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Epididymal specific, selenium-independent GPX5 protects cells from oxidative stress-induced lipid peroxidation and DNA mutation

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STUDY QUSTION: Can selenium (Se) independent, epididymal-specific glutathione peroxidase 5 (GPX5) protect CHO-K1 cells from oxidative damage and, more specifically, from lipid peroxidation and DNA mutation?

SUMMARY ANSWER: CHO-K1 cells expressing GPX5 have increased resistance to oxidative challenge and, more specifically, decreased levels of lipid peroxidation and decreased levels of the downstream DNA lesion 8-oxo-7,8-dihydroguanine (8-oxodG) compared with control cells.

WHAT IS KNOWN ALREADY: GPX5 associates with sperm during transit of the epididymis, and has been postulated to protect sperm from peroxide-mediated attack. However, its function as an active glutathione peroxidase has been questioned due to substitution of the classical selenocysteine residue at its active site. Indirect evidence for a functional role for GPX5 has been provided by *in vivo* studies, in particular from the GPX5 knockout mouse whereby offspring sired by $GPX5^{-/-}$ males have a higher rate of spontaneous abortion and developmental defects, attributed to increased oxidative injury (8-oxodG) to sperm DNA, but only when the $GPX5^{-/-}$ males are over 1 year of age. Interestingly, we have previously shown severely reduced levels of GPX5 in humans.

STUDY DESIGN, SIZE, DURATION: To look more directly at its role in protection against oxidative damage, we have used an *in vitro* system, generating a CHO-K1 mammalian cell line expressing recombinant rat GPX5.

PARTICIPANTS/MATERIALS, SETTING, METHODS: We have used the recombinant CHO-K1 cells to determine whether GPX5 is able to protect these cells from an administered oxidative challenge, using a range of approaches. We compared the viability of GPX5-expressing cells with control cells by both MTT and trypan blue exclusion assays. We next investigated whether GPX5 protects the cells specifically from lipid peroxidation, by using the fluorescent reporter molecule C11-$BODIPY^{(581/591)}$, and thus from downstream DNA mutation, by comparing levels of the DNA lesion 8-oxodG. We also investigated whether GPX5 can be transferred to rat sperm via epididymosomes.

MAIN RESULTS AND THE ROLE OF CHANCE: GPX5-expressing CHO-K1 cells had increased viability compared with control cells following oxidative challenge ($P < 0.005$). We also found that GPX5-expressing CHO-K1 cells had significantly lower levels of C11-$BODIPY^{(581/591)}$ oxidation, and hence lipid peroxidation, compared with control cells. Levels of 8-oxodG DNA damage were also markedly lower in the nuclei of GPX5-expressing cells than in control cells. Finally, we showed that GPX5 can be transferred to rat sperm via epididymosomes.

LIMITATIONS, REASONS FOR CAUTION: GPX5 is not active in glutathione peroxidase assays using H_2O_2 as the substrate. However, the related non-mammalian Se-independent GPXs show preference for electron donors other than glutathione, with a number utilizing thioredoxin as a reducing equivalent. Hence, the *in vitro* activity of GPX5 needs to be assessed using a range of alternative substrates and electron donors. GPX5 is secreted by the epididymis and associates with the sperm plasma membrane. We showed that this transfer can occur via epididymosomes; however, the mechanism for transfer and the identity of a potential binding partner in the sperm membrane needs to be determined. Finally, our study utilized an *in vitro* system that needs to be translated to human sperm.

WIDER IMPLICATIONS OF THE FINDINGS: Our study supports an important role for GPX5 as an antioxidant, possibly acting as a phospholipid hydroperoxidase and participating in the maintenance of cell and DNA integrity.

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Key words: GPX5 / antioxidants / sperm / lipid peroxidation / DNA damage

Introduction

Oxidative stress occurs when there is an imbalance between oxidants and antioxidants and can result in damage to biomolecules and severe cellular dysfunction. Peroxidation of lipids can be particularly detrimental as it results in the amplification of free radical species with far reaching consequences. Polyunsaturated fatty acids are particularly susceptible to peroxidation. Following abstraction of a hydrogen atom from a polyunsaturated fatty acid molecule, an organic lipid free radical is generated which can react with dioxygen to generate a lipid peroxyl radical. This in turn can oxidize further polyunsaturated fatty acid molecules giving rise to lipid hydroperoxides and secondary lipid radicals, generating a self-perpetuating chain reaction. Lipid peroxidation impairs membrane integrity and function, and lipid peroxidation products are a major cause of DNA damage (Marnett, 2002).

Sperm are particularly vulnerable to increased levels of reactive oxygen species (ROS) as their membranes contain a high content of polyunsaturated fatty acids that are extremely sensitive to peroxidative damage (Aitken *et al*., 1993; Storey, 1997). As sperm themselves possess only low levels of cytoplasmic antioxidants, having shed most of their cytoplasm, they are reliant for protection on antioxidant scavengers, synthesized and secreted by the epididymal epithelial cells, including extracellular superoxide dismutase (Williams *et al*., 1998a,b) and an unusual isoform of glutathione peroxidase, GPX5 (Ghyselinck *et al*., 1990; Perry *et al*., 1992; Lefrancois *et al*., 1993; Okamura *et al*., 1997; Vernet *et al*., 1997; Williams *et al*., 1998a,b).

Mammalian glutathione peroxidases are a family of enzymes originally named on their ability to catalyse the reduction of peroxides using glutathione as the reducing substrate. GPX5 is an unusual member of the mammalian GPX family, as, unlike the well-known mammalian GPXs 1–4 (Brigelius-Flohe, 1999), it does not contain a selenocysteine (SeCys) at its active site, having instead a cysteine residue (Perry *et al*., 1992); it is thus termed a selenium (Se)-independent GPX. GPX5 is expressed exclusively by the caput epididymis (Ghyselinck *et al*., 1990; Perry *et al*., 1992; Lefrancois *et al*., 1993; Williams *et al*., 1998a,b) and associates with sperm during transit (Okamura *et al*., 1997; Vernet *et al*., 1997; Williams *et al*., 1998a,b), specifically with the plasma membrane overlying the acrosome, which is particularly sensitive to peroxidative damage (Jones and Mann, 1977). It has therefore been postulated that GPX5 protects sperm from peroxide-mediated attack (Perry *et al*., 1992; Williams *et al*., 1998a,b).

Although an attractive hypothesis, the function of GPX5 as an active glutathione peroxidase has been questioned (Rocher *et al*., 1992; Okamura *et al*., 1997) as the SeCys at the active site of the four major mammalian glutathione peroxidase isozymes was thought essential for activity; the higher nucleophilicity and lower pK of Se compared with sulphur make for a more reactive redox centre. However, Se-independent GPXs from organisms outside the mammalian class exhibit catalytic activity (Herbette *et al*., 2007) and play a highly significant and physiological role in antioxidant defense as phospholipid hydroperoxidases (Beeor-Tzahar *et al*., 1995; Tang *et al*., 1995, 1996; Faltin *et al*., 1998; Tripp *et al*., 1998; Avery and Avery, 2001; Herbette *et al*., 2002; Gaber *et al*., 2004). Furthermore, indirect evidence for a functional role for GPX5 has been postulated from the increased levels of GPX5 in Se-deficient mice that may compensate for the decrease in Se-dependant GPX activity (Vernet *et al*., 1999).

