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MiniReview

The *Pig-a* Gene Mutation Assay in Mice and Human Cells: A Review

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Abstract: This MiniReview describes the principle of mutation assays based on the endogenous *Pig-a* gene and summarizes results for two species of toxicological interest, mice and human beings. The work summarized here largely avoids rat-based studies, as are summarized elsewhere. The *Pig-a* gene mutation assay has emerged as a valuable tool for quantifying *in vivo* and *in vitro* mutational events. The *Pig-a* locus is located at the X-chromosome, giving the advantage that one inactivated allele can give rise to a mutated phenotype, detectable by multicolour flow cytometry. For *in vivo* studies, only minute blood volumes are required, making it easily incorporated into ongoing studies or experiments with limited biological materials. Low blood volumes also allow individuals to serve as their own controls, providing temporal information of the mutagenic process, and/or outcome of intervention. These characteristics make it a promising exposure marker. To date, the *Pig-a* gene mutation assay has been most commonly performed in rats, while reports regarding its usefulness in other species are accumulating. Besides its applicability to *in vivo* studies, it holds promise for genotoxicity testing using cultured cells, as shown in recent studies. In addition to safety assessment roles, it is becoming a valuable tool in basic research to identify mutagenic effects of different interventions or to understand implications of various gene defects by investigating modified mouse models or cell systems. Human blood-based assays are also being developed that may be able to identify genotoxic environmental exposures, treatment- and lifestyle-related factors or endogenous host factors that contribute to mutagenesis.

Paroxysmal nocturnal haemoglobinuria (PNH) is a genetic disorder that affects 1–10 per million individuals and is caused by somatic *PIG-A* gene mutations within a bone marrow stem cell [1]. The *PIG-A* gene encodes a catalytic subunit of the N-acetylglucosamine transferase complex involved in the synthesis of glycosylphosphatidylinositol (GPI) cell surface anchors [2]. Inactivating mutations inhibit the biosynthesis of GPI anchors, causing a deficiency in GPI-anchored proteins on the cell surface, for example CD59, CD55 and CD24. PNH usually affects erythrocytes, granulocytes and monocytes. In a minority of cases, the lymphocyte lineage is also affected. As a cell surface phenotype present on readily accessible haematopoietic cells, flow cytometry-based techniques that measure the frequency of CD59- and/or CD55-deficient red blood cells have replaced the traditional HAM test (acid-induced disruption of the fragile red blood cells of patients with PNH) for PNH diagnosis [3,4]. Work directed at elucidating the aetiology of PNH and creating flow cytometry-based diagnostic tests provided an important starting point for the development of mutation assays that utilize the *Pig-a/PIG-A* loci as reporters of gene mutation.

The earliest reports that suggested that *PIG-A* may represent a useful reporter of gene mutation were from the group of Luzzatto [5] and later Chen [6]. These groups focused on human cells, often transformed lymphocytes grown in cell culture, or freshly isolated blood leucocyte population(s) [5–9]. Several extended this work to rodent blood erythrocytes and/or reticulocytes in order to provide regulatory safety assessment laboratories with a practical and efficient platform for evaluating chemical or physical agents' potential to cause gene mutation *in vivo* [10,11]. To date, this work has primarily been accomplished in rats, as a key rodent model used in regulatory toxicology studies [12,13]. However, the successful application of phenotypic *Pig-a*-mutant scoring methods in cell lines and mouse models is important given their prominent role in both regulatory toxicology and basic research. As much understanding has been accomplished and described for rats, the purpose of this MiniReview was to first introduce the Pig-a assay in detail followed by a description of the current status for cell lines, mouse models and human beings.

The scientific literature was searched in PubMed using keywords such as Pig-a, PIG-A, mutation, paroxysmal nocturnal haemoglobinuria, mouse, human beings, TK6 and followed by

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manual selection of relevant articles using a version of the *Pig-a/PIG-A* assays as main inclusion criteria. The studies are presented in tables 1 and 2 according to the publication year.

Principle of the Assay

Iida *et al.* [14] isolated the human *PIG-A* gene and found that it contains six exons over its 17 kb length. As demonstrated by Kawagoe *et al.* [15], there is a high degree of interspecies conservation of the gene's structure, function and position on the X-chromosome. The gene product plays a critical role in the first step in GPI anchor biosynthesis, and the entire process is thought to require at least two dozen genes. Mutation of any one of these genes could theoretically result in GPI anchor deficiency. However, all other genes involved in GPI anchor synthesis are autosomal. Mutations on both alleles would have to occur to ablate expression of GPI anchors, and this is expected to be an exceptionally rare event, at least when all other GPI anchor-associated gene alleles are both functional. Thus, the single-copy status of the *Pig-a/PIG-A* gene is a critical feature that makes it useful as a phenotypic reporter of gene mutation, because only one mutational event is sufficient to completely ablate expression of GPI anchors.

The evidence that GPI anchor deficiency represents a valid reporter of *Pig-a/PIG-A* mutation starts with investigations of PNH. One key finding is that with only extremely rare exceptions PNH clones exhibit mutation at the *PIG-A* locus. For instance, Nishimura *et al.* [16] analysed 146 patients with PNH and reported the types of mutations that lead to GPI anchor deficiency, all within the *PIG-A* locus. Single-base substitutions and frame-shift events were the most highly represented classes of mutations observed. They also found three examples of large deletions (entire gene, 4 kb and 737 base pairs), as well as a large insertion (88 base pairs). The mutations were widely distributed in the coding regions and splice sites, although Nafa *et al.* [17] found a somewhat higher frequency of missense mutations in exon 2 relative to other exons. Taken together, the PNH literature provides strong support for the concept that the GPI anchor-deficient phenotype represents a useful reporter of *Pig-a* mutations, and such assays would be sensitive to several important classes of mutations.

The cell surface of haematopoietic cells is dramatically affected by *Pig-a/PIG-A* mutations. For example, CD59, CD55 and/or CD24 are highly expressed on haematopoietic cells, and these expression levels are maintained throughout cells' lifespan. However, when bone marrow cells experience an inactivating *Pig-a/PIG-A* mutation, after a sufficient period of manifestation time, progeny cells show a complete absence of these markers. The significant changes to specific cell surface markers as a result of *Pig-a/PIG-A* mutations are readily detected using fluorescent antibodies in conjunction with flow cytometry. Furthermore, blood cells as well as lymphoblastoid cells in culture are highly compatible with flow cytometric analysis. These characteristics combine to make flow cytometry the method of choice for efficiently scoring *Pig-a/PIG-A*-mutant cells (fig. 1).

Variations in the basic approach of scoring mutant cells (mostly blood cells) via flow cytometry in conjunction with fluorescent antibody labelling have been described. For instance, for some cell types, it is possible to use the fluorescent-labelled aerolysin reagent (FLAER) instead of antibody(ies). Whereas antibodies recognize wild-type cells via their recognition of GPI-anchored protein(s), FLAER binds with high affinity to GPI anchors themselves. When the frequency of spontaneous mutant phenotype cells is very low, as in the case for rat and mouse erythrocytes and reticulocytes, the use of immunomagnetic separation technologies is convenient. In this approach, the number of reticulocytes evaluated for the mutant phenotype is enriched, thus decreasing the data acquisition time [18]. Alternatively, one may use immunomagnetic separation to specifically deplete the wild-type cells in each sample. In this scenario, flow cytometric analysis of immunomagnetically separated samples provides the means to efficiently evaluate orders of magnitude more erythrocytes and reticulocytes for the mutant phenotype than would otherwise be possible, leading to lower scoring errors [19]. These immunomagnetic separation techniques can be especially important when study designs seek to keep treatment group sizes modest while at the same time ensuring sufficient statistical power to detect modest changes to spontaneous mutant cell frequencies. This experimental refinement greatly adds to the sensitivity of the assay, due to the enrichment of unstained mutants and thus analysis of manyfold more potential mutant cells (fig. 2).

