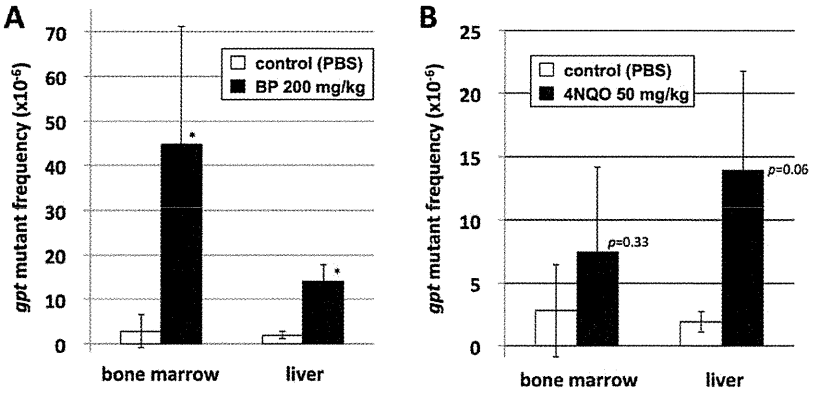

Fig. 3. Comparative analyses of *gpt* mutation in bone marrow and liver. Seven weeks after treatment, all mice were killed and liver samples were collected and analyzed by the *gpt* assay (bone marrow data are the same as in Fig. 2). The frequencies are the mean ± SD of data from five animals. $^{*}P < 0.05$.

power analyses indicating that the assay had probabilities of >99%, >60%, and <10% of rejecting the null hypothesis, respectively, i.e. to distinguish between the negative control and BP-treated group). Although significant increases were measured at all sampling times, a similar reduction with time was detected in the mice treated with 200 mg/kg BP (mean ± SD for mice treated with BP: 2 weeks after treatment, $15.80 \pm 8.20 \times 10^{-6}$; 4 weeks after treatment, $14.20 \pm 5.97 \times 10^{-6}$; and 7 weeks after treatment, $10.20 \pm 11.34 \times 10^{-6}$; with power analysis indicating that the assay had probabilities of >95%, >99%, and >30%, respectively, to distinguish between the treated and control groups) (Figs. 2C and 2D).

gpt Assay on Bone Marrow and Liver

Compared with the solvent control animals (MF for PBS control group, $2.83 \pm 3.68 \times 10^{-6}$), significant increases in bone marrow *gpt* MFs were observed in ENU- and high-dose BP-treated mice (ENU, $24.70 \pm 10.49 \times 10^{-6}$; BP 200 mg/kg, $44.86 \pm 26.37 \times 10^{-6}$) (Fig. 2E), but not in low-dose BP- or in 4NQO-treated mice (100 mg/kg BP, $7.64 \pm 3.12 \times 10^{-6}$; 4NQO, $7.45 \pm 6.75 \times 10^{-6}$) (Figs. 2B and 2C). *gpt* MFs also were increased in the liver of high-dose BP- and 4NQO-treated mice (MF for PBS control group, $1.97 \pm 0.83 \times 10^{-6}$; 200 mg/kg BP, $14.04 \pm 3.76 \times 10^{-6}$; 4NQO, $13.92 \pm 7.83 \times 10^{-6}$) (Fig. 3).

DISCUSSION

TGR mutation assays, such as the *gpt* gene mutation assay, are an established method for monitoring *in vivo* genotoxicity in multiple tissues concurrently [for review, see Nohmi et al., 2000; Lambert et al., 2005; OECD488, 2011]. While the *Pig-a* gene mutation assay analyzes only one type of cells, i.e., blood cells, it has the advantage of not requiring the use of transgenic animals [Miura et al., 2008a,b]. The *Pig-a* assay has been undergoing extensive development and validation studies are presently being conducted [see the special issue of *Environmental and Molecular Mutagenesis* 52, 2011]. Based on the recent studies, it has been suggested that the *Pig-a* assay could be integrated into repeat-dose toxicology studies. The majority of *Pig-a* work to date has been performed with rats, with very little data being generated in mice, and this data gap needs to be addressed. Therefore, in this study, we analyzed both transgene and *Pig-a* mutation using *gpt*-delta transgenic mice.

Our results using a single oral administration of ENU were consistent with those of previously reported mouse studies [Bhalli et al., 2011a; Horibata et al., 2011; Kimoto et al., 2011]. The ENU-induced MFs detected by the *Pig-a* and *gpt* assays were similar, suggesting that both assays were able to detect ENU genotoxicity equally well.

We also detected dose-dependent increases of *Pig-a* MF in BP-treated mice (Figs. 2C and 2D). The MFs in the mice treated with both low- and high- doses of BP peaked at 2 weeks after treatment and declined thereafter. These time-dependent reductions in *Pig-a* MF differed from the kinetics of ENU–induced *Pig-a* mutation. Phonethepswath et al. reported on the kinetics of *Pig-a* mutation in RBCs from Wister rats treated with ENU, 7,12-dimethyl-1,2-benz[*a*]anthracene, N-methyl-N-nitrosourea, 4NQO and BP [Phonethepswath et al., 2010]. While erythroid progenitors normally mature over a period of 7–14 days *in vitro* [Iscove and Sieber, 1975], it takes 4–6 months to re-establish stable hematopoietic stem cell numbers following bone marrow transplantation [Jordan and Lemischka, 1990]. These observations imply that ENU may effectively mutate hematopoietic stem cells, and that a large proportion of *Pig-a* mutants in BP-treated mice may be due to mutations induced in erythroid progenitors.

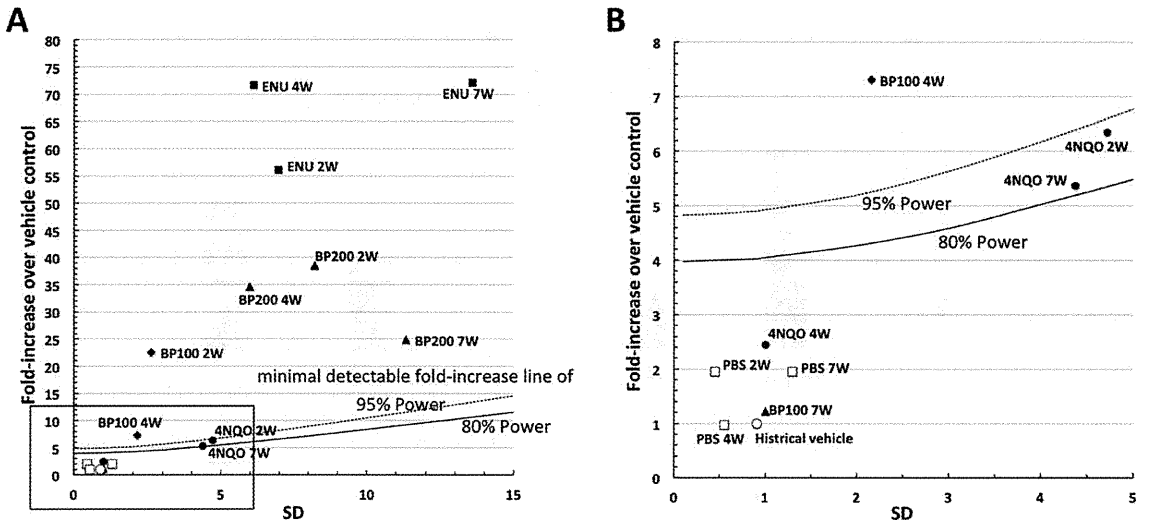

Fig. 4. Relationships between the power to detect fold-increases in *Pig-a* mutant frequency (MF) over the historical vehicle control MF and the effect of the SD of the mean measurements on the power of detection. The solid and dotted lines indicate the fold-increase over the historical vehicle control data detected with 80% and 95% power, respectively. All power estimates were based on an $n = 5$ comparing to the historical vehicle control ($n = 95$, SD = 0.91), $\alpha 5\%$, and use an unpaired two-tailed t-test. Closed square, closed triangle, closed diamond, closed circle, open square, and open circle indicate the fold-increase produced by treatment with ENU, high-dose BP, low-dose BP, 4NQO, and PBS and the historical vehicle control, respectively. The symbols located above each boundary line indicate that the fold-increases for these groups are detected with 95 and or 80% power by an unpaired t-test (two-tailed). Open-boxed area indicated in (**A**) is expanded in (**B**).