More recently, it has been shown that wild-type female mice mated with $GPX5^{-/-}$ males have a higher rate of spontaneous abortion and developmental defects, but only when the $GPX5^{-/-}$ males are over 1 year of age (Chabory *et al*., 2009), attributed to increased oxidative injury to sperm DNA in the absence of GPX5. Levels of other glutathione peroxidases and catalase are raised in the cauda epididymis of $GPX5^{-/-}$ animals, suggesting increased oxidative stress, presumably due to GPX5 deficiency and as a compensatory response, with no difference in total GPX activity between control and knockout animals. Collectively, the data from this study strongly support that GPX5 has a physiological role in oxidative defense. However, although this is a very important paper, gene deletion can be associated with compensatory effects, with the changes in phenotype being a secondary rather than primary consequence of the knockout. Indeed, the $GPX5^{-/-}$ model does not show a direct correlation between increased DNA damage in $GPX5^{-/-}$ sperm and increased rates of spontaneous abortion and embryonic defects. The data presented only show increased levels of 8-oxo-7,8-dihydroguanine (8-oxodG and increased levels of malondialdehyde) in sperm from 6 month old $GPX5^{-/-}$ mice, animals that were not associated with such birth defects.

Therefore, it is important that the conclusions of knockout experiments are validated by other, more direct, means. Introduction of the gene into other cells and demonstration that the expressed protein fulfils its expected function is a useful way to achieve this. Therefore, to look more directly at its role in protection against oxidative damage we have used an *in vitro* system, generating a mammalian cell line expressing recombinant GPX5.

Such a system has been attempted previously and some increased protection of CHOK1 cells to oxidative challenge was observed on expression of recombinant GPX5 (Vernet *et al*., 1996). However, a number of notable inconsistencies in the data, along with a lack of appropriate controls undermine its credibility. For example, although there was a significant difference in the ability of media from GPX5 recombinant cells, compared with control cell media, to metabolize H_2O_2, there was no difference with tert-butyl hydroperoxide as the substrate. More confusingly, the difference was lost in the presence of mercaptosuccinate (which inhibits Se-dependent glutathione peroxidases and thus not GPX5),

when it should have been at least as great, as the measured activity should have been confined to GPX5. Furthermore, there was no difference in the ability of control and recombinant cell lysates to metabolize either substrate, although recombinant cells showed increased resistance to both.

Hence far more robust, carefully executed and reliable data are required to confirm the role of GPX5.

We have used our *in vitro* system to determine whether GPX5 is able to protect cells from an administered oxidative challenge, using a range of approaches. Furthermore, we have investigated whether GPX5 protects cells specifically from lipid peroxidation, by using the fluorescent reporter molecule C11-BODIPY$^{(581/591)}$, and thus from downstream DNA mutations, by comparing levels of the DNA lesion 8-oxodG.

GPX5 associates with the sperm plasma membrane during transit of the epididymis; however, the molecular mechanism of this interaction has not been determined. GPX5 is known to be present in epididymosomes (Rejraji *et al.*, 2002), membranous vesicles found in the epididymal lumen originating from the surrounding epithelial cells. We have therefore investigated whether GPX5 can be transferred to rat sperm via these vesicles, an approach which may inform further studies addressing the interaction of GPX5 with the plasma membrane.

Materials and Methods

Construction of GPX5-CHOK1 (GPX5) and pEE-CHOK1 (pEE) cell lines

Rat epididymal cDNA was used as the template for PCR amplification (ExpandTM High Fidelity PCR system; Roche) of rat *GPX5* using the gene-specific primers (5′-CTCCAAGCTTGGATCCTAGTCATGGCTATA CAGCTAAGAGTC-3′ and 5′-GCCCGAATTCTAGATGCCTTCCTAT ATGGT TTTGAATTGATTC-3′) flanked by *Hind*III and *Eco*RI restriction sites, respectively. The resultant PCR product was gel purified and inserted into pGEM®-T-easy cloning vector (Promega) and the sequence was verified. The *GPX5* DNA was excised using *Hind*III and *Eco*RI restriction enzymes (Roche) and ligated into a *Hind*III and *Eco*RI restricted pEE-14 expression plasmid (CellTech Ltd).

CHO-K1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium without L-glutamine or pyruvate (Invitrogen) supplemented with 1% FCS, modified Eagles medium non-essential amino acids without L-glutamine, 1 mM sodium pyruvate (both Invitrogen), 0.06 mg ml^{-1} L-glutamic acid, 0.06 mg ml^{-1} asparagine, 0.007 mg ml^{-1} adenosine, 0.007 mg ml^{-1} guanosine, 0.007 mg ml^{-1} cytidine, 0.007 mg ml^{-1} uridine, 0.007 mg ml^{-1} thymidine, 50 units ml^{-1} of both penicillin and streptomycin (all Sigma; complete DMEM) at 37°C. Cells were transfected with the pEE-GPX5 construct DNA (GPX5 cells) or with empty pEE vector DNA (pEE cells) using FuGENE 6^{TM} transfection reagent (Roche). Stably transfected cells were selected and maintained in complete DMEM supplemented with 25–500 mM methionine sulphoxamine. Control CHO-K1 cells were cultured in complete DMEM without methionine sulphoxamine. For microscopy, cells were grown to 50% confluences on glass coverslips (25 mm).

SDS–PAGE and western blot analysis

Cells were lysed in SDS–PAGE sample buffer (200 mM Tris–HCL, pH 6.8, 25% (v/v) glycerol, 5% (w/v) SDS, 0.1% (w/v) bromophenol blue and 20 mM DTT). Following SDS–PAGE and western blot, membranes were probed with specific antisera (GPX5 antisera; Williams *et al.*, 1998a,b) for 1 h at room temperature (RT), washed with phosphate-buffered saline (PBS) and incubated for 1 h at RT with HRP-conjugated swine anti-rabbit Igs (Dako). Membranes were washed with PBS, incubated with enhanced chemiluminescence (ECL^{TM}) detection reagent and exposed to Hyperfilm^{TM}.

MTT cell viability assay

Cells (1×10^4) were cultured in a 96-well plate in complete DMEM for 16 h prior to assay. Cells were washed with PBS and incubated for 1 h at 37°C in 90 ml complete DMEM without FCS. Then 10 μl of 5 mg ml^{-1} MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well and cells were incubated for a further 4 h at 37°C. The MTT solution was removed and 200 μl DMSO added to each well with agitation to dissolve the formazan crystals and a further incubation for 10 min at 37°C. Absorbance was read at 550 nm using a VERSAmax tunable microplate reader (Molecular Devices). Data were analysed by Student's *t*-test.

