Detection of PIGO-Deficient Cells Using Proaerolysin: A Valuable Tool to Investigate Mechanisms of Mutagenesis in the DT40 Cell System

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Abstract

While isogenic DT40 cell lines deficient in DNA repair pathways are a great tool to understand the DNA damage response to genotoxic agents by a comparison of cell toxicity in mutants and parental DT40 cells, no convenient mutation assay for mutagens currently exists for this reverse-genetic system. Here we establish a proaerolysin (PA) selection-based mutation assay in DT40 cells to identify glycosylphosphatidylinositol (GPI)-anchor deficient cells. Using PA, we detected an increase in the number of PA-resistant DT40 cells exposed to MMS for 24 hours followed by a 5-day period of phenotype expression. GPI anchor synthesis is catalyzed by the series of phosphatidylinositol glycan complementation groups (PIGs). The PIG-O gene is on the sex chromosome (Chromosome Z) in chicken cells and is critical for GPI anchor synthesis at the intermediate step. Among all the mutations detected in the sequence levels observed in DT40 cells exposed to MMS at 100 μM, we identified that ~55% of the mutations are located at A:T sites with a high frequency of A to T transversion mutations. In contrast, we observed no transition mutations out of 18 mutations. This novel assay for DT40 cells provides a valuable tool to investigate the mode of action of mutations caused by reactive agents using a series of isogenic mutant DT40 cells.

Introduction

Research on gene function and regulation is of paramount importance for investigations into the causes of many diseases such as cancer. One very effective approach investigators have been using involves the reverse genetic technique making use of cell lines like the DT40 line which is derived from a chicken B-lymphocyte progenitor. DT40 cells were originated by making use of an avian leucosis virus to induce a bursal lymphoma and the cell line is considered to be isogenic [1]. The DT40 cell line has distinguished itself as being highly valuable as a higher eukaryotic model that exhibits a high ratio of targeted to random integration of transfected DNA [2,3]. This ease of genetic manipulation, along with the fact that DT40 mutants have been observed to display a strong phenotypic resemblance to murine mutants in DNA recombination and repair [4], has been the foundation that has created a steadily increasing interest in the usage of the DT40 cell line in genetic investigations into immunoglobulin diversification, DNA repair, DNA damage response, chromosome segregation, RNA metabolism and cell signaling [3–7].

One downside to the DT40 cell system, however, is that for the last two decades there has been no convenient mutation assay to investigate mechanisms of mutagenicity caused by exogenous reactive agents. One approach to detect mutations in mammalian cells is based on the endogenous gene, hypoxanthine-guanine phosphoribosyltransferase (HPRT), located on the X chromosome [8]. This method has been extensively utilized in toxicology [9,10]. The HPRT mutants from cultured cells and tissues of animals exposed to mutagens are resistant to the toxic nucleoside 6-thioguanine, which allows the selection of mutant cells. Thus, mutation rates and mutational sequences in the HPRT locus can be analyzed in 6-thioguanine-resistant cells. While the HPRT mutation assay has gained popularity in mutational analysis in mammalian cells, this assay is difficult to perform in DT40 cells due to the presence of the HPRT gene on the autosomal chromosome (chromosome 4) in chicken cells instead of the sex chromosome.

Recently, a novel mutation assay was developed in rats, making use of the endogenous phosphatidylinositol glycan complementation group A (PIG-A) gene for red blood cells and T-lymphocytes [11,12]. The PIG-A gene has several characteristics that make it well suited for the detection of somatic cell mutations. Although over 20 PIG genes, including PIG-A, were identified for GPI anchor synthesis (Figure 1), only the PIG-A gene is located on the X chromosome in mammalian cells (Table 1) [13]. As with the HPRT mutation, a single mutation on the PIG-A gene results in an altered cell phenotype in mammalian cells. PIG-A codes for an enzyme that, along with five other cores and one extra subunit (PIG-C/H/P/-Q/-Y and DPM2), is involved in the first step of GPI anchor synthesis (Figure 1) [13–17]. The functions of all six...
core subunits are essential or nearly essential for GPI anchor synthesis [17]. GPI anchors tether specific protein markers to the surface of various types of cells (e.g., hematopoietic cells) [18,19]. Thus, mutations in the PIG-A gene could disrupt GPI anchor synthesis which, in turn, would cause a deficiency in GPI-anchored proteins. Lack of the anchor or GPI-anchored proteins can be detected and quantified using a flow cytometer.