TABLE I. Power Analysis Using the Historical Control Data[a]

The number of animals of		Power to detect increase of				
experimental group	control group	twofold change over control	threefold change over control	fourfold change over control	fivefold change over control	sixfold change over control
5	5	10%	24%	47%	71%	88%
10	10	16%	48%	82%	97%	>99%
15	15	22%	66%	95%	>99%	>99%
20	20	28%	79%	>99%	>99%	>99%
25	25	35%	88%	>99%	>99%	>99%
80	80	81%	>99%	>99%	>99%	>99%

[a]The historical control data of *Pig-a* MF were $n = 95$, male mice of C57BL/6, 3–12 weeks of age and mean ± SD: 0.41 ± 0.91.

In this study, we found no significant increases in *Pig-a* MF in 4NQO-treated mice using the Steel multiple comparison test, although at each sampling time, the mean MF for the treated mice was greater than the negative control (Fig. 2B). Power analysis employing our historical background *Pig-a* MF ($n = 95$, male C57BL/6 mice, 3–12 weeks of age, mean ± SD: 0.41 ± 0.91) indicated that the assays conducted in this present study (sample size of 5, interrogating 1×10^6 cells/sample) lacked the ability to distinguish these two- to threefold differences between the treated and control groups. The plots shown in Figure 4 indicate that the assays had, at best, an 80–95% power to detect a true four- to fivefold increase of the control and that power depended on the variability of the measurements. When the SD for the MF measurement is small, that is, when the range in MFs for the individuals in the treatment group is small, the assay is capable of detecting about a fourfold increase in *Pig-a* MF with 80% of power (Fig. 4). But when the variability in within-group measurements increased, as it did for many of the treated groups and even some of the controls, then the power of the assay to detect true differences decreased. For instance, the range of MFs for the individual 4NQO-treated mice was relatively large, which contributed to the uncertainty in this measurement. It should be noted that the 2- and 7-week 4NQO responses fell into the 'gray area' for detection based on our power analysis. Performing unpaired t-tests comparing the historical vehicle control data and the responses produced by the 4NQO-treated groups indicated that the assays on 4NQO-

treated mice at 2 and 7 weeks were able to detect significant increases in *Pig-a* MF with over 80% of power (Fig. 4).

The statistical power of the assays can be increased by increasing either the number of mice per group, the number of RBCs interrogated per sample, or both. For instance, increasing the number of mice per group to 20 by itself will enable the assay to detect a threefold increase with nearly 80% power (Table I). Interrogating additional erythrocytes will reduce the number mice with "0" MFs (as can be seen in Fig. 2) and, potentially, the mouse to mouse variability in MF [Nowosiad et al., 2011]. With sufficient sample sizes, it is possible that 4NQO will test positive in the mouse *Pig-a* assay, even if the statistical analyses are conducted using multiple comparison tests. Similar to the *Pig-a* assay results, the mean bone marrow *gpt* MF in 4NQO-treated mice was only about two times higher than the frequency in the negative control, and these frequencies were not significantly different.

In contrast to the bone marrow *gpt* findings, we did detect a marginally significant increase in 4NQO-induced *gpt* MF in liver (Fig. 3B). Significant increases in liver mutagenicity also have been detected in MutaMouse following a single oral administration of 4NQO [Nakajima et al., 1999; Suzuki et al., 1999]. In these studies, the *lacZ* MF in the liver increased with time, but the MF in bone marrow peaked at 1 week and then decreased with time. These findings suggest that the peak bone marrow *gpt* MF in our present study could have been missed because the tissues were analyzed only 7 weeks after treatment. Additionally, in the previous MutaMouse studies, the *lacZ* MFs induced by BP and 4NQO were much higher in the bone marrow than in the liver [Hakura et al., 1998; Nakajima et al., 1999; Suzuki et al., 1999; Lemieux et al., 2011]. We also observed a greater response for BP in the bone marrow than liver. For 4NQO, however, we observed a higher *gpt* MF in the liver than in the bone marrow. The reason for these disparate results is unknown, but they may be related to the use of different TGRs.

Both the *Pig-a* and TGR assays have unique strengths and weaknesses. TGR assays are costly but they can be used for surveying mutation in various tissues. In this study, in fact, the *gpt* assay detected 4NQO genotoxicity in liver whereas the *Pig-a* assay did not detect the mutagenicity of 4NQO in peripheral blood. This differential response may, at least in part, be due to the target tissues for 4NQO mutagenesis because 4NQO also did not significantly increase *gpt*-mutation in bone marrow (although it is recognized that sampling times in the current study were not optimal for bone marrow mutation assessment).

The OECD guideline for TGR assays recommends a tissue sampling time of 3 days after 28 consecutive daily treatments [OECD488, 2011], making it difficult to integrate TGR assays it into standard repeat-dose toxicology studies. Since the *Pig-a* gene is an endogenous gene, the *Pig-a* assay does not require TGR animals. Thus, while the *Pig-a* assay can be combined with a TGR assay, as was done in this present study, it also potentially can be integrated into repeat-dose toxicology studies that do not use TGRs [Dertinger et al., 2010; Dobrovolsky et al., 2010; Bhalli et al., 2011b; Cammerer et al., 2011; Dertinger et al., 2011; Lemieux et al., 2011; Lynch et al., 2011; Schuler et al., 2011; Shi et al., 2011]. Currently, however, we need additional studies that compare mutational responses in the *Pig-a* gene and TGR transgenes in order to help validate the *Pig-a* assay.

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AUTHOR CONTRIBUTIONS

Drs. Horibata and Honma designed the study. Drs. Horibata, Kimoto, Masumura, Nohmi, and Honma critically discussed the study. Dr. Horibata, Ms. Ukai, Dr. Suzuki, and Ms. Kamoshita collected the data. Dr. Horibata and Ms. Ukai analyzed the data and prepared draft figures. Dr. Horibata prepared the manuscript draft with important intellectual input from Dr. Honma. All authors approved the final manuscript. Drs. Horibata and Honma had completed access to the study data.

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Interlaboratory trial of the rat *Pig-a* mutation assay using an erythroid marker HIS49 antibody

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ABSTRACT

The peripheral blood *Pig-a* assay has shown promise as a tool for evaluating *in vivo* mutagenicity. In this study five laboratories participated in a collaborative trial that evaluated the transferability and reproducibility of a rat *Pig-a* assay that uses a HIS49 antibody reacts with an antigen found on erythrocytes and erythroid progenitors. In preliminary work, flow cytometry methods were established that enabled all laboratories to detect CD59-negative erythrocyte frequencies (*Pig-a* mutant frequencies) of $<10 \times 10^{-6}$ in control rats. Four of the laboratories (the in-life labs) then treated male rats with a single oral dose of *N*-nitroso-*N*-ethylurea, 7,12-dimethylbenz[*a*]anthracene (DMBA), or 4-nitroquinoline-1-oxide (4NQO). Blood samples were collected up to 4 weeks after the treatments and analyzed by flow cytometry for the frequency of CD59-negative cells among total red blood cells (RBCs; RBC *Pig-a* assay). RBC *Pig-a* assays were conducted in the four in-life laboratories, plus a fifth laboratory that received blood samples from the other laboratories. In addition, three of the five laboratories performed a *Pig-a* assay on reticulocytes (RETs; PIGRET assay), using blood from the rats treated with DMBA and 4NQO. The four in-life laboratories detected consistent, time- and dose-related increases in RBC *Pig-a* mutant frequency (MF) for all three test articles. Furthermore, comparable results were obtained in the fifth laboratory that received blood samples from other laboratories. The three laboratories conducting the PIGRET assay also detected consistent, time- and dose-related increases in *Pig-a* MF, with the RET MFs increasing more rapidly with time than RBC MFs. These results indicate that rat *Pig-a* assays using a HIS49 antibody were transferable between laboratories and that data generated by the assays were reproducible. The findings also suggest that the PIGRET assay may detect the *in vivo* mutagenicity of test compounds earlier than the RBC *Pig-a* assay.

