Germline Stem Cell Gene PIWIL2 Mediates DNA Repair through Relaxation of Chromatin

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Abstract

DNA damage response (DDR) is an intrinsic barrier of cell to tumorigenesis initiated by genotoxic agents. However, the mechanisms underlying the DDR are not completely understood despite of extensive investigation. Recently, we have reported that ectopic expression of germline stem cell gene PIWIL2 is associated with tumor stem cell development, although the underlying mechanisms are largely unknown. Here we show that PIWIL2 is required for the repair of DNA damage induced by various types of genotoxic agents. Upon ultraviolet (UV) irradiation, silenced PIWIL2 gene in normal human fibroblasts was transiently activated after treatment with UV light. This activation was associated with DNA repair, because Piwil2-deficient mouse embryonic fibroblasts (mili−/− MEFs) were defective in cyclobutane pyrimidine dimers (CPD) repair after UV treatment. As a result, the UV-treated mili−/− MEFs were more susceptible to apoptosis, as characterized by increased levels of DNA damage-associated apoptotic proteins, such as active caspase-3, cleaved Poly (ADP-ribose) polymerase (PARP) and Blik. The impaired DNA repair in the mili−/− MEFs was associated with the reductions of histone H3 acetylation and chromatin relaxation, although the DDR pathway downstream chromatin relaxation appeared not to be directly affected by Piwil2. Moreover, guanine–guanine (Pt-[GG]) and double strand break (DSB) repair were also defective in the mili−/− MEFs treated by genotoxic chemicals Cisplatin and ionizing radiation (IR), respectively. The results indicate that Piwil2 can mediate DNA repair through an axis of Piwil2 → histone acetylation → chromatin relaxation upstream DDR pathways. The findings reveal a new role for Piwil2 in DNA repair and suggest that Piwil2 may act as a gatekeeper against DNA damage-mediated tumorigenesis.

Introduction

PIWIL2 (Pcwi-like 2) gene (alias mili in mouse or hili in humans), a member of AGO/PIWI gene family, is exclusively expressed in the germline stem cell (GSC) of testis but not in the adult tissue stem cells and somatic cells [1,2,3,4]. Recently, expression of PIWIL2 has been widely detected in a variety of tumor cell lines as well as in various stages of primary cancers [5,6,7,8,9,10,11]. Interestingly, PIWIL2 gene can be alternatively activated in tumor cells by intragenic promoters, resulting in a number of Piwil2 variants, namely Piwil2-like (PL2L) proteins with a potential function in tumorigenesis [11]. Especially, we have found that PIWIL2 expression is associated with the development of tumor stem cell (TSCs) [6,11,12,13,14]. However, the exact mechanisms PIWIL2-mediated cell transformation and tumor formation is unknown.

The AGO/PIWI family proteins containing PIWI and PAZ domains (PPD) [1,2] show multiple biological functions. Although it is known that the PAZ domain is bound by siRNA [15], the function of PIWI domain has not been clarified [16]. The Piwil2 protein is shown to be essential for gametogenesis in various organisms [3]. It controls gametogenesis through regulating self-renewal [17], RNA silencing [18,19], translational regulation [4], chromatin remodeling [20,21] and epigenetic modifications of GSCs [21,22]. Piwil2 binds piwi-interacting RNA (piRNA) to silence the selfish genetic elements such as retrotransposons through methylation of cytosine of CpG islands in the germ cells of testis [22,23,24]. Dysregulated or ectopic expression of Piwi family proteins, especially Piwil2, seems linked to cell transformation and tumorigenesis [6,11,12,13]. Elucidation of the role of Piwil2 in signaling cell transformation and tumorigenesis will provide new insights into the biological functions of PIWIL2 and potential therapeutic targets in cancer treatment.

