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Minireview

Physiological role of phospholipid hydroperoxide glutathione peroxidase in mammals

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Abstract

The redox enzyme phospholipid hydroperoxide glutathione peroxidase (PHGPx) has emerged as one of the most significant selenoenzymes in mammals, corroborated by early embryonic lethality of PHGPx null mice. PHGPx is one of five selenium-dependent glutathione peroxidases and the second glutathione peroxidase to be discovered in 1982. PHGPx has a particular position within this family owing to its peculiar structural and catalytic properties, its multifaceted roles during male gametogenesis, and its necessity for early mouse development. Interestingly, mice devoid of endogenous glutathione die at the same embryonic stage as PHGPx-deficient mice compatible with the hypothesis that a similar phenotype of embryonic lethality may be provoked by PHGPx deficiency and lack of its reducing substrate glutathione. Various gain- and loss-of-function approaches in mice have provided some insights into the physiological functions of PHGPx. These include a protective role for PHGPx in response to irradiation, increased resistance of transgenic PHGPx mice to toxin-induced liver damage, a putative role in various steps of embryogenesis, and a contribution to sperm chromatin condensation. The expression of three forms of PHGPx and early embryonic lethality call for more specific studies, such as tissue-specific disruption of PHGPx, to precisely understand the contribution of PHGPx to mammalian physiology and under pathological conditions.

Keywords: glutathione peroxidase 4; lipid peroxidation; selenoprotein; spermatogenesis; thiol peroxidase.

Introduction

A quarter of a century ago, Ursini et al. (1982) purified a protein from pig liver displaying peroxidative activity towards phosphatidylcholine in liposomes and biomembranes in the presence of glutathione (GSH). This peroxidation-inhibiting protein, designated as phospholipid hydroperoxide glutathione peroxidase (PHGPx, also called glutathione peroxidase 4), was later found to contain one mole of selenium per one mole of protein (Ursini et al., 1985), and to act as a monomer (Maiorino et al., 1991). After the gene encoding PHGPx was cloned, it became apparent that conserved motifs involved in its enzymatic activity are shared with glutathione peroxidases (GPx) (Schuckelt et al., 1991), even though the overall similarity is less than 40% compared to the other family members. In total, humans express five selenium-dependent glutathione peroxidases, whereas the recently discovered GPx6 is a Cys-containing variant in mice (Kryukov et al., 2003). Over the last couple of years, it has become increasingly clear that PHGPx is far more than a mere antioxidant device in cells and tissues, since it regulates a variety of cellular processes, including polyunsaturated fatty acid metabolism and cellular life and death decisions, and contributes to sperm development at various steps. Thus, it seems pertinent to review what further knowledge will be obtained through analyses of existing and forthcoming transgenic mouse models for PHGPx, including RNA interference approaches.

Biochemical and cellular functions of PHGPx

PHGPx differs significantly from other family members in terms of its monomeric structure, its limited overall homology with the homotetrameric glutathione peroxidases, its unique function in decomposing complex lipid hydroperoxides, its less restricted dependence on glutathione as a reducing substrate, and its indispensable role in early mouse development; key findings on PHGPx function are summarised in Table 1.

PHGPx shares common motifs with the other glutathione peroxidases, such as clusters involved in the active site and the catalytic triad, consisting of selenocysteine (Sec), glutamine and tryptophan. PHGPx displays a similar catalytic mechanism to the other glutathione peroxidases, involving oxidation of selenolate to selenenic acid, reduction thereof by GSH to a selenodisulfide intermediate, and regeneration by a second GSH substrate to the ground state (Ursini et al., 1995). Regarding its reducing and oxidising substrates, PHGPx has much broader substrate specificity than the other members of the glutathione peroxidase family. Besides hydroperoxide and lipid hydroperoxide, both common substrates for all glutathione peroxidases, only PHGPx reduces phospholipid-associated hydroperoxides in biological membranes to the corresponding alcohols. PHGPx activity does not rely on GSH as the sole reduc-

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Table 1 Summary of PHGPx functions identified *in vitro* and *in vivo.*

