Peroxiredoxin as a functional endogenous antioxidant enzyme in pronuclei of mouse zygotes

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Abstract. Antioxidant mechanisms to adequately moderate levels of endogenous reactive oxygen species (ROS) are important for oocytes and embryos to obtain and maintain developmental competence, respectively. Immediately after fertilization, ROS levels in zygotes are elevated but the antioxidant mechanisms during the maternal-to-zygotic transition (MZT) are not well understood. First, we identified peroxiredoxin 1 (PRDX1) and PRDX2 by proteomics analysis as two of the most abundant endogenous antioxidant enzymes eliminating hydrogen peroxide (H2O2). We here report the cellular localization of hyperoxidized PRDX and its involvement in the antioxidant mechanisms of freshly fertilized oocytes. Treatment of zygotes at the pronuclear stage with H2O2 enhanced pronuclear localization of hyperoxidized PRDX in zygotes and concurrently impaired the generation of 5-hydroxymethylcytosine (5hmC) on the male genome, which is an epigenetic reprogramming event that occurs at the pronuclear stage. Thus, our results suggest that endogenous PRDX is involved in antioxidant mechanisms and epigenetic reprogramming during MZT.

Key words: Hydrogen peroxide (H2O2), Mouse, Peroxiredoxin (PRDX), Zygotes

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mouse zygotes by proteomics analysis to uncover the antioxidant mechanisms in zygotes. As a result, peroxiredoxin 1 (PRDX1) was identified as the most abundant antioxidant enzyme in zygotes. PRDX proteins play a role of catalyzing the reduction of cellular hydrogen peroxide ($\text{H}_2\text{O}_2$) to $\text{H}_2\text{O}$ in the TRX/PRDX system [18]. Interestingly, immunocytochemical analysis showed that hyperoxidized PRDX family members including PRDX1 were localized in the male and female pronuclei of zygotes. Hyperoxidized PRDX proteins in the pronuclei of $\text{H}_2\text{O}_2$-treated zygotes at PN3 were significantly increased compared to those in untreated controls. Concurrently, 5-hydroxymethylcytosine (5hmC) was significantly decreased in the male pronuclei of zygotes. Thus, our results indicate the necessity to investigate the relationship between antioxidant enzymes and epigenetic reprogramming in further research.

**Materials and Methods**

**Animals**

All mice (ICR strain) were purchased from Kiwa Laboratory Animals (Wakayama, Japan) and maintained in light-controlled, air-conditioned rooms. This study was carried out in strict accordance with the recommendations in the Guidelines of Kindai University for the Care and Use of Laboratory Animals. Experimental protocols were approved by the Committee on the Ethics of Animal experiments of Kindai University (Permit Number: KABT-26-002). All mice were killed by cervical dislocation and all efforts were made to minimize suffering and to reduce the number of animals used in the present study.

**In vitro fertilization and embryo culture**

Collection of spermatozoa, oocytes, and zygotes were performed as described in previous studies [19–25]. Spermatozoa were collected from the cauda epididymis of male mice. The sperm suspension was incubated in human tubal fluid (HTF) medium for 1.5 h to allow for capacitation at 37°C under 5% CO$_2$ in air. Oocytes were collected from the excised oviducts of female mice (2–3 months old) that were superovulated with pregnant mare serum gonadotropin (PMSG; Serotropin, Teikoku Zoki, Tokyo, Japan) and 48 h later, human chorionic gonadotropin (hCG; Puberogen, Sankyo, Tokyo, Japan). Cumulus-oocyte complexes were recovered into pre-equilibrated HTF medium. The sperm suspension was added to the oocyte cultures and morphologically normal zygotes were collected 2 h after insemination. The zygotes were cultured in potassium simplex optimized medium (KSOM) [26] at 37°C under 5% CO$_2$ in air.