Genotoxic agents-induced DNA damage is a primary cause of tumorigenesis [25,26]. The resulted DNA damage response (DDR) is an anti-cancer barrier in early human tumorigenesis.
PIWIL2 gene is activated upon DNA damages

To determine the response of PIWIL2 gene to DNA damages, we treated human dermal fibroblasts (HDFs) with various doses of UV light, and examined the expressions of PIWIL2 transcripts and proteins in these cells at various time points by Western-blotting and RT-PCR. As shown in Figure 1, PIWIL2 protein expression in human dermal fibroblasts (HDFs) was induced by UV irradiation as early as one hour after treatment (Fig. 1A-B). The expression was dose-dependent and reached a peak between 10–20 J/m² UV irradiation 2 hrs after treatment (Fig. 1C-D). However, PIWIL2 expression was individually variable with experiments being at the high dose of 80 J/m² and sometime the level of PIWIL2 was lower than at 40 J/m², probably associated with more cell death at this time point (Fig. 1C and not shown). Consistently, Piwil2 transcripts were also up-regulated in HDFs as early as one hour after UV treatment (Fig. 1E-F). Interestingly, the level was temporarily reduced at 4 hrs, then reached a peak at 6 hrs after treatment and decreased thereafter (Fig. 1E-F). After 48–72 hrs of treatment, Piwil2 transcripts go back to the baseline, regardless of the level of PIWIL2 proteins (not shown). The results suggest that PIWIL2 gene can be activated temporarily upon DNA damages, and Piwil2 expression is transcriptionally regulated.

Piwil2-deficiency promotes DNA damage-induced cell death

To determine the significance of Piwil2 responding to DNA damage, we investigated effects of Piwil2 on DNA damage-induced cell death, using mouse embryonic fibroblasts (MEFs) derived from mili knockout (KO) mice. As observed in HDFs, Piwil2 expression was also up-regulated in MEFs upon UV irradiation (data not shown). To determine the susceptibility of mili-/- MEF to apoptosis induced by UV light, we evaluated cell survival rate after UV treatment. As shown in Figure 2, the survival rate at day 4 of mili-/- MEFs were significantly reduced in responding to various doses of UV light, compared to that of wild-type (WT) MEFs. This was associated with increased apoptosis of the UV-treated mili-/- MEF, because DNA damage-associated apoptotic proteins including activated caspase-3, cleaved Poly (ADP-ribose) polymerase (PARP) and Bik were up-regulated in the mili-/- MEFs; however, the expression of Bax and Bcl-XL, which are not specifically associated with DNA damage, was not significantly different between mili-/- and WT MEFs (Fig. 2B). Especially the up-regulation prominently occurred after 12 h of UV treatment when damaged DNA should have been repaired, suggesting that DNA repair might have failed in the mili-/- MEFs.

Piwil2 is essential for DNA repair

To verify that DNA repair was defective in the UV-treated mili-/- MEF, we treated mili-/- and WT MEFs with UV light, and examined cyclobutane pyrimidine dimers (CPD) and 6–4 pyrimidine photoproducts (6–4 PP), which can be induced by UV irradiation through covalent linkage between adjacent cytosine and thymine bases [35,36]. However, 6–4 PP is only 10–15% of the damaged DNA induced by UV light [37]. As shown in Figure 3, CPD repair was significantly reduced in mili-/- MEFs, compared to that in WT MEFs during DNA repair (Fig. 3A). Interestingly, 6–4 PP in mili-/- MEFs was reduced to the same level as observed in WT MEFs (Fig. 3B). Despite of this, the results suggest that Piwil2 activation upon DNA damage is responsible for DNA repair. Lack of Piwil2 may lead to defective DNA repair, resulting in decreased cell survival rate because of increased apoptosis (Fig. 2A).
The results suggest that Piwil2 is required for transforming condensed chromatin into a more relaxed structure, which is associated with active gene transcription [44].

It has been suggested that histone H3 acetylation is required for chromatin relaxation [41,44]. Thus, we hypothesized that histone acetylation might be inhibited in the DNA-damaged mili-/- MEFs. To verify the hypothesis, we examined the status of histone H3 acetylation in mili-/- MEFs. As expected, the acetylation of H3K9, 14 (acH3K9/14) and acH3K18 was reduced in mili-/- MEFs after UV treatment, while acH3K9/14 was increased in WT MEFs (Fig. 4C). It should be noted that mili-/- MEFs expressed higher level of acH3K9/14 than WT MEFs before UV treatment (Fig. 4C). The results confirm that decreased chromatin relaxation in mili-/- MEFs is associated with reduced acetylation of histone H3. However, Piwil2 had no effect on histone H3 phosphorylation, because the level of pH3(S10) was not significantly changed in mili-/- MEFs compared to that in WT MEFs (Fig. 4C).