One important aspect of the assay is that the mutants scored by antibody staining and flow cytometry are phenotypical mutants. Verification of these phenotypic mutants as true *Pig-a/PIG-A* mutants by DNA sequencing analyses is vital for confirmation of mutation frequencies measured and to demonstrate the true nature of the mutants. Such identification has been demonstrated in bone marrow erythroids of mice [20] and CD48-negative T cells of *N*-ethyl-*N*-nitrosourea (ENU)- and 7,12-dimethylbenz[a]anthracene (DMBA)-treated rats [21,22]. The latter was performed by flow cytometric sorting of mutant cells and sequencing of the *Pig-a* gene of expanded single cells [21,22]. The induced mutation spectra obtained were consistent with expected spectra obtained in other endogenously expressed genes, verifying the validity of the mutation frequencies measured. In human cells, point mutations as well as deletions in *PIG-A* have been identified in TK6 cell lines [23,24]. Recently, heterogeneous pools of *Pig-a*-mutant T cells derived from DMBA-exposed rats were efficiently sequenced by an elegant novel technique named mutation analysis with random DNA identifiers (MARDI), requiring no clone-by-clone analyses [25]. The MARDI technique facilitates verification of *Pig-a* mutants in a more feasible manner than previous strategies. Using the MARDI technique, nearly all previously found *Pig-a* mutations were identified, and new mutations were detected.

Pig-a in Mice

Searching the scientific literature, we identified 21 studies in mice (summarized in table 1). In 2008, the first study to

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Table 1.

Overview of Pig-a studies in mice.

Strain(s), genotype(s), sex, age	Agent/intervention (exposure, route, assessment time). Methodological details	Main outcome, mutation frequencies (Mf) in reticulocytes (RETs) or red blood cells (RBCs)	References
CD-1 females, 7–8 weeks	DMBA, ENU (three daily doses; i.p.; DMBA: 75 mg/kg/day, analyses 3 weeks after exposure; ENU: 40 mg/kg/day, analysed weekly)	Increased Mfs in RETs and RBCs. Results from week 2. ENU: RET mean Mf 600 × 10^{-6} (~43-fold) and RBC mean Mf 300 × 10^{-6} (~21-fold), maximum effect in RETs in week 1 and RBC in week 2; DMBA: RET mean Mf 286 × 10^{-6} (~21-fold) and RBC mean Mf 133 × 10^{-6} (~10-fold), vehicle control mean Mf 14 ± 21 × 10^{-6}	[26]
C57BL/6 males, 6–7 weeks	ENU (i.p.; single dose: 0, 10, 25, 45, 70, 100 and 140 mg/kg; single *versus* split doses: four weekly doses *versus* one single dose: 4 ×8 mg/kg *versus* 32 mg/kg and 4 × 40 mg/kg *versus* 160 mg/kg; assessment with 2- to 6-week intervals for 26 weeks after last exposure)	Single dose: increased RET and RBC mean Mfs with maximum effect at week 2 (RETs, 700 × 10^{-6}) and 4 (RBCs, 230 × 10^{-6}) weeks that was maintained for many weeks, and declined at weeks 20 and 26. Vehicle control mean Mfs ranged from 0.2 to 2.2 × 10^{-6} (RETs) and 0–2 × 10^{-6} (RBCs). Split *versus* single doses: groups receiving single doses had generally higher mean Mfs than equal total split doses. Increased RET mean Mfs at 2 weeks (32 *versus* 8×4 mg/kg: 180 *versus* 105 × 10^{-6}; 160 *versus* 40 × 4 mg/kg: 1150 *versus* 750 × 10^{-6}) and RBC mean Mfs at 4 weeks (32 *versus* 8 × 4 mg/kg: 66 *versus* 33 × 10^{-6}; 160 *versus* 40 × 4 mg/kg: 460 *versus* 400 × 10^{-6}). Vehicle control mean Mfs ranged from 0 to 5.5 × 10^{-6} (RETs) and 0.6–2.7 × 10^{-6} (RBCs)	[27]
CD-1[1] males, 6 weeks	ENU (single dose of 100 mg/kg, i.p.). Mf measured in erythroids from bone marrow (BM) and peripheral blood (PB) analysed pre-dosing and 1, 2 and 4 weeks after dosing.)	Increased erythroid Mf in BM occurred earlier and was higher than in PB. The Mfs in PB and BM erythroids reached 199 × 10^{-6} and 682 × 10^{-6} at 4 weeks after exposure, respectively. Mf in control mice was low (usually <5 × 10^{-6} in PB and 0–83.3 × 10^{-6} in BM)	[20]
Muta™Mouse males, 25 weeks	BaP (positive control ENU) (dosed with 0, 25, 50 and 75 mg/kg/day for 28 days, oral gavage, analysed 3 days after the last dose; positive control ENU was given as a single dose of 45 mg/kg bw. i.p., and analysed 2 weeks after dosing)	Significant dose-dependent increases in RET and RBC Mfs. At the highest BaP dose (75 mg/kg bw/day), the RET and RBC mean Mfs were ~240 × 10^{-6} and ~95 × 10^{-6}. Vehicle control mean Mfs were 0 in RETs and 0.4 × 10^{-6} in RBCs. Mf in ENU-positive control mice was ~190 × 10^{-6} (RETs) and ~30 × 10^{-6} (RBCs)	[33]
C57BL/6J males, 6 weeks	X-rays (160 kVp with 0.5-mm Cu and 0.5-mm Al filters) (0.52 Gy/min; single dose of 0.5, 1 and 2 Gy or fractionated dose of 4 × 0.5 Gy once a week; single exposures: analyses on days 0, 2, 7, 14, 21, 28, 35, 42, 49, 56, 70, 84, 105, 126, 148, 203, 232, 267, and 297 after exposure, for fractionated exposure analyses it was performed just before each exposure on days 0, 7, 14 and 21)	Dose-dependent increase in RBC Mf and increase in RET Mf, which over time returned to background levels. Equivalent single and fractionated doses gave rise to similar maximum RBC mean Mfs; however, the maximum of the fractionated dose appeared two to three weeks later than the single dose. At the highest single dose of 2 Gy, the maximum RET and RBC mean Mfs were on day 14 with 44.89 × 10^{-6} and ~150 × 10^{-6}. After the protracted dose of 4 × 0.5 Gy, the maximum RBC mean Mf was at day 42 with 49.97 × 10^{-6}, whereas the mean Mf of RETs varied considerably probably due to bone marrow toxicity. There were marked interindividual differences in response, both in the magnitude of the maximal response and in time until maximum response was evident	[35]
B6C3F1 males, 6–7 weeks	TiO_2-NP (positive control ENU) (i.v.; exposure for 3 days with 0.5, 5.0 and 50 mg/kg; positive control ENU was given as a single dose of 140 mg/kg bw. i.p.; analyses occurred on day −1 and weeks 1, 2, 4 and 6 after dosing)	No increased Mf in RETs or RBCs, despite availability to the bone marrow and bone marrow cytotoxicity. Over the study period, RET and RBC mean Mfs in vehicle control animals ranged from 0 to 1.2 × 10^{-6} and 0–4 × 10^{-6}. No significant increase was observed in treated animals. Positive control animals treated with ENU (140 mg/kg) had mean RET and RBC Mfs at 2 weeks of 864.00 × 10^{-6} and 304.80 × 10^{-6}, respectively	[38]

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Table 1. (continued)