PHGPx	*In vitro*	Reference
Not specified	Selenocysteine is indispensable for full PHGPx activity	Maiorino et al., 1995
	Reduction of hydroperoxy ester lipids generated by 15-lipoxygenase	Banning et al., 2004
	Knock-down of PHGPx leads to arachidonic acid metabolism up-regulation by 12-lipoxygenase and cyclooxygenase 1	Chen et al., 2000, 2003
	PHGPx polymerisation in the presence of H_2O_2 and absence of glutathione *in vitro*	Mauri et al., 2003
Cytosolic	Dampening of interleukin-1-induced NFκB activation in endothelial cells	Brigelius-Flohe et al., 1997
	Impaired leukotriene formation in RBL-2H3 cells by overexpression of rat PHGPx	Imai et al., 1998
	Overexpression in RBL-2H3 cells confers moderate protection towards oxidative stress	Arai et al., 1999
	Overexpression causes lower prostaglandin D2 production in RBL-2H3 cells	Sakamoto et al., 2000
	Porcine PHGPx prevents NF-κB activation, oxLDL-induced proliferation and linoleic acid-induced apoptosis	Brigelius-Flohe et al., 2000
	Protection from oxidative stress-induced death of Burkitt's lymphoma cells	Brielmeier et al., 2001
Mitochondrial	Expression mainly in somatic tissue and during embryonic stages	Borchert et al., 2006; Pushpa-Rekha et al., 1995; Schneider et al., 2006
	Import into mitochondria requires a leader sequence	Arai et al., 1996
	Protection from lipid hydroperoxide-mediated cell injury	Yagi et al., 1996, 1998
	Overexpression in RBL-2H3 cells confers high resistance towards stress	Arai et al., 1999
	Migration from the matrix to the outermost membrane of mitochondria	Haraguchi et al., 2003
	Protection from hypoglycaemia-induced apoptosis	Imai et al., 2003
	Prevention of geranylgeranoic acid-induced apoptosis	Shidoji et al., 2006
Nuclear	Occurrence mainly in sperm nuclei (snGPx)	Godeas et al., 1996; Pfeifer et al., 2001
	Expression in late spermatids; nucleolar localisation	Borchert et al., 2003; Maiorino et al., 2003; Moreno et al., 2003; Nakamura et al., 2003
	Ex vivo/in vivo	
PHGPx$^{-/-}$ mice	Embryonically lethal at gestational day 7.5	Imai et al., 2003; Yant et al., 2003
PHGPx$^{+/-}$ mice/cells	Decreased life span after exposure to irradiation/increased susceptibility to oxidative and genotoxic stress	Yant et al., 2003
Not specified	Purification of PHGPx as lipid peroxidation-inhibiting enzyme in pig liver	Ursini et al., 1982
	PHGPx is a selenoprotein/monomeric structure	Maiorino et al., 1991; Ursini et al., 1985
	High expression in testis/spermatogeneic cells	Maiorino et al., 1998; Roveri et al., 1992
	Major structural protein of mitochondrial capsule in sperm midpiece	Ursini et al., 1999
	Possible involvement in 12-lipoxygenase pathway in human platelets	Sutherland et al., 2001
	Transgenic mice overexpressing human PHGPx are protected against oxidative stress	Ran et al., 2004
Cytosolic	Overexpression in pancreatic tumour cell lines leads to growth inhibition in tumour transplantation experiments	Liu et al., 2006
Mitochondrial	Knock-down in isolated and *ex vivo* cultivated wild-type embryos causes increased degeneration of neuronal cells at midgestation	Borchert et al., 2006
	Overexpression in pancreatic tumour cell lines leads to growth inhibition in tumour transplantation experiments	Trachootham et al., 2006
Nuclear	Knock-out mice are fully viable and fertile, but display retarded chromatin condensation of male germ cells	Conrad et al., 2005
	Knock-down in isolated and *ex vivo* cultivated wild-type embryos causes abnormal heart formation at midgestation	Borchert et al., 2006

ing equivalent; it also reacts with thiols from proteins, in particular when GSH is no longer available, such as in late spermatogenic cells (we refer readers to the comprehensive review article on PHGPx and spermatogenesis by L. Flohé in this issue) (Ursini et al., 1997; Ursini et al., 1999; Roveri et al., 2001; Mauri et al., 2003). An *in vitro* mutational and biochemical approach by Maiorino and colleagues revealed that Sec to Cys conversion leads to strongly impaired PHGPx activity by approximately three orders of magnitude (Maiorino et al., 1995; Ursini et al., 1995). Similar findings have been reported for other selenoproteins such as thioredoxin reductases (Lee et al., 2000; Zhong et al., 2000).