Furthermore, proaerolysin (PA) has been successfully utilized in spleen T-cells to select for GPI anchor-deficient cells in the Pig-a mutation clonal assay [20]. PA is a protoxin form of aerolysin, which is a channel-forming microbial toxin [21–23]. After binding to GPI at the cell surface, PA is proteolytically cleaved into aerolysin, which ultimately forms transmembrane pores [24] and causes cell death. Mutations in the PIG-A gene abrogate GPI anchor synthesis and deplete GPI anchors at the surface of cells. Therefore, the GPI anchor-deficient mutant cells become resistant to aerolysin-induced cytotoxicity. In addition to determining the frequency of PIG-A gene mutations, it is possible to determine the PIG-A mutation spectrum through sequencing following RT-PCR of mRNA extracted from cells resistant to PA [20]. However, the PIG-A gene is also located on an autosome in DT40 cells (Table 1).

DT40 cells were established from B lymphocytes of female chickens having female heterogamety with Z and W sex chromosomes. Both PIG-G and PIG-O genes are involved in GPI anchor synthesis; however, in contrast to the PIG-A gene, both are located on chromosome Z. Modification of the GPI anchor by PIG-G is not essential for the function of the GPI anchor at the cellular level [25]. However, a complex of PIG-O and PIG-F catalyzes an attachment of phosphoethanolamine to the third mannose of the GPI anchor in the final step (Figure 1) immediately before attachment of the GPI anchor and cell surface protein. Since the phosphoethanolamine attached to the third mannose is the location where the surface protein binds, the PIG-O gene product is nearly essential for GPI anchor synthesis [26]. In the present study, we established a new PIG-O gene mutation assay to analyze the mutagenicity of reactive chemicals in DT40 cells through PA selection.

### Results and Discussion

#### Cytotoxicity of PA in DT40 cells

A concentration of PA suitable for the selection of GPI anchor-deficient DT40 cells was established using intact DT40 cells. In a low cell density experiment using a 24-well plate (2.5×10^3 cells/250 μL/well), the DT40 cells were exposed to PA at 0.125 nM or lower for three days. PA caused marked cell death in DT40 cells at 0.125 nM (Figure 2A). Furthermore, in a high cell density

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**Table 1. Chromosome location of GPI anchor synthesis genes.**

| Gene  | Chicken | Human | Rat  |
|-------|---------|-------|------|
| PIG-A | Chr 1   | Chr X | Chr X|
| PIG-C | Chr 8   | Chr 1 | Chr 13|
| PIG-P | Chr 1   | Chr 21| Chr 11|
| PIG-H | Chr 5   | Chr 14| Chr 6 |
| DPM2  | Chr 17  | Chr 9 | Chr 3 |
| PIG-Y | N.L.    | Chr 4 | Chr 4 |
| PIG-Q | Chr 14  | Chr 16| Chr 10|
| PIG-L | Chr 19  | Chr 17| Chr 10|
| PIG-W | Chr 19  | Chr 17| Chr 10|
| PIG-M | Chr 3   | Chr 1 | Chr 13|
| PIG-X | Chr 9   | Chr 3 | Chr 11|
| PIG-V | N.L.    | Chr 1 | Chr 5 |
| PIG-N | Chr 2   | Chr 18| Chr 13|
| PIG-B | N.L.    | Chr 15| Chr 18|
| PIG-F | N.L.    | Chr 2 | Chr 6 |
| PIG-O | Chr Z   | Chr 9 | Chr 5 |
| PIG-G | Chr Z   | Chr 4 | Chr 14|
| PIG-S | Chr 19  | Chr 17| Chr 10|
| PIG-T | Chr 20  | Chr 20| Chr 3 |
| PIG-U | Chr 20  | Chr 20| Chr 3 |
| PIG-K | Chr 8   | Chr 1 | Chr 2 |
| GAA1  | N.L.    | Chr 8 | Chr 7 |

Chr: Chromosome; N.L.: not identified.

