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Review

ROS, thiols and thiol-regulating systems in male gametogenesis ☆

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ABSTRACT

Background: During maturation and storage, spermatozoa generate substantial amounts of reactive oxygen species (ROS) and are thus forced to cope with an increasingly oxidative environment that is both needed and detrimental to their biology. Such a janus-faceted intermediate needs to be tightly controlled and this is done by a wide array of redox enzymes. These enzymes not only have to prevent unspecific modifications of essential cellular biomolecules by quenching undesired ROS, but they are also required and often directly involved in critical protein modifications.
Scope of review: The present review is conceived to present an update on what is known about critical roles of redox enzymes, whereby special emphasis is put on the family of glutathione peroxidases, which for the time being presents the best characterized tasks during gametogenesis.
Major conclusions: We therefore demonstrate that understanding the function of (seleno)thiol-based oxidases/reductases is not a trivial task and relevant knowledge will be mainly gained by using robust systems, as exemplified by several (conditional) knockout studies. We thus stress the importance of using such models for providing unequivocal evidence in the molecular understanding of redox regulatory mechanisms in sperm maturation.
General significance: ROS are not merely detrimental by-products of metabolism and their proper generation and usage by specific enzymes is essential for vital functions as beautifully exemplified during male gametogenesis. As such, lessons learnt from thiol-based oxidases/reductases in male gametogenesis could be used as a general principle for other organs as it is most likely not only restricted to this developmental phase. This article is part of a Special Issue entitled Redox regulation of differentiation and de-differentiation.

1. Introduction

Sperm development is a complex process involving a series of highly orchestrated steps including stem cell proliferation and renewal, genetic remodeling including genetic exchange between sister chromatids and properly controlled reduction of chromosomes, dramatic morphological alterations and eventually sperm activation, a process known as capacitation. During spermatogenesis, the diploid spermatogonial stem cells that originally derive from gonocytes either go through self-renewal proliferation in order to maintain the population of stem cells or differentiate into primary spermatocytes. These diploid spermatocytes undergo the first round out of meiotic division resulting in two haploid secondary spermatocytes, which upon the second meiotic division yield two haploid spermatids (spermatidogenesis). In a subsequent developmental process, known as spermiogenesis that can be further subdivided into 16 stages in mice according to morphological changes [1], the haploid spermatids undergo dramatic morphological alterations and elongate. These changes include the formation of the tail (from a centriole), of the axoneme (by microtubules) and of the acrosome (from the Golgi apparatus), replacement of the majority of histones by transition proteins and finally by protamines leading to tight packaging of the male haploid genome, and removal of unnecessary cytoplasm and organelles, leading to the formation of the so-called residual bodies. In a process called spermiation, elongated spermatozoa are eventually released into the lumen of the seminiferous tubule. The still immobile spermatozoa are then transported to the epididymis where they acquire motility and the capability to fertilize in addition to a further oxidation of sperm proteins as summarized below. Aberrant control of any these steps frequently contributes to male infertility, testicular cancer, germ cell mutations, miscarriages and progenies with disabilities. Although many of the steps involved in proper male gametogenesis are known and have been described in detail, a comprehensive knowledge about the role of ROS and systems generating partly reduced forms of oxygen and those keeping them at physiological concentrations is still scant. This is particularly astonishing in light of the very early and actually first observation in mammals that it is the spermatozoon that deliberately produces ROS [2]. This lack of knowledge is mainly due to inherent

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limitations to studying sperm development in vitro, the frequently observed stark discrepancies between highly divergent redox conditions in vitro and in vivo and still insufficient genetic animals models to unequivocally assign distinct redox-dependent processes to the individual steps of spermatogenesis in vivo.

Yet, a better knowledge about the molecular role of reactive oxygen species (ROS) and their surveillance systems in this vital process may not only guide us to predict and prevent diseases linked to ROS-induced aberrant spermatogenesis, but also might prove highly useful in the further improvement of assisted fertility techniques, which is of particular relevance in a steadily decreasing proportion of fertile men in the Western world. Hence, it is the goal of this review to critically portray the role of ROS and ROS-regulating systems during sperm development with special focus on the utilization of transgenic mouse models that has provided intriguing and often conclusive insights.