**Two-dimensional gel electrophoresis (2-DE)**

600, 10,000, and 15,000 MII oocytes for obtaining 2-DE master gels, and 600 MII oocytes and 600 zygotes for obtaining the gels for quantitative analysis of protein spots, in each case were sonicated three times at 4°C for 30 sec. The sonicated samples were resuspended in lysis buffer composed of 7 M urea, 2 M thiourea, 4% CHAPS, 0.05% tri-n-butylphosphate, 1 tablet per 10 ml complete mini protease inhibitor (Roche, Mannheim, Germany), and traces of bromophenol blue (BBP). The resuspended samples were precipitated with three volumes of 100% acetone and mixed with rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 0.05% tri-n-butylphosphate, IPG buffer, and traces of BPB). The 2-DE procedure was performed as described previously [27, 28]. The obtained proteins were separated in the first dimension on immobilized pH gradient IPG gels (Immobiline DryStrip, pH 3–11 nonlinear gradient, 13 cm, GE Healthcare). IPG gels were rehydrated overnight with rehydration solution containing protein samples. First-dimension electrophoresis was performed at 15°C under the following conditions: 1 min of linear gradient from 0 to 500 V, 8 h of constant voltage at 500 V, 1.5 h of linear gradient from 500 to 3,500 V, and 5.4 h of constant voltage at 3,500 V. Strips were then washed with distilled water and equilibrated at RT for 15 min in 5 ml of equilibration buffer composed of 6 M urea, 50 mM Tris-HCl (pH 8.0), 30% glycerol, 2% SDS, 50 mg DTT, and traces of BBP before being incubated for 15 min in an equilibration solution of the same composition except for 125 mg iodoacetamide replacing the DTT. The equilibrated strips were transferred onto the SDS-PAGE gel. Second dimension electrophoresis was performed on 12% polyacrylamide gels in CoolPhoreStar SDS-PAGE Tetra-200 (Anatech, Tokyo, Japan) with a constant current of 30 mA per gel for 1 h.

**Visualization and image analysis of 2-DE gels**

The gels were fixed with a fixation solution containing 10% methanol and 7% acetic acid for 30 min at room temperature (RT) and then transferred to distilled water for 20 min. The gels were stained with SYPRO Ruby (Invitrogen, Carlsbad, CA, USA) overnight and then washed with 10% ethanol. Gel images were obtained using an Alphalmager (Alpha Innotech, San Leandro, CA, USA) and analyzed using Progenesis PG220 and TT900 (Nonlinear Dynamics, Newcastle upon Tyne, UK) for spot detection, quantification, and comparative analysis.

**MALDI sample preparation**

For protein identification by mass spectrometry, protein spots in the 2-DE master gels from each 600, 10,000, and 15,000 MII oocytes were collected by gloved hand on a Safe Imager blue light transilluminator (Invitrogen). Gel pieces were incubated three times in destaining solution consisting of 50% acetonitrile (ACN) in 50 mM ammonium bicarbonate for 20 min at 37°C. The gel pieces were then incubated in 100% ACN for 1 min before being dried completely. The obtained proteins were digested with 1.67 μg/ml trypsin (Promega, Southampton, UK) in 25 mM ammonium bicarbonate at 30°C overnight. The digested proteins were purified and concentrated using ZipTipTMμC18 (Millipore, Bedford, MA, USA). The peptides absorbed in the gel were directly eluted onto the MALDI sample plate using 2.5 mg/ml α-cyano-4-hydroxycinnamic acid (Waters, Milford, MA, USA) in 70% ACN containing 0.1% trifluoroacetic acid.

**MALDI-TOF/MS**

MS spectrometric analysis of the tryptic was performed using a 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA). MS spectra were measured in the positive-ion reflector mode with a mass range from 700 to 3,500. Data were subjected to external calibration with five standard peptides (Sigma, St. Louis, MO, USA). The MS/MS spectra were measured in CID
mode. From a single parent MS spectrum, the five most abundant ions were selected for MS/MS analysis. The data were subjected to external calibration using fragment peaks of the human ACTH peptide 18-39 (MH1 2465, 1989, Sigma). The raw MS and MS/MS data were used in database searches using the MASCOT search engine (Matrix Science, London, UK, http://www.matrixscience.com/) and UniProt (http://www.uniprot.org/) with a mass tolerance of 0.2 Da.

Western blot analysis

Western blotting was performed as described previously [19-21, 23-25, 28, 29]. Samples (30 MII oocytes or zygotes for PRDX1 detection and 50 MII oocytes or zygotes for PRDX-SO2/3 detection) were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Each sample was treated with the same volume of 2 × SDS sample buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 0.02% BPB, 20% glycerol, and 10% 2-mercaptoethanol) before SDS-PAGE. Non-reduced samples were prepared without the 10% 2-mercaptoethanol [30]. Proteins were resolved in 12% running gels for 2 h and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (GE Healthcare) for 1.5 h. The membrane was washed with phosphate-buffered saline (PBS), incubated in Block Ace (Dainippon-Pharm, Osaka, Japan) at RT for 1 h, washed twice with PBS containing 0.2% Tween 20 (PBST) for 10 min, and incubated at 4°C overnight with anti-PRDX1 antibody (1:100,000; Abcam, Cambridge, UK; ab41906), anti-PRDX-SO2/3 antibody (1:2,000; Abcam; ab16830), and anti-Acin antibody (1:10,000; Sigma; A5441) as a loading control. The membrane was washed in PBST, incubated with donkey anti-rabbit IgG-horseradish peroxidase (HRP) conjugate (1:200,000; Millipore; AP180P) at RT for 1 h, washed three times with PBST for 10 min, and developed using ECL Prime Western Blotting detection reagent (GE Healthcare).