Glutathione peroxidase assay

Glutathione peroxidase activity was measured as described previously (Maiorino *et al.*, 1990) using H_2O_2 or *tert*-butyl hydroperoxide as the substrate and NADPH oxidation was monitored at 340 nm. GPX-independent peroxide detoxification was measured in parallel assays and GPX assay data were corrected for this. Recombinant GPX5 was concentrated by cation exchange chromatography. Culture medium was collected from cells and exchanged for chromatography buffer (50 mM HEPES, pH 7.4, 1 mM EDTA, 2 mM 2-mercaptoethanol) using a Vivispin 20 ultrafiltration concentrator (Sartorius). Samples were applied to a Mono S (FPLC) 5/5 ion exchange column and unbound protein was removed using the above buffer. Bound protein was eluted and a sample from each fraction was analysed for GPX5 by western blot using GPX5-specific antisera. Fractions containing GPX5 were pooled and concentrated using a centricon-10 spin column (Millipore). GPX1 from bovine erythrocytes (Sigma) was used as a positive control. Partially purified recombinant GPX5 was included in the assay at 100, 500 and 1000 ng and GPX1 was used at 25, 50 and 100 ng. Data were fitted to a Michaelis–Menton equation using Grafit (Erithacus Software Ltd).

Analysis of C11-BODIPY$^{581/591}$ oxidation

The lipid peroxidation reporter molecule C11-BODIPY$^{581/591}$ was used to examine the oxidation of membranes in GPX5 and pEE cells by fluorimetry and fluorescence micoscopy. Cells were labelled with 10 mM C11-BODIPY$^{581/591}$ for 30 min at 37°C and washed two times with enriched PBS (137 mM NaCl, 2.7 mM KCl, 0.5 mM MgCl, 0.9 mM Ca_2Cl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 5 mM glucose, pH 7.4). Oxidation of C11-BODIPY$^{581/591}$ was induced with cumene hydroperoxide or H_2O_2 in enriched PBS as described in the Results section. Oxidant was removed by washing with enriched PBS.

Fluorimetry

Fluorescence measurements were carried out at ambient temperature using a Fluorolog Tau-3 fluorimeter (Horiba). Initially, excitation and emission scans were performed with C11-BODIPY$^{581/591}$-labelled pEE cells to obtain wavelengths for the non-oxidized reporter and C11-BODIPY$^{581/591}$-labelled pEE cells were incubated for 1 h with 500 μM cumene hydroperoxide to obtain wavelengths for the oxidized reporter. Excitation and emission wavelengths were established as 542/592 nm (red; non-oxidized) and 495/513 nm (green; oxidized) and were used for all subsequent experiments. Slits were set to 5 nm band pass for all measurements. Oxidation of C11-BODIPY$^{581/591}$ was calculated as the ratio between green emission and

total emission (green plus red) to normalize for dye incorporation into cellular membranes as described by Jofre-Monseny *et al.* (2007). Data from cells challenged with oxidizing agent were then normalized to the respective controls without challenge.

Fluorescence microscopy

Images were captured using a Leica TCS-NT confocal laser scanning microscope attached to a Leica DM RBE upright epifluorescence microscope with phase contrast and a TRITC filter. V4.0.1 (Improvision) was used for compiling images.

Detection of 8-oxodG

For an oxidative challenge, cells were incubated with 500 μM H_2O_2 for 1 h in enriched PBS then washed two times with ice-cold PBS without calcium and magnesium, pH 7.4 (CMF-PBS). For the detection of 8-oxodG, cells were pre-treated as described by Soultanakis *et al.* (2000). Cells were fixed in 100% methanol for 30 min at −20°C then permeabilized with 0.1% Triton X100 for 15 min at RT, followed by two washes with CMF-PBS. Cells were next treated with 100 mg ml^{-1} ribonuclease (Sigma) for 60 min at 37°C, washed with CMF-PBS and incubated with 10 mg ml^{-1} proteinase K for 10 min at RT. Finally, DNA was denatured with 2 M HCl for 5 min at RT and neutralized with 2.5 volumes of 1 M Tris base for 7 min. Cells were blocked with 1% bovine serum albumin for 1 h at RT followed by overnight incubation at 4°C with 2 mg ml^{-1} 8-OHdG monoclonal antibody (15A3; Santa Cruz Biotechnology). Cells were washed three times with CMF-PBS and incubated with Alexa Fluor® 488 goat anti-mouse IgG (Life Technologies) for 1 h at RT. Finally, cells were incubated with DAPI for 5 min before coverslips were mounted on glass slides using Mowiol (Calbiochem) –DABCO [0.6% (w/v) 1,4-diazabicyclo-[2.2.2]octane] anti-photobleaching mounting media. Images were obtained by confocal laser scanning microscopy using a Leica TCS SP5-II AOBS confocal system (Leica Microsystems) attached to a Leica DMI 6000 CS AFC microscope with an Ar laser (488 nm) and 405 nm diode laser for excitation. A glycerol-objective lens (63×, NA 1.3) was used, and imaging parameters were selected to optimize confocal resolution and standardized to allow direct comparison between images.

Preparation of epididymosomes and transfer of GPX5 to sperm

Epididymosomes were collected from the rat caput and cauda epididymis as described (Frenette *et al.*, 2002). In brief, the epididymal fluid was collected from the two regions of the epididymis into 10 mM MES-PIPES, pH 6.5 plus 1.5 mM $ZnCl_2$ and centrifuged at 700*g* for 10 min, followed by 10 000*g* for 10 min. The resultant supernatant was then centrifuged at 45 000*g* for 2 h at 4°C. The supernatant was collected and stored at −20°C while the pellet was resuspended in the above buffer and again centrifuged at 45 000*g* for 2 h at 4°C. The resultant pellet was resuspended in 10 mM MES-PIPES, pH 6.5 plus 1.5 mM $ZnCl_2$. Sperm were collected from the initial segment (IS) of the rat epididymis and incubated with either caput epididymosomes or with the epididymal fluid supernatant for 4 h at 37°C. Following incubation the cells were washed extensively before analysis.

PCR analysis of thioredoxin and thioredoxin reductase

Rat epididymis, liver and testis cDNA was used as template for PCR amplification with rat thioredoxin (5′-GTAGTGGACTTCTCTGCCAC-3′ and 5′-TTAACCTGCTGGAGCTGGTC-3′) and thioredoxin reductase (5′-TTGCTCCTAGGTCACGTGAC-3′ and 5′-ACACTGTCGTTCTGATCTCAG-3′) gene-specific primers.

Results

Expression of recombinant GPX5 in CHO-K1 cells

CHO-K1 cells were transfected with the pEE-GPX5 construct or with empty pEE14 vector to generate recombinant GPX5-expressing (GPX5 cells) and control (pEE cells) stable cell lines. To confirm GPX5 expression, cell lysate and tissue culture medium from the cultured cells was analysed by SDS–PAGE and western blot probed with GPX5 antisera. A band of the expected size for GPX5 was detected in tissue culture medium (Fig. 1A) and cell lysate (Fig. 1B) from GPX5 clones. No band was detected in cell lysate or tissue culture medium of control CHO-K1 or pEE cells. The additional, higher bands seen in Fig. 1B are non-specific.

