PARK7 protein translocating into spermatozoa mitochondria in Chinese asthenozoospermia

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Abstract

PARK7 (DJ1) is a multifunctional oxidative stress response protein that protects cells against reactive oxygen species (ROS) and mitochondrial damage. PARK7 defects are known to cause various physiological dysfunctions, including infertility. Asthenozoospermia (AS), i.e. low-motile spermatozoa in the ejaculate, is a common cause of human male infertility. In this study, we found that downregulation of PARK7 resulted in increased levels of lipid peroxide and ROS, decreased mitochondrial membrane potential, and reduced mitochondrial complex I enzyme activity in the spermatozoa from AS patients. Furthermore, it was observed that PARK7 was translocated into the mitochondria of damaged spermatozoa in AS. Finally, we examined the oxidative state of PARK7 and the results demonstrated the enhancement of oxidation, expressed by increased sulfonic acid residues, the highest form of oxidation, as the sperm motility decreased. Taken together, these results revealed that PARK7 deficiency may increase the oxidative stress damage to spermatozoa. Our present findings open new avenues of therapeutic intervention targeting PARK7 for the treatment of AS.

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Introduction

Asthenozoospermia (AS), defined as low-motile spermatozoa in the ejaculate, is a frequent cause of human male infertility (Chemes *et al.* 1998). It is reported that more than 80% of the spermatozoa from infertile patients had motility defects and 19% of them were diagnosed with AS (Curi *et al.* 2003). Oxidative stress is one of the main issues associated with AS. When the physiological equilibrium between reactive oxygen species (ROS) production and elimination is perturbed, spermatozoa function may be compromised, and indeed oxidative stress is associated with male infertility (Sikka *et al.* 1995). Increased ROS levels have been implicated in spermatozoa DNA fragmentation, decreased motility, lipoperoxidation damage, and increased apoptosis (Agarwal *et al.* 2008). It has been proposed that the mitochondria are a major contributor to the oxidative stress followed by defective human spermatozoa (Koppers *et al.* 2008). Electron microscopy revealed that spermatozoa from AS patients have disordered mitochondria, significantly shorter midpieces, and fewer mitochondrial gyres when compared with their normozoospermic counterparts (Mundy *et al.* 1995). Furthermore, spermatozoa quality, particularly motility, is positively correlated with the

enzymatic activities of the electron transfer chain (ETC) complexes (Ruiz-Pesini *et al.* 1998, 2000) and the expression of ETC subunits (Amaral *et al.* 2007). The mitochondria are continuously protected against ROS damage by various antioxidants and multiple enzymatic defense systems, including Superoxide Dismutase, catalase, the glutathione peroxidase/reductase system, and non-enzymatic substances, such as ascorbic acid, glutathione, and α -tocopherol (Mannella 2008).

PARK7 (DJ1) is ubiquitously expressed in various human tissues, with a particularly high level of expression in the testes (Nagakubo *et al.* 1997). Although its precise biochemical function is not fully known, accumulated studies demonstrated that PARK7 is a multifunctional protein and plays essential roles in tissues with higher order biological functions, including cell transformation (Nagakubo *et al.* 1997), sperm maturation and fertilization (Klinefelter *et al.* 1997, Ooe *et al.* 2005, Hao *et al.* 2010), oxidative stress response (Mitumoto & Nakagawa 2001, Mitumoto *et al.* 2001, Kinumi *et al.* 2004, Taira *et al.* 2004), and Parkinson's disease (Bonifati *et al.* 2003). PARK7 was initially identified as a novel oncogene product that transforms NIH3T3 cell in cooperation with H-Ras