Immunocytochemistry of zygotes

The classification of pronuclear (PN) stages was performed according to previous studies [31], where the pronuclear morphology and hours post-insemination (hpi) were taken into consideration. The subcellular localization of PRDX1, PRDX-SO2/3, 5-methylcytosine (5mC), and 5hmC was determined by immunocytochemical analysis of zygotes, as described [19-22, 24, 25, 32]. Embryos were fixed in 10% formaldehyde neutral buffer solution (Nakalai Tesque, Kyoto, Japan) at RT for 15 min and the zona pellucida was removed with acid Tyrode’s solution. Samples were then washed three times in PBS containing 3% bovine serum albumin (PBS-BSA) and permeabilized with PBS-BSA containing 0.5% Triton X-100 (Nakalai Tesque) at RT for 15 min. For 5mC and 5hmC, the specimens were denatured with 4 N HCl at RT for 10 min and then neutralized with 40 mM Tris-HCl (pH 8.5) for 20 min. They were then incubated with anti-PRDX1 antibody (1:20,000), anti-PRDX-SO2/3 antibody (1:2,000; Abcam; ab16830), anti-5mC antibody (1:2,000; Calbiochem, Darmstadt, Germany; NA81), or anti-5hmC antibody (1:2,000; Active motif, Carlsbad, CA, USA; 39769) in PBS-BSA at 4°C overnight. After incubation, the samples were treated with Alexa Fluor 555-labeled donkey anti-rabbit IgG secondary antibody (1:2,000; Life Technologies, Carlsbad, CA, USA; A-21207) for anti-PRDX1, PRDX-SO2/3, and 5hmC antibodies, with an Alexa Fluor 488-labeled donkey anti-mouse IgG secondary antibody (1:2,000; Invitrogen; A21202) for detection of the 5mC antibody, all at RT for 1 h. PRDX1-, PRDX-SO2/3-, 5mC- and 5hmC-stained zygotes were each mounted into VECTASHIELD (Vector Laboratories, Burlingame, CA, USA) mounted on the slide glasses containing 3 µg/ml 4′-6-diamidino-2-phenylindole (DAPI) (Invitrogen; D1306). Finally, the PRDX1-, PRDX-SO2/3-stained zygotes were imaged using a conventional upright microscope (Axioplan2, Carl Zeiss, Jene, Germany) equipped with a mercury lamp (HBO 100, Carl Zeiss), and digital CCD camera (AxioCamMRc5, Carl Zeiss). The 5mC-, 5hmC-, and PRDX-SO2/3-stained zygotes were imaged using a confocal laser-scanning microscope (LSM 800, Carl Zeiss) equipped with 40 × and 63 × silicon oil-immersion objectives (Carl Zeiss).

Treatment of mouse zygotes with H2O2

Previously, it was shown that mouse zygotes treated with 200 µM H2O2 for 15 min results in the inhibition of cleavage and/or fragmentation [17]. To induce oxidative stress in early mouse zygotes, freshly fertilized oocytes at 1 hpi were incubated in KSOM including 10, 50, 100 or 200 µM H2O2 at 37°C under 5% CO2 for 6 hpi. For the control, the same protocol was used without H2O2. At 6 hpi, fertilized oocytes were washed with KSOM prior to further analysis. Preliminary we determined the H2O2 concentration that did not affect embryonic survival and pronuclei formation in freshly fertilized oocytes at 6 hpi, which is approximately PN3 [31]. As shown in Supplementary Table 1 (online only), the zygotes were able to form pronuclei in 100 µM H2O2-treatment at least until 6 hpi and maintained developmental ability to the 2-cell stage. In this study, we used 100 µM H2O2 as an optimal oxidative stress condition.

Statistical analysis

For statistical analysis, we used StatView version 5.0 (SAS Institute, Cary, NC, USA) and performed the analysis of Chi-square distribution and nonparametric test (Mann-Whitney U test) with an α level of 0.05 to determine possible statistically significant differences.

Results

Identification of endogenous antioxidant enzymes that are abundantly present in mouse MII oocytes and zygotes