GPX5 protects cells against oxidative challenge

Initially, the effect of GPX5 expression on cell viability following oxidative challenge was analysed using the MTT assay. GPX5 and control pEE and CHO-K1 cells were seeded and cultured for 16 h prior to addition of 0–1.5 mM H_2O_2 for 2, 6 or 24 h. Cell viability at each time point ($n = 8$) was evaluated using the MTT assay and normalized to the respective cell type without H_2O_2 challenge. The experiment was

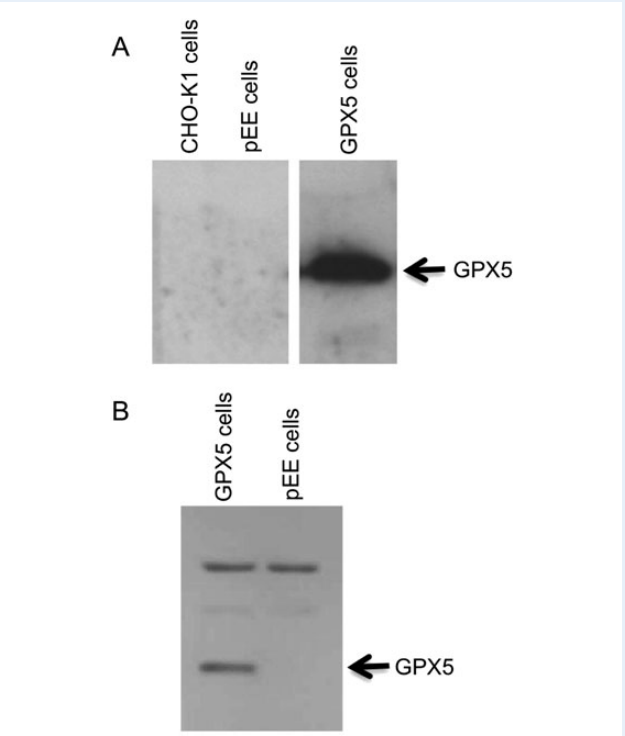

Figure 1 Expression and secretion of GPX5 by stable cell lines. Culture medium collected from CHO-K1, pEE14 and GPX5 cells following 3 days of incubation (**A**) and whole cell lysate from pEE14 and GPX5 cells (**B**) were resolved by SDS–PAGE and the proceeding western blot was probed with GPX5 polyclonal antibody, followed by HRP-conjugated secondary antibody and ECL.

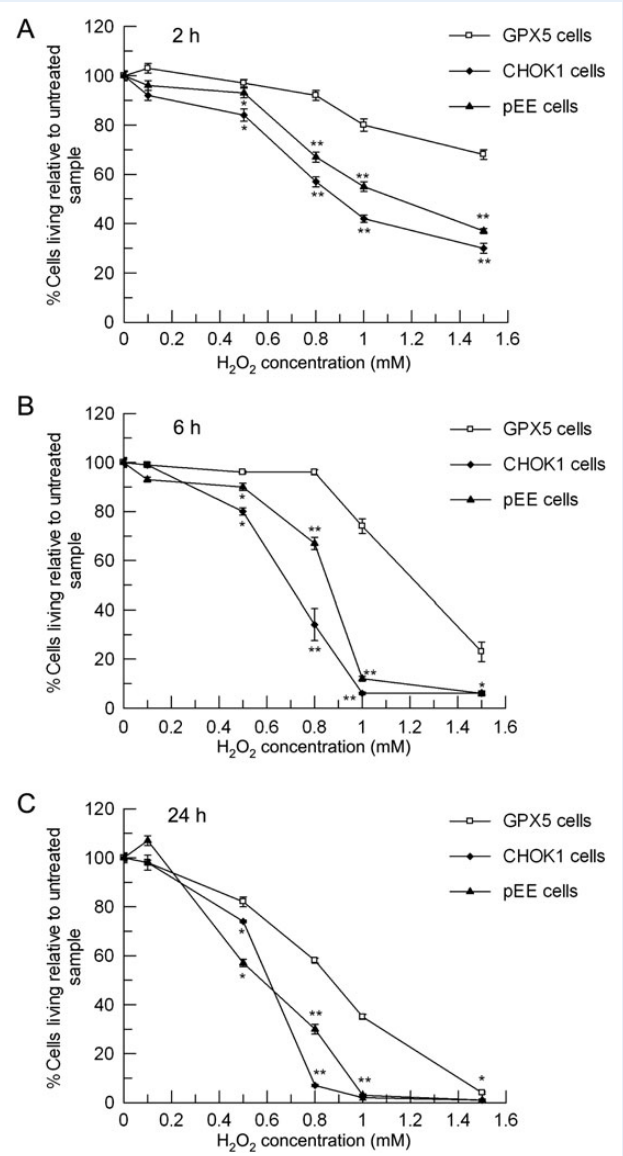

Figure 2 Viability of CHO-K1, GPX5 and pEE cells following 2, 6 and 24 h of H_2O_2 challenge. Cells were cultured with 0–1.5 mM H_2O_2 for 2, 6 and 24 h at 37°C before analysis of viability with the MTT assay. Data are shown as the mean Abs_{550nm} expressed as a % of Abs_{550nm} from 0 mM H_2O_2-treated sample for each cell type ($n = 8 \pm$ SEM). Data are presented from a single experiment and each experiment was repeated three times. *$P < 0.005$; **$P < 5 \times 10^{-7}$ for GPX5 cells compared with pEE/CHO-K1 cells.

performed on three separate occasions. As can been seen in Fig. 2, there was a greater number of viable GPX5 cells compared with control cells at all time points with concentrations of H_2O_2 from 0.5 to 1.5 mM, with the data being very highly statistically significant (GPX5 compared with pEE/CHO-K1 cells at all time points with 0.5 mM H_2O_2 $P < 0.005$; at all time points with 0.8–1 mM H_2O_2; 2 h with 1.5 mM H_2O_2, $P < 5 \times 10^{-7}$; 6 h and 24 h with 1.5 mM H_2O_2, $P < 0.005$). Similar results were obtained when cells were challenged with 0–1.5 mM *tert*-butyl hydroperoxide and when cells were analysed using the trypan blue exclusion assay (data not shown). Hence, GPX5 cells clearly have increased resistance to oxidative challenge. These data validated our *in vitro* system for further studies investigating how GPX5 protects cells from oxidative challenge.

Secreted soluble GPX5 does not protect cells against oxidative challenge by reducing peroxides

We next examined how the increased resistance of GPX5 cells to oxidative challenge might be attained. We reasoned that the secreted recombinant GPX5 might directly reduce the organic peroxide added to the culture medium. To examine this GPX5 and pEE cells were cultured for 16 h and their culture medium, termed GPX5- and pEE control-conditioned medium, was collected. GPX5, pEE and CHO-K1 cells were then incubated in GPX5- or control-conditioned medium supplemented with 0–1 mM H_2O_2 for 2 h, followed by analysis of viability using the MTT assay. As can be seen in Fig. 3A GPX5 cells showed a similar level of viability when incubated in GPX5- or control-conditioned medium, with no statistical difference in the data ($n = 8$). The viability of both CHO-K1 and pEE cells was also not statistically different when incubated in GPX5- or control-conditioned medium, but again was markedly less than that of the GPX5 cells on challenge with 0.8 and 1 mM H_2O_2 (GPX5 cells compared with pEE/CHO-K1 cells $P < 5 \times 10^{-5}$). The experiment was repeated on three separate occasions. Hence, soluble recombinant GPX5 does not protect control cells from oxidative challenge.