Strain(s), genotype(s), sex, age	Agent/intervention (exposure, route, assessment time). Methodological details	Main outcome, mutation frequencies (Mf) in reticulocytes (RETs) or red blood cells (RBCs)	References
C57BL/6J *gpt* delta males, 8 weeks	ENU, BaP, 4NQO (single oral doses of ENU (40 mg/kg), BaP (100 and 200 mg/kg) and 4NQO (50 mg/kg); analyses occurred at 2, 4 and 7 weeks after the treatment)	Increased RBC Mf for all three mutagens, with ENU giving an increase that was stable until week 7, BaP showed a decline after 2 weeks and 4NQO giving rise to variable, low response. At 2 weeks, the RBC mean Mfs were $23.00 \pm 6.96 \times 10^{-6}$ for ENU, $9.25 \pm 2.63 \times 10^{-6}$ for 100 mg/kg BaP, $15.80 \pm 8.20 \times 10^{-6}$ for 200 mg/kg BaP and $2.60 \pm 4.72 \times 10^{-6}$ for 4NQO. PBS vehicle control mean Mfs were 0.4–0.8×10^{-6}	[30]
CD-1 males, 7 weeks	Procarbazine (0, 37.5, 75 or 150 mg/kg/day, treatment for three consecutive days, oral gavage, analyses at days 15 and 30, the protocol included immunodepletion of wild-type cells)	Dose-related increases in RET and RBC Mfs. At day 15, the RET and RBC mean Mfs were $\sim 23 \times 10^{-6}$ and $\sim 5 \times 10^{-6}$ at the highest dose	[44]
C57BL/6J *gpt* delta males, 6–8 weeks	EMS (0, 5, 13, 20, 55 and 100 mg/kg/day, 28 days, oral gavage; analyses occurred at day 29, only RBCs were analysed)	Dose-related increase in RBC Mfs and a significant increase in mean RET Mf. The RET mean Mfs were $\sim 20 \times 10^{-6}$ for all doses ≥13 mg/kg/day, whereas the RBC mean Mfs increased with dose and were 5×10^{-6} at the highest dose. Vehicle control mean Mfs were maximum 2×10^{-6}	[32]
C57BL/6 *Ogg1*$^{+/+}$ and *Ogg1*$^{-/-}$ males, 10–11 weeks	BaP (positive control ENU) (dosed with 50 mg/kg bw for 3 days, i.p.; positive control ENU was given at 22 mg/kg bw for 3 days, i.p.; analysed 5 days prior to dosing and at days 16 and 34 after the last dosing, the protocol included immunodepletion of wild-type cells and comparisons with pre-dosing measurements were possible)	Small but statistically significantly increased RET and RBC Mfs, most evident at day 16 with no clear differences between genotypes At day 16, the mean Mfs of RETs were 17.43 and 13.64×10^{-6} and RBCs were 1.14 and 1.25×10^{-6} from Ogg1$^{+/+}$ and Ogg1$^{-/-}$, respectively. Day 5 prior to exposure, the mean Mfs were 0.29 and 0.78×10^{-6} in RETs and 0.13 and 0.08×10^{-6} in RBCs from Ogg1$^{+/+}$ and Ogg1$^{-/-}$, respectively. Vehicle control mean Mfs at day 16 were 7.70 and 0.27×10^{-6} in RETs and 1.26 and 0.09×10^{-6} in RBCs from Ogg1$^{+/+}$ and Ogg1$^{-/-}$, respectively. Mfs in ENU-positive control Ogg1$^{+/+}$ mice at day 16 were 125.80×10^{-6} (RETs) and 13.39×10^{-6} (RBCs)	[34]
B6C3F1 males, 7 weeks	AgNP (positive control ENU) (i.v.; single doses of 0.5, 1.0, 2.5, 5.0, 10.0 or 20.0 mg/kg; positive control ENU was given as a single dose of 140 mg/kg bw. i.p.; analyses occurred on day 2 and on weeks 2, 4 and 6 after dosing)	No increased Mf in RETs or RBCs, despite availability to the bone marrow and bone marrow cytotoxicity. Over the study period, RET and RBC mean Mfs in vehicle control animals ranged from 0 to 1.3×10^{-6} and 0 to 0.4×10^{-6}. No significant increase was observed in treated animals. Positive control animals treated with ENU had mean RET and RBC Mfs at 2 weeks of 550×10^{-6} and 190×10^{-6}, respectively	[39]
C57BL/6J *p53*$^{+/+}$ (WT) and *p53*$^{-/-}$ (KO) males[1, 7 weeks]	X-rays (160 kVp with 0.5-mm Cu and 0.5-mm Al filters) (1 Gy, 0.52 Gy/min, analyses occurred at days 0, 2, 7, 14, 21, 28, 35, 42, 49, 56, 63, 77, 91, 112, 133, 225, 323, 413 and 713 (only WT for the last 4 time-points due to death of KO); only RBCs were measured)	Increased RBC Mf in WT mice and two times higher increase in KO mice, which declined to background levels in WT mice. The maximum response occurred at day 28 with RBC mean Mfs in WT and KO mice of 10.37×10^{-6} and 21.59×10^{-6}, respectively. The RBC mean Mfs of unexposed WT and KO at day 28 were 2.26×10^{-6} and 2.65×10^{-6}, respectively. There were marked interindividual differences in response, both in the magnitude of the maximal response and in time until maximum response was evident	[36]

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Table 1. (continued)

Strain(s), genotype(s), sex, age	Agent/intervention (exposure, route, assessment time). Methodological details	Main outcome, mutation frequencies (Mf) in reticulocytes (RETs) or red blood cells (RBCs)	References
B6C3F1 males, 8 weeks	1,2-DCP and DCM (positive control ENU)(repeated inhalation, B6C3F1: 150, 300 and 600 ppm 1,2-DCP or 400, 800 and 1,600 ppm DCM. Coexposure groups were exposed to 150 + 400 ppm and 300 + 800 ppm of 1,2-DCP + CM for 6 hr/day, five consecutive days/week, for 6 weeks; analyses occurred in weeks 3 and 6 after inhalation of 1,2-DCP and/or DCM, only RBCs were analysed; positive control ENU was given as a single dose of 70 mg/kg bw i.p.)	No significant increase in RBC mean MF for any treatment. Positive control animals treated with ENU had RBC mean Mfs at $222 \pm 81 \times 10^{-6}$ at 3 weeks and $265 \pm 312 \times 10^{-6}$ at 6 weeks	[41]
C57BL/6 *Ogg1*$^{+/-}$ and *Ogg1*$^{-/-}$ males, 8–11 weeks	Selenium (Se)-deficiency through diet. (positive control ENU) (Se depletion through two generations. Mice were given a diet of either low (0.01 mg Se/kg diet) or normal (0.23 mg Se/kg diet) Se content. Positive control ENU was given 22 mg/kg bw for 3 days, i.p.; the protocol included immunodepletion of wild-type cells.)	Significantly increased RBC men Mf (p=0.008), but not RET mean Mf in mice given the low Se diet. No interaction was observed between diet and genotype. The RET mean Mf was insignificantly increased in *Ogg1*$^{-/-}$ mice. The mean RET and RBC Mfs were 179.9×10^{-6} and 33.4×10^{-6}, respectively.	[42]
Balb/c males, 3 week	Folate-deficient (D)/folate-supplemented (S) diet. (positive control ENU) Mice were fed a folate-deficient (0 mg/kg, D), control (2 mg/kg, C) or supplemented (6 mg/kg, S) diet from weaning for 18 weeks. Positive control ENU mice was given 80 mg/kg bw, i.p.; the protocol included immunodepletion of wild-type cells)	No significant change in RBC or RET mean Mfs for D or S compared to control. Increased RBC mean Mf and not RET Mf in D compared to S. D mice had 2.2 times higher RBC mean Mf than C, although not statistically significant. The RBC mean Mf was 3.8 times higher in D mice compared to S-mice ($p = 0.011$).	[43]
CD-1 males, 8 weeks	ENU, BaP, EC, P, MC (ENU: 0, 12.5, 25, 50 mg/kg/day; BaP: 62.5, 125, 250 mg/kg/day; EC: 100, 200, 400 mg/kg/day; P: 125, 250, 500 mg/kg/day; MC: 500, 1000, 2000 mg/kg/day; given on three consecutive days by oral gavage; analyses occurred on days 15 and 30; the protocol included immunodepletion of wild-type cells)	Dose-related significantly increased RET and RBC mean Mfs for the three known mutagens (ENU, BaP and EC), no change for the two known non-mutagens (P and MC)	[31]
C57BL/6J *gpt* delta males, 8 weeks	TiO_2-NPs (positive control ENU) (i.v.; 2, 10 or 50 mg/kg bw/week for 4 weeks; positive control ENU was given as a single dose of 70 mg/kg bw. i.p.; analyses occurred on day 30 after first injection, RBCs were assayed)	No increased Mf in RBCs, despite availability to the bone marrow and bone marrow cytotoxicity. RBC mean Mf in vehicle control animals was $0.40 \pm 0.55 \times 10^{-6}$. Positive control animals treated with ENU (70 mg/kg) had mean RBC Mf of $51 \pm 1.4 \times 10^{-6}$	[40]
B6C3F1 males, 8 weeks	Acrylamide (positive control ENU) (given at 0, 0.5, 1.5, 3.0, 6.0, 12.0 and 24.0 mg/kg/day in drinking water for 30 days; positive control ENU was given at 10 mL/kg via oral gavage on days 1–3 and 29–30, and assayed on day 31,; the protocol included immunodepletion of wild-type cells.)	No effect detectable in RET or RBC mean Mfs. The positive control ENU gave rise to significantly increased RET and RBC mean Mfs	[45]