We tried to explore the most abundant antioxidant enzymes in mouse zygotes in order to investigate the mechanisms of reducing ROS. However, it is more demanding to collect a large number of...
zygotes for proteomics analysis than to collect MII oocytes. Here, we decided to use MII oocytes instead of zygotes for proteomic analysis because zygote genome activation (ZGA) occurs from the late 1-cell to early 2-cell stages and maternal proteins are stored in oocytes until ZGA [33, 34]. First, we performed 2-DE using MII oocytes, and obtained the 2-DE gel photos (Fig. 1A). Next, we analyzed the 2-DE gel photos to construct a reference gel for comparing the protein spots (Fig. 1A). As a result, 449 protein spots were commonly detected on the gels in the 3 to 11 pH range and 10–200 kDa range (Fig. 1A), and 137 protein spots (64 protein species) were identified from these gels by MALDI-TOF/MS (Supplementary Table 2: online only). Furthermore, we examined changes in protein expression from MII oocytes to zygotes by comparing the 2-DE gels from 600 MII oocytes and 600 zygotes (Fig. 1B). PRDX1 and PRDX2 were identified as the dimeric form during pronuclear stages (Fig. 3B). Our findings at PN5 (Fig. 2A) showed that hyperoxidized PRDX was detected in pronuclear stages of zygotes, although pronuclear PRDX1 signals were clearly observed only in the cytoplasm of both treated and untreated zygotes (Fig. 4B, 4C, and Supplementary Fig. 1A: online only). The observation that hyperoxidized PRDX predominantly accumulated in the pronuclei of H2O2-treated zygotes was confirmed under oxidative damage conditions induced by H2O2 treatment, indicating that the PRDX functions as endogenous antioxidant enzymes in the pronuclei of zygotes.

Accumulation of hyperoxidized PRDX in pronuclei of H2O2-treated zygotes and the dynamics of 5hmC in male pronuclei of H2O2-treated zygotes

Since one of the specific events occurring in the pronuclei of zygotes is active DNA demethylation of the male pronucleus [40, 41], the possibility of zygotic-specific functions of pronuclear PRDX family members led us to examine the involvement of PRDX in active DNA demethylation of the male pronucleus of zygotes. To address this under oxidative stress conditions by H2O2-treatment, we performed localization assays using antibodies to 5mC, 5hmC, and PRDX-SO2/3 in freshly fertilized oocytes at 6 hpi (Fig. 4A), which corresponds to approximately PN3 [31]. A large amount of PRDX-SO2/3 was significantly observed in the male and female pronuclei of H2O2-treated zygotes compared to those of untreated ones (male pronucleus; P = 0.0038, female pronucleus; P = 0.0020), although PRDX-SO2/3 was clearly present in the pronuclei and cytoplasm of both treated and untreated zygotes (Fig. 4B, 4C, and Supplementary Fig. 1A: online only). The observation that hyperoxidized PRDX predominantly accumulated in the pronuclei of zygotes was confirmed under oxidative damage conditions induced by H2O2 treatment, indicating that the PRDX functions as endogenous antioxidant enzymes in the pronuclei of zygotes. Next, we found that there was a significant decrease in the level of 5mC in male pronuclei of H2O2-treated zygotes (P = 0.002; Fig. 4D, 4F, and Supplementary Fig. 1B), indicating that the accumulation of hyperoxidized PRDX in the male pronuclei of zygotes correlates with a decrease in the observed amount of 5mC, an oxidation product of 5mC by Tet methylcytosine dioxygenase 3 (Tet3). These results collectively suggest that endogenous PRDX is involved in both the antioxidant mechanisms and epigenetic reprogramming of freshly fertilized oocytes.

Discussion

In this study, we identified PRDX1 and PRDX2 as abundantly expressed endogenous antioxidants in MII oocytes and zygotes (Table 1 and Supplementary Table 3). Moreover, we also identified 9 reductases, which catalyze NAD (P) + to NAD (P) H, and glutathione S-transferase A4 (GSTA4) as the GSH system, although these proteins do not directly reduce ROS (Table 1 and Supplementary Table 2). In total, 12 redox proteins (19% of the identified 64 proteins) were abundantly expressed in MII oocytes and zygotes (Table 1 and Supplementary Table 3). In previous proteomics analysis, 7,349 proteins were identified from 28 mouse tissues, and various antioxidants such as the TRX system, GSH system, and SOD are...
Fig. 1. Proteins from mouse oocytes and zygotes as separated by 2-DE. (A) Representative 2-DE master gel from 600 MII oocytes. (B) Representative 2-DE gels from 600 MII oocytes and 600 zygotes. The original gel size was 16 × 16 × 0.1 cm. MW, molecular weight; pI: isoelectric point.
Table 1. The ranking of identified protein expression levels in mouse zygotes

| Ranking | Protein names | Accession No. | Entry name | Quantity |
|---------|---------------|---------------|------------|----------|
| 1       | L-lactate dehydrogenase B chain | P16122       | LDHB_MOUSE | 18.61854669 |
| 2       | Protein-arginine deiminase type-6 | Q8K3V4       | PARD6_MOUSE | 9.947958597 |
| 3       | Ubiquinol-cytochrome-c reductase 1 | Q9RPF9       | UQCR1_MOUSE | 7.70796872 |
| 4       | Actin, cytoplasmic 1 | P07010       | ACTB_MOUSE | 3.48409797 |
| 5       | Tubulin beta-4B chain | P68372       | TUBB4B_MOUSE | 3.392538556 |
| 6       | Heat shock protein HSP 90-alpha | P09901       | HSPA1A_MOUSE | 3.348318752 |
| 7       | Tubulin alpha-1C chain | Q2L877       | TUBA1C_MOUSE | 2.62285433 |
| 8       | KL domain-containing protein 3 | Q9CW6U5     | Q9CW6U3_MOUSE | 1.891422066 |