To directly determine whether recombinant GPX5 was able to reduce peroxide, medium from cultured GPX5 cells was analysed for enzyme activity using an *in vitro* glutathione peroxidase assay. As background oxidation of NADPH occurred with H_2O_2 as substrate in this assay, we used the alternative non-physiological but conventional substrate *tert*-butyl hydroperoxide. GPX1 was used for comparison as a positive control. No peroxidase activity was observed with GPX5 medium (data not shown). As we were concerned that the concentration of recombinant GPX5 in the culture medium might be below the level of detection of the assay, we increased the concentration by ion exchange chromatography. Following concentration of the GPX5 containing fractions, the only visible contaminating band detected on a Coomassie stained gel was albumin. The proportion of albumin to recombinant GPX5 was taken into account when estimating the concentration of GPX5. Concentration-dependent activity was recorded for GPX1 but again no activity was detected for recombinant GPX5. Figure 3B shows the activity for 100 ng of GPX1 and 1000 ng of recombinant GPX5 ($n = 3$). Finally to ensure there was no inhibitory derivative from the culture medium, the assay was repeated using GPX1 diluted in the buffer containing GPX5. The activity of GPX1 was not altered (data not shown).

GPX5-expressing cells have reduced levels of lipid peroxidation following oxidative challenge

We next explored the possible mechanism by which the GPX5 cells were protected from oxidative damage. *In vivo* GPX5 associates with sperm membranes and is hypothesized to protect them from such oxidative damage. Furthermore, many Se-independent GPXs outside the mammalian kingdom predominantly act as phospholipid hydroperoxidases (Beeor-Tzahar *et al.*, 1995; Tang *et al.*, 1995, 1996; Faltin *et al.*, 1998;

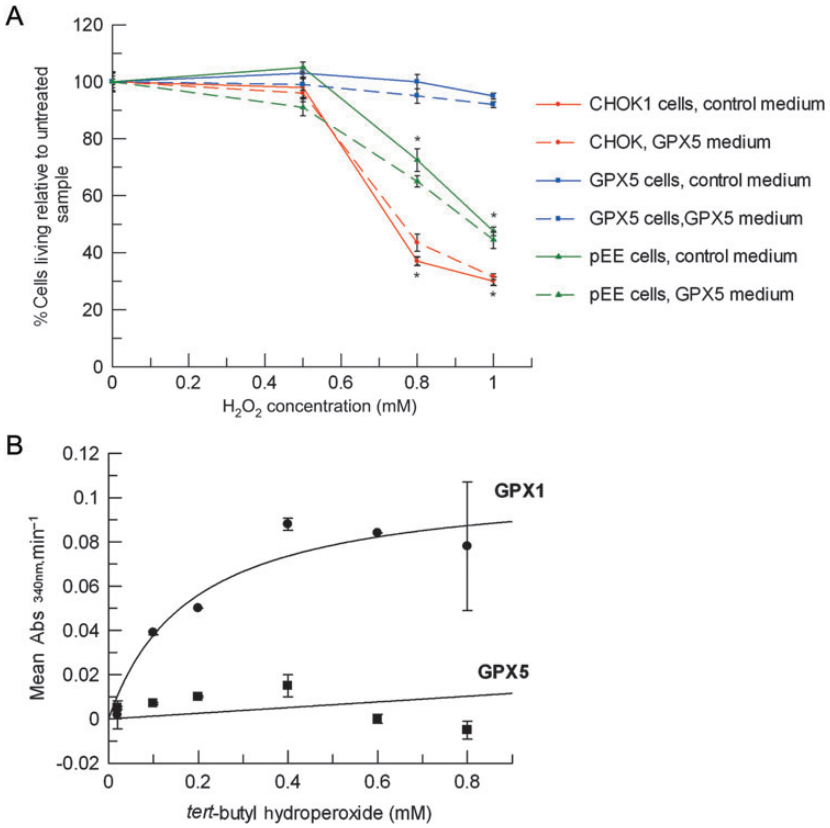

Figure 3 Secreted, soluble recombinant GPX5 does not confer resistance to oxidative challenge by reducing peroxide. (**A**) CHO-K1, pEE and GPX5 cells were incubated in GPX5- or pEE-conditioned medium with 0–1 mM H_2O_2 for 2 h at 37°C before analysis of viability using the MTT assay. Data are shown as the mean Abs_{550nm} expressed as a % of Abs_{550nm} from 0 mM H_2O_2-treated sample for each cell type ($n = 8 \pm$ SEM). *$P < 5 \times 10^{-5}$ for GPX5 cells compared with pEE/CHO-K1 cells. Data are presented from a single experiment and each experiment was repeated three times. (**B**) Activity of GPX1 (100 ng) and recombinant GPX5 (1000 g) was assayed with 3 mM GSH and 0.02–0.8 mM *tert*-butyl hydroperoxide and ΔAbs_{340nm} monitored for 10 min. GPX-independent *tert*-butyl hydroperoxide detoxification was measured in parallel assays, and GPX assay data were corrected for this activity ($n = 3 \pm$ SEM). Data were fitted to a Michaelis–Menton equation using Grafit (Erithacus Software Ltd).

Tripp *et al.*, 1998; Avery and Avery 2001; Herbette *et al.*, 2002; Gaber *et al.*, 2004). We therefore looked at the correlation between GPX5 expression and levels of lipid peroxidation, in cells following oxidative challenge, using the fluorescent probe C11-BODIPY$^{(581/591)}$ to determine whether GPX5 protects the cells from lipid peroxidation. C11-BODIPY$^{(581/591)}$ is a thermal- and photo-stable lipophilic fluorescent probe that incorporates readily into cell membranes and can be used to detect lipid peroxide formation. It contains a high degree of conjugated double bonds that render it susceptible to oxidation revealed as a decay in emission (Pap *et al.*, 1999) at 592 nm and a simultaneous increase in emission at 513 nm that displays first order kinetics (Naguib, 1998). The loss in fluorescence at 592 nm reflects indirectly the oxidation of unsaturated fatty acids (Borst *et al.*, 2000) with the peroxidation rate comparable to that of endogenous fatty acids (Pap *et al.*, 1999).

GPX5 and pEE cells were labelled with C11-BODIPY$^{(581/591)}$ and subjected to oxidative challenge with 0.5 or 1 mM H_2O_2 or 100 μM or 500 μM cumene hydroperoxide for 1 h at 37°C ($n = 3$). Cumene hydroperoxide was included as a radical-generating system for this assay as it easily penetrates the lipid moiety of biological membranes and as such is very well suited to study lipid peroxidation in cellular systems (Drummen *et al.*, 2004). Significantly, more lipid peroxidation was detected in pEE cells compared with GPX5 cells, as determined by oxidation of C11-BODIPY$^{(581/591)}$, at both concentrations of H_2O_2 and cumene hydroperoxide (Fig. 4A). The levels of C11-BODIPY$^{(581/591)}$ oxidation in pEE cells challenged with 100 and 500 μM cumene hydroperoxide were 2.01 ± 0.36- and 2.87 ± 0.12-fold greater, respectively, than in untreated pEE cells compared with being 1.25 ± 0.18 and 1.63 ± 0.21 greater in treated GPX5 cells than in untreated GPX5 cells. Levels of oxidation in pEE cells challenged with 0.5 and 1 mM H_2O_2 were 1.4 ± 0.06- and 1.49 ± 0.1-fold greater than in untreated cells compared with being 0.82 ± 0.04 and 1.07 ± 0.12 greater in H_2O_2-treated GPX5 cells than in untreated GPX5 cells (Fig. 4A).

