, there were 5 mice (out of 8) in the group of irradiated *p53*^{+/+} mice that displayed high mutant frequencies once (#2, #4, #5, and #7) or twice (#8) before Day 77. In contrast, 7 irradiated *p53*^{-/-} mice (all except #27) displayed high mutant frequencies, with two or three rebounds by Day 133 after irradiation. For example, frequencies were increased on Days 35, 63, 91, and 133 for the mouse #21; Days 28, 49, and 63 for the mouse #24; Days 21, 28, 56, and 63 for the mouse #25; Days 28–42, 56, and 112 for the mouse #26; and Days 42, 77 and 91 for the mouse #28, respectively (Table 1). In addition, there were also 6 mice (out of 8) in the group of unirradiated *p53*^{-/-} mice displaying high frequencies.

The average life-spans of irradiated *p53*^{-/-} mice and unirradiated *p53*^{-/-} mice were 110.0 d ($n = 8$) and 170.9 d ($n = 8$), respectively ($P < 0.01$); the life-spans of *p53*^{+/+} mice could not be calculated because some of the mice were alive at the end of 2-year study.

Comparison of the mean *Pig-a* mutant frequencies between irradiated *p53*^{+/+} and *p53*^{-/-} mice from Day 28 to Day 56 after irradiation

On Day 28 after irradiation, the mean *Pig-a* mutant frequencies peaked in irradiated *p53*^{+/+} and *p53*^{-/-} mice (Fig. 2A), with the *p53*^{-/-} mice having about twice higher *Pig-a* mutant frequencies than the *p53*^{+/+} mice. On

Fig. 3. Half-life of *Pig-a* mutant frequencies in (A) irradiated $p53^{+/+}$ and (B) irradiated $p53^{-/-}$ mice. The horizontal axis shows the length of time (d) after Day 28 after irradiation and the vertical axis shows the average number of CD24/TER-119⁺ events [RBCs $\times 10^6$ cells]. Five plots of *Pig-a* mutant frequencies from Day 28 to Day 56 were fit using an exponential function for both strains of mice. The plots for (A) irradiated $p53^{+/+}$ and (B) irradiated $p53^{-/-}$ are shown as closed circles and closed triangles, respectively.

Day 35 after irradiation, the frequencies in irradiated $p53^{-/-}$ mice were significantly higher than that in irradiated $p53^{+/+}$ mice (Fig. 2B). To calculate the distinct rates by which *Pig-a* mutant frequencies decreased, we compared the rates from Day 28 to Day 56 after irradiation between irradiated $p53^{+/+}$ and $p53^{-/-}$ mice. The mutant frequencies in irradiated $p53^{+/+}$ and $p53^{-/-}$ mice showed approximately exponential changes, which can be expressed by the formulas $y = 7.981 e^{-0.084x}$ and $y = 21.645 e^{-0.033x}$ (Fig. 3). The half-life of affected RBCs with *Pig-a* mutation was 8.3 days in irradiated $p53^{+/+}$ mice and 21 days in $p53^{-/-}$ mice.

DISCUSSION

To clarify the connection between *Pig-a* mutant frequency and p53 which protects the genome and induces apoptosis, in the present study, we examined the temporal change in *Pig-a* mutant frequencies after radiation exposure in relation to the presence or absence of p53 function. We compared the kinetics of *Pig-a* mutant frequency in RBCs from irradiated and unirradiated $p53^{+/+}$ and $p53^{-/-}$ mice (Table 1 and Fig. 1). The mutation frequencies decreased gradually after the peak in irradiated $p53^{-/-}$ mice than those in irradiated $p53^{+/+}$ mice (Figs. 1 and 3). Moreover, in the late-life, *Pig-a* mutant frequencies increased in most $p53^{-/-}$ mice with or without irradiation (Fig. 1B).