Piwil2-mediated DNA repair is of broad significance

To determine whether Piwil2-mediated DNA repair is universal to DNA damage induced by different genotoxic agents, we investigate the DNA repair in mili-/- MEFs treated by cisplatin and ionizing radiation (IR), respectively. As shown in Fig. 5A, cell survival rate of mili-/- MEFs was significantly reduced compared to WT counterparts after treatment with various doses of cisplatin. Cisplatin can cause intrastrand crosslinking of DNA to form adducts such as guanine-guanine (Pt-[GG]), which can be detected by mAbs [45]. Consistently, the level of Pt-[GG] was not significantly reduced in the cisplatin-treated mili-/- MEFs at 8 and 24 hrs of treatment, as compared to the level of Pt-[GG] in the cisplatin-treated WT MEFs (Fig. 5B). The Piwil2-responding to cisplatin was also observed in vivo (Fig. 5C). Piwil2 was detected in the kidney and liver of mice treated with cisplatin but not with vehicle (Fig. 5C). The results suggest that Piwil2 can respond to cisplatin-induced DNA damage.

Similar results were also observed in the mili-/- MEFs treated by X-ray radiation or IR, which can induce DNA DSBs (Fig. 5D–E). The cell survival rate of X-ray-treated mili-/- MEFs was significantly decreased in a dose-dependent manner, as compared to their WT counterparts (Fig. 5C). The reduced survival rate appeared to be associated with their reduced capacity of DNA repair, as revealed by Comet assay (Fig. 5E). Moreover, consistently with the observation that phosphorylation of H2AX was not affected in the mili-/- MEFs treated by UV and cisplatin, phosphorylation of H2AX was neither affected in the X-ray treated mili-/- MEFs, because the size of γH2AX foci was comparable between the mili-/- MEFs and WT MEFs at 1 hour after the treatment (Fig. 5G). However, the size of γH2AX foci in the majority of mili-/- MEFs was much smaller than that in the WT MEFs at 3 hrs of X-ray treatment (Fig. 5F–G), suggesting that Piwil2 did not affect phosphorylation of H2AX, but did affect the...
formation of chromatin remodeling complexes [46], which mediate DNA DSB repair [29]. Taken together, these results confirm that Piwil2 is essential for DNA repair in the cells insulted by various types of genotoxic agents, including UV, IR, and chemotherapeutic agents such as cisplatin.

Discussion

Normally, PIWIL2 gene is silent in adult tissue stem cells and somatic cells except for testis [1,4,5,11]. Recently we and others have found that Piwil2 may play important roles in tumor development, despite the fact that the underlying mechanisms are not yet clear [5,7,8,9,10,11,13,14]. In this study, we have for the first time revealed that PIWIL2 gene can be activated upon DNA damages induced by genotoxic agents. The finding suggests that the usually silent PIWIL2 gene in adult tissue cells is responsible for cell stresses and thus can be activated upon DNA damage. The notion is further supported by our observation that variable levels of Piwil2 transcripts and proteins were sometimes detected in HDFs and other cell lines in the long-term cultures, probably associated with increased stressing in the cultures such as high density or over growth of cells (not shown). This activation is critical for DNA repair, because DNA repair was defective in the mili⁻/⁻ MEFs treated by various types of genotoxic agents, including UV, IR and cisplatin. Consistently with the failure to repair damaged DNA, increased apoptosis or decreased cell survival was observed in mili⁻/⁻ MEFs treated by these agents. Interestingly, activated caspase-3, cleaved PARP and Bik but not Bax were up-regulated in mili⁻/⁻ MEFs after UV treatment, suggesting that the DNA damage-associated apoptotic pathway is activated preferentially [47,48,49]. Therefore, Piwil2 is required for DNA repair.