In addition, oxidation of C11-BODIPY$^{(581/591)}$ was observed using fluorescence microscopy, which provides information on intracellular localization of the reporter. GPX5 and pEE cells were labelled with C11-BODIPY$^{(581/591)}$ and cell images were recorded before and 30 min after addition of 1 mM H_2O_2. Predominant staining with C11-BODIPY$^{581/591}$ was seen in the perinuclear region of cells (Fig. 4B) with staining reminiscent of plasma membranes also being observed (Fig. 4B arrow). Image enhancement with over illumination

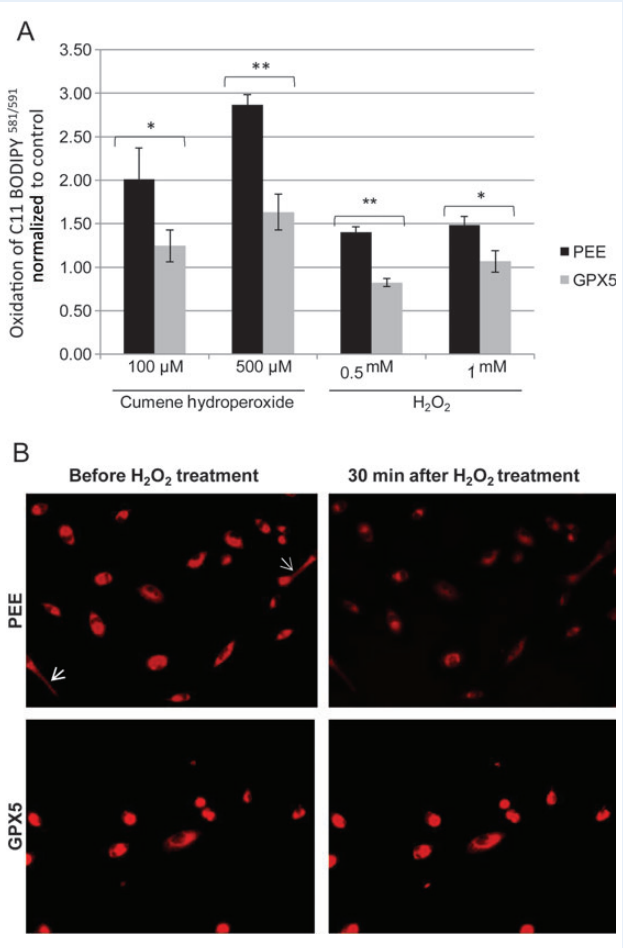

Figure 4 Comparison of C11-BODIPY$^{581/591}$ oxidation in GPX5-expressing and control cells. Cells were labelled for 30 min at 37°C with 10 mM C11-BODIPY$^{581/591}$. (**A**) Oxidation was induced by addition of cumene hydroperoxide (100 μM and 500 μM) or H_2O_2 0.5 mM and 1 mM) for 1 h at 37°C and fluorescence intensity was measured at green (495/513 nm) and red (542/592 nm) wavelengths. Oxidation of C11 BODIPY$^{581/591}$ is expressed as the ratio between green fluorescence (oxidized) and total fluorescence (oxidized plus reduced). Data from cells were normalized to the respective controls without oxidative challenge. Data were compared using the Student's unpaired *t*-test. Values are means with their standard errors depicted by vertical bars. Statistical significance was considered when *P* was <0.05* or 0.005**; $n = 3$. (**B**). Fluorescence micrographs of C11-BODIPY$^{581/591}$ labelled pEE and GPX5 cells. Images were recorded before and 30 min after addition of 1 mM H_2O_2. Predominant staining with C11-BODIPY$^{581/591}$ is seen in the perinuclear region of cells, with staining reminiscent of plasma membranes also observed (arrow). Images were captured using a Leica TCS-NT confocal laser scanning microscope attached to a Leica DM RBE upright epifluorescence microscope with phase contrast and a TRITC filter. V4.0.1 (Improvision) was used for compiling images. All images were taken with an exposure time of 50 ms.

of the perinuclear region revealed similarly stained regions in other cells (data not shown). No detectable cytosolic or nuclear staining was observed. Following incubation with H_2O_2, C11-BODIPY$^{(581/591)}$ was clearly oxidized in pEE but was oxidized to a lesser extent in GPX5 cells as determined by the observed reduction in red fluorescent intensity.

GPX5 protects cells from oxidative DNA damage

Lipid peroxidation generates highly reactive aldehydes that are a major source of DNA damage (Marnett, 2002) with 8-oxodG being the most common lesion produced. As our GPX5 cells had lower levels of lipid peroxidation on oxidative challenge compared with control cells, we next compared the level of 8-oxodG in these cells following oxidative challenge to determine whether the expression of GPX5 also protects cells from such downstream DNA mutation. Cells were incubated with 500 μM H_2O_2 for 1 h followed by detection of 8-oxodG with a specific antibody. As illustrated in Fig. 5, the level of 8-oxodG was markedly increased in both pEE and CHO-K1 control cells compared with GPX5 cells after oxidative challenge. An overlay of DAPI stained nuclei images (red) with the respective 8-oxodG images (green) clearly demonstrates DNA lesions in the nuclei.

GPX5 can be transferred to sperm via epididymosomes

It has previously been shown that a number of proteins synthesized by epididymal epithelial cells are transferred to the sperm membrane via small membranous vesicles called epididymosomes, which originate from the formation of apical cytoplasmic blebs that detach from the cells (Frenette *et al.*, 2002; Sullivan *et al.*, 2007; Frenette *et al.*, 2010). Epididymosomes have been identified in the epididymal fluid of many mammalian species including bull (Frenette and Sullivan, 2001; Frenette *et al.*, 2002), hamster (Yanagimachi *et al.*, 1985), human (Frenette *et al.*, 2005), mouse (Rejraji *et al.*, 2006) ram (Gatti *et al.*, 2005) and rat (Fornes *et al.*, 1995; Eickhoff *et al.*, 2001). GPX5 has been shown to be associated with epididymosomes in the mouse (Rejraji *et al.*, 2002), hence it may also be transferred to sperm via these vesicles. We therefore performed preliminary experiments to address this hypothesis.

Sperm from the IS of the rat epididymis, a location proximal to the caput epididymis where GPX5 is synthesized, do not have GPX5 associated. To determine whether GPX5 can be transferred to rat sperm via epididymosomes, IS sperm were incubated with either rat caput epididymosomes or with the epididymal fluid supernatant following isolation of epididymosomes. Association of GPX5 with the isolated epididymosomes as well as in the soluble epididymal fluid fraction was verified (Fig. 6). Following incubation, GPX5 was detected on rat IS sperm incubated with epididymosomes but not those incubated with the soluble GPX5 fraction (Fig. 6). GPX5 was not detected on CHO-K1 cells following incubation with epididymosomes (data not shown), presumably due to lack of the necessary interacting partner on these cells.