2. Reactive oxygen species: the source and nature of generation

ROS is an "umbrella" term and describes partly forms of molecular oxygen that in part show high reactivity towards cellular components, including DNA, proteins and lipids. Some highly relevant forms of ROS in biological systems include singlet oxygen, superoxide anion ($O_2.^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (OH•), lipid hydroperoxide (LOOH), phospholipid hydroperoxide (PLOOH) among others. ROS are produced at virtually all subcellular compartments where they perform specific functions. Relevant sites of ROS generation include mitochondria, endoplasmic reticulum (ER), cytosol, lysosomes, peroxisomes and at the plasma membrane [3]. Besides a number of environmental exposure conditions including toxic compounds and γ-irradiation that lead to ROS generation and that impair or even abrogate male fertility, a series of enzymatic sources are present in the aforementioned compartments that deliberately, or as a metabolic side-product, generate ROS. Examples include the different complexes of the mitochondrial respiratory chain (here, in particular complexes I and III) [4], α-ketoglutarate dehydrogenase and pyruvate dehydrogenase in mitochondria, the endoplasmic reticulum oxidoreductase system (ERO) [5], lipoxygenases and cyclooxygenases in the cytosol, xanthine oxidase, cytochrome P450 monoxygenase, and NADPH oxidases in the plasma membrane [6].

However, a direct involvement of these potential ROS-generating systems in male gametogenesis has been experimentally demonstrated for only a subset of them up until now. For instance, ROS generated by one of the NADPH oxidase family members, NADPH oxidase 1 (NOX1), was implicated in the facilitation of stem cell self-renewal of spermatogonial stem cells (SSC) [7]. NOX1 along with NOX3 and NOX4 are the sole members expressed in germline stem cells (GSC), and knockdown and inhibitor experiments suggested that ROS derived from the NOX1 complex through the engagement of p38 MAPK and c-jun N-terminal kinase (JNK) contribute to GSC proliferation. Although NOX1-deficient mice were previously shown to be fertile and normal in appearance [8], a detailed analysis performed by Morimoto and colleagues indicated a reduced number of spermatogonia and an impaired colonization capacity of SSCs upon serial transplantation, indicative of a reduced rate of SSC self-renewal during colony regeneration [7].

In a model of testicular ischemia–reperfusion (I/R), which is inevitably linked to high ROS production it was shown that xanthine oxidase, the lipid peroxide breakdown product malondialdehyde and the nuclear factor (erythroid-derived 2)-like 2 (Nrf2)-regulated gene heme oxygenase (HO) contribute to the extent of I/R induced tissue injury that could be blunted by the strong lipophilic antioxidant curcumin [9]. Besides this, the xanthine oxidase system was frequently used to experimentally manipulate ROS levels and to address the impact of ROS in the individual steps of sperm development and fertilization, albeit knockout studies could not yet provide any direct evidence of an implication of this system in male gametogenesis [10].

Mitochondrial respiratory activity is essential for the rapid movement of released sperm for the fertilization process [11], and mitochondria are still being considered as a major site of ROS production in sperm cells. Yet one needs to consider that mitochondrial respiration really picks up only in caudal sperm as glycolysis is not only an important process for delivering the sperm's energy demand at earlier stages of sperm development [12,13], but also during its rapid movement [14,15]. Notwithstanding, mitochondrial inhibitors have been shown to impair sperm motility, and thus fertilizing capacity; and incomplete electron transfer and electron leakage mainly at complexes I and III are known to ultimately lead to the generation of substantial amounts of ROS and thus oxidative stress, a major cause of defective sperm function and male infertility [16,17]. For instance, challenging human sperm with respiratory chain inhibitors specific for the individual complexes revealed that ROS generated at complex I had the most significant adverse impact such as peroxidative damage of sperm midpiece and a loss of sperm movement [16]. Notably, these effects could be prevented by the lipophilic antioxidant α-tocopherol. The latter is in line with the high abundance of poly unsaturated fatty acids (PUFAs) in sperm that can be easily modified by oxidation leading to peroxidative breakdown of lipid bilayers [18], and the induction of subsequent apoptotic or non-apoptotic cell death paradigms. In this context, the naturally occurring electrophilic lipid peroxidation breakdown 4-hydroxynonenal (4-HNE) and acrolein were recently reported to target succinate dehydrogenase in isolated human spermatozoa, leading to an increased ROS production and a concomitant decrease in sperm motility both of which could be compensated for by treatment of aldehyde-exposed spermatozoa with thiol containing nucleophiles [19,20].

Although lipid-associated peroxides severely impair sperm motility, their generation by enzymatic systems in sperm cells has not been studied in detail. Up until now, it has only been considered being secondary to H_2O_2— and thus OH•-triggered lipid peroxidation. Yet arachidonic acid metabolizing enzymes such as lipoxygenases and cyclooxygenase, which have been recently implicated in non-apoptotic cell death signaling in somatic cells [21,22], have not been put to the test for their capability to trigger lipid hydroperoxides directly in sperm cells with adverse impacts on sperm motility and survival. Only an implication of 12/15-lipoxygenase in the acrosome reaction [23] or sperm cytoplasmic droplet (CD) migration has been shown thus far [24,25].