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Table 1. (continued)

Strain(s), genotype(s), sex, age	Agent/intervention (exposure, route, assessment time). Methodological details	Main outcome, mutation frequencies (Mf) in reticulocytes (RETs) or red blood cells (RBCs)	References
C57BL/6N *Ogg1*$^{+/-}$ (functional wild-type, WT) and *Ogg1*$^{-/-}$ (KO) males, 5–16 weeks	γ-Irradiation (^{60}Co, 1.4 mGy/h for 45 days, 1.5 Gy total dose). Se deficiency through diet: (two-generation depletion through diet; mice were given a diet of either low (0.01 mg Se/kg diet) or normal (0.23 mg Se/kg diet) Se content. Positive control ENU was given at 40 mg/kg bw for 3 days, i.p.; the protocol included immunodepletion of wild-type cells.)	Small statistically significantly increased RBC mean Mf, but not RET mean Mf ($p = 0.009$), of chronic contiguous exposure to low dose rate γ-irradiation. No significant effect of diet/genotype alone. Interaction between radiation and diet in RET ($p = 0.015$) and RBC ($p = 0.013$) mean Mfs. The mean RET and RBC Mfs were 96.1×10^{-6} and 23.75×10^{-6}, respectively	[37]
C57BL/6J males, 6–52 weeks	Diet-induced obesity. (six-week-old mice had been given either a high-fat calorie content (60%) diet or a normal-fat calorie content (10%) diet until week 24 and were further maintained on the respective diets. Positive control mice of both groups were treated with ENU (40 mg/kg bw for 3 days) and were analysed 3.5 weeks after. Mice not exposed to ENU were assayed at week 52. The protocol included immunodepletion of wild-type cells.)	Significantly higher and sustained RBC mean Mf, but not RET mean Mf, in diet-induced obese mice given a high-fat diet compared to lean mice given a normal-fat diet. The obese mice had significantly higher and sustained RBC mean Mf (increased 2.5- to 3.7-fold, $p < 0.02$) compared to non-obese mice	[46]

Mf × 10^{-6}, mutation frequency; RET, reticulocytes; RBCs, red blood cells; DMBA, 7,12-dimethyl-1,2-benz(a)anthracene; ENU, *N*-ethyl-*N*-nitrosourea; BaP, intraperitoneal injection (i.p.), benzo[a]pyrene; TiO_2-NP, 10-nm titanium dioxide anatase nanoparticles; i.v., intravenous injection; 4NQO, 4-nitroquinoline-1-oxide; EMS, ethyl methanesulphonate; Ogg1, 8-oxoguanine DNA glycosylase; PVP, 5-nm polyvinylpyrrolidone; AgNP, coated silver nanoparticles; p53, tumour protein p53, 1,2-DCP, 1,2-dichloropropane; DCM, dichloromethane; EC, ethyl carbamate; P, pyrene; MC, methyl carbamate.
[1]Measured in erythroids in peripheral blood (PB) and bone marrow (BM).

demonstrate cross-species potential from rats to mice was published [26]. In this study, CD-1 mice were exposed to the carcinogen DMBA and the alkylating agent ENU and showed clear induction of *Pig-a* mutations in both reticulocytes (RET, immature red blood cells) and red blood cells (RBCs) [26]. The conclusion was that the *Pig-a* assay could be effectively applied to *in vivo*-exposed mice. The temporal dynamics of mutation induction and potential selection of mutant cells were important issues to comprehend also in mice, to conduct appropriate dosing regimens and to determine the optimal timing of measuring mutation frequencies. Hence, mutation frequencies were determined weekly for 5 weeks after exposure to ENU, showing that mutant RETs preceded mutant RBCs, as expected, and that the effect persisted for the 5 weeks in both RBCs and RETs. This study was followed by two studies in C57BL/6 [27] and CD-1 mice [20]. In both studies, mice were exposed to ENU and showed significant induction of *Pig-a* mutations in RETs and RBCs (i.e. increased RET mean Mf at day 2 of 700 × 10^{-6} compared to vehicle control mean Mf ranging from 0.2 to 2.2 × 10^{-6}). These initial studies contributed with information regarding important descriptors of the assay which includes understanding the kinetics of the mutagenic process: mutation induction, maximum response time and potential persistence. For information regarding magnitude of effects, see table 1. *Pig-a* mutants appear slightly faster in mice [26,27] than in rats [28]. After exposure to ENU in mice, the maximum response appeared in week 2 after dosing in RETs and in week 4 in RBCs [26,27]. In rats, the maximum response in RBCs started 6 weeks after the end of exposure to ENU [28,29]. Moreover, in mice, the RBC-mutant frequencies decrease slowly after reaching maximum, whereas they persist in rats for at least 6 months [27,28]. In both mice and rats, ENU-induced *Pig-a* mutants clearly accumulate, as shown by two studies comparing single doses with split doses [27,28]. In rats, the accumulation was nearly additive (57.3 × 10^{-6} in the 8.9 mg/kg single exposure group and 176.8 × 10^{-6} in the split group given four weekly doses of 8.9 mg/kg) [28], whereas in mice, the relative mutant numbers were somewhat lower than in the rat in the split dosing regime compared with single dosing (a single dose of 32 mg/kg compared to four weekly doses of 8 mg/kg gave rise to mean Mfs of 180 *versus* 105 × 10^{-6}, respectively) [27]. Other studies have confirmed the mutagenicity of ethylating agents such as ENU [20,30,31] and ethyl methanesulphonate (EMS) [32].

Several polycyclic aromatic hydrocarbons (PAHs) have been tested using the *Pig-a* assay in mice [26,30,31,33,34]. Both DMBA [26] and the benzo[a]pyrene (BaP) [30,31,33,34] showed clear induction of *Pig-a* mutants. Mutagenic effect of BaP was evident after different exposure scenarios in several mouse strains. This includes 28-day dosing by oral gavage in the Muta™ mouse model [33], a single oral dose in *Gpt* delta mice [30], intraperitoneal injection for 3 days in C57BL/6 wild-type and DNA repair-deficient (*Ogg1*$^{-/-}$) mice [34] and recently also after 3 days of exposure by oral gavage in CD-1 mice [31]. This demonstrates that for the prototypical mutagenic PAHs DMBA and BaP, the mouse *Pig-a* gene mutation assay is able to reveal their genotoxic potential.

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Table 2.

Overview of PIG-A studies in human cells.

Study classification[1]	Cell type(s)	Experimental details	Main outcome, mutation rates and mutation frequencies (Mf)	References
A	Eight mutator colon cancer lines (defective in mismatch repair) and four non-mutator cancer lines	Enrichment of mutants using the GPI anchor-reactive bacterial toxin aerolysin	All mutator colon cell lines have higher Mf ($42–74 \times 10^{-6}$) than control cell lines. Verification of mutant cells by DNA sequencing	[6]
B, C	B-lymphoblastoid cell lines from normal donors, Fanconi anaemia (FA) and ataxia telangiectasia (AT) patients	Existing mutants were omitted by cell sorting of anchor-positive cells	The mutation rate in normal donors were 10.6×10^{-7} per cell division (range 2.4–29.6) and was clearly elevated in cell lines from the cancer-prone FA (mean 411×10^{-7}) and AT patients (mean 40.1×10^{-7})	[50]
A, B	B-lymphoblastoid cell lines (BLCL) from normal donors, transformed lymphoma (TL), Mantle cell lymphoma (MCL), T-cell ALL (TCA), marginal zone lymphoma (MZL)	Depletion of existing mutants by flow sorting of anchor-positive cells	BLCL and MZL exhibit low mutation rates with 11–17 mutations per 10^7 cell division compared to the mutation rates of TL, MCL and TCA ranging from 170 to 3286 mutations per 10^7 cell division	[47]
B	Red blood cells	Blood from 97 normal individuals and 10 individuals undergoing chemotherapy	Mean Mf in RBC^{CD59-} from normal donors was 5.1 ± 4.9, with 75% showing <8. Two individuals, however, showed very high Mf of 100 and 300. No clear correlation with age and smoking status. Patients undergoing chemotherapy had generally weak responses, up to threefold increases	[51]
D	Lymphocytes isolated from full blood. Exposure occurred in 1994; blood collection occurred in 2009	35 Gulf War veterans exposed to depleted uranium. Individuals were divided into low-U (<0.10 μg U/g creatinine) and high-U groups (≥0.10 μg U/g creatinine)	*PIG-A* Mfs ranged from 2.9 to 113×10^{-6} (mean = 14.91×10^{-6}). Group means ($18.13 \times 10^{-6} \pm 4.85$ for low U and $9.45 \times 10^{-6} \pm 0.81$ for high U) were not significantly different between the two exposure groups, although a non-significant reduction in the mean mutation frequency in the high-exposure group was apparent (numbers not adjusted for age). Trend for a positive association with age	[8]
A	Myeloid cells	$CD34^+$ cells transduced with either AML-ETO or MLL-AF9 fusion genes and expanded with cytokines	The median mutation rate in AML-ETO cells was 9.4×10^{-7} (range $3.6–23 \times 10^{-7}$) per cell division. Only few mutations were observed in the MLL-AF9-transduced cells	[48]
B	Peripheral blood granulocytes from 142 individuals.	Mutants identified as CD59/CD55/CD24-negative cells	The median Mf was 4.9 with significant interindividual variability with Mf ranging between <1 and 37.5	[9]
A,C	Lymphoblastoid cell lines (LCL) with mutations in the MUTYH DNA repair protein from patients with colorectal polyposis cancer	Mutants identified as CD59/CD55/CD24-negative cells	The cell lines exhibited a fourfold increased Mf (mean Mf of 87.6) compared with LCLs from healthy donors (mean Mf 21.7). The mutant cells were also hypermutable after exposure to the oxidative stressor KBrO3	[49]