PHGPx protects cells against various apoptotic triggers. For instance, cells overexpressing PHGPx are highly resistant to hydroperoxide-mediated injury, including dilinoleoyl phosphatidylcholine monohydroperoxide, linoleic acid hydroperoxide (Yagi et al., 1996, 1998), geranylgeranoic acid (Shidoji et al., 2006), linoleic acid (Brigelius-Flohe et al., 2000), and *tert*-butylhydroperoxide

(Arai et al., 1999). In particular, cells expressing the mitochondrial form were shown to be more resistant to the effects of inhibition of the respiratory chain by rotenone and KCN, whereas cells expressing the short form failed to do so (Arai et al., 1999). Overexpression of mitochondrial, but not cytosolic PHGPx suppressed hypoglycaemia-induced apoptosis in RBL-2H3 cells (Nomura et al., 2000). PHGPx was also identified in an expression cloning approach as an enzyme protecting Burkitt's lymphoma cells from oxidative stress-induced cell death brought about by seeding the cells at low cell density in culture (Brielmeier et al., 2001).

Beyond its antioxidant/antiapoptotic activity, overexpression of PHGPx was shown to dampen interleukin-1-induced NF-κB activation (Brigelius-Flohe et al., 1997), and to counteract oxidised low-density lipoprotein-stimulated proliferation of rat aortic smooth muscle cells (Brigelius-Flohe et al., 2000). Overexpression of PHGPx in a pancreatic tumour cell line with low endogenous PHGPx levels resulted in strong growth inhibition by approximately 80–95% (Trachootham et al., 2006). Similarly to the other glutathione peroxidases, PHGPx has been considered to govern arachidonic acid-metabolising enzymes, such as lipoxygenases (Lox) and cyclooxygenases (Cox). Both types of enzymes require peroxides and/or hydroperoxy intermediate metabolites for activation and full activity (Ivanov et al., 2005), referred to as the cellular peroxide tone (Weitzel and Wendel, 1993; Smith, 2005). PHGPx might represent an ideal candidate to counteract Lox and Cox activities by regulating the cellular peroxide tone. Early evidence of an *in vivo* antagonistic role for PHGPx in arachidonate metabolism was provided by selenium depletion and repletion studies, indicating that down-regulation of PHGPx due to selenium depletion was inversely correlated with Lox activity (Weitzel and Wendel, 1993). PHGPx knock-down in a human carcinoma cell line leads to up-regulation of 12-Lox and Cox 1 (Chen et al., 2003), whereas overexpression of PHGPx impairs arachidonic acid metabolism and leukotriene secretion (Imai et al., 1998; Chen et al., 2000; Sakamoto et al., 2000). PHGPx has been shown to control 5-, 12-, and 15-Lox activities (Weitzel and Wendel, 1993; Sutherland et al., 2001; Banning et al., 2004).