**Peroxiredoxin family proteins are highlighted by red. The glutathione system is highlighted by blue. The proteins catalyzing NAD (P) + to NAD (P) H are highlighted by yellow.**
ROLE OF PRDX IN ZYGOTES

Included in the 100 most abundant proteins in those tissues [42]. In particular, PRDX1 is included in the 100 most abundant proteins in 20 of these tissues [42]. On the other hand, PRDX2 is included in the 100 most abundant proteins in only 2 tissues, and the proteins of GSH system that directly eliminate H\textsubscript{2}O\textsubscript{2}, such as GPX, are not included in the 100 most abundant proteins in any tissues, while 7 types of GST are included in the 100 most abundant from 1-9 tissues [42]. In addition, SOD1 or SOD2 are included in the 100 most abundant proteins in 19 tissues [42]. According to another proteomics analysis using zygotes, SOD1 is indicated as the most abundant antioxidant in the identified proteins [43]. However, regarding the antioxidant enzymes involved in eliminating H\textsubscript{2}O\textsubscript{2}, PRDX1 is the most abundant antioxidant [43], which is in good agreement with our data. Thus, our results and these previous reports strongly

![Fig. 2. Localization of PRDX1 and the existence of oxidized PRDX1 in zygotes at pronuclear stages. (A) Immunostaining for localization of PRDX1 at pronuclear stages (PN) 1–5. Shown are representative images of zygotes stained with DAPI (blue) and anti-PRDX1 antibody (red). Key: ♂, male pronucleus; ♀, female pronucleus; PB, polar body; scale bars = 50 µm. (B) Reduced and non-reduced immunoblots for PRDX1 at pronuclear stages. The bands predicted as nonspecific signal or covalent complexes are indicated by *]. β-ACTIN was used as a loading control in immunoblot analyses. MW, molecular weight.]

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included in the 100 most abundant proteins in those tissues [42]. In particular, PRDX1 is included in the 100 most abundant proteins in 20 of these tissues [42]. On the other hand, PRDX2 is included in the 100 most abundant proteins in only 2 tissues, and the proteins of GSH system that directly eliminate H\textsubscript{2}O\textsubscript{2}, such as GPX, are not included in the 100 most abundant proteins in any tissues, while 7 types of GST are included in the 100 most abundant from 1-9 tissues [42]. In addition, SOD1 or SOD2 are included in the 100 most abundant proteins in 19 tissues [42]. According to another proteomics analysis using zygotes, SOD1 is indicated as the most abundant antioxidant in the identified proteins [43]. However, regarding the antioxidant enzymes involved in eliminating H\textsubscript{2}O\textsubscript{2}, PRDX1 is the most abundant antioxidant [43], which is in good agreement with our data. Thus, our results and these previous reports strongly
Fig. 3. Localization of PRDX-SO_{2/3} in zygotes and somatic cells. (A) Immunostaining for localization of PRDX-SO_{2/3} at pronuclear stages (PN) 1–5. Shown are representative images of zygotes stained with DAPI (blue) and anti-PRDX-SO_{2/3} antibody (red). Key: ♂, male pronucleus; ♀, female pronucleus; PB, polar body; scale bars = 50 µm. (B) Reduced and non-reduced immunoblots for PRDX-SO_{2/3} at pronuclear stages. The bands predicted as nonspecific signal or covalent complexes are indicated by *. β-ACTIN was used as a loading control in immunoblot analyses. MW, molecular weight. (C) Immunostaining for localization of PRDX-SO_{2/3} in cumulus cells. Shown are representative images of cumulus cells stained with DAPI (blue) and anti-PRDX-SO_{2/3} antibody (red). Scale bars = 10 µm.
Fig. 4. Hyperoxidation of PRDX and accumulation of 5hmC on the male genome induced by H$_2$O$_2$-treatment. (A) Schematic diagram of the experimental procedure. (B) Immunostaining images of PRDX-SO$_{2/3}$ in untreated and H$_2$O$_2$-treated zygotes. Key: ♂, male pronucleus; ♀, female pronucleus. PB, polar body; scale bar = 50 µm. (C) Quantification of the ratio of PRDX-SO$_{2/3}$ intensities in untreated and H$_2$O$_2$-treated zygotes at 6 hpi. Red bars indicate the median values. The number of zygotes is 20 for each group. (D) Immunostaining images of 5mC (green) and 5hmC (red) in untreated and H$_2$O$_2$-treated zygotes. Key: ♂, male pronucleus; ♀, female pronucleus. Scale bar = 20 µm. (E) and (F) Quantification of the ratio of 5mC and 5hmC intensities in the pronucleus of untreated and H$_2$O$_2$-treated zygotes at 6 hpi. Red bars indicate the median values. The number of zygotes is 15 for each group.
support that PRDX1 is abundantly expressed as an antioxidant enzyme eliminating H$_2$O$_2$ in a wide range of cells, including MII oocytes and zygotes. PRDX1 and SOD1 may play a role in eliminating H$_2$O$_2$ and superoxide, respectively, in various cells. Not only PRDX1 and PRDX2 identified by our investigation but also all isoforms of the PRDX family (PRDX1-6) in MII oocytes and zygotes are identified in the previous report [43]. Furthermore, another proteomics analysis indicates that PRDX2 and PRDX4 are highly expressed proteins in porcine fibroblast cell nuclei incubated in extracts from MII oocytes, as compared with those in germline vesicle (GV) oocytes or fibroblast cells [44]. Taken together, PRDX family members might be actively involved in eliminating H$_2$O$_2$ in fertilized oocytes and early embryos as well as somatic cells.