As we expected that the *Pig-a* mutant frequencies in

$p53^{-/-}$ mice would decline slower than those in $p53^{+/+}$ mice, the decrease in the ratios of *Pig-a* mutant frequencies from Days 28 to 56 after irradiation were distinct in irradiated $p53^{+/+}$ and $p53^{-/-}$ mice (Fig. 3), and there was also a significant decrease of *Pig-a* mutant RBCs in irradiated $p53^{+/+}$ mice on Day 35 after irradiation (Fig. 2B). The calculated half-life of RBCs with *Pig-a* mutation in irradiated $p53^{-/-}$ mice (21.0 d) was longer than that of affected RBCs in irradiated $p53^{+/+}$ mice (8.3 d) when the *Pig-a* mutant frequencies from Day 28 to Day 56 were fit using an exponential function (Fig. 3). This disparity in the half-life of RBCs might arise from the different mechanisms by which they are eliminated in irradiated $p53^{-/-}$ and $p53^{+/+}$ mice. We also expected that the affected RBCs in irradiated $p53^{+/+}$ mice should be eliminated rapidly after Day 28 after irradiation, because the normal degradative and processing functions of p53 protein would induce apoptosis in the affected hematopoietic stem cells and/or erythroid progenitor cells (Igari *et al.*, 2006). In contrast, the affected RBCs in the irradiated $p53^{-/-}$ mice would remain in the blood and decrease gradually, because of the lack of p53 function in hematopoietic stem cell and erythroid progenitor cells. As a result, more RBCs with *Pig-a* mutation were thought to be pooled and accumulated in peripheral blood due to functional loss of apoptosis.

As another reason for the disparities in the elimination

of RBCs, we suggested that the dissimilar persistence of *Pig-a* mutant RBCs in ENU-treated and irradiated mice was caused by the occurrence of distinct types of mutation in hematopoietic organization, because ENU is a mutagen that induces point mutations in mice (Russell *et al.*, 1979). When mice are exposed to irradiation, many types of gene mutations such as point mutations and/or multiple/large deletions would be induced in cells. Miura *et al.* (2009) reported that ENU-treated rats have increased *Pig-a* mutant frequencies that accumulate in an almost additive manner and persist for as long as 6 months after the treatment. Conversely, although RBCs in ENU-treated mice maintained high *Pig-a* mutant frequencies until 26-week post-treatment, the frequencies decreased slightly after reaching the maximum (Bhalli *et al.*, 2011). This study suggested that the slight decline detected in ENU-treated mice was associated with the life-span of RBCs. Therefore, the persistence of affected RBCs in irradiated *p53*^{-/-} mice in our study may also depend on the life-span of RBCs. The average life-span of RBCs in mice was identified as 47 days (Abbrecht and Littell, 1972). In irradiated *p53*^{-/-} mice, we found that the progression from Day 28 (maximal frequencies) to Day 77 (almost back to background frequencies after peaking) coincides with the life-span of mouse RBCs in this study. The data indicated that the life-span of RBCs would be influenced by the gradual decrease of the affected RBCs in irradiated *p53*^{-/-} mice.

Besides, rapid RBCs production to recover RBCs diminished by irradiation may cause gradual decrease of *Pig-a* mutant frequencies. We confirmed that RBCs were produced rapidly because CD71⁺ reticulocyte frequencies of *p53*^{+/-} mice showed acute increases at Day 7 after irradiation, following the decreasing of CD71⁺ frequencies at Day 2 (Ohtani *et al.*, 2012). It suggests that many normal RBCs were produced and were pooled in blood. The series of blood-forming mechanism lead to the gradual decrease of *Pig-a* mutant frequencies. Taking together, the lack of p53-dependent apoptosis, different types of mutation, the life-span of RBCs with *Pig-a* mutation, and rapid normal RBCs production all play crucial roles in gradually eliminating the RBCs affected by irradiation.