Figure 2. Mili⁻/⁻ MEFs are more susceptible than WT MEFs to apoptosis induced by UV light. A. Survival rate was significantly decreased in the mili⁻/⁻ MEFs treated with various doses of UV (p<0.01). The relative cell survival rate was determined by methylene blue staining 72 or 96 hours after treatment. Shown are the data derived from 96 hrs after irradiation. B. DNA damage-associated apoptotic proteins were up-regulated in the UV-treated mili⁻/⁻ MEFs. The data shown are a representative of three experiments in triplicate. Caspase-3: activated caspase-3; cPARP: cleaved PARP. doi:10.1371/journal.pone.0027154.g002
Genotoxic agents-induced DNA damage is immediately followed by complex DDR cascades, including two major events: chromatin relaxation and the recruitment of DDR proteins, i.e., DNA damage signaling proteins and DNA repair proteins, to the sites of DNA damage [28,32,41,46]. Chromatin relaxation allows the DDR proteins to be recruited to the site of DNA damage and thus is a prerequisite for DNA repair [41]. There are multiple pathways for DNA-damage repair, including direct reversal (DR), base-excision repair (BER), nucleotide excision repair (NER) and DNA mismatch repair (MMR) for single-strand break (SSB), and homologous recombination (HR) and non-homologous end joining (NHEJ) for double-strand break (DSB) repair [50,51] (Fig. 6). In this study, we demonstrated that DNA repair in mili-/- MEFs was defective and this defect is associated with compact structure of chromatin but not with activation of signaling transduction proteins for DNA damage. Piwil2 modulates chromatin relaxation through promoting histone H3 acetylation during DDR, because acH3K9/14 and acH3K18 were reduced in mili-/- MEFs after DNA damage. It is well known that histone acetylation is associated with transcriptional activation and euchromatin formation or chromatin relaxation [29,44]. The unwound heterochromatin allows the damaged DNA to be accessible for the signaling transduction proteins of DNA damage as well as DNA repair proteins [25,26,52]. The finding is consistent with the functions of Piwi proteins to promote chromatin remodeling in Drosophila [20,53]. It is unlikely that Piwil2 is directly involved in the activation of DDR proteins, because we did not observe any effect of Piwil2 on the activation of p53 and H2AX, two hallmarks for the signaling transduction pathway of DNA damage. However, the size of γH2AX foci in the IR-treated mili-/- MEFs was greatly reduced compared to that in the WT counterparts, suggesting that the formation of chromatin remodeling complexes was defective in mili-/- MEFs during DSB repair. This may be caused by defective chromatin decondensa-

Figure 3. Piwil2 is required for repair of DNA damage induced by UV light. Mili-/- (KO) and WT MEFs were treated with 10 J/m² UV light and examined for CPD (A) and 6–4 PP abducts (B) at various time points, using Immuno-slot blotting. The data shown are a representative of two experiments. **, p<0.01.
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A

| UV (10 J/m²) | Mili ^+/+ MEF | Mili ^− MEF |
|-------------|----------------|-------------|
| -           | +              | +           |
| +           | +              | -           |
| Time post-treatment (h) | 1 | 4 | 8 | 24 |
| γH2AX       |                |             |
| pp53        |                |             |
| Tubulin     |                |             |

B

| MNase (0.1 u/ml) | Mili ^+/+ | Mili ^− | Mili ^+/− | Mili ^− |
|-----------------|------------|----------|-----------|----------|
| -               | -          | -        | -         | -        |
| UV (100 J/m²)   | +          | +        | +         | +        |

C

| UV (10 J/m²) | Mili ^+/+ MEF | Mili ^− MEF |
|-------------|----------------|-------------|
| -           | +              | +           |
| Time post-treatment (h) | 1 | 4 |
| pH3(S10)    |                |             |
| AcH3 (K9,14)|                |             |
| AcH3 (K18)  |                |             |
| Tubulin     |                |             |
irradiation by UV light. Expression of phosphorylated histone H3 [pH3 (S10)] and acetylated histone H3 [AcH3 (K9, 14) and AcH3k18] in mili-/- and WT MEFs were analyzed by Western blotting after UV irradiation. Tubulin expression was monitored as an internal control. Shown are the data from one of two experiments. doi:10.1371/journal.pone.0027154.g004

Figure 4. Piwil2 promotes chromatin relaxation through regulation of histone H3 acetylation in responding to DNA damage. A. Piwil2 has no effect on activation of H2AX and p53 in MEFs after treatment with UV light. The γH2AX and pp53 in mili-/- and WT MEFs were analyzed by Western blotting. B. Piwil2 is required for chromatin relaxation in MEFs irradiated by UV light, as revealed by Mune assay. Top panel: micrograph of DNA ladders; bottom panel: quantitation of DNA fragments in the top panel. **, p<0.01. C. Piwil2 up-regulate histone H3 acetylation in MEFs irradiated by UV light. Expression of phosphorylated histone H3 [pH3 (S10)] and acetylated histone H3 [AcH3 (K9, 14) and AcH3k18] in mili-/- and WT MEFs were analyzed by Western blotting after UV irradiation. Tubulin expression was monitored as an internal control. Shown are the data from one of two experiments.