(continued)

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Table 2. (continued)

Study classification[1]	Cell type(s)	Experimental details	Main outcome, mutation rates and mutation frequencies (Mf)	References
C	Lymphoblastoid cells lines (LCL) expressing biallelic or monoallelic variants of the *MUTYH* DNA repair gene	Mutants identified as CD59/CD55/CD24-negative cells.	LCLs homozygous for two *MUTYH* mutations exhibited mutator phenotypes with three- to sixfold increases. Exposure of cells to the oxidative stressor $KBrO_3$ leads to increased Mf	[53]
E	TK6 cells	EMS: 30 μM over night. Assayed at days 1, 3, 6, 8 and 10	Selection of pro-aerolysin GPI anchor-deficient isolates in the TK6 cell line. Spontaneous and EMS-induced mutants are characterized by DNA sequencing. Discover that the parent TK6 line is hemizygous for *PIG-L*. EMS-treated cells GPI-a Mf of 20×10^{-6} (background) to 470×10^{-6} (day 10). Few mutants were verified as *PIG-A* mutants, while many as *PIG-L* mutants	[23]
B	Peripheral blood erythrocytes from 52 non-smoking, self-reported healthy adults	Mutants were identified as CD59/CD55-negative RBC and RET. Immunomagnetic enrichment of mutants. Technical replicated included to achieve sufficient cell numbers and for investigations of assay variability	The mean frequency of Mf was 6.0 in RETs and 2.9 in RBCs, consistent with a modest selective pressure in circulation. Intraindividual variability was low, while interindividual variability was relatively high (RET>30-fold). The Mf increased with increasing age. Cold whole blood can be held for a week without changing the Mf	[52]
E	B-lymphoblastoid TK6 cells	EMS: 200–800 μM (4 hr) and 100–200 μM (24 hr); UVC: 2–8 J/m^2; 4-NQO: 50–200 nM; Pyr: 1–10 mM; Cyhex: 1–10 mM. Incubation for 4 hr or 24 hr (EMS), assayed 10 days or 11 days after exposure, respectively. Mutants were identified as CD59/CD55− negative cells. Elimination of pre-existing mutants was made using antibody-coated cell dishes. Generation of mutant suspension was performed by pro-aerolysin lysis of WT cells.	Dose-related increases in Mf after exposure to EMS, UV-C and 4-NQO (2- to 7.5-fold increases compared to control). No increases in Mf for the negative controls Pyr and Cyhex. Mf increases reached a plateau at day 10 which was maintained at least until day 14	[54]
E	B-lymphoblastoid TK6 cells and TK6-related p53-deficient WI-L2-NS cells	EMS: 200–800 μM, 4-hr incubation; UVC: 2–8 J/m^2	Dose-related increases in Mf after exposure to EMS or UVC in both cell lines, lower response in WI-L2-NS cells for both mutagens. Identification of a heterozygous deletion in one copy of the autosomal *PIG-L* in TK6 cells that may serve as a second target for mutation detection by one hit	[24]

Mf $\times 10^{-6}$, mutation frequency; GPI, glycosylphosphatidylinositol; LCL, lymphoblastoid cells lines; MUTYH, MutY DNA glycosylase; RBCs, red blood cells; RETs, reticulocyte; EMS, ethyl methanesulphonate; UVC, ultraviolet radiation C; 4NQO, 4-nitroquinoline-1-oxide; Pyr, pyridine; CyHex, cycloheximide.

[1]The studies are classified using letter codes. Studies in A: cancer cells; B cells from normal donors; C: cells with impaired DNA damage response or DNA repair defects; D: human lymphocytes exposed to environmental stressors; E: the TK6 cell line.

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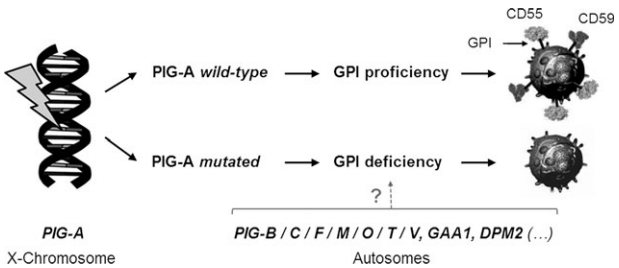

Fig. 1. Glycophosphatidylinositol (GPI)-dependent genotype-to-phenotype relationship. Inactivating mutations in the *PIG-A* gene cause the GPI-deficient phenotype identified as *PIG-A* mutants by the lack of immunostaining of anchor proteins such as CD55 and CD59. This is due to the X-chromosomal location of *PIG-A*, which requires inactivation of one allele only (adapted from Krüger *et al.*, 2015).

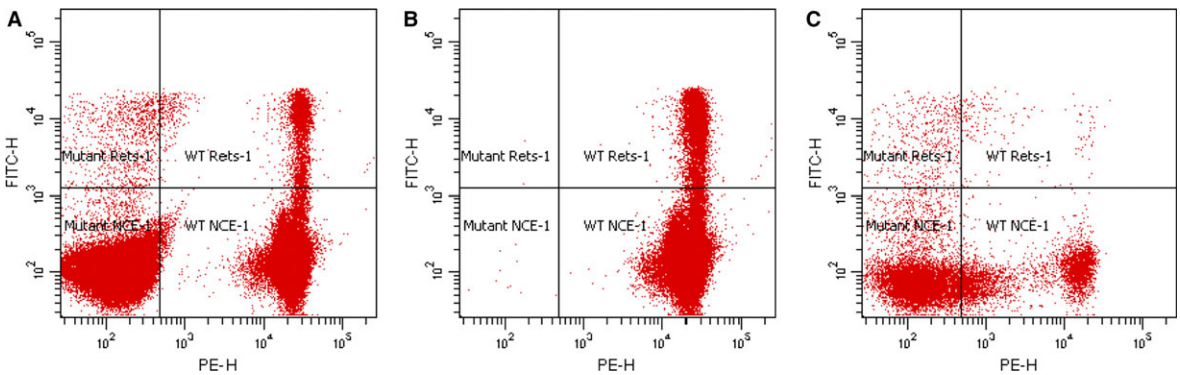

Fig. 2. Bivariate plots showing nucleic acid dye *versus* anti-CD24-PE fluorescence profiles (mouse blood example). Note that only gated events (i.e. erythrocytes) are shown. Key to quadrants: upper right = wild-type reticulocytes (WT Rets-1); lower right = wild-type mature erythrocytes (mutant normochromatic erythrocytes; WT NCE-1); upper left = mutant phenotype reticulocytes (mutant Rets-1); lower left = mutant phenotype mature erythrocytes (mutant normochromatic erythrocytes; mutant NCE-1). Plot A: instrument calibration standard (ICS); blood that was fully processed was spiked with mutant-mimicking cells (i.e. erythrocytes that were not incubated with anti-CD24-PE). This ICS is used to set PMT voltages and compensation settings. The ICS also provides enough cells in order to rationally and consistently setting the position of the vertical demarcation line that discriminates mutant phenotype erythrocytes from wild-type erythrocytes. Plot B: blood from an N-ethyl-N-nitrosourea (ENU)-treated mouse, pre-column analysis (i.e. before immunomagnetic separation); this blood sample was obtained from a mouse 15 days after the last of three administrations of ENU. A brief analysis of the pre-column blood sample is used to determine the numbers of total reticulocytes and total erythrocytes that are applied to the immunomagnetic separation procedure (i.e. these are the denominators for mutant cell frequency calculations). Plot C: blood from the same ENU-treated mouse, post-column analysis (i.e. after immunomagnetic separation); this sample was depleted of wild-type erythrocytes via immunomagnetic separation. With a subsequent centrifugation step, mutant phenotype cells become highly concentrated. The number of mutant reticulocytes and mutant erythrocytes is directly determined from this sample.