As shown in Fig. 3A and 3C, our results suggest the possibility of specific functions of PRDX family members as endogenous antioxidant enzymes in pronuclei of zygotes. In the previous reports, as for the antioxidant enzyme in nuclei of cells, nucleoredoxin (NRX) has been discovered by its overexpression [45, 46]. In fact, it has been reported that endogenous NRX exists predominantly in the cytoplasm of cultured cells by cell fractionation [47]. While the endogenous antioxidant enzymes actively existing in the nucleus has been uncertain, our data provide the first evidence that PRDX1 and other PRDX family members are pronuclear-localized antioxidant enzymes in zygotes. The pronuclear PRDX1 signals were observed at PN5 compared to other stages while PRDX1 functions before fertilization (Fig. 2B). PN5 (largely during 10–12 hpi) zygotes are mostly in G2 phase [31, 33], and according to the previous report, the H$_2$O$_2$-treated zygotes in G1 phase (at 7 hpi) are shown to significantly delay their entry to G2/M phase and to decrease the ratio of embryos that develop to the 2-cell stage [48, 49]. Thus, not to delay G2/M checkpoint activation induced by H$_2$O$_2$ as much as possible, PRDX1 might play a role in eliminating H$_2$O$_2$ in the pronucleus with the other PRDX family for normal embryo development. However, Prdx1 knockout mice are viable, although they have a shortened lifespan due to the development of severe haemolytic anemia and several malignant cancers beginning at about 9 months of age, and the DNA damage is significantly elevated in some murine tissues [50, 51]. In addition, Prdx2 knockout mice have been reported to show similar phenotypes [52]. Thus, some PRDX proteins might compensate for eliminating H$_2$O$_2$ even though one kind of Prdx is lacking in an early embryonic development. While pronuclear PRDX1 was observed at a later pronuclear stage, hyperoxidized PRDX signals were observed in pronuclei from PN2 (Fig. 2A and 3A). The other report shows that PRDX2 is not observed in pronuclei at PN3 [53]. Therefore, prionuclear hyperoxidized PRDX proteins around PN3 may be any of PRDX3-6. On the other hand, we could not observe any hyperoxidized PRDX signals in the nuclei of cumulus cells despite their exposure to the same stress environment as the zygotes (Fig. 3C). Therefore, PRDX proteins appear to specifically function as endogenous antioxidant enzymes in pronuclei of zygotes.

We also observed that treatment of zygotes at the pronuclear stage with H$_2$O$_2$ impaired the generation of 5hmC on the male genome but showed no significant effect on the existing 5mC (Fig. 4D, 4E, 4F, and Supplementary Fig. 1B). Since spermatid 5mC is removed independently of Tet3, and then de novo 5mC by DNA methyltransferases (DNMTs) is converted to 5hmC by Tet3 in zygotes [54], we speculate that the activity of Tet3 and DNMTs might be depressed under the oxidative stress induced by the H$_2$O$_2$-treatment. Indeed, H$_2$O$_2$ exposure results in lowered Tet3 activity in cultured mammalian cells [55]. As mentioned above, we found that PRDX proteins appear to specifically function as endogenous antioxidant enzymes in the pronuclei of zygotes. Taken together, our results suggest that epigenetic reprogramming might be regulated under ROS conditions moderated by PRDX-mediated antioxidant mechanisms during the maternal-to-zygotic transition (MZT). Correspondingly, the observation that preventing oxidative stress is important for epigenetic reprogramming, is shown in a recent report, in which treatment with the antioxidant, vitamin C after exposure to the HDAC inhibitor, trichostatin A, dramatically improves cloning efficiency in somatic cell nuclear transfer (SCNT) embryos [56]. Further studies are needed to elucidate how PRDX-mediated antioxidant mechanisms are involved in epigenetic reprogramming during MZT.