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**Figure 1. Biosynthesis of the glycosylphosphatidyl inositol (GPI)-anchored protein.** Synthesis of GPI-anchored proteins involves multiple reaction steps. Briefly, the first step of GPI anchor biosynthesis is catalyzed by a multi-subunit GPI-N-acetylglucosaminyltransferase comprised of at least 6 different proteins (PIG-A, PIG-C, PIG-H, PIG-P, PIG-Q, PIG-Y). In addition, DPM2 appears to regulate this first step, followed by de-N-acetylation by the PIG-L. PIG-W then attaches an acyl chain to form glucosamine-(acyl)PI. In the next step, three mannose (Man) residues are added sequentially to glucosamine-(acyl)PI, generating Man-Man-Man-glucosamine-(acyl)PI by PIG-M/PIG-X complex, PIG-V, and PIG-B. After the Man-1 and Man-2 conjugation, PIG-N adds ethanolamine phosphates (EtNP) to the Man-1. In the final step of GPI anchor synthesis, PIG-G/PIG-F and PIG-G/PIG-F complexes attach EtNP to the Man-3 and Man-2, respectively, to generate the mature GPI anchor protein.

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experiment using a 96-well plate (4×10⁴ cells/50 μL/well), nearly all cells were killed by PA treatment at 0.8 nM or higher during a 7-day cultivation period (Figure 2B). Based on these results, we chose 1.2 nM PA for the selection of GPI anchor-deficient cells for the rest of the study.

Characterization of PAr DT40 cells

In order to confirm whether the DT40 cells that survived PA exposure were indeed PAr cells, we re-challenged the cells to different concentrations of PA. Compared to intact DT40 cells, six different clones of DT40 cells that survived the first PA treatment at 1.2 nM were resistant to cell death induced by PA (Figure 3A), indicating that accurate selection of PAr DT40 cells is feasible using 96-well plates. Using one of the PAr clones, we seeded different numbers of PAr cells (0 to 80 cells/plate) onto 96-well plates containing intact DT40 cells (4×10³ cells/well) to validate the accuracy of the PA selection step of the assay. We detected a linear relationship between expected and

![Figure 2. Cell survival after PA exposure.](image1)

(A) In a low cell density experiment using a 24-well plate (2.5×10³ cells/250 μL/well), DT40 cells were exposed to PA (0.0221–0.125 nM). After a three-day cultivation, cell viability was determined by XTT. Each point represents the mean and S.D. (bars) from three independent experiments. (B) In a high cell density experiment using a 96-well plate (4×10⁴ cells/50 μL/well), DT40 cells were exposed to PA (0.5–1.2 nM). After a seven-day incubation, colony formation was scored visually using an inverted microscope. Each point represents the mean and S.D. (bars) from three independent experiments.

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![Figure 3. Characterization of PA-resistant (PA⁺) DT40 cells and validation of PA selection-based GPI anchor-deficient cell detection assay.](image2)

(A) In a low cell density experiment using a 24-well plate (2.5×10³ cells/250 μL/well), the intact DT40 cells and six different clones of DT40 cells that survived from the first PA treatment at 1.2 nM were exposed to PA (0.0221–1.2 nM). After a three-day cultivation, cell viability was determined by XTT. Each point represents the mean and S.D. (bars) from three independent experiments for DT40 cells and single experiment for six different clones of DT40 cells resistant to PA. (B) Using one of the PAr clones used for Figure 3A, different numbers of PAr cells (0 to 80 cells/plate) were seeded onto 96-well plates containing intact DT40 cells (4×10³ cells/well) to validate the accuracy of the PA selection step of the assay. The cells were exposed to PA at 1.2 nM. After a seven-day incubation, colony formation was scored visually using an inverted microscope. Plating efficiency was also determined using PAr cells.

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observed frequencies of PA<sup>+</sup> cells ($R^2 = 0.99$, $y = 0.501x + 1$) (Figure 3B).