Mutagenic effects of ionizing radiation such as X-rays and gamma irradiation have been studied [35–37] and provide valuable information concerning timing of mutant induction and elimination in mice, as well as responses after acute *versus* protracted/chronic exposure to mutagens. Moreover, in contrast to many chemicals, ionizing radiation does not depend on metabolic activation to exert its effects. For X-rays, a version of the *Pig-a* assay using anti-CD24, anti-TER-119 and anti-CD71 to detect the *Pig-a*-mutant frequencies in RBCs and RETs was used. Single and protracted exposures (single dose of 0.5, 1 and 2 Gy or fractionated dose of 4 x 0.5 Gy once a week) to X-ray (0.52 Gy/min) resulted in comparable dose- and time-dependent increases in *Pig-a*-mutant frequencies, which subsequently declined over time returning to background frequencies [35]. After single dosing, the maximum response occurred at days 7–21, whereas after protracted dosing the maximal response occurred at day 42. There were marked interindividual differences, both in the magnitude of the maximal response and in time until maximum response was evident. This study was followed up by investigations of the role of p53 for X-ray-induced *Pig-a* mutants, showing that the persistence of mutated RBCs was slower in $p53^{-/-}$ mice than in wild-type mice and that *Pig-a* mutations increased with increasing age of $p53^{-/-}$ mice [36]. These studies used acute high dose rates (0.52 Gy/min) of X-rays. A pertinent issue is to understand the outcome of low dose rate exposures to gamma irradiation, which is more relevant to the general population, either alone or in combination with other natural co-stressors. We recently published a study on single and combined effects of continuous chronic low dose rate gamma exposure and selenium deprivation via diet in a mouse model with deficient repair of oxidized DNA (8-oxoguanine DNA glycosylase-deficient mice; $Ogg1^{-/-}$) and control animals. The main result of this study was that gamma radiation given in a more environmentally relevant exposure manner (1.48 mGy/hr for 45 days to a total dose of 1.5 Gy) is indeed genotoxic in mice [37]. One of several methods used to determine genotoxic effects was the *Pig-a* gene mutation analyses. This assay

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was assessed 2 weeks after the end of continuous irradiation. In general, gamma irradiation caused a moderate but significant increase in the frequency of mutant RBC and no significant effect of the genotype or diet alone. The frequency of mutant RET was not significantly affected by any factor (i.e. irradiation, diet or genotype) alone, whereas radiation and diet together had a statistically significant impact on the frequencies of mutant RET and RBC, suggesting an interaction between the two factors. Micronucleated blood cells were clearly induced by the chronic continuous low dose rate of gamma irradiation, irrespective of mouse genotype and diet. This study demonstrated that prolonged low dose rate exposures to ionizing radiation are barely detectable in the *Pig-a* assay when measured 2 weeks after cessation of treatment, with significant interindividual variation. In mice, the maximum response after protracted exposures seems to occur later than after acute exposures [35], so in the study by Graupner *et al.* [37], an additional measurement time-point at 4 weeks after exposure cessation would have given valuable information. Acute exposure to ionizing radiation, on the other hand, clearly induces *Pig-a* mutants [35].

Other agents besides ionizing radiation inducing DNA damage through the production of reactive oxygen species are represented by the carcinogen 4-nitroquinoline 1-oxide (4-NQO), investigated in the *Pig-a* assay in mice [30]. Comparisons were made between *Pig-a*- and *Gtp* delta-mutant frequencies in transgenic *Gtp* delta mice. 4-NQO induced weak insignificant increases in bone marrow cells in both the *Pig-a* and *Gtp* assays, whereas a significant increase in *Gtp*-mutant frequency was observed in the liver, which is target tissue for 4-NQO. These results are in line with recommendations from the International Workshop on Genotoxicity Testing (IWGT) [13] that blood-based *Pig-a* assays will tend to benefit from immunomagnetic separation strategies allowing scoring of more cells than are otherwise practical, and taking care not to interpret negative *Pig-a* results as evidence of no *in vivo* mutagenic potential.

Genotoxicity of two kinds of nanoparticles has been characterized using the *Pig-a* assay [38–41]. The first study investigated the genotoxic properties of titanium dioxide anatase nanoparticles (TiO_2-NPs, intravenous (i.v.) injection) in mice observing no significant elevation of *Pig-a* mutants or micronucleated blood cells [38]. In the second study, silver nanoparticles (AgNP) were injected into mice [39]. Both studies confirmed – using analytical methods – that the nanoparticles reached the bone marrow, but no increases in mutant RET or RBC nor micronucleated RETs were detected when measured 1, 2, 4 or 6 weeks after injection. However, the levels of DNA lesions, measured using the alkaline comet assay, were elevated. The third study exposed *Gpt* delta transgenic mice to TiO_2-NP by i.v. injections for up to 4 weeks [40] to study genotoxicity using an array of genotoxic end-points in blood including the *Pig-a* assay. All end-points were negative, indicating that nanoparticles are not genotoxic in blood cells as shown by the lack of induction of RET or RBC *Pig-a* gene mutations or micronucleated RETs as well as other genotoxic end-points.

Nutrient deficiencies such as vitamin or essential element deficiencies may give rise to genotoxic effects in mice investigated using the *Pig-a* assay [42,43]. Mice were deprived of the antioxidant and essential element selenium through depletion starting in the parental generation [42]. Genotoxicity was monitored in offspring where DNA lesions were clearly induced in lung and testicular cells using the alkaline comet assay. However, no increased level of DNA lesions was evident in blood cells and *Pig-a*-mutant frequencies in RETs and RBCs remained unchanged. This indicates that selenium deprivation is not genotoxic to bone marrow or blood cells and that testicular and lung cells as more susceptible cells. Folate is a B vitamin and folate deficiency leads to mutations and genome instability. Mice were fed a diet that was deprived, adequate or supplemented with folate [43]. The mutant RET and RBC frequencies were similar in mice given the deprived and the adequate diets, whereas the mutant RBC frequency in mice given the deprived diet was higher than in mice given the supplemented diet. The mutant RBC frequencies of the mice given the deprived diets varied significantly. The authors suggest that the results indicate that adequate folate protects against mutagenesis.

Other compounds that have been tested in mice using the *Pig-a* assay include the antineoplastic hydrazine derivative procarbazine showing a significant induction of both mutant RETs and RBCs [44]. The chlorinated organic solvents 1,2-dichloropropane and dichloromethane, suggested to be involved in the induction of cholangiocarcinoma in workers, were tested in two mouse strains giving negative results for RBC^{CD24-} cells [41]. In the liver, DNA damage as measured in the alkaline comet assay as well as *Gpt* mutation frequencies was increased.

Importantly, Labash *et al.* [31] compared the response in *Pig-a* mutation frequencies after exposure of mice to known mutagens [ENU, BaP and ethyl carbamate (EC)] as well as two structurally related non-mutagens (pyrene and methyl carbamate). The results clearly demonstrate that the mutagens gave statistically significant increases in mutant RET and RBC mutation frequencies, whereas the two non-mutagens did not.

Acrylamide is a compound known to induce tumours in rodents. However, there is still controversy regarding the mode of action for the tumour induction. Recently, Hobbs and coworkers published a study where they measured *Pig-a* mutation frequencies in rats and mice exposed to acrylamide in drinking water for 30 days [45]. The applied doses spanned and exceeded the doses used for the determination of the carcinogenic effects. They assayed *Pig-a* mutations the day after the last dosing, a timing that may not be the most suitable to reveal effects, showing equivocal effects. Given humans' low but widespread exposure to acrylamide, further work with this compound may be warranted.