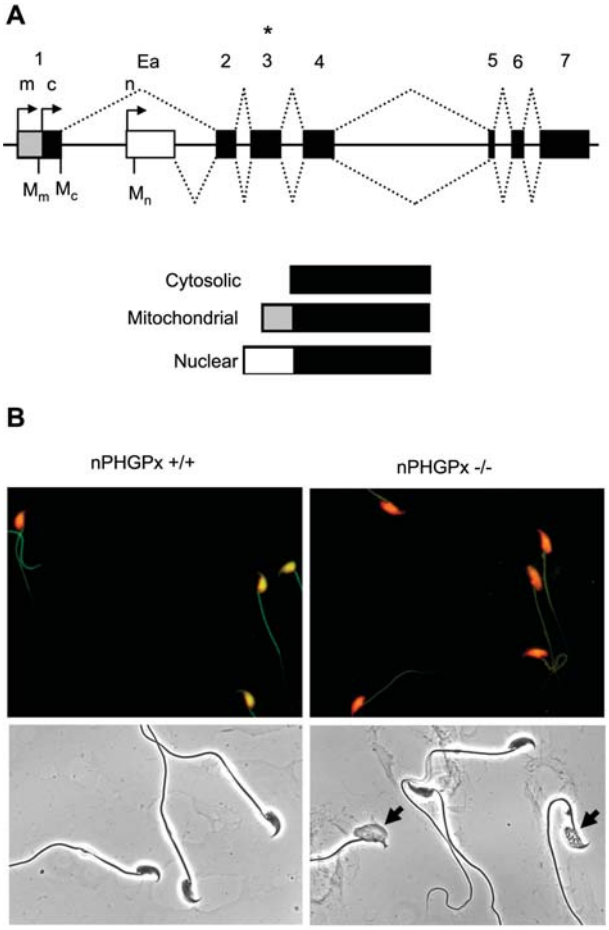

Figure 1 Targeted disruption of the nuclear form of PHGPx in mice.
(A) The wild-type PHGPx gene consists of seven ('classical') exons and one alternative exon (Ea) (upper part). The Sec codon is localised on exon 3 (asterisks), and the SECIS element is encoded by exon 7. Dashed lines indicate splicing of the mitochondrial (m), cytosolic (c) (above) and nuclear forms (below). Transcription initiation sites for the three forms are depicted with arrows and translational start codons with M. The cytosolic, mitochondrial and nuclear forms differ only in their N-termini. (B) Isolated spermatozoa from caput epididymis of mice lacking nuclear PHGPx show delayed chromatin condensation as determined by acridine orange staining (upper part; red and green fluorescence indicates partially or fully condensed sperm DNA, respectively) and lesser resistance to physical stress imposed by drying and hydration (arrows) (Conrad et al., 2005).

PHGPx expression in mice

Cloning and sequencing of the porcine PHGPx gene initially revealed the existence of seven exons, with exon 3 harbouring the selenocysteine (Sec) codon and exon 7 encoding the *cis*-acting selenocysteine insertion sequence (SECIS) element (Figure 1A) (Brigelius-Flohe et al., 1994). Depending on which transcription initiation site in exon 1 is used, either the mitochondrial form (also called the long form) or the cytosolic form (short form) is produced. S1 nuclease protection experiments indicated that the cytosolic form of PHGPx prevails in adult somatic tissues, whereas the mitochondrial form is mainly present in testicular tissue (Pushpa-Rekha et al., 1995). Recently, gene expression studies using different techniques produced somewhat controversial results. In one study, expression of mitochondrial PHGPx was detected throughout the embryonic stages and in neonatal brain and cardiac tissue (Borchert et al., 2006), whereas the second study showed that expression of the mitochondrial form was mainly confined to testis (Schneider et al., 2006).

Purification of a 34-kDa selenoprotein from rat epididymal sperm and protein sequencing and database analyses unravelled the existence of an alternative exon (Ea) localised in intron 1 of the PHGPx gene (Figure 1A) (Pfeifer et al., 2001). Since the 34-kDa form was found to be present only in late sperm nuclei, as shown by tracing rats with [^{75}Se], it was named sperm nuclei-specific glutathione peroxidase (snGPx). These findings corroborated previous immunogold studies that had suggested the presence of PHGPx in sperm nuclei (Godeas et al., 1996),

although it was unclear at that time that it was the nuclear form of PHGPx. Since expression of the nuclear form of PHGPx in tissues other than sperm nuclei could not be formally excluded, we thus refer to it as the nuclear form of PHGPx (nPHGPx) in the following. RT-PCR analyses, immunoblotting and immunohistochemistry suggested expression of the nuclear form during mouse development and to a minor extent in adult kidney (Borchert et al., 2003, 2006). However, the conclusions drawn from immunoblotting and immunohistochemical studies should be regarded with caution. The antibody used throughout these studies recognised a protein of approximately 34 kDa in kidney (Borchert et al., 2003), yet this protein migrated at an isoelectric point of approximately 7, in contrast to the finding of Pfeifer et al. (2001), who reported that nPHGPx is a highly basic protein (pI>13).

Transcription initiation at the start of the alternative exon leads to expression of the nuclear form of PHGPx (Maiorino et al., 2003; Moreno et al., 2003), which differs from the cytosolic and mitochondrial forms only in its N-terminus (Figure 1A). nPHGPx displays a similar spatio-temporal expression pattern to the protamines. The coding region of the alternative exon confers nuclear (nucleolar) localisation of nPHGPx (Moreno et al., 2003; Nakamura et al., 2003), and the alternative exon encodes clusters rich in arginines and lysines, also reminiscent of protamines. Protamines are small (approx. 5 kDa), highly basic and cysteine-rich proteins that replace the majority of histones during the final steps of sperm maturation. Binding of protamines to DNA leads to dramatic condensation of sperm DNA. Subsequent oxidation of cysteines in protamines results in introduction of intra- and intermolecular disulfide bridges into protamines, ensuring a tightly packed male haploid genome. This protects sperm DNA from physical, chemical and mechanical stress-induced insults, including irradiation. Owing to the high homology of the N-terminal part of nPHGPx to protamines (approx. 50%), it may be hypothesised that the nuclear form of PHGPx binds to DNA in a manner similar to protamines (Vilfan et al., 2004), whereby nPHGPx is in close proximity to protamines. The coincidence of protamines and PHGPx in sperm nuclei, the virtual absence of GSH in late sperm cells and the less restricted substrate specificity for the oxidising substrate implies that the nuclear form of PHGPx may be the enzyme responsible for oxidising, and thus cross-linking, protamines. Data supporting this proposed model were obtained by measuring the extent of DNA condensation in sperm nuclei, indicating that sperm DNA from selenium-depleted rats was less resistant to heat and acid denaturation (see below) (Pfeifer et al., 2001).