1. Introduction

An *in vivo* gene mutation assay using the phosphatidylinositol glycan, class A gene (*Pig-a* in rodents, *PIG-A* in humans) as an endogenous reporter (*Pig-a* assay) is currently anticipated as being useful in quantitatively assessing genotoxicological risk including a radiation [1–3]. *Pig-a* is necessary for the synthesis of glycosylphosphatidylinositol (GPI), which binds a variety of proteins expressed on the cell surface. In mammals, *Pig-a* is the only gene necessary for GPI synthesis that is located on the X chromosome. Thus, a single mutation in the *Pig-a* gene can result in a deficiency in GPI-anchored proteins on the cell surface and GPI deficiency is virtually equivalent to *Pig-a* mutation [4,5]. These features of the GPI anchor system are exploited in the design of the *Pig-a* gene mutation assay that measures loss of a GPI-anchored protein marker by flow cytometry as a rapid assay for *in vivo* gene mutation.

Recent *Pig-a* assay development has focused on rats, resulting in various approaches for measuring *Pig-a* mutant cells in rat peripheral red blood cells (RBCs), reticulocytes (RETs), bone marrow erythroids, and spleen T lymphocytes [6–11]. Assays using peripheral blood erythrocytes have several advantages that make it possible to measure *in vivo* somatic mutation in a high-throughput

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manner. One advantage is that toxicant-induced RBC *Pig-a* mutant frequencies (MFs) persist in rat peripheral blood for at least months and accumulate as a result of repeat dosing [12,13]. Another advantage is that the assay can be performed with only a small volume of peripheral blood. These attributes benefit the integration of the *Pig-a* assay into other toxicology studies, especially repeated-dose toxicology studies.

An international interlaboratory trial has evaluated the transferability, reproducibility, and relative sensitivity of the rat *Pig-a* assay [14]. In this trial, anti-CD59-PE and SYTO 13 dye were used to label leukocyte-depleted blood samples to identify GPI-deficient *Pig-a* mutant cells. This system makes it possible to distinguish RETs from RBCs and to evaluate *Pig-a* MF in both populations simultaneously. The results from the trial indicated that the *Pig-a* assay was highly transferable and reproducible, and that the *Pig-a* assay could be a useful tool for evaluating *in vivo* mutagenicity.

While the protocol using the SYTO 13/anti-CD59 PE with leukocyte depletion is a well-known approach for detecting *Pig-a* mutations, a protocol developed by Dobrovolsky et al. also may have value as a screening tool for evaluating *in vivo* mutagenicity [10]. In this procedure, an antibody against the rat erythroid marker, HIS49, is employed to positively identify RBCs and RETs. A subsequent study on the HIS49 *Pig-a* assay in rat RBCs (referred to here as the RBC *Pig-a* assay) confirmed its potential for detecting mutagenic chemicals [15]. In addition, a recently developed assay for measuring *Pig-a* mutant cells in rat RETs (the PIGRET assay), which employs magnetic enrichment of CD71-positive RETs, is capable of interrogating more than 1×10^6 CD71/HIS49-positive RETs for *Pig-a* mutation [16]. However, it remains unknown whether these approaches are technically transferable between laboratories.

Collaborative work on the *Pig-a* assay has been conducted in Japan as part of a project entitled 'Study on the development of an integrated system of toxicology assays that can evaluate multiple endpoints,' supported by the Japan Health Sciences Foundation, Grants Nos. KHB1006 and KHB1209. The objective of the Grant KHB 1006 project is to develop a protocol that can acquire *in vivo* genetic toxicity data in a manner consistent with the '3Rs.' To this end, the *Pig-a* assay group has evaluated the transferability of the rat *Pig-a* assay using the HIS49 and CD59 antibodies. First, rat RBC *Pig-a* assay procedures developed by Teijin Pharma Limited (Teijin) were shared among four other participating labs, which included laboratories with no prior experience with assaying *Pig-a* mutation. The collaborating laboratories defined a standardized gating rule to reduce variability in the measurement of spontaneous CD59-negative cells (*Pig-a* mutants). They then confirmed the transferability of the protocol that was developed by measuring mutation in rats dosed with *N*-nitroso-*N*-ethylurea (ENU). In addition, they performed multilaboratory experiments using 4-nitroquinoline-1-oxide (4NQO) and 7,12-dimethylbenz[*a*]anthracene (DMBA) to evaluate interlaboratory portability and reproducibility. As part of the 4NQO and DMBA experiments, three of the five participants also conducted the PIGRET assay to test its transferability and reproducibility and to compare the responses in this assay with those of the RBC *Pig-a* assay.

2. Materials and methods

2.1. Reagents

ENU and DMBA were purchased from Sigma–Aldrich (St. Louis, MO, USA); 4NQO, olive oil and 0.5% (w/v) methylcellulose (400 cps) solution were purchased from Wako Pure Chemical Industry Co., Ltd. (Osaka, Japan). Anti-rat CD59 antibody (clone TH9, FITC-conjugated), anti-rat CD71 antibody (clone OX-26, PE-conjugated), anti-rat erythroid marker (clone HIS49, biotin-conjugated), and streptavidin-APC were purchased from BD Biosciences (Tokyo, Japan).

2.2. Animals

All animal experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals in each participant laboratory, and all experimental protocols were approved by the appropriate institutional review boards. Male CD (SD) rats were obtained from Charles River Laboratories (Kanagawa, Japan) (Table 1). The animals were given commercial feed and water *ad libitum* throughout the acclimation and experimental periods.

2.3. Administration of test agents and blood collection

After an acclimation period, the rats were administered a single dose of a test agent (ENU, 4NQO, or DMBA) by gavage. ENU was dissolved in warm (37 °C) PBS (pH adjusted to 6.0–6.1) at a concentration of 1.0 or 4.0 mg/mL (the ENU concentration was adjusted to account for moisture and stabilizer in the commercial preparation). 4NQO was suspended in 0.5% (w/v) methylcellulose solution at a concentration of 2.5, 5.0, or 10 mg/mL. DMBA was dissolved in olive oil at a concentration of 2.0, 4.0, or 6.0 mg/mL.

The description for study design is shown in Table 3. In the ENU study, test rats were administered a single dose of 0 (PBS vehicle), 10, or 40 mg/kg ENU by gavage ($n = 3$/group). In 4NQO study, rats were administered a single dose of 0 (0.5%, w/v MC vehicle), 25, 50, or 100 mg/kg 4NQO by gavage ($n = 5$/group). In DMBA study, rats were treated with a single dose of 0 (olive oil vehicle), 20, 40, or 60 mg/kg DMBA by gavage ($n = 5$/group). All test agents and the vehicle control were administered in a volume of 10 mL/kg rat weight. An ENU 40 mg/kg dosing group was used as a positive control group in the 4NQO and DMBA experiments for a confirmation of reactivity in rats (data not shown). Blood was collected from the tail vein or abdominal aorta and was promptly mixed with K2-EDTA to prevent clotting.