Recently, a study concerning mutagenicity as a result of diet-induced obesity was published [46]. These investigators observed significantly higher and sustained mutant RBC frequency but not RET frequency in diet-induced obese C57BL/6J mice given a high-fat diet compared to lean mice given a normal-fat diet. They attributed the significant finding in RBC and not RET as owing to the much larger number of RBCs

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interrogated for the mutant phenotype (>150 million) *versus* RET (approximately 3 million).

PIG-A in Human Cells

The literature search performed identified 13 studies with *PIG-A* in human cells apart from those involved in studies of paroxysmal nocturnal haemoglobinuria (PNH). The studies are summarized in table 2 according to the year of publication. The studies can be broadly classified (classification indicated by letters in table 2) into studies in cancer cells (A [6,47–49]), in cells from normal donors (B; [9,47,50–52]), in cells with impaired DNA damage response or DNA repair defects (C [49,50,53]), in human lymphocytes exposed to environmental stressors (D [8]) and recently also in the TK6 cell line that is widely used and relevant for chemical risk assessment (E [23,24,54]).

A pioneering study involved colon cancer cell lines that exhibited significantly higher mutation frequencies than control cell lines [6], using *PIG-A* mutants as a marker of mutator phenotypes in cancer. The mutants were enriched by use of the GPI anchor-reactive bacterial toxin that lyses cells that express anchor proteins. In this experiment, sorted and cloned mutants were verified by DNA sequencing.

Several of the initial studies concentrated on determining mutation rates (mutations per cell division) rather than measuring mutation frequencies (here the incidence of mutant phenotype cells, often expressed as number per 10^6 cells) [47,48,50]. It was shown that the mutation rates from donors that were cancer prone (Fanconi anaemia (FA) and ataxia telangiectasia (AT) were elevated compared to cell lines from normal donors [50], as well as marked differences in mutation rates among B-lymphoblastoid cell lines from normal donors and lymphoma cancer patients [47] and transformed myeloid cells [48]. These studies have given rise to important new information regarding the rate of spontaneous mutations in human cells.

The group of Bignami *et al.* developed a flow cytometry-based *PIG-A* gene mutation assay using three anchor molecules (CD48, DC55 and CD59) to investigate the role of the DNA repair protein MUTYH, and different spontaneous *MUTYH* mutants, for mutagenesis [49,53]. They measured *PIG-A* mutants in lymphoblastoid cell lines derived from patients with *MUTYH*-associated colon polyposis. Pre-existing *PIG-A*-mutant cells were selectively removed by flow sorting to ascertain that the *PIG-A* mutation frequency measured was due to the *MUTYH* defect or to $KBrO_3$ treatment (oxidative stress). The spontaneous *PIG-A* mutation frequency in the *MUTYH*-mutated lymphoblastoid cell lines was 3–6 times higher compared to wild-type levels depending on type of *MUTYH* mutation and allelic presence (mono- or biallelic mutation), suggesting that the type of MUTYH mutation contributes to determine the magnitude of the mutator phenotype. After oxidative stress ($KBrO_3$ exposure), the *PIG-A* mutation frequencies in homozygous *MUTYH* cell lines were more pronounced than in wild-type or heterozygous cell lines.

Several approaches have been initiated to investigate normal human donor cells. One study used the *PIG-A* assay, along with a battery of other tests to assess the genotoxicity in blood lymphocytes from Gulf War veterans exposed to depleted uranium. Selected due to urine U exposure, individuals were divided into low- and high-U groups, and results in the two groups were compared [8]. *PIG-A* mutation frequencies ranged from 2.9 to 113 $\times$ 10^{-6} (mean = 14.9 $\times$ 10^{-6}). Group means of the low-U group (18.1 $\times$ 10^{-6} $\pm$ 4.9) were not significantly different from the high-U group (9.5 $\times$ 10^{-6} $\pm$ 0.8), but interestingly the high-U group exhibited apparently lower Mf with less variation. No clear association with age was detected (age 43.62 $\pm$ 5.35). Dobrovolsky *et al.* [51] investigated 97 normal individuals (mean age 39.9 $\pm$ 13.4; span 18–73) and 10 individuals undergoing chemotherapy (age not stated). They found that the mean mutant RBC frequency was 5.1 $\pm$ 4.9 $\times$ 10^{-6} and that some individuals expressed very high mutation frequencies. They detected no clear correlation with age and smoking status, and the patients undergoing chemotherapy showed generally low responses. Rondelli *et al.* [9] reported similar findings for granulocytes with respect to mutation frequencies, with significant variation between individuals. To facilitate studies of human blood samples, Dertinger *et al.* [52] recently reported on the development and initial tests of a human blood *PIG-A* assay analogous to one of the most used protocols to determine mutant RET and mutant RBC frequencies in rodent blood samples. Expression of two GPI-anchored proteins (CD59 and CD55) served as reporters of *PIG-A* mutation, and immunomagnetic depletion of wild-type cells was included that gives a dramatic enrichment of mutant cells. This appears to be especially important for human samples, as there is evidence that mutant RETs represent a better indicator of mutation compared to mutant RBCs in humans, as there is evidence of selective pressure against circulating human *PIG-A*-mutant erythrocytes due to complement-mediated lysis. The data suggested several important characteristics of human blood-based *PIG-A* assays. The mutant RET frequencies are consistently higher than the corresponding mutant RBC frequencies, and this suggests that the former may serve as a more accurate representation of mutation frequency. The variation within each individual was relatively low but similar to the two preceding reports in normal human blood cells, while variability between individuals was high. An effort has been initiated to explore factors that may explain the large variation observed in healthy volunteers, such as age, smoking habits. Analyses show that in the very young (15 umbilical cord blood samples from healthy full-term babies and five from infants), mutant RET frequencies are consistently low, an observation that warrants confirmation. With increasing age, mutant RET increased and was accompanied with increased variation, suggesting that age is a factor that leads to increased *PIG-A* mutation frequencies. Investigation of the role of human exposure to external factors such as environmental stressors is warranted before the assay can be used for human biomonitoring or clinical applications.

Recently, the *PIG-A* assay has been successfully established *in vitro* B-lymphoblastoid TK6 cells, commonly used for *in vitro* mutagenicity testing, were applied [23,24,54]. The GPI status of the cells was determined by multicolour flow

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cytometry. The GPI-linked proteins CD55 and CD59 were stained to differentiate between mutant and wild-type cells. To exclude unspecific events during the flow cytometric analysis, the GPI-independent B-cell marker CD19 was stained with allophycocyanin-conjugated antibodies to increase the sensitivity and reproducibility of the assay. Ten days of phenotype expression time was found to be suitable after 4-hr incubations with respective mutagens. The *in vitro* methodology was thoroughly validated by mutant spiking experiments as well as by comparing the new flow cytometric analysis with the classical approach based on surviving of mutant cells in a selective media, as applied, for example, in the hypoxanthine-guanine phosphoribosyltransferase (HPRT) or mouse lymphoma assay [54]. To assess the sensitivity and the selectivity of the test system, different positive and negative controls were examined. Cells were treated with known mutagens such as ethyl methanesulphonate (EMS), NQO or ultraviolet C (UVC) irradiation and, after phenotype expression, mutant frequencies were determined via flow cytometry. In all experiments, mutant frequencies of treated samples were found to be increased in a dose-dependent and statistically significant manner when compared to untreated controls. Treatments with the non-mutagenic compounds pyridine and cycloheximide did not increase the mutant frequencies, which were comparable to untreated controls in both experiments [54]. A subsequent investigation of the genotype-to-phenotype relationship in TK6 cells revealed that this cell line exhibits a heterozygous, autosomal deletion of the PIG-L gene on chromosome 17 [23,24]. PIG-L is, like PIG-A, essential for the GPI biosynthesis. This leads to the unique situation that inactivating mutations in two genes, the X-linked PIG-A and the autosomal PIG-L, result in a mutant, GPI-deficient phenotype in TK6 cells. It explains the comparatively high spontaneous mutation rate in this assay, requiring the depletion of existing mutant cells via antibody-coated cell culture dishes. However, on the other hand, it could provide a distinct advantage: it should enable the TK6 cell line not only to pick up point mutations, but also clastogenic alterations, if deletions affect the *PIG-L* gene on chromosome 17, as – in contrast to X-chromosomal events – adjacent genes required for viability would still be present on the second copy of chromosome 17. However, more investigations are needed to verify this assumption. First experiments in this direction revealed that the p53-proficient TK6 cell line is more sensitive towards the mutagens EMS and UV-C compared to the closely related, but p53-deficient cell line WI-L2-NS, vice versa, than expected from more efficient cell cycle regulation in case of p53 proficiency. Thus, one explanation may be the fact that WI-L2-NS cells do not carry the *PIG-L* deletion described for TK6 cells [24]. Future studies will have to clarify whether the *in vitro* variant can also be conducted in other cell lines, such as epithelial cells.