Materials and Methods

Animals, antibodies and cell lines

Piwil2 (mili) gene knockout mice with C57BL/6 background provided by Dr. Haifan Lin at Department of Cell Biology &Yale Stem Cell Center, Yale University School of Medicine, New Haven, CT, were bred and maintained in the animal pathogen-free facility at The Ohio State University Medical Center. Male C57BL/6 mice were purchased from Jackson Laboratories. The protocol of animal experiments for the study was approved by the Institutional Animal Care and Use Committee (IACUC), OSU (Protocol number: 2006A0250). The following antibodies were used in this study. Rabbit polyclonal antibody to Piwil2 (1:1000) was generated in our laboratory [11]. Mouse anti-cleaved PARP (1:1000), rabbit anti-cleaved caspase-3 (1:1000), rabbit anti-Bik (1:1000), rabbit anti-Bax (1:1000), rabbit anti-Bcl-XL (1:1000), etc.
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A

Cell survival rate (%)

B

Remaining Pk-GG (%)

C

Testis
Kidney
Liver

Cisplatin
- 
+ 
- 
+ 
- 
+

Piwil2

100 KDa

Tubulin

D

Cell survival (%)

E

Relative Units of DNA Tails

F

Mili KO

WT

G

% of γH2AX⁺ cells

Time after irradiation (h)
Figure 5. Piwil2 is required for repair of DNA damage induced by IR and cisplatin. A, B & C. Piwil2 is required for repair of DNA damage induced by cisplatin. (A) The survival rate of mili−/− MEFs was significantly reduced in a dose-dependent manner as compared to WT MEFs after cisplatin treatment in various doses. The relative cell survival rate was determined by methylene blue staining (n = 3). **, p < 0.01. (B) DNA repair in the cisplatin-treated mili−/− and WT MEFs. The MEFs were treated with cisplatin for 1 h, cultured and harvested at the indicated time for ISB assay to determine amounts of Pt-GG in the cells (n = 3). (C) Cisplatin induced Piwil2 expression in vivo. Male mice were treated i.p. with cisplatin (20 mg/kg) or vehicle (PBS) for 5 consecutive days and kidney, liver and testis were harvested and whole cell lysates from the tissue were prepared and subjected to Western blotting with monoclonal anti-piwil2 IgM antibody (Kaoz2 supermatant; 1:50). The data shown were a representative of two experiments. D & E. Piwil2 is required for repair of DNA damage induced by IR. (E) mili−/− and WT MEFs were seeded at 1 x 10^5/well in 6-well plates in triplicates. When cells grew to 50–60% confluence (2 days) they were exposed to various doses (0, 0.5, 2, 5, 10 Gy) of X-ray (RS 2000 Biological Irradiator; Rad Source Technologies, Inc. Alpharetta, GA). Four days after irradiation, cells were harvested and counted with trypan blue exclusion of dead cells. Cell survival rate was calculated as percentage of viable cells of each dose normalized to untreated counterparts (n = 3). **, p < 0.01; *, p < 0.05. (E) DNA repair in IR-treated MEFs. Mili−/− and WT MEFs were X-rayed at exponential growth phase and comet assay was performed with standard protocol. DNA damage was estimated by measuring the distance of the tail against the edge of far side of the nuclei for 50 random selected cells (n = 50; **, p < 0.001). The data shown are representative of two experiments. F & G. Different size of γH2AX foci in Mili−/− MEFs versus WT MEFs irradiated by X-ray. (F) Representative micrographs of γH2AX foci in MEFs at 3 h after X-ray irradiation (3 Gy). Arrows indicate the MEFs with large γH2AX foci. (G) Quantitation of γH2AX foci in MEFs at 1 and 3 h after X-ray irradiation (n = 3). **, p < 0.01 compared between Mili−/− and WT MEFs. Note that there is no significant difference between Mili−/− and WT MEFs in the formation of large γH2AX foci at 1 h after irradiation.