**Optimization of detection of GPI anchor-deficient DT40 cells exposed to MMS**

MMS is a well-studied mutagen; therefore, we utilized this reactive agent to optimize the PA-selection-based GPI anchor-deficient cell detection assay. The effect of 24-hour MMS exposure at 100 μM on cell growth was first evaluated over three days after MMS exposure. While the first 24-hour incubation with 100 μM MMS decreased the growth rate to ~80% compared to the control, the cell growth rate recovered to control level after replenishing the medium without MMS (Figure 4A). It has been well established that, following a mutation at the HPRT locus, time is required before the new thioguanine-resistant phenotype is completely expressed [27]. We next optimized the length of the phenotype expression period in the PA<sup>+</sup> DT40 cells after MMS treatment. The length of expression time showing a maximum mutant frequency depends on segregation of the mutant allele from the normal allele by cell proliferation; loss of the original, normal gene activity due to dilution or degradation of wild-type protein; and loss of original, wild-type mRNA due to its dilution or degradation.
degradation [27]. An increase in PA' cells was detected 3 days after MMS treatment, and the number of PA' cells reached nearly maximum at 5 days after MMS treatment (Figure 4B). This indicates that a 4- to 5-day cultivation is required for full phenotypic expression (GPI anchor deficiency) following MMS treatment. We also detected a decrease in the frequency of PA' DT40 cells after a 6-day expression period. This reduction appears to be due to slower proliferation rates of mutant than non-mutant cells; therefore, propagation longer than five days may lead to further decreases in the PA' cell frequencies. Based on this time-course experiment, we chose a 24-hour MMS exposure plus a 5-day phenotype expression period for most of the experiments in this study. While the PA' cells were allowed to grow for 5–7 days in the presence of PA before identifying resistant cells, small colonies of PA' cells were usually detectable as early as 3 days after the PA treatment.

Frequent of GPI anchor-deficient DT40 cells exposed to MMS at different concentrations

MMS has recently been evaluated for its low dose effects in the HPRT and tk genes in the AHH-1 human lymphoblastoid cell line [28]. MMS exposure for 24 hours caused a nonlinear (hockey-stick-shaped dose-response) curve containing a range of non-mutagenic low doses in both mutation assays. We next addressed whether the appearance rate of PA' cells after MMS treatment showed a non-linear dose response curve in DT40 cells. DT40 cells were exposed to different concentrations of MMS to determine an increase in the rate of PA' cell formation. Cell growth inhibition was observed at 80 or 100 μM after 24-hour MMS exposure, but not at 60 μM or lower (Figure 4A and data not shown). We detected the hockey stick-like dose-response curve in the incidence of PA' cells (Figure 4C). These results indicate that a PA-based selection is a useful tool to detect GPI anchor synthesis gene mutations in DT40 cells. When we compared different mutation assays in different cell lines, we recognized that the PIG-O mutation assay in DT40 cells required slightly higher mutation assays in different cell lines, we recognized that the PIG-O mutation assay in DT40 cells required slightly higher concentrations of MMS compared to the HPRT mutation assay in AHH-1 cells [28]. This difference could be due to the variation of the functionality of various DNA repair pathways against DNA lesions caused by MMS.