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References

1. Tiwari M, Prasad S, Tripathi A, Pandey AN, Singh AK, Shrivastava TG, Chauhe SK. Involvement of reactive oxygen species in meiotic cell cycle regulation and apoptosis in mammalian oocytes. Reactive Oxygen Species 2016; 1: 110–116. [CrossRef]
2. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. Cell Signal 2012; 24: 981–990. [Medline] [CrossRef]
3. Reczek CR, Chandel NS. ROS-dependent signal transduction. Curr Opin Cell Biol 2015; 33: 8–13. [Medline] [CrossRef]
4. Guerin P, El Mouatassim S, Ménézo Y. Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. Hum Reprod Update 2001; 7: 175–189. [Medline] [CrossRef]
5. Takahashi M. Oxidative stress and redox regulation on in vitro development of mammalian embryos. J Reprod Dev 2012; 58: 1–9. [Medline] [CrossRef]
6. Drevet JR. The antioxidant glutathione peroxidase family and spermatogenesis: a complex story. Mol Cell Endocrinol 2006; 250: 70–79. [Medline] [CrossRef]
7. Tsunoda S, Kawano N, Miyado K, Kinura N, Fujii J. Impaired fertilizing ability of superoxide dismutase 1-deficient mouse sperm during in vitro fertilization. Biol Reprod 2012; 87: 121. [CrossRef]
8. Wu G, Fang YZ, Yang S, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. J Nutr 2004; 134: 489–492. [Medline] [CrossRef]
9. Shi ZZ, Osei-Frimpong J, Kala G, Kala SV, Barrios RJ, Habib GM, Lukin DJ, Danney CM, Matzuk MM, Lieberman MW. Glutathione synthesis is essential for mouse development but not for cell growth in culture. Proc Natl Acad Sci USA 2000; 97: 5101–5106. [Medline] [CrossRef]
10. Lu J, Holmgren A. The thioredoxin antioxidant system. Free Radic Biol Med 2014; 66: 75–87. [Medline] [CrossRef]
11. Matsui M, Oshima M, Oshima H, Takaku K, Murayama T, Yodoi J, Taketo MM. Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. Dev Biol 1996; 178: 179–185. [Medline] [CrossRef]
12. Naso-Esfahani MM, Johnson MH. The origin of reactive oxygen species in mouse embryos cultured in vitro. Development 1991; 113: 551–560. [Medline] [CrossRef]
13. Lopers AS, Lane M, Thompson JG. Oxygen consumption and ROS production are increased at the time of fertilization and cell cleavage in bovine zygotes. Hum Reprod 2004; 19: 31–37. [Medline] [CrossRef]
view of novel mechanisms and emerging concepts in cell signaling. Free Radic Biol Med 2005; 38: 1543–1552. [Medline] [CrossRef]

56. Neumann CA, Cao J, Mageswaran V. Peroxiredoxin 1 and its role in cell signaling. Cell Cycle 2009; 8: 4072–4078. [Medline] [CrossRef]

57. Rhee SG, Woo HA, Kill IS, Bae SH. Peroxiredoxin functions as a peroxidase and a regulator and sensor of local peroxides. J Biol Chem 2012; 287: 4403–4410. [Medline] [CrossRef]

58. O'Neill JS, Reddy AB. Circadian clocks in human red blood cells. Nature 2011; 469: 498–503. [Medline] [CrossRef]

59. Gu TP, Guo F, Yang H, Wu HP, XG GF, Lu W, Xie ZG, Shi L, He X, Jin SG, Iqbal K, Shi YG, Deng Z, Szabó PE, Pfeffer GP, LI J, Xu GL. The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature 2011; 477: 606–610. [Medline] [CrossRef]

60. Wossidlo M, Nakamura T, Lepikov K, Marques CJ, Zakharchenko V, Boiani M, Arand A, Nakano T, Reik W, Walter J. 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. Nat Commun 2011; 2: 241. [Medline] [CrossRef]

61. Geiger T, Velec A, Macke B, Lundberg E, Kampf C, Nagaraj N, Uhlen M, Cox J, Mann M. Initial quantitative proteomic map of 28 mouse tissues using the SILAC mouse. Mol Cell Proteomics 2013; 12: 1709–1722. [Medline] [CrossRef]

62. Wang S, Kou Z, Jing Z, Zhang Y, Guo X, Dong M, Willmut I, Gao S. Proteome of mouse oocytes at different developmental stages. Proc Natl Acad Sci USA 2010; 107: 17639–17644. [Medline] [CrossRef]