Usefulness in Basic Science

In addition to use in applied sciences such as chemical risk assessment, the investigations using the *Pig-a* mutation assay have proven useful also for basic research, both *in vitro* in cell lines and in mouse models. Most advantage has been in understanding roles of different DNA repair enzymes [49,53]. The role of the MutY DNA glycosylase (MUTYH), its different mutant versions (both monoallelic and biallelic) and the role they have as mutator phenotypes involved in colorectal polyposis cancer have been envisioned using the *PIG-A* assay. Also other proteins involved in DNA damage response and repair have been studied by measuring *PIG-A* mutation frequencies, such as cell lines derived from patients exhibiting Fanconi anaemia and ataxia telangiectasia showing that the mutation rate in these two patient groups is higher than in normal donors [50].

Mice exhibiting different deficiencies in DNA repair proteins involved in protection of the genome from oxidized DNA have been studied [34,36,37,42, Rolseth *et al.*, unpublished]. The DNA repair proteins investigated are involved in base excision repair eliminating oxidized DNA damage and comprise 8-oxoguanine DNA glycosylase (Ogg1), the MutY DNA glycosylase (MutY) and three endonuclease VIII-like DNA glycosylases (Neil1, Neil2 and Neil3) as well as the tumour protein p53 (p53). Ogg1 and MutY work in concert to prevent mutations from being induced after oxidative stress. Oxidative stress leads to induction of the mutagenic 8-oxoguanine (8-oxoG) that is removed by Ogg1. If not removed, the DNA polymerases may misincorporate adenine opposite 8-oxoG, which may be removed by MutY that excises adenine from 8oxoG:A base pairs, giving a new chance for Ogg1 to remove 8-oxoG. Studies of Ogg1-deficient mice have shown no significant elevation of the spontaneous *Pig-a* mutation frequency [34,37,42], which correlates well with the lack of cancer phenotype in this mouse model. The Ogg1-deficient mice have been subjected to BaP, to Se deficiency and to low dose rate gamma irradiation without the *Pig-a*-mutant cell frequency exceeding the wild-type levels, suggesting that the DNA damage levels induced are being repaired via other backup DNA repair systems. In line with this, when several DNA repair enzymes are eliminated, such as in the cancer-prone model $Ogg1^{-/-}MutY^{-/-}$ double knockout, the *Pig-a* mutation frequencies are elevated (Rolseth *et al.*, unpublished).

Concluding Remarks

The *Pig-a/PIG-A* methodology detects mutations that result in functional inactivation of the *Pig-a/PIG-A* gene, as opposed to other much used genotoxicity assays such as the alkaline comet assay that detects pre-mutagenic DNA lesions. The test is rapid and requires only a minute volume peripheral blood, it is easily incorporated into experiments within regulatory toxicology, and it has been demonstrated as useful for identifying and investigating genetic factors that underlie the variation in the somatic mutation rate, as well as environmental factors that may affect it, and factors involved in cancer development. Besides rats where the *Pig-a* assay has been used more extensively, the assay is useful both in mice, with the advantage of the variety of mouse models that exist, and in human cells. Compared to the rat, the kinetics of mutant

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induction and persistence in mice and human beings is not similar to the rat and seems to vary with the investigated chemical. In ENU-treated mice, the mutant RET and RBC appear slightly faster than in rats, and contrary to the long persistence of mutants in rats, the mutants in mice slowly decline after reaching maximum, indicating a moderate selection of GPI-negative cells. However, based on a limited number of studies so far, the specificity of the *Pig-a* assay both in mice [31] and in human cells [23,54] appears to be good as several known non-mutagens did not show any mutagenic effects as opposed to (structurally related) potent mutagenic carcinogens that showed clear mutagenic effects. Other less potent agents such as ionizing radiation were found to cause moderate induction of *Pig-a* mutants. More experiments with low-potency agents are required to characterize in more detail the sensitivity (the proportion of correctly identified positive stressors) of the *Pig-a* assay in mice. Variability between individuals seems to be an issue both in mice and in human cells, and needs to be taken into account during experimental design by ensuring that the power of the study is adequate by including sufficient numbers of individuals, or by making use of an experimental design that takes the variability into account (repeated measurements in the same individual to identify temporal differences) or conduct pre-exposure assessments that facilitate omittance of individuals with abnormal spontaneous Mfs. The *Pig-a* assay is clearly a valuable complement to other genotoxicity assays that can be included in the same experiment, such as *in vivo* micronucleus assay detecting clastogens and the *in vivo* comet assay in blood detecting DNA lesions. Several studies have used a battery of genotoxicity tests [30,32–34,37,42], including the comet assay and mutation detection in transgenic rodents (LacZ, Big Blue™). On the other hand, using assays such as the haematopoietic cell-based *Pig-a* assay, normal rodents can be assessed, which may be more cost- and time-efficient, without killing the animals. One limitation of blood-based genotoxicity assays such as the *Pig-a* and the MN assays is that they depend on the fact that the compound, or metabolite, of interest is present in the bone marrow in levels reflecting those of target tissues. In the *in vivo* comet assay or mutation analyses in transgenic rodents, target tissues can be studied directly. Positive comet assay results in the liver or a target tissue may indicate that a negative result with *Pig-a* or MN may be due to non-availability of the compound to the bone marrow. Moreover, understanding the timing of mutation induction in mice and human beings is vital for correct detection of a mutagenic response.

The *Pig-a* assay can easily be integrated into repeat-dose toxicology studies and has been proposed as an alternative to transgenic rodent (TGR) mutation assays [30]. On the other hand, the TGR mutation assay can measure mutations in the target organ but is dependent on the fact that the compound induces the type of mutations that the TGR system can reveal. The combination of different genotoxicity assays covering different modes of action of genotoxicants is beneficial and highly recommended. A combination of *Pig-a*, micronucleus and target tissue *in vivo* comet analyses may serve as a good combination of genotoxicity assays, as this combination covers a range of DNA lesions and tissue compartments. An important issue in chemical risk assessment is to identify germ cell mutagens. Comparison of results from the *Pig-a* assay with analyses of DNA lesions or mutations in germ cells by the use of transgenic reporter gene mutation assays (i.e. Big Blue) may serve useful, to understand the predictability of *Pig-a* mutations in blood for germ cell mutagenesis.

Recently, new strategies of sequencing *Pig-a* mutants have emerged facilitating more feasible verification and characterization of *Pig-a* phenotypic mutants [25]. This is important validation work that addresses the phenotype–genotype assumptions of the assay. These advanced sequencing strategies may also be useful in cases where mutational spectra data would be beneficial, for example, when the *Pig-a* mutation frequency is only marginally increased.

In human beings, *PIG-A* mutation analyses have obvious potential. *PIG-A* is clearly suitable as a sentinel gene to study somatic mutations and mutagens in human beings experiencing real-world exposures. The initial studies performed to date report low intraindividual differences and rather large interindividual differences (>30-fold; [52]), which warrants confirmation and characterization. This variation should inform study designs; for instance, whenever possible, pre-exposure samples should be collected. Wide interindividual variation has other important implications, as it suggests important lines of future research because the variation may be a result of mutagenic factors. For example, the human blood *PIG-A* assay may provide a platform to study and identify environmental, lifestyle or other host factors that influence mutation frequency. The cross-species nature of the end-point would be valuable in such investigations, as host or environmental factors causing elevated *PIG-A* mutation in human beings could be systematically studied in highly controlled animal experiments.

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Conflict of Interest

SDD is an employee of Litron Laboratories. Litron holds patents covering flow cytometric methods for scoring GPI anchor-deficient erythrocytes and sells kits based on this technology (*In Vivo* MutaFlow®).

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