PIG-O gene is mutated in PA' DT40 cells

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hematopoietic stem-cell disorder resulting from the clonal expansion of hematopoietic cells with somatic mutations in the X-linked PIG-A gene in humans. The affected cells with PIG-A gene mutations are deficient in GPI-anchored proteins. PNH is frequently associated with hemolytic anemia, a hypercoagulable state, and bone marrow failure [29]. Many proteins on the eukaryotic cell surface are anchored by GPI [30]. While the PIG-A gene is located in the autosome in DT40 cells, there is a single copy of the PIG-O gene located on the Z chromosome in the cells derived from the female chicken. The chicken PIG-O gene codes for a protein that contains 1145 amino acids (XP_001232870). The percent amino acid sequence identity of the chicken PIG-O with the human PIG-O (1089 amino acids, NP_116923) is 94%. To address whether PA' DT40 cells possess a PIG-O gene mutation, we next sequenced PIG-O cDNA in both normal DT40 cells and DT40 cells resistant to PA. Figure S1 shows the comparison between the PIG-O cDNA sequences found in DT40 cells and those found in chicken (XM_001232869). The cDNA sequence in parental DT40 cells was used as a reference for determining DNA alterations in PA' DT40 cells. Among all the mutations detected in the sequence levels observed in DT40 cells exposed to MMS, we identified that ~55% of the mutations are located at A/T sites, including a high frequency of A to T transversion mutations and three frameshift mutations (Table 2). In contrast, there were no transition mutations out of the 18 mutations. We also observed three clones with deletions ranging from 20 to 204 bases and three clones with insertions ranging from 2 to 6 bases. When we compared our results in the mutational spectrum of the PIG-O gene in DT40 cells and the previously published spectrum of the hprt gene in CHO cells, there was a drastic difference in the mutational spectrum caused by MMS exposure. We detected no G/C→A/T transition mutations in our study whereas predominant G/C→A/T transition mutations at the hprt gene have been frequently reported in Chinese hamster fibroblast/ovary cells [31,32]. This can be explained by a O6-methylguanine-DNA-methyltransferase deficiency in these Chinese hamster fibroblast/ovary cells, which leads to the accumulation of mutagenic O6-methylguanine. In contrast, the high frequency of A/T→T/A trans-version mutations in the PIG-O gene in DT40 cells is in agreement with the mutational spectrum of target genes obtained from in vivo exposure to MMS. For example, mutations with a high frequency of A/T→T/A trans-versions have been demonstrated at the hprt gene in splenic T-lymphocytes from N-methylpurine-DNA glycosylase knockout mice exposed to MMS and the vermillion locus of Dro sophila melanogaster exposed to MMS [33–35]. The A/T→T/A trans-version mutations found in MMS exposure-derived PA' DT40 cells appear to be due to N3-methyladenine or base excision repair intermediates, such as abasic sites and single strand breaks originated from N3-methyladenine [36,37]. Although more work needs to be done to characterize the spectrum of spontaneous mutations (Table 3), most of the PIG-O gene mutations in DT40 cells exposed to MMS represent mutations derived from DNA damage caused by MMS due to the increase of mutation frequency by more than 45 times over the spontaneous mutation rate (Figure 4C). Furthermore, it is worthwhile to mention that our preliminary study showed a high background of PIG-O mutations in some of DNA repair gene-deficient DT40 cells (e.g., XPA-deficient cells), confirming the important value of this new mutation assay in DT40 cell system. It has been previously reported that PIG-O and PIG-F act together in transferring phosphoethanolamine to the third mannose during GPI anchor synthesis in CHO cells [26]. In the same study, the Pig-o gene mutant cells showed a pronounced decrease, but not a complete loss, in the expression of Thy-1, a GPI-anchored protein, in CHO cells. The Pig-o gene mutant CHO cells also expressed no detectable CD59 and only 10% of normal levels of decay-accelerating factor (both CD59 and decay-accelerating factor are GPI-anchored proteins) [38], indicating that Pig-o is involved in and nearly essential for GPI anchor biosynthesis [26]. We attempted to detect PIG-O mutants using fluorescent-tagged proaerolysin (FLAER)-coupled FACS analysis; however, the separation between FLAER-positive and negative DT40 cells was not accomplished in our study. This could be due to partial expression of GPI-anchored proteins on the membrane in PIG-O mutants as described above. Therefore, we chose the PA selection-based method instead of the FACS-based analysis to analyze the frequency of GPI-anchor deficient DT40 cells with high specificity. As described above, PA selection-based GPI-deficiency cell analysis demonstrated a reasonable dose-response relationship between MMS concentrations and the frequency of GPI-deficient cells, indicating that this assay is a useful tool for characterizing mutation events in the DT40 cell system.
Materials and Methods

Materials
Penicillin/streptomycin and methyl methanesulfonate (MMS) were obtained from Sigma (St. Louis, MO). Trizol, Taq DNA polymerase, RPMI 1640 culture medium and chicken serum were acquired from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from PAA laboratories Inc (Etobicoke, Canada). iScript cDNA synthesis kit and PCR primers were acquired from Bio-Rad Laboratories (Hercules, CA) and Thermo Fisher Scientific (Waltham, MA), respectively. Wizard SV Gel/PCR Clean-Up System and PA were purchased from Promega (Madison, WI) and the University of Saskatchewan (Saskatoon, Canada), respectively.