(Nagakubo *et al.* 1997). Around the same time, PARK7 and its homologues, sperm protein 22 (SP22) and contraception-associated protein 1, were identified as the first proteins to be correlated with male infertility (Klinefelter *et al.* 1997, Wagenfeld *et al.* 1998, Welch *et al.* 1998). In a study conducted on rats, it was reported that the amount of PARK7 in spermatozoa and the efficiency of fertilization decreased with an increasing dose of spermatozoa toxicant such as ornidazole and epichlorohydrin, to which the rats were exposed (Wagenfeld *et al.* 1998, Welch *et al.* 1998). It was also demonstrated that PARK7 began to be expressed after the appearance of a second spermatocyte during spermatogenesis and is finally located in the sperm head, implying an important function of PARK7 in spermatogenesis (Wagenfeld *et al.* 1998, Welch *et al.* 1998). Then it was found that an anti-SP22/DJ1 antiserum abrogated the fertilization activity of sperm (Klinefelter *et al.* 2002, Okada *et al.* 2002). Importantly, PARK7 can protect cells against oxidative stress damage, in which ROS was eliminated *in vitro* and *in vivo* by oxidizing PARK7 itself (Yokota *et al.* 2003, Taira *et al.* 2004). PARK7 possesses three cysteines at amino acid positions 46, 53, and 106 (Cys46, Cys53, and Cys106). Oxidation of Cys106 is essential for PARK7 to exert its full activities (Takahashi-Niki *et al.* 2004), but unoxidized PARK7 and oxidized PARK7 in the sulfonic (SO₃H) form are both inactive (Yokota *et al.* 2003). PARK7 is mainly located in the nucleus and cytoplasm (Nagakubo *et al.* 1997, Shinbo *et al.* 2006). When cells were treated with an inducer of ROS, PARK7 was translocated into the mitochondria for maintenance of mitochondrial complex I (CI) activity and was co-localized with ND1 and NDUFA4, subunits of mitochondrial CI encoded by mitochondrial and nuclear DNA respectively. This oxidation-induced mitochondrial relocation of PARK7 and protection against cell death were abrogated in the mutating cysteine residue Cys106A but not Cys53A or Cys46A (Canet-Aviles *et al.* 2004). Furthermore, the CI activity was reduced in two types of PARK7-knockdown NIH3T3 and HEK293 cells (Hayashi *et al.* 2009).

Although PARK7 has been reported to be related to infertility of rats and mice and to participate in fertilization for the spermatozoa to penetrate into the zonae pellucidae of eggs (Welch *et al.* 1998, Klinefelter *et al.* 2002, Okada *et al.* 2002, Yoshida *et al.* 2003), the characteristics of PARK7 in spermatozoa from AS patients are still not clear, and the function of mitochondria-resident PARK7 is still not known.

In this study, we analyzed the expression of PARK7 in human spermatozoa and its effect on mitochondrial function. The results showed that downregulation of PARK7 in spermatozoa from AS patients depressed the mitochondrial function and that PARK7 was translocated into the mitochondria.

Materials and methods

Patients

From June 2012 to October 2013, we enrolled 150 males (aged 21–45 years) diagnosed with infertility in the Department of Andrology, Peking University Third Hospital, Beijing, China, controlled by 86 age-matched control subjects with normal semen parameters (Supplementary Table 1, see section on supplementary data given at the end of this article). Semen samples of patients with pyospermia, varicocele, or smoking history were not eligible. According to the severity of AS, patients were divided into two groups: mild AS patients with A-grade spermatozoa percentage >10% (*n*=81) and moderate AS patients with A-grade spermatozoa percentage <10% (*n*=69). All participants provided informed consent for participation in the study approved by the Ethics Institutional Review Board of Peking University Third Hospital.

Semen analysis

Seminal samples were collected by masturbation after 2–7 days of sexual abstinence. Each semen sample was liquefied for 30 min at 37 °C before sample preparations. The volume, pH, and spermatozoa concentration and motility of sample were assessed according to the World Health Organization guidelines (Organization 2010) by a computer-assisted semen analyzer (Weili Company, Beijing, China).

Total spermatozoa protein extraction

After analysis of semen parameters, the total spermatozoa protein was extracted as described previously (An *et al.* 2011). In brief, the remaining semen was washed twice with Hanks balanced salt solution by centrifugation. The combined spermatozoa pellets, resuspended in 80 mM Tris–HCl buffer (pH 7.4), were oscillated using an ultrasonic apparatus and then homogenized for 1 h on ice. Finally, the mixtures were centrifuged to remove the insoluble debris, and the supernatant fraction was frozen at –80 °C until use.