2.4. Blood processing for the RBC Pig-a assay

The sample preparation and the gating strategy for the RBC *Pig-a* assay were described previously [15]. The blood samples for the rat RBC *Pig-a* assay were processed within 72 h after the blood sampling. If shipping was required, the blood samples were refrigerated during shipment. Three microliters of blood/EDTA mixture were suspended in 200 μL of phosphate-buffered saline (PBS), and the cells were labeled with 0.125 μg of biotinylated HIS49 antibody and 1 μg of FITC-conjugated anti-rat CD59. After incubation for 1 h in the dark at room temperature, the samples were washed with PBS, centrifuged for 5 min at 1680 × *g*, and resuspended in 200 μL of PBS. Then, 0.2 μg of APC-conjugated streptavidin were added, and the samples were incubated for 15 min in the dark at room temperature. The specimens then were centrifuged for 5 min at 1680 × *g* and resuspended in approximately 1 mL of PBS.

2.5. Blood processing for the PIGRET assay

The sample preparation and the gating strategy for the PIGRET assay were described previously [16]. The blood samples for the PIGRET assay were processed within 24 h after the blood sampling. Approximately 80–150 μL of blood/EDTA were mixed with 200 μL of PBS, and then the cell suspension was layered onto Lympholyte-Mammal (Cedarlane Laboratories; Burlington, Ontario, Canada) and centrifuged to separate the nucleated cells and platelets from the RBCs. The RBC fraction was washed and then gently mixed with 100 μL of PBS. The resulting suspension was placed in a new tube containing 1 μg of PE-conjugated anti-rat CD71 and incubated for 15 min on ice. After being washed with 2 mL of 1× IMag™ Buffer (BD Biosciences) and centrifuged (1680 × *g*, 5 min), the cells were mixed with 50 μL of BD IMag™ PE Particles Plus-DM (BD Biosciences) and incubated for 15 min in a refrigerator. The samples were enriched for CD71-positive cells by processing with a BD IMagnet™ magnetic stand (BD Biosciences) according to the manufacturer's instructions. The enriched samples were labeled with HIS49 and anti-CD59 antibodies as indicated for total RBC labeling, with the exception that the incubation time for labeling enriched RETs was half that for the total RBCs. The final volume of the cell suspension was 500 μL.

2.6. Flow cytometry

Flow cytometric analysis to enumerate CD59-negative RBCs and RETs was conducted using flow cytometers equipped with 488-nm blue and 635-nm red lasers. The types of flow cytometers and software for data acquisition/analysis used in this study are shown in Table 1. The gating strategy for enumerating CD59-negative cells has been described previously [15,16]. All gates for the detection of CD59-negative RBCs and RETs were set using unstained and single-stained samples before evaluating the experimental samples. Approximately 1 million HIS49-positive RBCs and 1 million HIS49- and CD71-positive RETs were analyzed for expression of CD59 on the cell surface.

2.7. Statistical analysis

All data were expressed as the frequency of CD59-negative RBCs or RETs per million cells. Dunnett's multiple comparison *t*-test (non-parametric, two-sided) was

Table 1
Information of the participating laboratories in the collaborative study.

Participant laboratories (abbreviation)	Animals used in the experiments	Instruments, Software
Teijin Pharma Limited (Teijin)	Crl: CD(SD); 7 weeks (male)	FACSCanto II, FACSDiva ver. 6.1 (FACSAria I, FACSDiva ver. 4.1.2)[a]
Daiichi Sankyo Co., Ltd. (Daiichi Sankyo)	Crl: CD(SD); 7 weeks (male)	FACSCanto, FACSDiva ver. 6.0
Kaken Pharmaceutical Co., Ltd. (Kaken)	Crl: CD(SD); 6 weeks (male)	FACSCallibur, Cell Quest ver. 3.3
Mitsubishi Tanabe Pharma Corporation (Mitsubishi Tanabe)	Crl: CD(SD); 6 weeks (male)	FACSCallibur, Cell Quest ver. 3.3 (FACSCallibur, Flow Jo ver. 7.2.2)[b]
National Institute of Health Sciences (NIHS)	Not used (NIHS received blood samples from other participant laboratories and processed them into the *Pig-a* assay.)	FACSCanto II, FACSDiva ver. 6.1

[a] For the ENU study shown in Figs. 2 and 3.
[b] For the transferability study shown in Table 2.

Table 2
The spontaneous frequency of CD59-negative peripheral red blood cells in rats.

Region setting rule for CD59 negative red blood cells	Animal No.	Spontaneous *Pig-a* mutant frequency ($\times 10^{-6}$)				
		Teijin	Daiichi Sankyo	Kaken	Mitsubishi Tanabe	NIHS
No rule	01	1	7	12	11.1	13
	02	1	8	8	18.4	16
	03	4	4	1	11.2	25
	04	–	–	–	16.5	–
	Mean	2.0	6.3	7.0	14.3	18.0
	S.D.	1.7	2.1	5.6	3.7	6.2
After the rule applied	01	1	–	6	1.8	7
	02	0	–	7	5.5	5
	03	4	–	1	0.0	10
	04	–	–	–	0.0	–
	Mean	1.7	–	4.7	1.8	7.3
	S.D.	2.1	–	3.1	2.6	2.5

used for comparisons between the vehicle control group and each of the treatment groups at each time point. The levels of significance were set as $p < 0.05$ or $p < 0.01$.

3. Results

3.1. Transferability of the RBC Pig-a assay using the HIS49 antibody

Five laboratories took part in the collaborative study (Table 1). The protocol for the assay was transferred from the Teijin lab (Reference lab) to other participant labs, and then participants attempted the RBC *Pig-a* assay in untreated rats to establish a common gate setting for the appropriate measurement of CD59-negative cells. In the first trial, the means of the spontaneous frequency in 2 of the 5 laboratories were more than 10×10^{-6} (Table 2), which was considered excessively high. Following discussions, a rule was agreed to in which the height of the gate region for CD59-negative RBCs was set to capture $99.0 \pm 0.1\%$ of the cells in the flow cytometry standard that was stained only with HIS49 antibody (Fig. 1). This rule was determined based on the evidence that most of the cells in the flow cytometry standard were included in this region according to the density plot analysis (Fig. 1). All participants, with the exception of the Daiichi Sankyo lab, adjusted their gate settings according to the rule and reprocessed their flow cytometry acquisition data. After the rule was applied, the spontaneous MFs of all the participating labs were $<10 \times 10^{-6}$ (Table 2). This rule was used in all labs to analyze samples in all subsequent studies.

3.2. Interlaboratory study with ENU

The participating labs conducted an ENU study whose design is shown in Table 3. Test rats were administered a single dose of 0 (PBS vehicle), 10, or 40 mg/kg ENU by gavage ($n = 3$/group). Blood samples were collected before the treatment and at 2 and 4 weeks after the ENU treatment. The National Institute of Health Sciences (NIHS) lab evaluated blood samples sent from the Teijin lab and conducted the *Pig-a* assay within 3 days after the blood collection. Fig. 2 shows the *Pig-a* MFs detected in each laboratory. The *Pig-a* MFs produced by a single dose of 40 mg/kg increased over time in all participating labs. We further analyzed the results of the ENU study by combining the data generated by the individual labs. Significant increases in *Pig-a* MF were produced by 10 mg/kg and 40 mg/kg ENU at both 2 and 4 weeks after the treatment (Fig. 3b and c). The *Pig-a* MF in the 10 mg/kg ENU group at 2 and 4 weeks after the treatment was 2.0- and 3.3-fold higher than in the control group, respectively. The *Pig-a* MF in the 40 mg/kg ENU group at 2 and 4 weeks after the treatment was 7.5- and 16.7-fold higher than in the control group, respectively. The *Pig-a* MF in the vehicle control group was stable and averaged less than 10 per million ($4.6–6.1 \times 10^{-6}$) (Fig. 3).