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Figure 6. Schematic diagram of the role of Piwil2 for DNA repair. Once DNA damage is induced by genotoxic agents, silent Piwil2 gene can be activated in the stressed culture (unpublished observation), we used subconfluent HDF for experiments. The cell lines were cultured and maintained in D10 F medium (DMEM plus 10% fetal calf serum supplemented with 5 mM glutamine, 50 mM 2-mecaptoethanol, 100 U/ml penicillin, and 100 mg/ml streptomycin). Genotyping of mili−/− mice
To obtain mili−/− and wild-type (WT) littermates, male mili−/− mice were crossed with female wild-type mice. Offsprings were genotyped by genomic DNA Polymerase chain reaction (PCR) [3]. Genomic DNAs of tails were extracted using a silica-gel method with modifications [6,63,64] following overnight digestion with 200 µl of DNA lysing buffer (100 mM NaCl, 10 mM Tris-HCl, 25 mM EDTA, 1% SDS, and 50 µg/ml proteinase K) at 56°C. The conditions for genomic DNA PCR were as follows: 10 cycles of initial denaturation at 95°C for 5 min followed by 94°C for 30 s, annealing at 65°C for 1 min, touchdown −1°C/cycle, and extension at 72°C for 1 min; and then 25 cycles of 94°C for 30 s, 55°C for 1 min, and 72°C for 1 min with the final step of extension at 72°C for 10 min. All PCR products were separated on 1.0% agarose gel at the 5 v/cm for 90 min. The primer sequences used for PCR were: 5′-ACA TAG CGT TGG CTA CCC GTG ATA-3′ (Neo forward); 5′-TTG ATG CCC ACC TAC CCT GTG CAT-3′ (mili forward); and 5′-GAA AGC TGG CTG TTG TGC CAG TTA-3′ (mili reverse). The expected PCR products were 1250 bp for WT mice and 900 bp mili−/− mice. PCR Master Mix (Promega, Cat No. M7502) was used for all PCR reactions.

Establishment of mouse embryonic fibroblast (MEF) lines
MEFs were generated from mouse embryos at day 13 post coitum of mili KO and WT mice. Briefly, each embryo was ground in the presence of 1 ml 0.25% trypsin/1 mM EDTA (Gibco, Carlsbad, CA) per embryo, passed through 18 G syringe twice, and incubated at 37°C for 15 min. Trypsin was inactivated by addition of equal volume of DMEM (Gibco) containing 10% fetal bovine serum (FBS; HyClone, South Logan, UT) and the cells of each embryo were then plated in 10 cm culture dishes and allowed to adhere for 24 h. Non-adherent cells were then discarded and the adherent MEFs were expanded by passaging pre-confluent cultures at a ratio 1:3 or 1:5. The cell lines were cultured at 37°C in a humidified atmosphere of 3% CO2. The cultures were split at the log phase of cell growth to prevent over population-induced cell death. The cytology was examined at

rabit anti-phosphorylated H3 (S10) (1:1,000) and rabbit anti-AcH3 [K18] (1:1,000) antibodies were purchased from Cell Signal Technology Inc (Danvers, MA). Rabbit anti-AcH3 (K9,14) (1:20,000), rabbit anti-AcH3 (K9) (1:1,000), and rabbit antihistone H3 (1:1,000) antibodies were purchased from Millipore (Billerica, MA). Mouse anti-β-Actin (1:1,000) and mouse anti-Tubulin (1:2,000) antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Rabbit anti-CPD antibody (1:1000) was purchased from Sigma. Mouse mAb to anti-64PP (1:1000) was purchased from MBL International Corporation, Woburn, MA, and rat anti-Pt-GG (1:1000) was provided by Dr. Jürgen Thomale, Institut für Zellbiologie, Universitätsklinikum Essen, Germany.

Human dermal fibroblasts (HDF)-Al and OSU-2 were used. The HDF-AI were a gift from Dr. Andrew Issekutz, Dalhousie University, Halifax, NS, Canada. Since PIWIL2 gene can be

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various time points by Giemsa-staining of cytopsin preparations, or directly monitored under a phase contrast microscope.