Western blotting

The concentration of total protein in the spermatozoa lysates was measured using a Bradford reagent (Cellchip Biotechnology Co., Ltd, Beijing, China). Proteins (20 mg) were separated by 12.5% SDS–PAGE and transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk in Tris-buffered saline solution with Tween 20 with agitation. Proteins were sequentially incubated with the anti-DJ1 MAB (Abcam, Cambridge, UK) and HRP-conjugated IgG (KPL, Gaithersburg, MD, USA). Western Blotting Substrate (Applygen Technologies, Beijing, China) was used for the detection of ECL on an X-ray film. GAPDH was used as an internal standard. For quantification of PARK7 expression levels, Quantity One Software (Bio-Rad) was used to quantify the optical density of each band. Levels of PARK7 were normalized against GAPDH.

Lipid peroxide assay

Lipid peroxidation (LPO) was measured using the thiobarbituric acid (TBA) assay according to the method described previously (Searle & Willson 1983) with some modifications. Spermatozoa total protein of 200 mg was treated with 4 µl of 0.01 M cysteine and 8 µl of 0.001 M FeSO₄. After the mixture was incubated, 100 µl of 20% TCA along with a chromogenic agent of 100 µl of 0.1% TBA was added to terminate the reaction. The TBA reactive substance – MDA in the resultant mixture – was measured by the absorbance at 532 nm.

ROS assay

The level of ROS in spermatozoa was quantified using the ROS assay kit. Spermatozoa were washed with Biggers, Whitten, and Whittingham medium (BWW; Organization 2010), suspended in DCFH-DA diluted to a final concentration of 10 mM at a density of 1×10^6 spermatozoa/ml, and incubated in the dark. After being washed three times with PBS, the fluorescence was analyzed quantitatively by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The excitation and emission wavelengths were set at 488 and 525 nm.

Indirect immunofluorescence

Spermatozoa were collected by density gradient centrifugation (WHO 2010). Then, 1 ml of semen was layered on the density gradient media composed of 1 ml of 40% (v/v) Percoll solution (Sigma) above 1 ml of 80% (v/v) Percoll solution. All Percoll solutions were buffered with BWW solution. Following centrifugation, the spermatozoa accumulated at the bottom of the tube were collected.

An aliquot of spermatozoa washed with Percoll solution and resuspended in PBS was spotted on glass slides and air-dried. These preparations were incubated with 200 nM MitoTracker Deep Red FM (MT-DR FM; Invitrogen), fixed with 4% paraformaldehyde, and then permeabilized with ice-cold acetone, according to the operation manual recommended by Life Technologies Corporation. These preparations were blocked with 10% goat serum, and then sequentially incubated with the anti-DJ1 MAB (Abcam) or normal rabbit IgG as a negative and FITC-conjugated anti-rabbit antibody (Proteintech, Chicago, IL, USA). The nuclei were counterstained with Hoechst 33342 and mounted with antifade mounting medium. All samples were observed under a laser scanning confocal microscope (Leica, Mannheim, Germany).

Flow cytometric analysis

The spermatozoa were mixed gently in 200 nM MT-DR FM solution and incubated for 45 min in the dark. After staining was complete, the spermatozoa were pelleted by centrifugation and resuspended in fresh prewarmed BWW solution. Their fluorescence was measured using a FACS-Calibur flow cytometer (BD Biosciences) with excitation at 644 nm and emission at 665 nm. The amount of fluorescence was also quantified using the 'CELL QUEST' program with a value of 'median'.

Measurement of mitochondrial CI activity

The mitochondrial CI activity was determined by spectrophotometric assay using a Quantitative Detection Kit of mitochondrial respiratory chain CI activity (Genmed Scientifics, Inc., Wilmington, DE, USA). According to the provided protocol, 50 µg (25 µl) of total proteins isolated from spermatozoa and 25 µl Reagent C (negative control) were treated with Reagent A (buffer), B (reaction solution), and D (substrate solution). The absorbance was measured at 0 and 5 min at a wavelength of 340 nm. The values were corrected with non-specific absorption at 380 nm.

Immunoprecipitation and mass spectrometry analysis

The oxidative state of Cys106 of PARK7 was identified by LTQ Orbitrap mass spectrometry analysis as described previously (Gao *et al.* 2011) with some modifications. The spermatozoa proteins were concentrated by centrifugation in ultrafiltration tubes (Millipore) at about 8 µg/µl. The proteins (3.5 mg) were immunoprecipitated with a rabbit anti-DJ1 polyclonal antibody and separated on 20% polyacrylamide gel. After the gel had been stained with Coomassie brilliant blue, the band corresponding to PARK7 was excised for measurement by LTQ Orbitrap Velos Pro (Thermo, Waltham, MA, USA) mass spectrometry.