Dissecting the physiological role of PHGPx *in vivo*

Transgenic mouse models to study the overall function of PHGPx

Reverse genetics techniques provided early insights into the importance of PHGPx in physiology and under pathological conditions in mice. Loss of exons 2–7 of the PHGPx gene or the entire gene results in early embryonic lethality at E7.5 (Imai et al., 2003; Yant et al., 2003); the reasons for embryonic death, however, still remain enigmatic. PHGPx, cytosolic thioredoxin reductase and mitochondrial thioredoxin reductase are the only selenoenzymes that have proven to be indispensable for mouse embryogenesis so far (Conrad et al., 2004; Jakupoglu et al., 2005). Only mice with targeted deficiency in the gene of the Sec-specific tRNA (Trsp) die somewhat earlier than PHGPx knockout mice (Bosl et al., 1997). Interestingly, mice specifically lacking γ-glutamyl-cysteine synthetase, the enzyme catalysing the rate-limiting step in GSH synthesis, fail to develop beyond E7.5 (Shi et al., 2000). This is compatible with the hypothesis that lack of PHGPx or of its reducing substrate glutathione results in the same lethal phenotype early in embryonic development. Thus, PHGPx may represent one of the most important enzymes not only within the selenoprotein family, but also within the GSH-dependent system.

Mouse embryonic fibroblasts (MEFs) with only one functional PHGPx allele are more susceptible to various stressors, such as *tert*-butyl-hydroperoxide, hydrogen peroxide, paraquat and γ-irradiation, which fits well with its discovery as a lipid peroxidation-inhibiting enzyme (Yant et al., 2003). Mice with one copy of the PHGPx gene have reduced PHGPx protein levels in tissues such as brain, heart, kidney, liver and testis, which are obviously sufficient to maintain full viability and male fertility. Only when challenged with relatively high doses of γ-irradiation do $PHGPx^{+/-}$ mice die earlier than their wild-type littermates, but this holds true only when mice are kept on a mixed C57BL/6/129SvJ background (Yant et al., 2003).

Gain-of-function approaches were used to investigate whether augmented PHGPx protein levels provide increased resistance to oxidative stress *in vivo*. Transgenic mouse lines were created with a genomic clone containing the human PHGPx gene plus 30 and 20 kb of the 5′- and 3′-flanking regions, respectively, that overexpress PHGPx (Ran et al., 2004). In fact, these mice were more resistant to diquat-induced liver damage due to decreased lipid peroxidation. Likewise, MEFs derived from the transgenic mice were more resistant to oxidative stress-induced cell death than those from wild-type mice. Importantly, the PHGPx transgene was able to rescue the lethal phenotype of $PHGPx^{-/-}$ mice (Ran et al., 2004).

Specific approaches for dissecting the physiological functions of PHGPx

The aforementioned approaches, however, did not allow dissection of the role of PHGPx in embryonic development or tissue development and function, and did not provide any insight into the individual roles of the specific isoforms of PHGPx. Thus, more focussed studies on the individual forms of PHGPx, including spatio-temporal disruption of PHGPx function, were required. The first approach to specifically address the individual role of one of the three forms of PHGPx was accomplished by targeted disruption of the nuclear form in mice (Conrad et al., 2005). Since expression of the nuclear form is driven by its own promoter (Figure 1A), removal of the alternative exon (Ea) abolishes nPHGPx expression without per-

turbing expression of the two other forms. Mice carrying a targeted deletion of the nuclear variant of PHGPx were born at a Mendelian ratio, ruling out any significant contribution of nPHGPx to murine development. This was not unexpected owing to the largely restricted expression of this form in testicular tissue (Moreno et al., 2003; Schneider et al., 2006). In contrast, wild-type embryos (isolated at E7.5 and cultivated for 3 days *ex vivo*) in which expression of the nuclear form was abrogated by siRNA-mediated knock-down apparently display features of cardiac malformation (Borchert et al., 2006), a finding that still has to be confirmed by studying nPHGPx null embryos at various gestational stages.