Cell growth assay after MMS exposure
DT40 cells [6] were cultured in a manner previously reported [6,7]. We performed two different types of cell survival assays:

Low cell density experiment. One of the cell survival assays was conducted as described previously for the DNA damage response analysis [6,7]. Briefly, suspended DT40 cells (2.5 × 10^3 cells per 250 μL per well) were seeded into 24-well plates, exposed to either PA or MMS, and allowed to divide for 7 to 8 cycles (approximately 3 days). After cultivation, cell viability was determined by the XTT assay [6,7].

High cell density experiment. We performed a cell growth assay during/after MMS treatment. The assay was performed under the exposure conditions used for the PA-selection-based GPI anchor-deficient cell detection assay described below. Briefly, suspended DT40 cells (1.25 × 10^6 cells/10 ml) were seeded into 10-cm Petri dishes, exposed to MMS, and allowed to cultivate for 24 hours. The number of viable cells was quantitated by the trypan blue exclusion assay. The culture medium was replenished and the cells (1.25 × 10^6 cells/10 ml) were further incubated for 24 hours. This cycle was repeated for 5 days.

PA-selection-based GPI anchor-deficient cell detection assay
The overall scheme of this assay is shown in Table 4. The cells were maintained at a concentration of 0.125 to 1.5 × 10^6/mL. Since the background PA-resistant (PA') cell population was low, we directly used DT40 cells for the assay without re-populating with PA-sensitive DT40 cells in this study. Cell suspension at 1.25 × 10^6 cells/10 ml was exposed to MMS at different concentrations for 24 hours at 39.5°C, 5% CO_2. The cells were then washed to remove the MMS and were resuspended in 10-mL

Table 2. Mutational spectrum of PIG-O gene in DT40 cells exposed to MMS at 100 μM.

| Mutation (cDNA position) | Target sequence (5'→3') | Amino acid change (codon) |
|-------------------------|-------------------------|--------------------------|
| Transversion            |                         |                          |
| AT to TA (840)          | CCA(T)GGT               | His to Gln (280)         |
| AT to TA (2009)         | GCC(T)GAG               | Leu to Gln (670)         |
| AT to TA (2011)         | CTG(A)GAA               | Arg to stop (671)        |
| AT to TA (2332)         | GCA(A)AGG               | Lys to stop (778)        |
| AT to TA (2941)         | TCC(A)AGA               | Lys to stop (981)        |
| AT to CG (689)          | ACC(A)CTG               | His to Pro (230)         |
| AT to CG (950)          | ACC(T)GGT               | Leu to Arg (317)         |
| GC to CG (2771)         | ACT(G)GAA               | Trp to Ser (924)         |
| GC to TA (2911)         | TGT(G)AGA               | Glu to stop (971)        |
| Frameshift              |                         |                          |
| +4 (1915)               | CTI(T)CTG               |                          |
| +4 (241)                | GAA(T)TTG               |                          |
| +4 (941)                | CCC(A)GGT               |                          |
| Insertion/Deletion      |                         |                          |
| +6 bases (1092)         | CAA(GCAGTT)GCA          |                          |
| +2 bases (2786)         | TGT(GG)TTG              |                          |
| +4 bases (2873)         | CAG(TTAT)TTG            |                          |
| −145 bases (497–641)   | GAA(GAA—GAA)CAG        |                          |
| −20 bases (1746–1765)  | AGC(TGA—CAT)TCC         |                          |
| −204 bases (2669–2872) | CTG(AGC—CAG)TTG         |                          |

Table 3. Spontaneous mutational spectrum of PIG-O gene in DT40 cells.

| Mutation (cDNA position) | Target sequence (5'→3') |
|-------------------------|-------------------------|
| Deletion                |                         |
| −171 bases (923–1093)   | AGG(AGC—AGC)AGT         |
| −4 bases (1084–1051)    | GTG(TCTG)AGG            |
| −4 bases (1093–1096)    | AAG(CAGG)TTG            |
| −4 bases (1092–1095)    | CAA(GCAG)GTG            |