Statistical analysis

Data are expressed as means $\pm$ s.d. Statistical analysis was performed using a one-way ANOVA for different groups of measurements. Comparisons with $P < 0.05$ were generally considered to be significant although $P < 0.01$ was also given for those comparisons with more significant difference as indicated in figures.

Results

Decrease in spermatozoa PARK7 expression in AS

PARK7 was detected as a single band with a molecular weight of ~ 20 kDa by western blotting (Fig. 1A). A significant difference in the expression level of PARK7 was demonstrated between AS and normal spermatozoa ($P < 0.001$; Fig. 1B), and the positive relationship between the decrease in PARK7 and the severity of AS was revealed by the Kruskal–Wallis Test (Fig. 1C).

Increase in LPO and ROS in AS

Two indicators, LPO and ROS, were selected as evaluation indexes of the spermatozoa peroxide level. Thiobarbituric acid reactive substances (TBARS) content is an index of LPO and is usually assayed for indicating oxidative damage to plasma membranes. The data reported in Fig. 2A indicate that the TBARS content increased significantly in spermatozoa from AS patients. As shown in Fig. 2B, there is a considerable increase in the intracellular ROS production in AS spermatozoa compared with the normal spermatozoa by flow

Figure 1 The expression level of PARK7 was analyzed by western blotting. (A) Before considering the severity of AS, seven bands on the left side represent AS, while the other four represent the normal group. GAPDH was used as a loading control; (B) histogram showing the quantification of the expression level of each sample using GAPDH as a loading control before considering the severity of AS. Significant differences are shown between the two groups; and (C) The samples of AS were divided into two subgroups in line with the percentage of A-grade sperm: MiA and MoA. The results shown in the histogram indicate that the expression level of PARK7 decreased more aggressively with exacerbation of AS. All data are expressed as mean $\pm$ s.d. *n*, number of samples used. *** $P < 0.001$ vs normal group by the Kruskal–Wallis test. AS, asthenozoospermia; MiA, mild asthenozoospermia; MoA, moderate asthenozoospermia.

cytometry. In fact, a lower percentage of spermatozoa with low levels of ROS was detected, along with a concomitant higher percentage of those with high levels of ROS.

Immunocytochemical localization of PARK7 in spermatozoa in AS

Preliminary experiments showed that PARK7 was translocated into the midpiece of spermatozoa, where the mitochondria exist (Supplementary Fig. 1, see section on supplementary data given at the end of this article). To indicate the location of the mitochondria, spermatozoa were incubated with MT-DR FM, which passively diffuses across the plasma membrane and accumulates in the active mitochondria. Spermatozoa were also treated with the anti-DJ1 MAB and then with a FITC-conjugated anti-rabbit IgG, and the nuclei were counterstained with Hoechst 33342. These reactions were represented by red, green, and blue fluorescence respectively, and these images were merged.

From the imaging results, we found that PARK7 was visualized as a scarf-like structure in the damaged spermatozoa midpiece, in randomly selected areas. Moreover, the more serious the disease, the greater the

number of 'scarfs', and there was nearly no scarf in the normal spermatozoa (Fig. 3).

The intensities of red fluorescence from the excited MT-DR FM corresponding to the levels of mitochondrial membrane potential (MMP) showed that the MMP of spermatozoa gradually disappeared with the increasing severity of AS.

MMP analysis by flow cytometry

In this study, the mitochondria of spermatozoa were incubated with MT-DR FM and measured using flow cytometry. By doing so, we were able to isolate two different subpopulations: MT-DR FM-positive and -negative spermatozoa (Fig. 4A and B). The percentage of MT-DR FM-positive and -negative spermatozoa was distinct in the three groups (48.7 ± 15.87 and $51.35 \pm 15.9\%$ for normal samples, 43.25 ± 20.45 and $56.81 \pm 20.46\%$ for mild AS, and 21.24 ± 12.15 and $78.89 \pm 12.22\%$ for moderate AS; Fig. 4C). The moderate AS group contained the least MT-DR FM-positive and the most MT-DR FM-negative spermatozoa ($P < 0.01$), which suggested that the percentage of MT-DR FM-positive spermatozoa of a given sample was positively correlated with its quality.