3. Peroxides, redox-controlling systems and antioxidants in the course of sperm development

As early as 1946, spermatozoa were recognized as the first mammalian cells to generate hydrogen peroxide [2]. Prior to this observation, a landmark finding described the detrimental impact of molecular oxygen on the motility of human spermatozoa [26]. Nowadays, it is conceived that ROS impact on spermatozoa both at the functional as well as at the quality level: Firstly, lipid peroxidation as an inducer of sperm motility impairment through a not fully understood mechanism possibly involving ATP depletion, and secondly the oxidative modification of DNA, as demonstrated by accumulation of the guanine nucleotide 8-hydroxy,2′ deoxyguanosine (8OHdG) [27]. Counterintuitively, a certain threshold of ROS is required to enable specific developmental steps of sperm development, such as stem cell-renewal as outlined above, sperm hyperactivation and capacitation, acrosome reaction during fertilization and presumably throughout all the oxidative steps essential for acrosome formation and formation of the rigid structures of the midpiece, the tail and the nucleus (as discussed under Section 4). For these reasons, it is evident that spermatozoa must have developed a highly sophisticated antioxidant network ensuring a tightly controlled window of ROS generation and action and at the same time preventing the potentially deleterious effects of excessive ROS. The situation in spermatozoa seems to be more complex than in somatic cells, because (i) most of the cytoplasm is stripped off during spermiogenesis, thereby losing virtually most of the main cellular

antioxidant glutathione (GSH) and also many cytosolic small molecule and enzymatic ROS scavengers, (ii) there is virtually no gene expression and adaptation possible due to the tight package of the sperm DNA during the final stages of sperm maturation, and (iii) the remodeling of the plasma membrane increases the content of PUFAs, in particular docosahexaenoic acid, with the already mentioned risk for incurring increased oxidative damage of this predisposed sensitive compartment.

During testicular and epididymal sperm development, spermatozoa express and lose a series of redox enzymes and exploit the antioxidant endowment of the different surroundings that they experience on their journey from the epididymis to the female duct [28]. In fact, the seminal fluid is among the body fluids most highly enriched in low molecular antioxidants [18,29]. Unfortunately, only a subset of knockout studies has provided conclusive evidence that enzymes directly or indirectly contributing to redox homeostasis indeed confer an important role in male fertility. This is mainly owed to the fact that some of the respective enzymes simply cause embryonic lethality or early postnatal lethal phenotypes or do not fulfill expectations. For instance, mice lacking catalase that specifically detoxifies H_2O_2 only develop subtle phenotypes [30], whereas mice deficient in mitochondrial manganese superoxide dismutase (SOD2) die early after birth and display defects in cardiac and neuronal tissues and aberrant lipid accumulation and metabolic acidosis [31,32]. The subtle effects of catalase null animals may also be explained based on the fact that peroxisomes are present in Sertoli cells and spermatogonia, but disappear in later developmental stages during sperm maturation [33]. Cu/Zn superoxide dismutase (SOD1) deficient spermatogenic cells were shown to be more sensitive to heat stress [34], and *SOD1*$^{-/-}$ spermatozoa reveal an impaired in vitro fertilizing capacity due to a time-dependent decrease in ATP levels coinciding with an increase in lipid peroxidation [35].

Another class of enzymes that involves a (seleno)thiol-mediated mechanism for peroxide detoxification and/or protein sulfoxidation (as discussed under Section 4) are enzymes of the thioredoxin- and glutathione-dependent systems. These include thioredoxins (Txn) and the respective thioredoxin reductases (Txnrd), a thioredoxin-glutathione reductase (TGR, Txnrd3) [36], spermatid-specific thioredoxins (Sptrx), peroxiredoxins (Prdx), glutaredoxins (Grx) and glutathione peroxidases (Gpx).

As reported in the "Redox Atlas of the Mouse" (http://www.online.uni-marburg.de/redoxatlas/), the thioredoxins and thioredoxin-related genes as well as glutaredoxins are mainly expressed in testicular tissue at distinct developmental stages [37]. While expression of cytosolic thioredoxin (Txn1), mitochondrial thioredoxin (Txn2) and mitochondrial thioredoxin reductase (Txnrd2) appears to be expressed in the entire seminiferous tubule, cytosolic thioredoxin reductase (Txnrd1) expression is rather confined to spermiogenic stages. The spermatocyte/spermatid-specific thioredoxin Txndc2 (Txndc2, thioredoxin-domain containing 2, also named Sprtx-1) is transiently expressed during spermiogenesis (steps 9–18), but is lost just before spermiation [38]. Similarly, Txndc3 (also named Nme8 and Sptrx-2) expression starts at the spermatid stages 14–15, is incorporated in the fibrous sheath and is highest at step 19 and remains associated with epididymal spermatozoa [39]. The Txn1-related spermatocyte/spermatid-specific thioredoxin-3 (Sptrx-3 or Txndc8) is expressed mainly in the Golgi apparatus of pachytene spermatocytes, in round/elongating spermatids and transiently in the acrosome of spermatids [40].