**Cell survival assay**

The sensitivity of mili⁻/⁻ and WT MEFs to genotoxic agents including IR, UV light, and cisplatin were evaluated by cell survival assay. Cells were seeded into 96-well (3×10³/well for mili⁻/⁻ MEFs and 5×10³/well for WT MEFs) for UV light and cisplatin treatment or 6-well plates (1×10⁴/well for both mili⁻/⁻ and WT MEFs) for X-ray irradiation. The cells were mock treated or treated with various doses of UV light, IR (X-ray), and cisplatin. UV irradiation was performed with a germicidal lamp at a dose rate of 0.8 J/m²/s as measured by a Kettering model 65 radiometer (Cole Palmer Instrument Co., Vernon Hill, IL, USA), and X-ray treatment was performed with RS 2000 Biological Irradiator (Rad Source Technologies, Inc. Alpharetta, GA). For cell viability assay of UV light or cisplatin-treated cells, cells were washed in PBS 3 times, fixed in methanol/acetic acid (3:1) for 1 hr, followed by staining with methylene blue for 1 hr. The plates were then rinsed in cold water, and a 100 µl solution containing 40% methanol, 10% acetic acid was added. Absorbance was measured at 660 nm. For cell survival assay of IR-treated cells, cells were harvested, and counted with trypan blue exclusion of dead cells. The cell survival rate of each sample was normalized to mock-treated counterparts.

**Genomic DNA isolation**

Genomic DNA was isolated by using standard techniques described by Sambrook et al [65]. Briefly, cell pellet was lysed in buffer containing 10 mM Tris–HCl (pH 8.0), 0.1 M EDTA, 0.5% SDS during 20 min. Lyzates were incubated with proteinase K (final concentration 100 µg/ml) at 50 °C for 3 h, and extracted twice with phenol and twice with chloroform. Genomic DNA was precipitated with 0.2 volume of ammonium acetate and 2 volumes of ethanol. DNA was washed with 70% ethanol and dissolved in TE buffer. The DNA concentration was determined by spectrophotometry and its integrity was checked by 1.5% agarose gel electrophoresis.

**Immuno-slot blot (ISB) analysis**

ISB was used to determine the amounts of CPD, 6–4 PP and Pt-GG. Briefly, DNA (20 µg) isolated from each samples was sonicated and then denatured at 100°C for 10 minutes. The heat-denatured DNA was quickly chilled on ice and immediately slot-blotted onto nitrocellulose membranes using a Convertible Filtration Manifold System (GibcoBRL, Carlsbad, CA). The membranes were baked for 2 hours at 80°C. After the single-stranded DNA was immobilized onto the nitrocellulose membranes, the membranes were blocked with 5% milk–1×TBST and then incubated with antibodies to CPD (1:1000 diluted), 6–4 PP (1:10000 Dilution), or Pt-GG (1:10000 Dilution) overnight at 4°C. The membrane was then incubated with horseradish peroxidase-conjugated goat anti-mouse or rat IgG (1:5000 diluted) (Chemicon, Temecula, CA) for 1 hour at 37°C. Chemiluminescent substrate (Super Signal West Dura Extended Duration Substrate, 34075; Pierce Biotech) was used to detect positive bands, which were visualized on X-ray film. The relative amounts of CPD, 6–4 PP and Pt-GG were determined by quantification of the intensity of each band of the lesions and normalization to a reference standards run at the same experiment. The intensity of each band was quantified by scanning images and processing with Alphaimager-2000 software.

**RT-PCR**

RT-PCR was performed as previously described [6,66]. Total RNA was extracted from HDFs and reversely transcribed into cDNA, using Superscriptase II (Invitrogen, CA) and oligo (dT) in a 20 µl reaction containing 1 µg of total RNA, which was pretreated with RNase-free DNase I (Invitrogen, CA) to eliminate contaminating genomic DNA. For PCR, an aliquot of 0.5 µl cDNA was used in each 20 µl PCR reaction, using PCR Master Mix (Promega, MI). The sequences of human Pwll2 primers were as follows: forward 5’-TTCCGAGTGTGCCCAGAAGATTT-3’ and reverse 5’-ACAGTTCCAGGATGGGAGTTCACA-3’ with a 499 bp product. The following conditions were used: an initial denaturation at 95°C for 5 min followed by denaturation at 94°C for 30 seconds, annealing at 53°C for 1 min, touchdown – 1°C per cycle, and extension at 72°C for 1 min for a total of 10 cycles. Then the condition was fixed for 25 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 1 min, and extension at 72°C for 1 min with a final extension at 72°C for 10 min. PCR products were analyzed by 1.5% agarose gel.