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fresh medium (1.25×10^6 cells/10 mL). The culture medium was replenished daily to maintain appropriate cell concentrations as described above. The 96-well plates were then loaded with 4×10^4 cells/50 μL/well containing 1.2 nM PA (2 plates per sample). Nonselection plates were prepared by inoculating 2 target cells and 4×10^4 lethally lactic acid pre-treated (50 mM lactic acid for 30 min at 39°C) DT40 cells as feeder cells in 50 μl medium in each well to determine the plating efficiency in the absence of PA treatment (2 plates per sample). All plates were incubated for approximately 7 days at 37°C, in 5% CO₂, and in a humidified atmosphere. Subsequent colony formation was scored visually using an inverted microscope to determine the mutation frequency of each dose, calculated as described by Furth et al. [39].

Reverse transcription-polymerase chain reaction
RNA was prepared using Trizol reagent and was reverse-transcribed using a mixture of random hexamers and Oligo d(T) according to standard procedures. Primers for PIG-O PCR were designed using Primer-BLAST (National Center for Biotechnology Information) based on a predicted chicken PIG-O cDNA sequence (XM_001232869.1). cDNA (150 ng/25 μl) was PCR-amplified in 20 mM Tris (pH 8.4), 50 mM KCl, 1 mM MgCl₂, 0.1 mM of each primer, calculated as described by Furth et al. [39]. using an inverted microscope to determine the mutation frequency at 37°C, in 5% CO₂, and in a humidified atmosphere. Subsequent colony formation was scored visually using an inverted microscope to determine the mutation frequency of each dose, calculated as described by Furth et al. [39].

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Table 4. Scheme of PIG-O mutation assay in DT40 cells.

| Step | Description |
|------|-------------|
| 1.   | Seed cells into a 10-cm dish at 1.25 million/10 ml. |
| 2.   | Expose cells to MMS for 24 hours. |
| 3.   | Replenish medium with cells at 1.25 million/10 ml everyday after MMS exposure. |
| 4.   | Seed approximately 40,000 cells/well in 96-well plate at five days after MMS treatment. |
| 5.   | Treat cells with proaerolysin at 1.2 mM. |
| 6.   | Incubate cells for seven days. |
| 7.   | Count colonies under the microscope. |

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Table 5. Primers for the RT-PCR and Sequencing of Chicken PIG-O cDNA.

| Primers | Position of primers | Nucleotide sequence (5’→3’) |
|---------|---------------------|-----------------------------|
| 1F      | -48 to -26          | GCTGGAGAT AGAGGCCTGG GGGCT |
| 1R      | 450 to 472          | GCACGTTGCTCTCCCTTGATCCGA |
| 2F      | 369 to 388          | TGCCACCATGCGAGCCATCA |
| 2R      | 1287 to 1306        | CCGGTCGCTCCGCGAGTAG |
| 3F      | 1127 to 1146        | TGGGCTGAGGCTGAGGCA |
| 3R      | 1666 to 1685        | AAGGCAGGCGAAAAGCAC |
| 4F      | 1593 to 1612        | GCATCCCAGGCGAGGCGGTT |
| 4R      | 2075 to 2094        | CAGCGAAGGCTGACGCTAT |
| 5F      | 1968 to 1988        | CAGTGCCGCGCCTCGGT |
| 5R      | 2505 to 2524        | GGCGTCGCGAGTACACTG |
| 6F      | 2470 to 2489        | AGCTGCCAGGCCCCAGTGC |
| 6R      | 2980 to 2999        | GGGACCTCCCGAGCCTCAT |

*Primer annealing sites relative to the A of the ATG initiation codon (XM_001232869).

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Statistical analysis
A one-way ANOVA, followed by a Dunnett’s posthoc test, was used to determine if any of the treatment doses were significantly different from the zero dose in the 24-hour MMS exposure experiment.

Supporting Information

Figure S1 Comparison between the PIG-O cDNA sequences found in DT40 cells and those found in chicken (XM_001232869).

(DOCX)

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Author Contributions
Conceived and designed the experiments: JN JAS. Performed the experiments: JN HG XT SJB. Analyzed the data: JN. Contributed reagents/materials/analysis tools: SJB. Wrote the paper: JN.
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