3.3. Interlaboratory study with 4NQO

The design for the 4NQO study is shown in Table 3. Four laboratories administered a single dose of 0 (0.5%, w/v MC vehicle), 25, 50, or 100 mg/kg 4NQO by gavage ($n = 5$/group). Three laboratories collected blood samples from the treated rats before and at 1, 2, and 4 weeks after the 4NQO treatment; one laboratory (Kaken) collected blood only at 2 and 4 weeks after dosing. All laboratories conducted the rat RBC *Pig-a* assay. The NIHS lab analyzed blood samples collected at the other four laboratories in an effort to evaluate data variability between the laboratories.

The results of multilaboratory experiments using 4NQO are shown in Fig. 4. All the in-life laboratories observed clear time- and dose-related increases in *Pig-a* MF after the treatment; for the high dose, the increases were statistically significant by 1 or 2 weeks

Table 3
Summary of experiments measuring *Pig-a* mutant cells in rats treated with genotoxic compounds.

Laboratories	Test compounds[a]	Dose (mg/kg)	Blood collection after the treatment (week)	Conducted assay
Teijin	ENU[b]	0, 10, and 40	Pre, 2 and 4	*Pig-a* assay in total RBCs
	4NQO[c]	0, 25, 50, and 100	Pre, 1, 2 and 4	PIGRET assay
	DMBA[d]	0, 20, and 60	Pre, 1, 2 and 4	
Daiichi Sankyo	ENU	0, 10, and 40	Pre, 2 and 4	*Pig-a* assay in total RBCs
	4NQO	0, 50, and 100	Pre, 1, 2 and 4	PIGRET assay
	DMBA	0, 20, and 60	Pre, 1, 2 and 4	
Kaken	ENU	0, 10, and 40	Pre, 2 and 4	*Pig-a* assay in total RBCs
	4NQO	0, 25, 50, and 100	2 and 4	PIGRET assay
	DMBA	0, 20, 40, and 60	2 and 4	
Mitsubishi Tanabe	ENU	0, 10, and 40	Pre, 2 and 4	*Pig-a* assay in total RBCs
	4NQO	0, 25, 50, and 100	Pre, 1, 2 and 4	
	DMBA	0, 20, and 60	Pre, 1, 2 and 4	
NIHS	ENU	Not conducted	Not conducted	*Pig-a* assay in total RBCs[e]
	4NQO			
	DMBA			

[a] The route of administration was gavage.
[b] *N*-nitroso-*N*-ethylurea.
[c] 4-Nitroquinoline-1-oxide.
[d] 7,12-Dimethylbenz[*a*]anthracene.
[e] NIHS analyzed blood samples delivered from other laboratories.

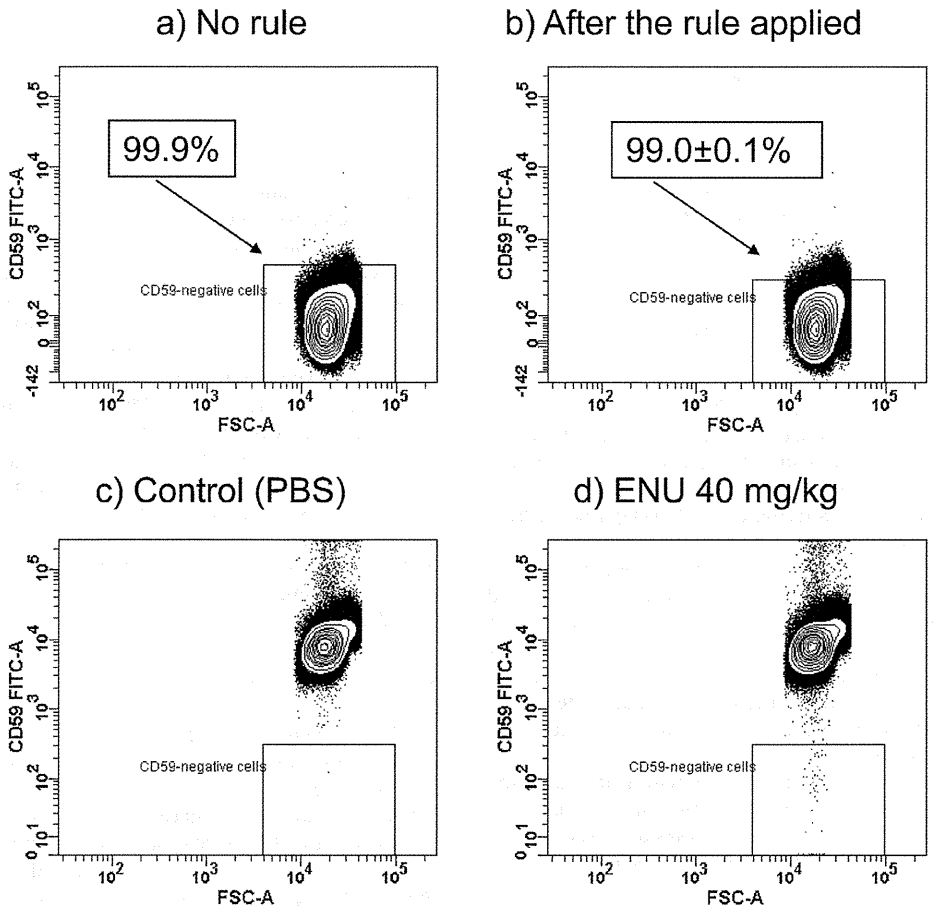

Fig. 1. Gating strategy for measuring CD59-negative cells. The red blood cells were defined by light scatter, and the positive expression of rat erythroid marker reacted with HIS49 antibody. (a) Plot data for pre-adjustment of the gate region using an HIS49 single-stained specimen. Most of the cells (99.9%) mimicked CD59-negative cells without anti-CD59 antibody. (b) Gate region setting after the rule was applied. The same specimen was used for the analysis in both (a) and (b). The height of the gate region for CD59-negative cells was set to include 99.0 ± 0.1% of cells. (c) and (d) Typical cytograms for measuring CD59-negative cells from the vehicle control (PBS) or 40 mg/kg *N*-nitroso-*N*-ethylurea groups.