Although the isolated knockouts for Txndc2 and Txndc3 surprisingly did not produce any apparent impairment of male fertility, the compound mutant knockout of both enzymes resulted in an age-dependent increase in oxidative stress, DNA damage and impaired sperm motility, as well as decreased sperm chromatin compaction [41]. These studies clearly showed that there must be extensive redundancy among some of these thiol-containing redox proteins in sperm development in order to avoid sperm development deterioration that otherwise might have a major impact on male fertility and on male genomic stability.

Concerning the thioredoxin-dependent peroxidases (nowadays referred to as peroxiredoxins), all but Prdx4 appear to be weakly or moderately expressed in the seminiferous tubule without any distinct expression to certain cell types, whereas Prdx4 expression is clearly augmented in very late stages of sperm maturation [37]. Cytosolic glutaredoxin (Glrx) was described to be expressed ubiquitously with strong expression in spermatogonial cells, while mitochondrial glutaredoxin (Glrx2; Glrx2a) appears to be increased in spermiogenic cells but to a lesser extent in spermatozoa. Notwithstanding, Grx2 expression seems to be rather complex in testis, as three mRNA variants (designated as mGLRX2_v3, v4 and v5) lead to the generation of the testis-specific Glrx2 isoforms, Grx2c and Grx2d, with cytosolic/nuclear localization [42]. Similarly, glutaredoxin 3 expression surfaces during spermiogenesis and is maintained until the spermatozoan stage. In contrast, mitochondrial monothiol glutaredoxin 5, which is involved in the maturation of cellular Fe/S proteins and regulation of cellular iron, is only weakly expressed in the seminiferous tubule with a slightly increased expression during spermiogenesis.

Interestingly, the Nrf2 target gene and enzyme catalyzing the rate-limiting step in glutathione (GSH) synthesis, glutamate-cysteine ligase catalytic subunit (Gclc), was found to be only very weakly expressed in spermatogonial cells but to a much higher extent in vessels of testicular tissue. Whether this functions to prevent high amounts of GSH that would otherwise prevent all the oxidative steps required to enable proper sperm development (see below) still needs to be put to the test. Although mice lacking Gclc die during early embryogenesis [43], a conditional knockout model for Gclc [44] with sperm-specific inactivation would certainly be useful to address the role of GSH during the individual steps of spermatogenesis. Nevertheless, mice lacking Nrf2 reveal dysfunctional sperm development with age [45]. Although young mice have normal spermatogenesis, mice show increased lipid peroxidation after two months of age. This is associated with a reduced number of testicular and epididymal sperm head counts (approx. 50% of that of wildtype mice) and impaired sperm motility when compared with wild-type males [45], consistent with many previous observations that exogenous lipid peroxides damage sperm motility.

Despite knowledge gained by the aforementioned knockout models, only little is known about a potential contribution of these redox enzymes in male gametogenesis. This is certainly also accounted for by the fact that the specific loss of some of the enzymes yield lethal phenotypes and conditional knockout mouse models are still limited. For instance, mice lacking Txn1 and Txn2 die at the blastocyst stage and mid-gestation [46,47], respectively, and mice null for the respective reductases, the selenoenzymes Txnrd1 and Txnrd2, produce lethal phenotypes during early embryogenesis and the fetal phase, respectively [48,49]. Using conditional deletion of both enzymes, it was also shown that Txnrd1 is required for cerebellar development [50], whereas Txnrd2 plays an essential role in cardiac development and function [48,51]. Yet no study has so far addressed their putative contribution to spermatogenesis despite their apparent expression in the seminiferous tubule. The role of another related selenoprotein, TGR (i.e. Txnrd3) [52], an enzyme mainly expressed in elongating spermatids, in male gametogenesis has remained unclear, but is believed to co-operate with glutathione peroxidase 4 (Gpx4) during the oxidation process and maturation steps of elongating spermatids (see Section 4.).