**Western Blot**

Total cellular proteins were isolated from cultured cells or animal tissues using lysis buffer. Protein concentration was determined by protein assay (D, Protein Assay System; Bio-Rad, Hercules, CA), as described by the manufacturer. A total of 40 µg of protein was loaded per well, separated on an SDS-PAGE [8% (w/v) polyacrylamide gel] and then transferred by electrophoresis to nitrocellulose membranes. The membranes were blocked with 5% milk in Tris-buffered saline Tween (M-TBST; 20 mM Tris, 0.5 M NaCl, and 0.05% Tween 20 [pH 7.4]) for approximately 60 minutes at 37°C, incubated overnight at 4°C with a primary antibody appropriately diluted in M-TBST; and rinsed four times in M-TBST. Then, the membranes were incubated with appropriate horseradish peroxidase-conjugated secondary antibody in M-TBST for 1 h at 37°C, rinsed four times with TBST, and developed with chemiluminescent substrate (Super Signal West Dura Extended Duration Substrate, 34075; Pierce Biotech). The positive bands were visualized on X-ray films. Tubulin or β-Actin on the same membrane was used as a loading control.

**Chromatin relaxation assay**

Chromatin relaxation was evaluated by MNase digestion [43]. Mili⁻/⁻ MEFs and WT MEFs were cultured in 6-well plates and irradiated when they became subconfluent. The cells were harvested immediately after UV irradiation and the nuclei were isolated from mili⁻/⁻ and WT MEFs, respectively, before and after UV irradiation, which were subjected to MNase digestion as described [43]. The genomic DNA was isolated and the fragments are separated by a 1.8% agarose gel.

**Single-cell gel electrophoresis (Comet assay)**

Exponentially growing Mili⁻/⁻ and WT MEFs cells with 70 – 80% confluence were exposed to radiation at room temperature using a Cabinet X-rays System Faxitron Series (dose rate: 0.997 Gy/min; 130 kVp; Hewlett Packard, McMinnville, OR). Cells shielded from radiation were included as the sham-IR control. The comet assay was conducted using the Trevigen’s CometAssay kit (Alkaline version). Briefly, 1×10⁵/ml cells were mixed with molten LMAgarose (at 37 °C) at a ratio of 1:10 (v/v) and immediately pipetted 50 µl onto the cometSlide and stayed in the dark for 10 min. The slides were then immersed in prechilled lysis solution for 30 min at 4°C. Excess buffer was drained from the slides and the slides were then immersed in freshly prepared
alkaline unwinding solution (pH>13) in dark for 30 min at room temperature before electrophoresis at 21 volts for 30 min. The slides were then immersed twice in dH2O for 5 min each, then in 70% ethanol for 5 min followed by drying at room temperature for 15 min, staining with DAPI for 5 min and then drying completely at room temperature in the dark. The slides were then viewed by fluorescence microscopy (maximum excitation and emission are respectively 330 nm/470 nm). DNA damage and repair were estimated by measuring the distance of the tail against the edge of far side of the nuclei for 50 random selected cells.

Detection of γH2AX foci in X-ray treated MFES

MFES [Mili+/− and Mili−/−] were grown in D10 F medium in an incubator at 37°C with 5% of CO2. The cells were seeded (1×10⁶/ml) on coverslips in a 100 mm culture dish for 2 hrs, grew up to repair were estimated by measuring the distance of the tail against emission are respectively 350 nm/470 nm. DNA damage and completely at room temperature in the dark. The slides were then viewed by fluorescence microscopy (maximum excitation and emission are respectively 330 nm/470 nm). DNA damage and repair were estimated by measuring the distance of the tail against the edge of far side of the nuclei for 50 random selected cells.

Statistics analysis

Data of multiple group observations were statistically analyzed by the one-way analysis of variance (ANOVA), and two groups of observations were compared by student-T test. A value of p≤0.05 was considered significant. Data are expressed as mean±SD. * p≤0.05; ** p≤0.01.

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Author Contributions

Conceived and designed the experiments: JXG DTY. Performed the experiments: DTY QW LC MYL QY WD CH JYL. Analyzed the data: DTY QW JYL. JXG. Contributed reagents/materials/analysis tools: RS GH AW. Wrote the paper: JXG.

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