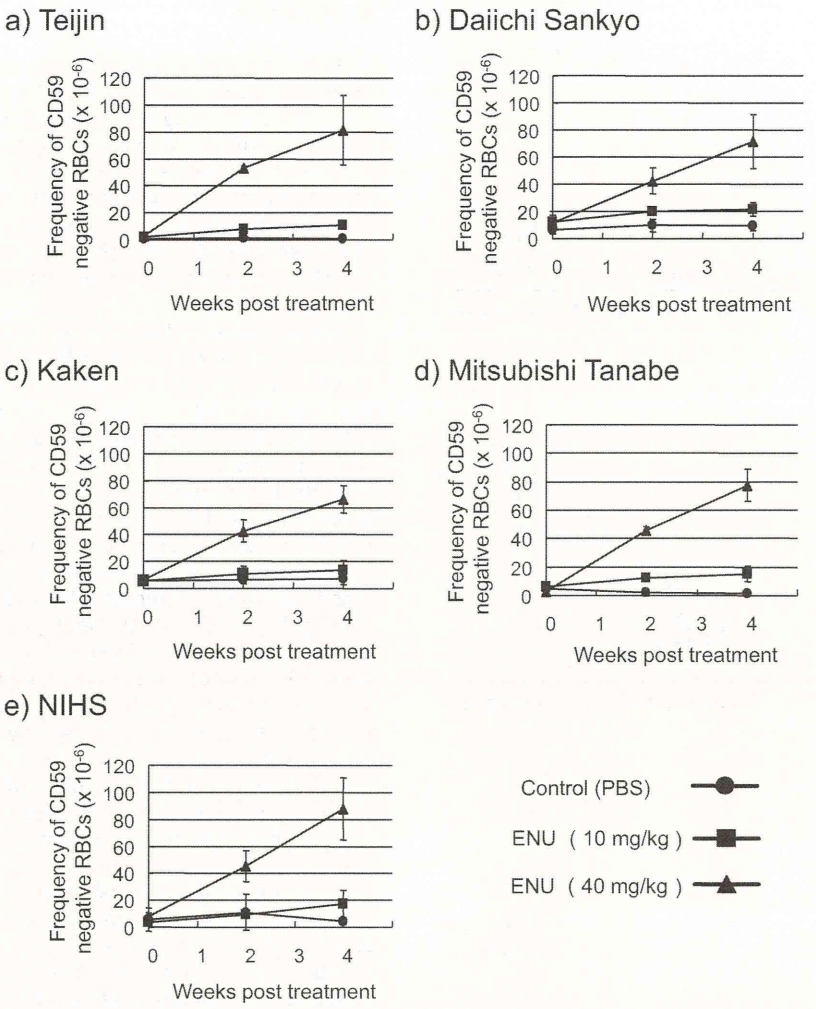

Fig. 2. *Pig-a* mutant frequencies measured by the participating laboratories in rats administered *N*-nitroso-*N*-ethylurea (ENU). The mutant frequencies were analyzed by enumerating the CD59-negative red blood cells (RBCs). Male SD rats were administered a single dose of ENU *via* gavage as shown in Table 3 ($n = 3$/group). Each graph shows the results by participating laboratory. The National Institute of Health Sciences (NIHS) received the blood samples from Teijin Pharma and conducted the RBC *Pig-a* assay within 3 days after the blood collection.

after the treatment (Fig. 4a, d, g, and j). Furthermore, comparable results were obtained at the NIHS lab, which analyzed the blood samples from the other participating labs (Fig. 4b, e, h, and k). The mean background *Pig-a* MFs in the vehicle control groups were generally less than 10 per million (64.3% of the samples had a MF less than 5.0×10^{-6}); two vehicle control means that were processed at the NIHS lab (pretreatment samples from the Teijin lab and 4-week post-treatment samples from the Kaken lab) were exceptions (Fig. 4b and h). Also, despite the uniformly low *Pig-a* MFs that were detected by the Mitsubishi Tanabe lab in their pretreatment samples (Fig. 4j), the NIHS lab detected non-significant increases in *Pig-a* MF in the same samples (Fig. 4k).

Fig. 4c, f, and i shows the results of PIGRET assays conducted on rats treated with 4NQO. Three of the five labs performed the PIGRET assay with the same blood samples used for the RBC *Pig-a* assay. The Teijin and Daiichi Sankyo labs produced comparable results, with apparent dose-related increases in the number of *Pig-a* mutant RETs by 1 week after the treatment (the Kaken lab performed the PIGRET assay only on 4 week samples). At 4 weeks after the treatment, all laboratories found significant increases in the 100 mg/kg dosing group. The mean RET *Pig-a* MFs in the vehicle controls were $0.0–1.8 \times 10^{-6}$, with all individual rat samples having MFs $<5.0 \times 10^{-6}$.

3.4. Interlaboratory study with DMBA

The design of the DMBA study is shown in Table 3. Four laboratories treated animals with a single dose of 0 (olive oil vehicle), 20, 40, or 60 mg/kg DMBA by gavage ($n = 5$/group). Similar to the 4NQO study, three laboratories collected blood samples from rats before the treatment and at 1, 2, and 4 weeks after dosing; the Kaken laboratory sampled blood only at 2 and 4 weeks after the dosing. All in-life laboratories conducted the rat RBC *Pig-a* assay, while the NIHS lab performed the RBC *Pig-a* assay on blood samples from three of these laboratories to evaluate data variability between the laboratories.

Fig. 5 shows the results of the multilaboratory experiments using DMBA. Clear time- and dose-related increases in *Pig-a* MF were observed in all laboratories, and the increases were statistically significant for the high-dose group by 1 or 2 weeks after the treatment (Fig. 5a, d, g, and j). Furthermore, comparable results were obtained at the NIHS lab, which analyzed the blood samples from other participating labs (Fig. 5b, e, and h). The mean *Pig-a* MFs in the vehicle control groups generally were stable and less than 10 per million (70.0% of the samples had a MF $<5.0 \times 10^{-6}$). The exceptions were the mean pretreatment sample from the Teijin lab and the 4 week vehicle control sample mean from the Teijin lab,

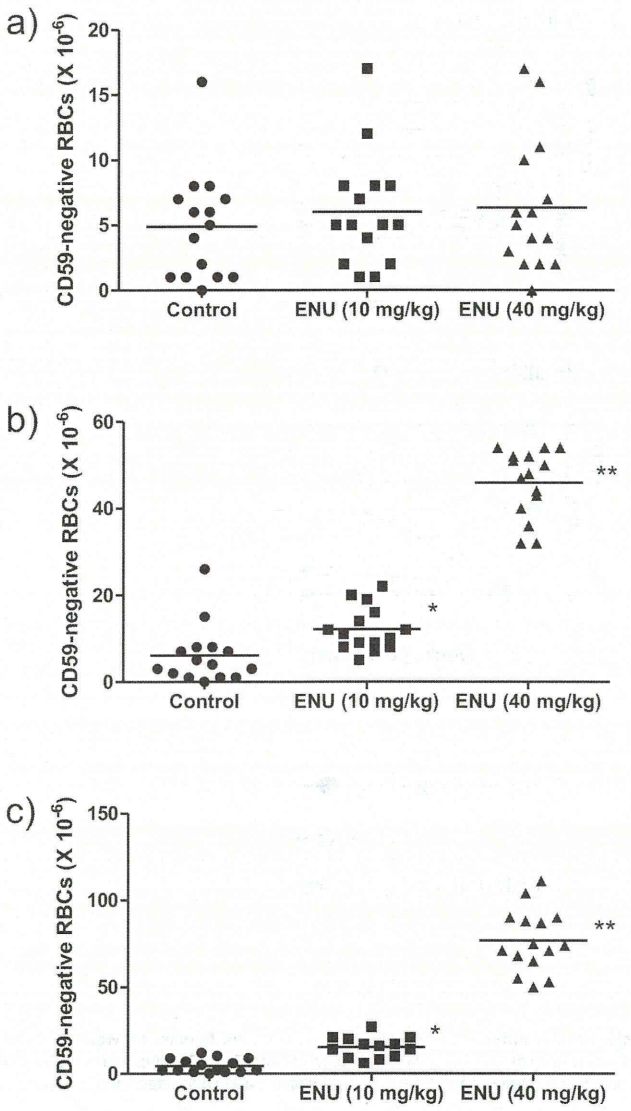

Fig. 3. Summary of *Pig-a* mutant frequencies in rats administered *N*-nitroso-*N*-ethylurea. The mutant frequencies were analyzed by enumerating CD59-negative red blood cells. (a), (b), and (c) display the combined data from assays conducted before and at 2 and 4 weeks after the first dosing, respectively. All individual data are displayed as scatter plots, and a bar indicates the mean of each group ($n = 13$ or 15). Statistically significant differences from vehicle controls are indicated at the $p < 0.05$ or 0.01 levels (*, or **, respectively).

analyzed at NIHS (Fig. 5a and b). For their assays on pretreatment blood, the first sample analyzed by flow cytometry by the Teijin lab had a MF of 65×10^{-6}; however, no increases of *Pig-a* MF were found for the same rat when blood was analyzed at NIHS and at other sampling points at Teijin.