Concerning the family of glutaredoxins, knockout studies have been published for Glrx1 and Glrx2 [53–55], however, with so far no reported phenotype in spermatogenesis despite marked immunostaining of Glrx2 in spermatogonia and elongating spermatids [42]. A gene trap for mouse Glrx3 (also called PICOT, PKC-interacting cousin of thioredoxin) presented a lethal phenotype during the fetal stage (E12.5) and heterozygous mice showed aggravated cardiac hypertrophy in response to pressure overload [56]; however, no conditional loss of function model is available to date that allows the investigation of the contribution of this member in male gametogenesis.

Some recent studies have shed more light on the role for some of the peroxiredoxins during sperm development. Among the six peroxiredoxins, peroxiredoxin IV (Prdx4) has been studied in more detail than the others in sperm development [57] (Table 1). It was already recognized that Prdx4, which in the testis exists in a soluble and ER-localized membrane-bound form, is down-regulated upon experimental cryptorchidism [58]. Conditional inactivation of Prdx4 by flanking exon 1 (meanwhile designated as exon 1B) with loxP sites and Cre-mediated removal of the loxP-flanked exon leads to Prdx4 knockout mice (designated as −/y; Prdx4 is X chromosome encoded) [59]. Though these animals sire litters of normal size indistinguishable from their wild-type counterparts, −/y animals develop testis atrophy, present signs of increased oxidative stress, as a measure of increased thiobarbituric acid reactive substance (TBARS) levels and 8OHdG-positive staining, reduced levels of reactive free thiols and an approx. 50–60% decline in epididymal sperm counts over age. Despite a clear decrease in sperm counts, in vitro fertilization capacity of isolated spermatozoa was not affected consistent with the findings that −/y mice produce offspring of normal litters [59]. In a subsequent study, however, it was found that there is a sperm-specific exon located further downstream (exon 1A) of exon 1B and closer to exon 2 that gives rise to a testis-specific longer isoform (31 kDA) that was not fully affected by the targeted strategy used in the first knockout approach [60]. Yet it remains to be shown whether the complete loss of the entire *Prdx4* gene may have an even more severe impact on spermatogenesis than reported in the first knockout study [59].

Besides Prdx4, Prdx2 is also expressed in a soluble form in spermatids and a Triton-insoluble form in mature spermatozoa [61]. In the latter, Prdx2 was found to be confined to the mitochondrial sheath of the sperm tail midpiece associated with mitochondrial sheath proteins including sperm mitochondria-associated cysteine-rich protein (SMCP) and Gpx4, indicating a functional relationship during formation of the mitochondrial capsule (see below). Furthermore, bovine Prdx5 was just recently described to be secreted in the cauda epididymis and to bind to the sperm plasma membrane during epididymal transit [62]. Unlike Prdx4, no specific phenotypes in male gametogenesis have been reported for Prdx1 [63], Prdx2 [64], Prdx3 [65], Prdx5 and Prdx6 [66] knockout mutants so far. For their preeminent importance for male gametogenesis, the role of glutathione peroxidases in sperm maturation shall be discussed in the following section.

4. Glutathione peroxidase dependent thiol-disulfide mechanisms in shaping sperm morphology and in conferring sperm stability

Despite the low abundance of cellular GSH in testicular and epididymal tissue, some members of the glutathione peroxidase family of proteins have been demonstrated to exert unique roles in sperm maturation [67]. It is actually one of the main prerequisites that developing sperm cells have low levels of GSH that eventually allow the oxidative steps to shape spermatozoa in the different subcompartments including the acrosome, the nucleus, the midpiece and the flagellum. In accordance with this during sperm elongation more than 95% of sperm cell GSH is actively removed and lost [68,69].

Mammals express eight glutathione peroxidases, yet not all of them are evidently present in sperm cells [70]. Although Gpx1 is expressed in testis, no effect on spermatogenesis has been reported by knockout studies thus far. Gpx2 is mainly expressed in intestinal tissue, Gpx3 and Gpx5 are abundantly expressed in epididymal tissue, Gpx4 in virtually all testicular compartments and mature sperm cells and Gpx6 in epithelial olfactorium — still very little is known about the expression of the ER-resident Gpx7 and Gpx8 in vivo. Gpx1-4 and Gpx6 are selenoproteins in humans (the latter being a cysteine-containing variant in mice), whereas Gpx5, Gpx7 and Gpx8 are cysteine-containing enzymes. In humans there are a total of 25 selenoproteins (in mice 24) [71], which are characterized by the co-translational incorporation of the 21st and rare amino acid selenocysteine (Sec) into the nascent polypeptide chain [72]. Sec discriminates from Cys in only one atom, selenium replacing sulfur. As Sec is encoded by the opal codon UGA that normally represents a STOP codon, a highly complex and orchestrated machinery is not only essential for the synthesis of Sec-loaded tRNA, but also for the successful incorporation of Sec into selenoproteins, employing a series of factors acting in cis and trans [72].