Another distinctive feature of each group, namely the mean fluorescence intensity, was observed. Especially in the MT-DR FM-negative spermatozoa, the fluorescence

Figure 2 Increase in LPO and ROS in AS. (A) The ordinate value represents the normalized OD against the mean OD of normal group. Significant enhancement of lipid peroxide in AS compared with normal group was shown. All data are expressed as mean $\pm$ s.d. *n*, number of samples used. ** $P < 0.01$ vs normal group by the Kruskal–Wallis test and (B) flow cytometry analysis of reactive ROS in sperms. It was demonstrated that along with the aggravation of AS, the formation and accumulation of ROS obviously increased. AS, asthenozoospermia; MiA, mild asthenozoospermia; MoA, moderate asthenozoospermia.

Figure 3 Immunofluorescence localization of PARK7 and mitochondria in ejaculated spermatozoa. Spermatozoa were treated with MT-DR FM (red) (as described in 'Materials and methods' section), and then spermatozoa were immunostained with an anti-DJ1 antibody (green). The nuclei were counterstained with Hoechst 33342 (blue). The images were merged (merge). Yellow arrows indicate the depolarized mitochondria and white arrows represent the electrochemically active mitochondria that were permeated with MT-DR FM in the MiA or MoA merge. PARK7, resembling a 'scarf', appeared in the depolarized mitochondria (yellow arrows) and was then visualized using a confocal laser microscope. Scale bars=25 μ m. MiA, mild asthenozoospermia; MoA, moderate asthenozoospermia; MT-DR FM, MitoTracker Deep Red FM.

intensity exhibited significant differences among these three groups ($3.53 \pm 0.86\%$ for normal samples, $4.00 \pm 0.52\%$ for mild AS samples, and $4.55 \pm 0.61\%$ for moderate AS samples; Fig. 4D). It is interesting that in moderate AS, the fluorescence intensity of the MT-DR FM-negative spermatozoa was highest compared with normal and mild samples ($P < 0.01$). This finding indicated that the mitochondrial function of these spermatozoa was improved to a certain extent via some unknown compensatory mechanisms.

Impairment of mitochondrial CI activity

Mitochondrial CI is the first catalytic system in the respiratory chain. Specific reduction in mitochondrial CI activity inhibited sperm motility through regulation of the NAD^+/NADH redox balance. In this study, fresh ejaculated semen samples were collected, from which the total protein of sperm was isolated, and the CI enzyme activity was measured. In comparison to normal samples, CI enzyme activity was significantly reduced in AS samples and showed a positive correlation as the severity of AS paralleled the PARK7 expression (Fig. 5).

Cys106 oxidation modification of PARK7 in spermatozoa of AS

PARK7 contains three cysteine residues (Cys46, Cys53, and Cys106) whose oxidation states regulate its function. Of these, Cys106 is particularly susceptible to gradual oxidation to sulfenic acid (SOH), sulfinic acid (SO_2H), and finally sulfonic acid (SO_3H). The function of PARK7 is closely related to its oxidation state, which may cause the inactivation of PARK7. We observed Cys106 in the

sequence of sperm PARK7, which was obtained by immunoprecipitation followed by SDS-PAGE. The percentage of each form was used to reflect the oxidative state of PARK7. As shown in Fig. 6, the value of oxidized Cys106, especially the SO_3H form, has been shown to intensify in PARK7 proteins derived from sperms of AS compared with the normal ones.

Discussion

This study has revealed a new molecular mechanism underlying PARK7-mediated human spermatozoa motility by protecting the mitochondrial function. We demonstrated that PARK7 expression was reduced in ejaculated spermatozoa from AS patients compared with normal donors. We also found that the content of TBARS-MDA in spermatozoa was significantly increased in AS patients whose PARK7 expression was blocked using TBA and that the decreased expression of PARK7 was consistent with the level of MMP. Importantly, we confirmed that PARK7 was translocated into the mitochondria and improved the activity of CI against oxidative stress. Finally, we analyzed the oxidation state of the three cysteine residues of PARK7, Cys46, Cys53, and Cys106, and the results demonstrated that the level of superfluous oxidation was increased in AS patients.