Expression of thioredoxin and thioredoxin reductase in the rat epididymis

Finally, as GPX5 can clearly protect cells from oxidative damage but does not exhibit activity in a glutathione peroxidase assay, we reasoned that it may use an alternate electron donor. Thioredoxin is utilized as electron donor by a number of Se-independent GPXs and by mammalian GPX3, and both thioredoxin and thioredoxin reductase are secreted by

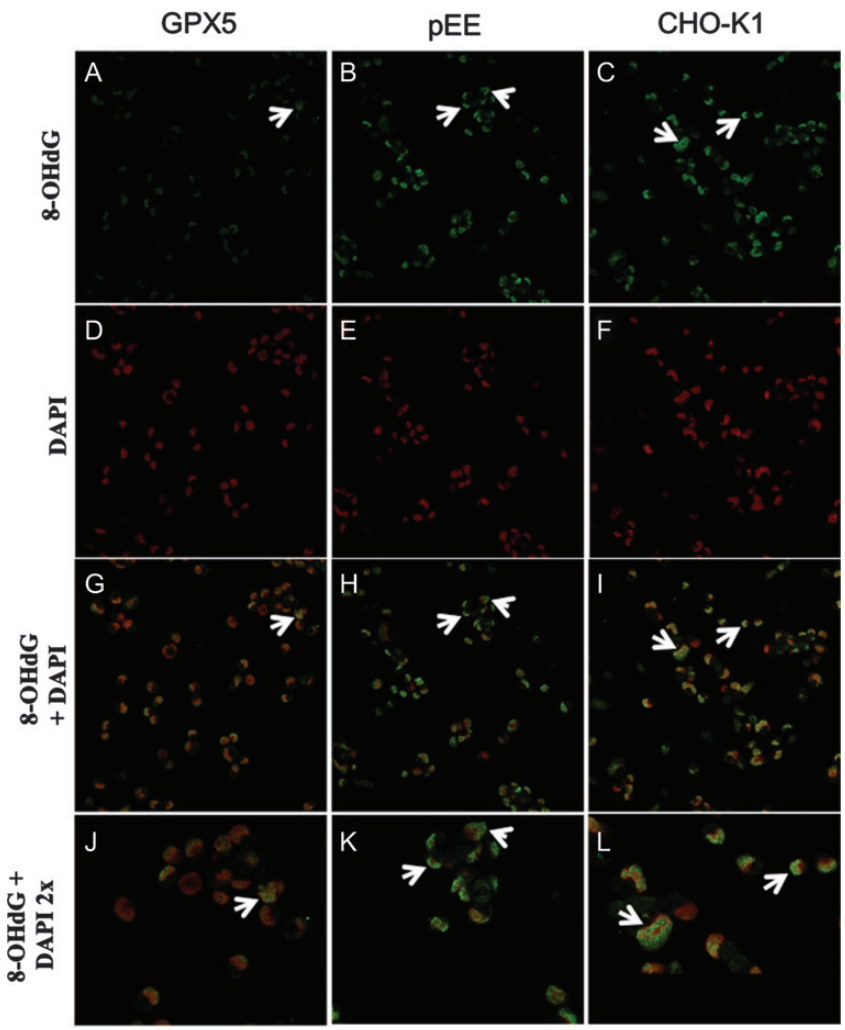

Figure 5 Detection of 8-oxodG in H_2O_2-treated GPX5 and control cells. GPX5 (**a**, **d**, **g**, **j**), pEE (**b**, **e**, **h**, **k**) and CHO-K1 (**c**, **f**, **i**, **l**) cells were incubated for 1 h with 500 μM H_2O_2. Then 8-oxodG was detected with an 8-OHdG monoclonal antibody (a, b, c) and nuclei were stained with DAPI (d, e, f). Image overlays of 8-oxodG and DAPI staining were generated (g, h, i). Field of view: 246 × 246 μm. Images j, k and l show a further 2× magnification of the respective overlay images. Images were obtained by confocal laser scanning microscopy using a Leica TCS SP5-II AOBS confocal system attached to a Leica DMI 6000 CS AFC microscope with an Ar laser (488 nm) and 405 nm diode laser for excitation. DAPI nuclear stain is in red. Arrows indicate examples of cells with 8-oxodG staining within the nucleus.

mammalian cells (Rubartelli *et al.*, 1992; Sahaf *et al.*, 1997; Di Trapani *et al.*, 1998; Soderberg *et al.*, 2000); however, their expression in the male reproductive tract has not been determined. We therefore determined the expression of both thioredoxin and thioredoxin reductase in epididymal cells, and secretion of thioredoxin reductase into the epididymal fluid. Transcripts for both thioredoxin and thioredoxin reductase were detected (Fig. 7A), as was the reductase in the epididymal fluid (Fig. 7B). Rat liver and testis cDNA and the total protein were included as controls.

Discussion

In this study, we have used an *in vitro* system to investigate the role of GPX5 as a physiological antioxidant. We generated a mammalian cell line expressing recombinant GPX5 and show that GPX5-expressing cells have increased resistance to an administered oxidative challenge. More specifically, using the lipophilic reporter molecule C11-BODIPY$^{(581/591)}$, we show that GPX5 cells had decreased levels of lipid peroxidation and decreased levels of the downstream DNA lesion 8-oxodG than control cells following challenge. In addition, we show that GPX5 can be transferred to rat sperm via epididymosomes, membranous vesicles originating from the epididymal epithelial cells that synthesize the enzyme.

During epididymal transit, sperm are vulnerable to attack from both intracellular and extracellular ROS (Vernet *et al.*, 2004) and are reliant on antioxidant scavengers synthesized by the epididymis for protection. Our data support a significant role for GPX5 as part of this protective system. In addition, as GPX5-expressing cells have increased protection from lipid peroxidation, it is tempting to speculate that GPX5 acts as a phospholipid hydroperoxidase. Such a function would not be

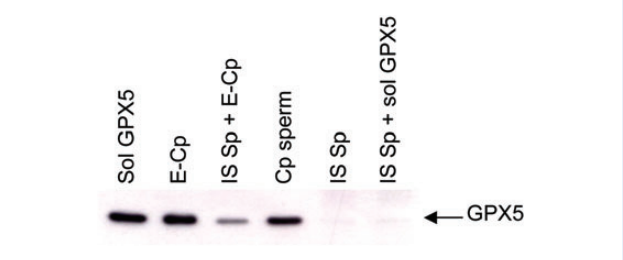

Figure 6 Transfer of GPX5 to rat sperm via epididymosomes. Western blot of initial segment rat sperm (IS Sp) incubated for 4 h at 37°C with epididymosomes isolated from the caput epididymis (E-Cp) or with the epididymal fluid remaining after collection of epididymosomes, which contain soluble (sol) GPX5 probed with GPX5 polyclonal antibody followed by HRP-conjugated secondary antibody and ECL. Caput (Cp) sperm were included as a positive control.