Fig. 5c, f, and i shows results from PIGRET assays conducted on blood from rats treated with DMBA. Three of the five labs conducted the PIGRET assay using the same blood samples used for the RBC *Pig-a* assay. Both the Teijin and Daiichi Sankyo labs found an apparent dose-related increase in the number of *Pig-a* mutant RETs by 1 week after the treatment. By 2 and 4 weeks after the treatment, all three laboratories found significant increases in the 60 mg/kg dosing group. The mean *Pig-a* MFs for RETs in the vehicle control rats from this study were $0.3–8.4 \times 10^{-6}$, with 79.6% of the samples having MFs $< 5.0 \times 10^{-6}$.

4. Discussion

Data from the five laboratories that participated in this collaborative study indicated that the protocol for the *Pig-a* assay using HIS49 antibody was transferable among different laboratories. First, a rule for setting flow cytometer gating was established that, in most cases, resulted in mean background MFs in the RBC *Pig-a* assay of $< 10 \times 10^{-6}$. This rule was applied to a gate in the FSC/CD59 cytograms for the purpose of accurately enumerating CD59-negative cells. After the rule was applied, more than 60% the *Pig-a* MFs in the vehicle control group were no greater than 5×10^{-6} (Table 2).

It was important that *Pig-a* mutants were identified consistently in the different labs and ideally that the background *Pig-a* MFs were reasonably consistent with previous measurements of background *Pig-a* MFs, and MFs of other X-linked reporter genes (like *Hprt*). A low spontaneous MF also was anticipated to benefit the assay in terms its power to detect the mutagenicity of test chemicals. With the HIS49 *Pig-a* assay, it was expected that the different laboratories could achieve equivalent assay power by adopting the rule. In fact, all laboratories demonstrated similar dose-dependent increases of *Pig-a* MF in the ENU experiment, the first experiment that was conducted after the rule was adopted (Fig. 2). The data that were collected (Fig. 3) indicated that the ENU treatment resulted in a consistent, statistically significant increase in *Pig-a* MF in each of the participant labs. This result indicates that the protocol was successfully transferred between participants, and that the participating labs had equivalent test power in the evaluation of ENU mutagenicity.

Based on the results of the ENU experiment, we then confirmed the reproducibility of the *Pig-a* assay between the participating laboratories by testing two other mutagenic agents, 4NQO and DMBA. Four of the participating labs carried out both the *Pig-a* assay and the animal experiment while the NIHS lab only carried out the RBC *Pig-a* assay, using blood samples shipped from other participating labs. The results indicated very small differences among the laboratories in terms of the RBC *Pig-a* MFs (Figs. 4 and 5). Both 4NQO and DMBA are known to increase the *Pig-a* MF in rats [13,15,17,18]. In this study, the participants found *Pig-a* MF responses that were similar to those in the previous studies. A recent report by Bhalli et al. demonstrated that both the RBC *Pig-a* assay and PIGRET assay produced similar results to the Litron's high throughput method which is the *Pig-a* mutant enrichment protocol in a Cis-platin study [19]. These results suggest that both the RBC *Pig-a* assay and PIGRET assay using HIS49 antibody are reproducible and sensitive methods for the detection of *in vivo* mutagenicity.

A second finding from this study was that the PIGRET assay can detect increases in *Pig-a* MF sooner after exposure than can the RBC *Pig-a* assay. In the 4NQO and DMBA experiments, 3 of the 5 participating labs also conducted the PIGRET assay, and most of their results indicated statistically significant increases in the treated animals (Figs. 4 and 5). It was notable that the PIGRET assay consistently detected increases in *Pig-a* MF at 1 week after the dosing, whereas the RBC *Pig-a* assay only occasionally detected an agent-induced increase at this sampling time. The PIGRET assay uses magnetic enrichment of RETs to increase the number of RETs that can be interrogated for mutations in a reasonable length of time [16]. The PIGRET assay not only has a good correlation with *Pig-a* MF measurements made in bone marrow erythroids, but it also has a low background *Pig-a* MF (usually $< 5 \times 10^{-6}$) [16], which benefits its ability to detect small increases in agent-induced MF. Although it is a challenging work, the PIGRET assay with a sorting instrument might be useful for a sequencing analysis of *Pig-a* gene because reticulocytes usually contain mRNA.

While the interlaboratory differences found for the HIS49 *Pig-a* assay were relatively small, it should be noted that some samples