Repression in PARK7 expression in AS diminishes the antioxidant capacity of spermatozoa

Spermatozoa are particularly vulnerable to oxidative stress because of their characteristics, high polyunsaturated fatty acid content, intrinsic deficiencies of intracellular antioxidant enzymes, and limited capacity for DNA repair (Talevi *et al.* 2013). Under physiological

Figure 4 Mitochondrial membrane potential (MMP) analysis by flow cytometry. (A) Two subpopulations were obtained and sorted: MT-DR FM-negative spermatozoa (light unstained, left) and MT-DR FM-positive spermatozoa (deep stained, right) using a confocal laser microscope. For imaging purposes, nuclear DNA was stained with the DNA dye Hoechst 33342; (B) representative histograms obtained using flow cytometry; and (C) comparison between different group samples for the percentages of two subpopulations of spermatozoa. (D) Comparison between different group samples for the fluorescence intensity of MT-DR FM-negative spermatozoa. Data are expressed as mean $\pm$ s.d. n , number of samples used. ** $P < 0.01$ vs normal group. MiA, mild asthenozoospermia; MoA, moderate asthenozoospermia; MT-DR FM, MitoTracker Deep Red FM.

conditions, spermatozoa possess various antioxidant strategies for protecting themselves against ROS damage.

PARK7 has already been identified as a key redox-reactive protective protein implicated in the regulation of oxidative stress. According to the distribution in ejaculated spermatozoa, the surface of the posterior part of the head, the anterior part of the midpiece, and spermatozoa flagella, PARK7 was considered to play two putative roles in fertilization: one is binding to the egg, and the other is flagella movement (Yoshida *et al.* 2003). It has been reported that the levels of PARK7 in AS seminal plasma are significantly lower than that from healthy donors (Wang *et al.* 2009). In this study, we analyzed the content of PARK7 in ejaculated spermatozoa and obtained similar results showing that the levels of PARK7 in moderate AS spermatozoa were about half of those in the control. These data, together with the observation that the levels of LPO and ROS were obviously higher in the AS patients, strongly suggest that downregulation of the PARK7 protein could result in impaired antioxidant capacity of spermatozoa, which would then affect their viability and/or motility, as is observed in AS.

PARK7 translocates into the damaged mitochondria to rescue the spermatozoa in AS

The major finding in this study is the demonstration that PARK7 is translocated into the mitochondria of spermatozoa, where it participates in an efficient endogenous protection mechanism against mitochondrial defects.

Spermatozoa consume ATP provided by the mitochondria mainly to sustain motility, and also for various cellular events including hyperactivation, capacitation, and acrosome reaction (Ramalho-Santos *et al.* 2009, Pelliccione *et al.* 2011). In human spermatozoa, the mitochondria differ from the corresponding organelles of somatic cells in morphology. The mitochondrial sheath is organized in a helix of about 13 gyres, with two mitochondria per gyre, surrounding the axoneme at the midpiece level. Mitochondrial dysfunction is an important contributor to male infertility, including AS (Ferramosca *et al.* 2008). Recently, it has been demonstrated that a subpopulation of human spermatozoa showing better fertilization potential is characterized by high mitochondrial functionality (Sousa *et al.* 2011). In

Figure 5 Impairment of mitochondrial CI activity. The enzyme activity of CI in sperm was measured by spectrophotometric assay. The activity of CI was determined by the NADH content catalyzed by the enzyme in 1 mg total protein/min at 30 °C and pH 7.5. Compared with the normal samples, the enzyme activity of CI was significantly reduced in MiA and MoA samples, while a positive relationship was observed in the severity of AS. Data are expressed as mean $\pm$ s.d. $n=9$ in each group. * $P < 0.05$ and ** $P < 0.01$ vs normal group. AS, asthenozoospermia; MiA, mild asthenozoospermia; MoA, moderate asthenozoospermia.