63. Miyamoto K, Nagai K, Kitamura N, Nishikawa T, Ibegbun B, Binn NT, Tsukamoto S, Matsumoto M, Tsukiyama T, Minami N, Yamada M, Ariga H, Miyake M, Kawa-saka T, Matsumoto K, Imai H. Identification and characterization of an oocyte factor required for development of porcine nuclear transfer embryos. Proc Natl Acad Sci USA 2011; 108: 7040–7045. [Medline] [CrossRef]

64. Kuronoka H, Kato K, Minoguchi S, Takashashi Y, Ikeda J, Habu S, Osawa N, Buchberg AM, Moriwaki K, Shiu H, Honjo T. Cloning and characterization of the nucleoredoxin gene that encodes a novel nuclear protein related to thioredoxin. Genomics 1997; 39: 331–339. [Medline] [CrossRef]

65. Hirot A, Matsui M, Murata M, Takashima Y, Cheng FS, Itoh T, Fukuda K, Yoido J. Nucleoredoxin, glutaredoxin, and thioredoxin differentially regulate NF-kappaB, AP-1, and CREB activation in HEK293 cells. Biochem Biophys Res Commun 2008; 274: 177–182. [Medline] [CrossRef]

66. Funato Y, Michiue T, Aasahima M, Mikli H. The thioredoxin-related redox-regulating protein nucleoredoxin inhibits Wnt-β-catenin signalling through dishevelled. Nat Cell Biol 2006; 8: 501–508. [Medline] [CrossRef]

67. Qiao D, Li Z, Zhang Y, Huang Y, Wu Q, Rui G, Chen M, Wang B. Response of mouse zygotes treated with mild hydrogen peroxide as a model to reveal novel mechanisms of oxidative stress-induced injury in early embryos. Oxtrod Med Cell Longev 2016; 20152482. [Medline] [CrossRef]

68. Zhang Y, Qian D, Li Z, Huang Y, Wu Q, Ru G, Chen M, Wang B. Oxidative stress-induced DNA damage of mouse zygotes triggers G2/M checkpoint and phosphorylates CDK1 and Cdc2. Cell Stress Chaperones 2016; 21: 687–696. [Medline] [CrossRef]

69. Neumann CA, Kramme DS, Carman CV, Das S, Dubey DP, Abraham JL, Bronson RT, Fujiwara Y, Orkin SH, Van Etten RA. Essential role for the peroxiredoxin Prdx1 in erythroid antioxidant defence and tumour suppression. Nature 2003; 424: 561–565. [Medline] [CrossRef]

70. Eger RA, Fernandes E, Rothermund K, Sereika S, de Souza-Pinto N, Jaruga P, Dizdaroglu M, Prochownik E. Regulation of reactive oxygen species. DNA damage, and eG-specific function by peroxiredoxin 1. Oncogene 2005; 24: 8038–8050. [Medline] [CrossRef]

71. Lee TH, Kim SU, Yu SL, Kim SH, Park DS, Moon HB, Dho SH, Kwon KS, Kwon HJ, Han YH, Jeong S, Kang SW, Shin HS, Lee KK, Rhee SG, Yu DY. Peroxiredoxin II is essential for sustained life span of erythrocytes in mice. Blood 2005; 101: 3033–3038. [Medline] [CrossRef]

72. Egler RA, Fernandes E, Rothermund K, Sereika S, de Souza-Pinto N, Jaruga P, Dizdaroglu M, Prochownik E. Regulation of reactive oxygen species. DNA damage, and eG-specific function by peroxiredoxin 1. Oncogene 2005; 24: 8038–8050. [Medline] [CrossRef]

73. Lee TH, Kim SU, Yu SL, Kim SH, Park DS, Moon HB, Dho SH, Kwon KS, Kwon HJ, Han YH, Jeong S, Kang SW, Shin HS, Lee KK, Rhee SG, Yu DY. Peroxiredoxin II is essential for sustained life span of erythrocytes in mice. Blood 2005; 101: 3033–3038. [Medline] [CrossRef]

74. Egler RA, Fernandes E, Rothermund K, Sereika S, de Souza-Pinto N, Jaruga P, Dizdaroglu M, Prochownik E. Regulation of reactive oxygen species. DNA damage, and eG-specific function by peroxiredoxin 1. Oncogene 2005; 24: 8038–8050. [Medline] [CrossRef]

75. Amouroux R, Nashun B, Shirane K, Nakagawa S, Hill PW, DSouza Z, Nakayama M, Matsuura M, Turp A, Ndjette E, Encheva V, Kudo NR, Koehse H, Sasaki H, Hijikawa P. De novo DNA methylation drives Sca1 accumulation in mouse zygotes. Nat Cell Biol 2016; 18: 225–233. [Medline] [CrossRef]

76. Niu Y, DesMarais TL, Tong Z, Yao Y, Costa M. Oxidative stress alters global histone modification and DNA methylation. Free Radic Biol Med 2015; 82: 22–28. [Medline] [CrossRef]