For more than 30 years, the trace element selenium has been recognized as an essential factor for male fertility in rodents [73–75]. Notably, human mutations in one of the factors acting in trans, selenocysteine insertion sequence-binding protein 2 (SBP-2), were recently shown to cause a multisystem selenoprotein deficiency disorder with azoospermia and complete lack of spermatids and spermatozoa, whereas spermatogonia and spermatocytes appeared to be unaffected [76], indicating that one or several selenoproteins are vital also for human male gametogenesis. Along the same line, male mice lacking the selenium transport protein selenoprotein P (Sepp) are infertile and

Table 1
Summary of specific knockout mouse models for thiol-related redox enzymes with reported phenotypes in male gametogenesis.

Gene/ protein	Author	Method	Major pathophysiologic phenotype	Ref.
Txndc2/ Txndc3	Smith et al., 2013	Systemic knockout of Txndc2 and site-directed mutation of the active site of Txndc3	Increased age-dependent oxidative stress and 4-HNE-modified sperm; decreased (progressive) sperm motility and oxidative DNA damage; yet no impairment on overall fertility	[41]
Prdx4	Iuchi et al., 2009	Systemic deletion (knockout) of exon 1B of Prdx4	Increased age-dependent oxidative stress and cell death in spermatogenic cells and consequently reduced sperm counts; partial testicular atrophy but no impact on overall male fertility	[59]
nGpx4	Conrad et al., 2005	Systemic knockout of the alternative exon encoding nuclear Gpx4	Male fertility is not impaired, but isolated knockout sperm show giant heads due to delayed protamine sulfoxidation	[94]
nGpx4	Puglisi et al., 2012	See Conrad et al., 2005	Faster sperm nucleus decondensation upon oocyte fertilization	[102]
mGpx4	Schneider et al., 2009	Systemic knockout of mitochondrial Gpx4	Male infertility; massive loss of sperm motility and progressivity; severe abnormalities in the midpiece of sperm reminiscent of severe selenium-deficiency; decreased protein sulfoxidation	[95]
Gpx4	Imai et al., 2009	Conditional knockout of Gpx4 using Pgk2-Cre mice	Testis atrophy; germ cell loss at the spermatocyte stage; morphological abnormalities of isolated sperm	[98]
Gpx4	Liang et al., 2009	Transgenic expression of cGpx4 in *Gpx4*$^{-/-}$ mice	Morphological abnormalities of isolated sperm and male infertility	[96]
Gpx5	Chabory et al., 2009	Systemic knockout of Gpx5	Increased oxidative stress in sperm and epithelium of cauda epididymidis; no impairment in male fertility, but sperm from old male mice (>1 year) led to miscarriages and developmental defects mainly at the fetal stage are evident	[82]
nGpx4/ Gpx5	Noblanc et al., 2012	Double knockout for nGpx4 and Gpx5	Epididymal up-regulation of members of the thioredoxin/peroxiredoxin system and glutathione-S-transferases; increased DNA oxidation/fragmentation and sperm nuclei are more susceptible to nuclear decondensation	[103]

present flagellar defects, such as a truncated mitochondrial sheath, extrusion of axonemal microtubules and outer dense fibers from the principal piece and hairpin-like bends at the midpiece–principal piece junction [77]. Likewise, male mice lacking the cognate Sepp receptor in the testis, apolipoprotein E receptor-2 (ApoER2), precisely mimic the phenotype of sperm defects as observed in Sepp null mice [78]. Interestingly, male infertility provoked by Sepp deficiency could be bypassed by simple liver-restricted transgenic Sepp expression, indicating that hepatic Sepp is the major source for physiological selenium in the body [79].

Of the eight glutathione peroxidases, important functions during male gametogenesis have been ascribed so far only for Gpx3, Gpx4 and Gpx5. Gpx3 is expressed throughout the epididymis [80], while secreted Gpx5 is expressed in caput epididymis and secreted into the lumen. Hence, both enzymes are considered to protect the paternal genome from potentially deleterious effects of ROS generated during the epididymal transit. Although no phenotype in sperm development has been reported for Gpx3 [81], mice lacking Gpx5 present increased oxidative stress in the sperm and epithelium of the cauda epididymidis as a measure of 8OHdG-positive staining, and a compensated expression of Gpx1, Gpx3 and cytosolic Gpx4 in cauda epididymidis of *Gpx5*$^{-/-}$ animals [82]. Surprisingly, loss of Gpx5 did not impact on male fertility as litter sizes were indistinguishable between wild-type and knockout males. But when 1-year-old or even older male mice were used for breeding, miscarriages and developmental defects mainly at the fetal stage were manifested, in line with the findings that *Gpx5*$^{-/-}$ sperm inherently show increased DNA oxidation. As the caudal sperm nuclei of *Gpx5*$^{-/-}$ animals were found to be more compacted as determined by Chromomycin A3 (CMA3) staining, it can be concluded that ROS in the epididymal duct facilitate sperm chromatin compaction as further outlined below [82]. However, as Gpx5 does not rely on selenothiol-mediated Gpx catalysis, it is clear that it cannot account for the effects observed under experimental selenium deficiency.