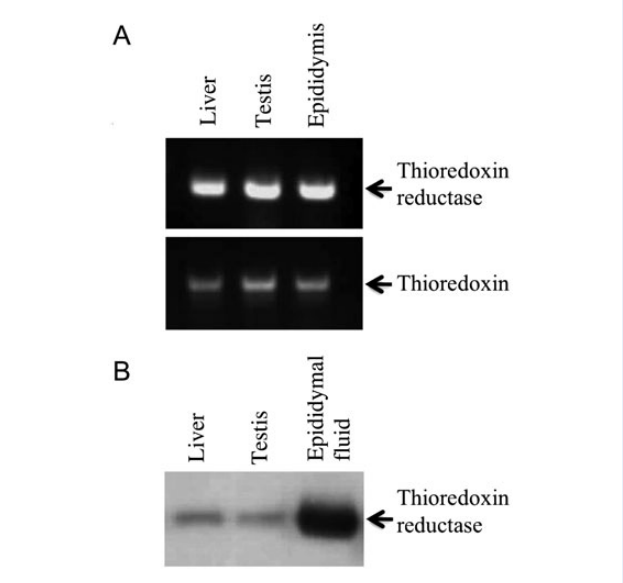

Figure 7 Expression of thioredoxin reductase and thioredoxin in the rat epididymis. (**A**) PCR amplification of rat liver, testes and epididymis cDNA with thioredoxin reductase or thioredoxin-specific primers. (**B**) Western blot of 5 μg of the total protein from rat liver, testes and epididymal fluid probed with thioredoxin reductase polyclonal antibody followed by HRP-conjugated secondary antibody and ECL.

unexpected as GPX5 associates with sperm membranes as they transit the epididymis and the related Se-independent GPXs from other organisms act predominantly as phospholipid hydroperoxidases (Beeor-Tzahar *et al.*, 1995; Tang *et al.*, 1995, 1996; Faltin *et al.*, 1998; Tripp *et al.*, 1998; Avery and Avery, 2001; Herbette *et al.*, 2002; Gaber *et al.*, 2004).

In keeping with this, we also observed that a substantial proportion of endogenous recombinant GPX5 in the GPX5-expressing cells was associated with the membrane fraction following sub-cellular fractionation (data not shown). However, exogenous recombinant GPX5 did not associate with the cell membrane of control cells following incubation and control cells incubated with exogenous recombinant GPX5 were not afforded the increased protection against oxidative challenge seen for the GPX5 cells. It is possible that GPX5 requires membrane association or proximity for activity, although the mechanism of membrane association is unclear. Interestingly, our data suggest that association of GPX5 with sperm membranes *in vivo* may be mediated via epididymosomes, rather than directly from the soluble fraction in the epididymal fluid. Transfer of other proteins synthesized by epididymal epithelial cells to sperm via epididymosomes has previously been described (Frenette *et al.*, 2002; Sullivan *et al.*, 2007; Frenette *et al.*, 2010); however, the mechanisms involved have not yet been elucidated.

In addition, the protection afforded to the GPX5-expressing cells on oxidative challenge may be attributed to endogenous recombinant GPX5 utilizing an alternative electron donor to glutathione, explaining its lack of activity in the conventional glutathione peroxidase assay (present study and Okamura *et al.*, 1997). Indeed, most of the amino acids predicted to bind glutathione in cytosolic GPX1 (Rubartelli *et al.*, 1992) are substituted in GPX5. Moreover, the concentration of glutathione in the epididymal fluid (~20 μM) is at least two orders of magnitude lower than the concentration used for *in vitro* glutathione peroxidase assays (3–4 mM) and than intracellular levels (1–10 mM; Rubartelli *et al.*, 1992), making its use as electron donor for GPX5 in the epididymis questionable. In addition, other, non-mammalian Se-independent GPXs show preference for electron donors other than glutathione, with a number utilizing thioredoxin as reducing equivalent (Di Trapani *et al.*, 1998; Soderberg *et al.*, 2000; Leisinger *et al.*, 2001). As a prelude to investigating thioredoxin as an alternative electron donor for GPX5, we show that both thioredoxin and thioredoxin reductase are expressed by the epididymis.

Overall, our data strongly support a role for GPX5 as an important antioxidant. Such a role for GPX5 may be pertinent to human fertility, as we have previously shown that the majority of human GPX5 transcripts are aberrantly spliced, containing an 118 bp frame shift deletion (Hall *et al.*, 1998), and likely subject to nonsense-mediated decay. Although GPX5 protein should be translated from the very small amount of full-length transcript available, protein levels are clearly so low that they were undetectable on human sperm and in human epididymal fluid and seminal plasma by the approaches used by both us and others (Dacheux *et al.*, 2006). We postulate that such a severe reduction in GPX5 contributes to the particular vulnerability of human sperm to oxidative stress. Indeed, the quality of human spermatozoa is notoriously poor compared with all other mammalian species (Aitken and Sawyer, 2003) with the most common cause of sperm dysfunction and DNA damage being oxidative stress (Aitken and Koppers, 2011).

Interesting parallels can be drawn between human sperm and sperm from GPX5$^{-/-}$ mice (Chabory *et al.*, 2009). Human sperm exhibit similar traits of oxidative DNA damage to those of GPX5$^{-/-}$ mice and also exhibit a positive correlation between oxidative DNA damage and age (Trisini *et al.*, 2004; Moskovtsev *et al.*, 2006). It has been shown in an animal model of ageing that the activity of sperm antioxidant scavengers decrease with age (Weir and Robaire, 2007) resulting in an increased risk of epididymal sperm to oxidative damage. As in the GPX5$^{-/-}$ mouse, such stress factors may be exacerbated in ageing humans with sperm integrity further compromized by the lack of GPX5. However, it is important to note that other factors also contribute to human sperm DNA damage and poor sperm quality (reviewed by Aitken and De Iuliis, 2010), for example, deficient protamination coupled

with unresolved DNA strand breaks during chromatin remodelling and defects in cell remodelling, during spermiogenesis. Coupled with abortive apoptosis such defective sperm persist in the epididymis, and are inherently even more susceptible to oxidative stress. Furthermore, lifestyle factors such as diet contribute to sperm quality and fertility in ageing men. A study by Schmid *et al*. (2012) demonstrated that, in older men, a higher intake of certain antioxidants and micronutrients is associated with a decreased incidence of DNA damage, possibly by reducing oxidative stress in the epididymis.

For humans, as for the GPX5$^{-/-}$ mouse, the clinical consequences of sperm damage are reflected in the increased incidence of subfertility, spontaneous abortion, developmental defects and pathologies in offspring with advanced paternal age (Schmid *et al*., 2007). Together with previous data (Hall *et al*., 1998; Chabory *et al*., 2009), our results support an important role for GPX5 participating in the maintenance of such cell and DNA integrity.

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Authors' roles

A.T. performed experiments and analysed data. A.R., B.C.H. and C.A.J. performed experiments and assisted with data analysis. W.C.L.F. assisted in manuscript preparation and analysis of data and provided critical discussion. J.F. designed the study, performed experiments, analysed data and prepared the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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