Figure 6 Cys106 oxidation modification of PARK7 by mass spectrometry assay. The SO₃H-modified Cys106 form of PARK7 in AS accounted for a proportion of 61.2%, compared with 46.6% for the normal form, while the sulfhydryl form decreased from 41.3 to 21.3%. No significant difference in the SOH and SO₂H forms was found between the two groups. AS, asthenozoospermia.

addition, several observations have confirmed that PARK7 is translocated from the cytoplasm into the mitochondria when MMP is decreased (Ooe *et al.* 2005, Junn *et al.* 2009, Hao *et al.* 2010, Krebichl *et al.* 2010, Heo *et al.* 2012, Kaneko *et al.* 2013), and it maintained the CI activity by directly binding to NDUFA4 and ND1, nuclear and mitochondrial DNA-encoding subunits of the mitochondria respectively (Hayashi *et al.* 2009). Along this line, we detected the location of PARK7 by staining the spermatozoa with an anti-DJ1 antibody. The immunocytochemical results demonstrated that PARK7 appeared in the midpiece of seemingly abnormal spermatozoa, in which the mitochondria exist, and the mitochondria enriched with PARK7 showed a reduced membrane potential. However, all of these spermatozoa had high motility, because the sample was treated by density gradient centrifugation, a standard preparation technique to obtain high-motility spermatozoa samples.

To test the MMP, the mitochondria were labeled with the specific fluorescent marker, MitoTracker probe. The cell-permeant MitoTracker probes contain a mildly thiol-reactive chloromethyl moiety for labeling the mitochondria. Most MitoTracker probes such as MT-DR FM, used in this study, are well retained in the mitochondria following fixation and permeabilization. It is useful to

subsequently probe for other intracellular structures by immunocytochemistry. In this experiment, we found that the moderate group of AS samples possessed the least number of MT-DR FM-positive spermatozoa and the most MT-DR FM-negative spermatozoa. We also accidentally discovered that the fluorescence intensity of the MT-DR FM-negative spermatozoa in moderate AS samples was clearly higher than its counterpart in the normal samples.

The phenomenon described above could be explained as follows: without any therapeutic intervention, the translocation of PARK7 into the injured mitochondria initiated the endogenous repair mechanisms under which PARK7 improved the CI activity and thus enhanced the MMP levels. Although the repair capacity of PARK7 was not sufficient to completely compensate for the decreased MMP because of the decreased amount of PARK7, it probably improved the gently injured spermatozoa motility to a certain extent. Further experiments are required to investigate these possibilities.

In addition, we examined the CI activities in order to reveal the difference among AS sample groups. CI is essential for oxidative phosphorylation, ROS, and electron transport system of the mitochondria for ATP production. It is highly sensitive to oxidative stress, which contributes to a range of pathologies (Wu *et al.* 2010). Of particular note is that we did not respectively analyze the CI activity of MT-DR FM-negative and -positive spermatozoa, given that the result reflected both cell types. Reduced CI activity was highly correlated with decreased expression of PARK7, further supporting the view that mitochondrial targeting of PARK7 provided significantly stronger cytoprotection.

Superfluous oxidation of cysteine residues inhibits the function of PARK7

The cysteine residues in PARK7, including Cys46, Cys53, and Cys106, appear to be very important for PARK7-mediated cytoprotection. Of these, Cys106 is the major oxidation site (Canet-Aviles *et al.* 2004). The oxidation of Cys106 necessarily consumes ROS and thus it has been proposed that PARK7 acts as a direct scavenger of ROS (Andres-Mateos *et al.* 2007). Furthermore, oxidation of Cys106 with SO₂H is necessary for the mitochondrial

Figure 7 Proposed mechanism of PARK7 in maintaining the sperm motility. PARK7 maintains the sperm motility by protecting against oxidative stress damage and mitochondrial translocation to protect the mitochondrial function. However, PARK7 may lose these functions when oxidized excessively or when its expression is decreased.

translocation of PARK7, but this effect is greatly diminished upon further oxidation (Canet-Aviles *et al.* 2004). In this study, we examined the oxidative levels of PARK7 and the results showed the enhancement of oxidation, expressed by increased SO₃H residues, the highest form of oxidation, as the sperm motility decreased.

In this study, we confirmed that PARK7 is deficient in AS spermatozoa, resulting in increased LPO levels and reduced antioxidation ability of spermatozoa. We further demonstrated that under oxidative stress conditions, PARK7 was translocated into the mitochondria. In addition, we examined the oxidation levels of PARK7 protein by combined application of immunoprecipitation and LTQ Orbitrap mass spectrometry, which established a direct relationship between oxidation of PARK7 and sperm motility.

The present results provide evidence that PARK7 is closely involved in cytoprotection against ROS damage and protection of the mitochondrial function, as shown in Fig. 7, suggesting new avenues of therapeutic intervention targeting PARK7 for the treatment of AS.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0222>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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