As shown in Fig. 2A, the mean *Pig-a* mutant frequencies in irradiated *p53*^{-/-} mice were 2-times higher than the frequencies in irradiated *p53*^{+/-} mice on Day 28 after irradiation. These data implicated that hematopoietic stem cells, erythroid progenitor cells, and/or erythroblast with the *Pig-a* mutation would not be eliminated by apoptosis in *p53*^{-/-} mice. Additionally, the significant increase in *Pig-a* mutant frequencies in irradiated *p53*^{-/-} mice may be caused by the lack of repair of damaged DNA,

which would normally occur through the function of p53 and downstream signaling such as p53R2 (Tanaka *et al.*, 2000), in hematopoietic stem cells, erythroid progenitor cell, and/or erythroblast. These results suggested that the lack of p53 function would affect directly accelerating accumulation of RBCs with *Pig-a* mutation till Day 28 after irradiation.

To identify the late effects of ionized radiation on *Pig-a*, we measured the mutant frequencies in irradiated and unirradiated mice up to 2 years (Fig. 1). Our results showed that the mean *Pig-a* mutant frequencies in *p53*^{-/-} mice with or without irradiation were re-elevated after Day 112 after irradiation. Similar elevation was observed as the late effect of TCR variants in unirradiated *p53*^{-/-} and *p53*^{+/-} mice (Igari *et al.*, 2006). Therefore, these elevations of mutant frequencies with aging are probably caused by lack of p53 function. On the other hand, we did not detect statistically significant differences in the late increase in frequencies between irradiated and unirradiated *p53*^{+/-} and *p53*^{-/-} mice, although TCR mutant frequencies were re-elevated earlier in *p53*^{+/-} mice at about 44-week after irradiation (Igari *et al.*, 2006). In spite of using different cell types, both T-lymphocytes and RBCs belong to hematopoietic system. Therefore, we expected that *Pig-a* mutant frequency in irradiated mice might be increased earlier than those in unirradiated mice, in terms of the late effects of irradiation. However, we did not observe the late effect between irradiated and unirradiated mice in this study. One reason is that there were some differences in terms of experimental approaches between the measurements of TCR variants and *Pig-a* mutant frequency. While Igari *et al.* (2006) exposed 3 Gy radiation and analyzed TCR mutant frequencies (about 2-3 events per 10,000 cells in wild type mice), we exposed 1 Gy radiation and analyzed the *Pig-a* mutant frequencies (about 2-3 events per 1,000,000 cells). The irradiation dose in our study were less than that of Igari's study, so re-elevation could not be detected in both irradiated and unirradiated *p53*^{+/-} mice. Another reason could be that *p53*^{-/-} mice die considerably earlier than the *p53*^{+/-} mice used in the Igari study, because *p53*^{-/-} mice develop substantially more tumors than normal mice (Donehower *et al.*, 1992). Moreover, the *Pig-a* mutant frequencies in late life of *p53*^{+/-} mice could not be compared precisely because some *p53*^{+/-} mice were still alive at the end of the study and their exact life-span was not determined. Although our data did not provide conclusive evidence regarding the effect of age on *Pig-a* mutant frequencies in irradiated and unirradiated mice, the data suggest that the functional loss of p53 accelerates the increase in *Pig-a* mutant frequencies in late life of *p53*^{-/-} mice with or without irra-

diation.

Our study suggests that the different response of *Pig-a* mutant frequencies to irradiation would be caused by lack of p53 protein in hematopoietic stem cell and/or in erythroid progenitor cells. As a result, RBCs with *Pig-a* mutant frequencies was a gradual decrease in irradiated *p53*^{-/-} mice but a rapid decrease in irradiated *p53*^{+/-} mice after peaking the frequencies. These results indicated that p53 played a crucial role in the kinetics of RBCs with *Pig-a* mutation when mice were irradiated. Although we could not confirm that the *Pig-a* mutant frequencies were associated with aging in *p53*^{+/-} mice, *Pig-a* mutant frequencies were increased with aging in *p53*^{-/-} mice due to the lack of p53 function. The persistence of *Pig-a* mutant frequencies in irradiated mice was affected by the major functions of p53, by the region of the mutation in *Pig-a*, and/or by the life-span of RBCs. These results not only contribute to a better understanding of the effect of radiation on GPI-anchored proteins in hematopoietic cell systems but also are useful for radiobiology and study of aging.

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