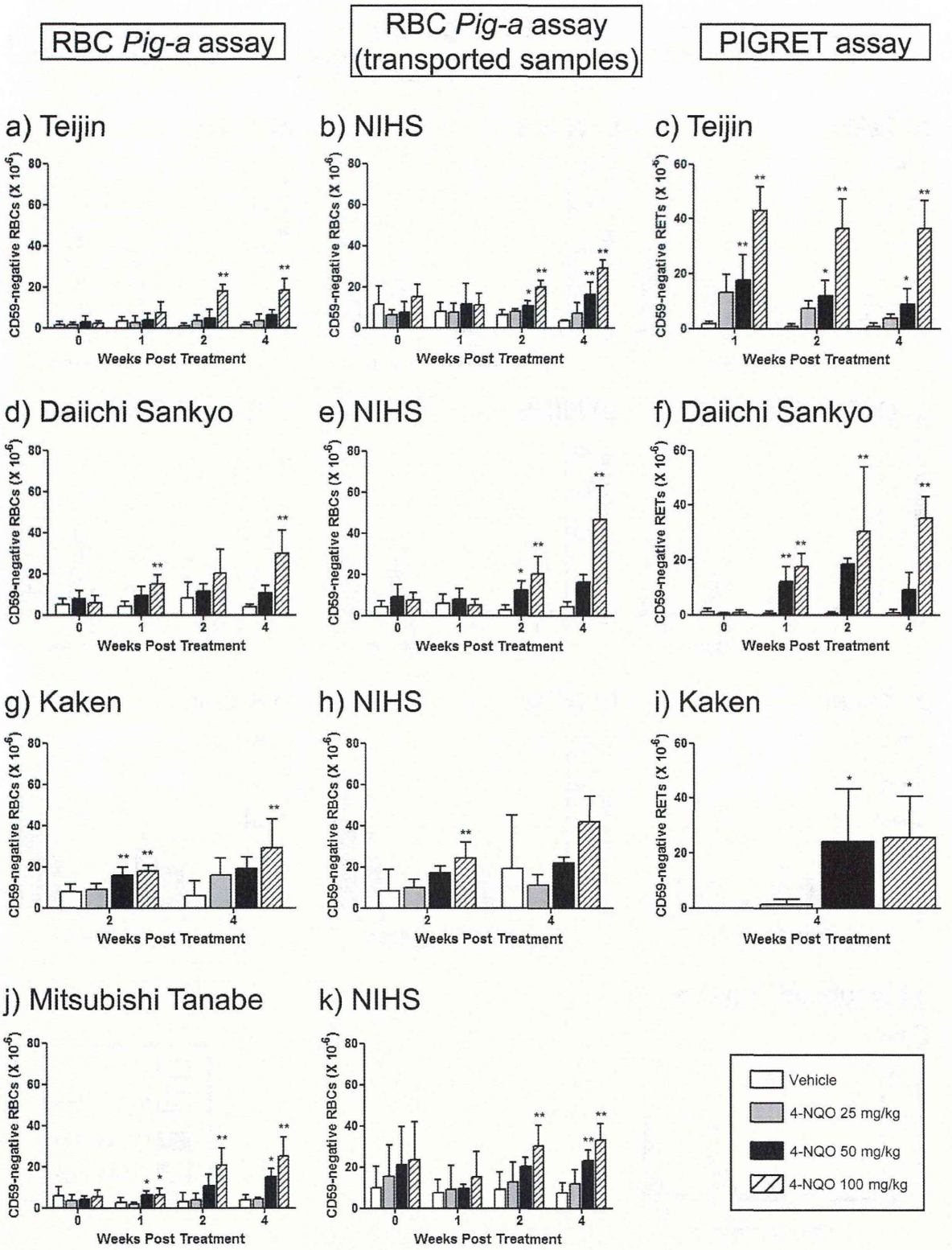

Fig. 4. *Pig-a* mutant frequencies in rats administered 4-nitroquinoline-1-oxide (4NQO). The mutant frequencies were analyzed by enumerating CD59-negative red blood cells (RBCs) or CD59-negative reticulocytes (RETs). Male SD rats were administered a single dose of 4NQO *via* gavage as shown in Table 3 ($n = 5$/group). Each graph shows the results by participating laboratory. The National Institute of Health Sciences (NIHS) analyzed blood samples from the other four laboratories using the RBC *Pig-a* assay ((b), (e), (h), and (k)). Statistically significant differences from vehicle controls are indicated at the $p < 0.05$ or 0.01 levels (*, or **, respectively).

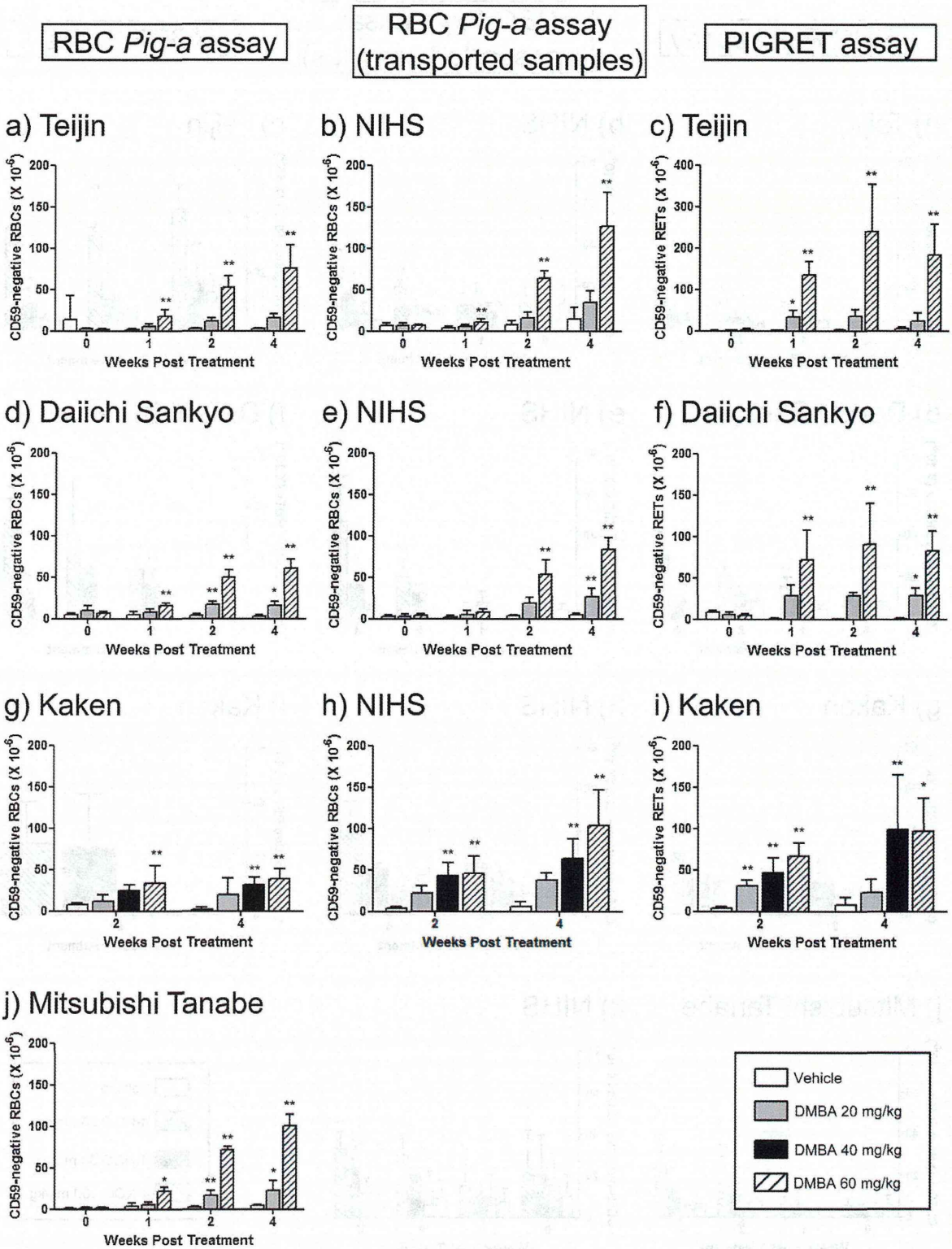

Fig. 5. *Pig-a* mutant frequencies in rats administered 7,12-dimethylbenz[*a*]anthracene (DMBA). The mutant frequencies were analyzed by enumerating CD59-negative red blood cells (RBCs) or CD59-negative reticulocytes (RETs). Male SD rats were administered a single dose of DMBA *via* gavage as shown in Table 3 ($n = 5$/group). Each graph shows the results by participating laboratory. The National Institute of Health Sciences (NIHS) analyzed the blood samples from the other three laboratories using the RBC *Pig-a* assay (Fig. 4(b), (e), and (h)). Statistically significant differences from vehicle controls are indicated at the $p < 0.05$ or 0.01 levels (*, or **, respectively).

from the vehicle control groups had unusually high background frequencies. In particular, the RBC *Pig-a* MF of one of the pre-treatment samples from the Teijin lab was 65×10^{-6} (Fig. 5a). We speculate that this high value was caused by contamination from an unstained flow cytometry standard that was inadvertently left on a sample injection tube without cleaning. Other RBC *Pig-a* MF data from the same animal examined at 1–4 weeks after the dosing, and from this same blood sample at the NIHS lab, all had MFs of $<5 \times 10^{-6}$. Furthermore, some RBC *Pig-a* MFs measured by the NIHS lab indicated a high background frequency, although the other participating labs measuring MFs in these samples found that they had MFs of $<10 \times 10^{-6}$. These discrepancies are thought to be related to the shipment that these samples had undergone. It was noticed that some of these samples had dispersed within the tube, and portions of the blood dried. In addition, although it was exceedingly rare, some animals had consistently high spontaneous *Pig-a* MFs without dosing (data not shown). Therefore, it is important to clarify the way that outliers with a high spontaneous *Pig-a* MF are dealt with in designing and interpreting assays. As examples, a preliminary study might be conducted to identify animals with unusually high spontaneous *Pig-a* MFs so that they can be excluded from the experiment, or an experiment record sheet might be developed that identifies operational mistakes that could compromise the data. Also, further refinement of the protocol may ensure that the background *Pig-a* MF is consistently low.

Taken together, we found low variability in the *Pig-a* gene mutation data generated by the participating laboratories. In addition, in terms of measuring *in vivo* mutagenicity, the PIGRET assay, although technically more challenging than the RBC *Pig-a* assay, more consistently detected agent-induced increases in *Pig-a* MF at early sampling times than did the RBC *Pig-a* assay.

Conflict of interest statement

None.

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