A landmark study performed by Ursini and colleagues provided first evidence that Gpx4 is the most important target for testicular selenium [83], and the enzyme responsible for the defects and male infertility as previously demonstrated for severe selenium deficiency and as demonstrated by a series of knockout studies in mice in the meantime. Gpx4, previously also named phospholipid hydroperoxide glutathione peroxidase (PHGPx), was first identified as a seleno-peroxidase that efficiently decomposes phospholipid hydroperoxides and other peroxides to their respective alcohols using GSH as electron donor [84]. Yet in contrast to many other glutathione peroxidases monomeric Gpx4 is not only less restricted to its oxidizing substrates, but it is equally ambiguous when it comes to its reducing substrates. Besides GSH, Gpx4 also uses a wide range of thiol containing compounds such as cysteine, DTT and even protein thiols when GSH concentrations are limiting as particularly evident in maturing sperm (Fig. 1) [85]. In the study by Ursini and colleagues, Gpx4 was found to be the most abundant selenoprotein in sperm mitochondrial capsules, an amorphous and rigid keratin-like matrix that surrounds the elongated mitochondria in the midpiece of mature spermatozoa and that ensures full mitochondrial sperm midpiece stability [83]. Thereby, Gpx4 is linked to other sperm capsular proteins including SMCP and is practically devoid of any measureable peroxidase activity [86]. Gpx4-specific enzyme activity of capsular Gpx4, however, could be regained when isolated mitochondrial capsules were treated with high concentrations of the thiol reducing agent ß-mercaptoethanol [83]. This strongly suggests that Gpx4 is a key enzyme in the oxidation process of sperm capsule proteins, whereby it becomes cross-linked to capsular proteins both via its cysteine residues and eventually through its selenothiol moiety when all other protein thiols are consumed (Fig. 1).

Independent and direct proof for this oxidizing and structural function of Gpx4 in mitochondrial capsules was obtained by reverse genetic studies in mice [87]. The Gpx4 gene is composed of 7 classical exons plus one alternative exon (Ea) localized in the intron between

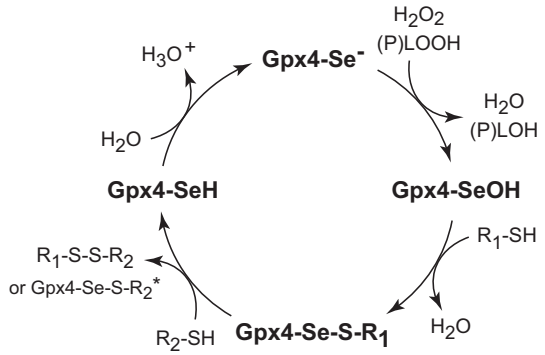

Fig. 1. The catalytic cycle of Gpx4 in the absence of cellular GSH. The selenolate anion of Gpx4 is first oxidized by a peroxide. Whether a selenenic acid (–SeOH) or a selenylamide (–SeN, not shown) with an adjacent amino group is generated is debated. In the absence of GSH, Gpx4 accepts a thiol group from a first interacting partner yielding a selenylsulfide bridge (–Se-S-R_1). A second thiol group from the same or different protein or Gpx4 itself leads to the formation of a disulfide (R_1-S-S-R_2) bridge. The selenol of Gpx4 is deprotonated, thus closing the catalytic cycle. When no further (seleno)thiol group is available suicidal inactivation of Gpx4 occurs, then becoming cross-linked to other sperm proteins (indicated by an asterisks).

exons 1 and 2. Alternative transcription initiation at exon 1 determines whether the mitochondrial form (long form, 22 kDa) or the cytosolic form (short form, 19 kDa) is expressed [88]. The alternative exon encodes for a sperm-nucleus specific variant of Gpx4 (34 kDa) and is expressed from its own promoter [89,90]. Targeted and systemic disruption of the *Gpx4* gene causes embryonic lethality at the gastrulation stage [91], for what reason more specific knockout approaches have been conducted in the meantime.