Besides full viability of nPHGPx-deficient mice, male knockout mice are fully fertile and isolated sperm did not reveal any morphological alterations, which was surprising and unexpected. Multiple malformations, especially in the head region and midpiece of mature spermatozoa, were frequently observed under severe selenium deficiency in rodents (Wu et al., 1973; Wallace et al., 1983a,b). Since PHGPx is highly expressed in testis and strongly retained after experimental selenium deprivation, it has been considered as the major molecular target for seleno-deficiency in mice and male infertility. The analysis of nPHGPx-deficient mice provided conclusive evidence that it is not the nuclear form of PHGPx that is essential for sperm development and male fertility. Despite this rather disappointing finding, nPHGPx-deficient mice served as an ideal tool to unmask its hypothetical protein thiol peroxidase function *in vivo*, an activity that had been predicted by Ursini and collaborators based on structural and biochemical data (Ursini et al., 1995; Roveri et al., 2001; Mauri et al., 2003). In fact, the grade of sperm DNA condensation (equivalent to protamine oxidation) was clearly diminished in nPHGPx knockout sperm derived from caput epididymis, as monitored by the resistance of DNA to heat and acid denaturation and treatment with detergents and physical stress (Figure 1B). Labelling of free thiol groups in sperm showed that knockout sperm had a significantly higher ratio of free sulfhydryl groups to oxidised cysteines. These experiments provided for the first time definitive proof that (n)PHGPx indeed harbours protein thiol peroxidase activity *in vivo* (Conrad et al., 2005). Besides its antioxidant and structural roles (Ursini et al., 1999), these findings add another distinct function to PHGPx in male gametogenesis as a protamine thiol peroxidase during sperm chromatin condensation. The question as to whether the observed delay in chromatin condensation might be of pathophysiological relevance can be addressed by monitoring the frequency of genetic abnormalities in offspring of nPHGPx$^{-/-}$ versus wild-type males that have been exposed to irradiation or DNA-damaging chemotherapeutic drugs.

Since we have been unable to define any phenotype in nPHGPx$^{-/-}$ mice other than delayed sperm condensation, the proposed role for nPHGPx in the development of other organs such as heart and kidney (Borchert et al., 2006) remains questionable and awaits confirmation in nPHGPx$^{-/-}$ mice.

In a first attempt to decipher the contribution of the mitochondrial form of PHGPx (mPHGPx) to early embryonic development, Borchert et al. (2006) have used a sequence specific for the 5′-region of mPHGPx mRNA to downregulate mPHGPx expression in *ex vivo* cultivated wild-type embryos using an RNA interference approach. They described that siRNA-mediated knockdown of mPHGPx resulted in minor microencephaly and abnormal hindbrain development caused by increased apoptosis.

Objectives

While many of the biochemical traits of PHGPx have been analysed in great detail in the past, many unanswered questions in terms of its physiological role remain. Conditional gene disruption of PHGPx function therefore seems most suitable for exploring its role in tissue development and homeostasis, as well as under pathological conditions. Equally important, it will provide a unique source of various *ex vivo* cellular knockout systems. Conditional gene disruption involves insertion of two loxP sites in intronic regions of the PHGPx gene, which leads to viable and normal offspring (Seiler et al., in preparation). Conditional ablation of PHGPx in various cells and tissues, however, will not distinguish between the roles of individual isoforms such as the mitochondrial and cytosolic forms of PHGPx, assuming from our present work that the nuclear form plays a negligible role, if any, in somatic tissues.

Targeted removal of mPHGPx might provide a unique tool to further confine PHGPx functions and to be able to unequivocally assign distinct functions to cytosolic and mitochondrial PHGPx in various cellular processes. Targeting of the mitochondrial form will be far more difficult compared to the nuclear form. The transcription initiation site for the cytosolic form lies within the mitochondrial localisation signal, and disruption of the mPHGPx leader sequence may lead to perturbed expression (decreased, increased or unregulated) of the cytosolic form.

Ten years ago, Ursini et al. (1997) first formulated the question: phospholipid hydroperoxide glutathione peroxidase (PHGPx): more than an antioxidant enzyme? Over the last couple of years, a great leap has been made towards understanding the physiological role of PHGPx, and thus the question mark is certainly no longer required. These advances have been made possible mainly by improved biochemical techniques and the implementation of novel loss- and gain-of-function approaches. Conditional disruption of PHGPx combined with pathological models and *ex vivo* knockout systems will provide the most powerful tools to address its precise role in many cellular processes. These studies might also answer whether PHGPx is the long-suspected limiting selenium-dependent enzyme in sperm development.

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