To specifically inactivate the mitochondrial form, an in-frame STOP codon was inserted into the first exon downstream of the mitochondrial ATG but upstream of the putative transcription initiation site of the cytosolic variant because it was already expected from previous expression analysis studies [92,93] and knockout studies for the nuclear form [94] that it is the cytosolic form of Gpx4 that is essential for early embryonic development and for many other somatic tissues. Indeed, mice lacking the mitochondrial form of Gxp4 (mGpx4) are fully viable and male mice are infertile [95]. Isolated sperm exactly phenocopied the structural abnormalities in the midpiece of spermatozoa as observed under severe selenium deficiency in rodents and genetic *Sepp* and *ApoER2* deficiency mice. These include irregularly aligned mitochondria, bends between the midpiece and the tails, swollen mitochondria, and structural abnormalities between the head and the midpiece. Furthermore, epididymal sperm were less oxidized than their wild type counterparts [95], supporting the idea that Gpx4 acts as a thiol peroxidase by introducing disulfide and selenylsulfide bridges into capsular proteins [83]. From this one may assume that the selenolate anion of Gpx4 becomes first oxidized (i.e. activated) by a peroxide (Fig. 2). Then, due to the low abundance of their reducing equivalent, GSH, oxidized Gpx4 oxidizes a neighboring thiol of a capsular protein or one of the eight (plus one considering the one in the mitochondrial leader sequence [MLS]) cysteine residues within Gpx4, thus forming a mixed selenylsulfide. A second thiol leads to formation of a disulfide and fully reduced selenolate, the latter being oxidized again by peroxide. Yet, since Gpx4 lacks isomerase and disulfide reductase function, probably another testis-restricted enzyme, the dimeric selenoenzyme TGR, may contribute to proper disulfide bond formation once a first disulfide is catalyzed by Gpx4 [52]. Unfortunately, no knockout data on TGR is available like for another selenoprotein V, which is also highly expressed in testicular tissue [71]. Furthermore, it also remains to be explored how other thiol-related proteins like Prdx2 (mitochondrial sheath and shown to associate with Gpx4), Txndc2 (fibrous sheath, transient) and Txndc3 (fibrous sheath) may functionally cooperate with Gpx4 in disulfide formation and isomerization. In this context, it should be mentioned

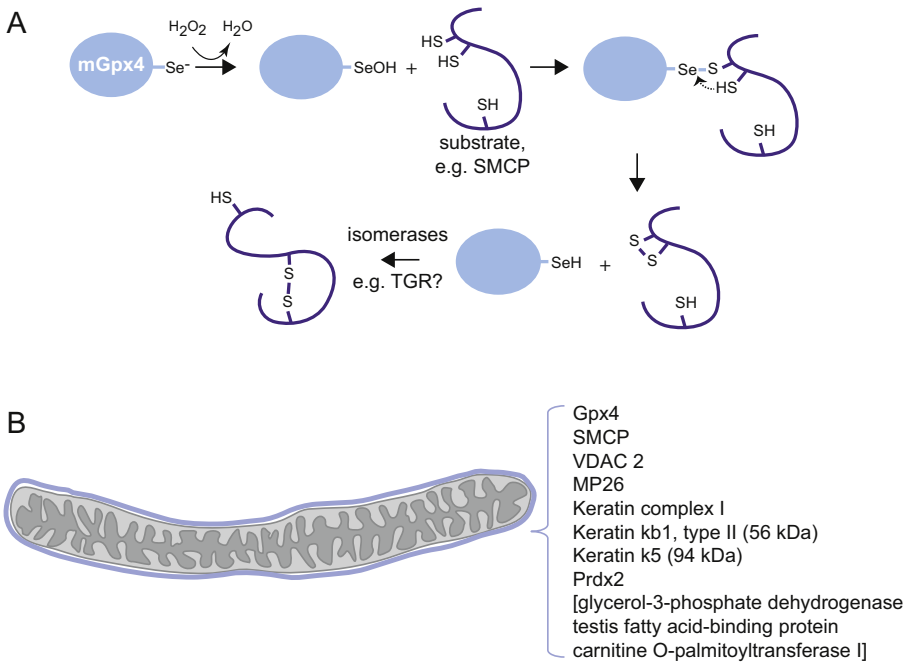

Fig. 2. Functional interaction of Gpx4 in the midpiece of maturing spermatozoa. (A) As illustrated in Fig. 1, Gpx4 initially forms a mixed selenylsulfide with capsular proteins, such as SMCP. Inter- or intramolecular disulfide exchange reactions release reduced Gpx4. Protein disulfide isomerases, such as TGR, may help to reshuffle disulfide bridges in capsular proteins, leading to their mature stage. (B) Capsular protein candidates identified by LC/MS are given [61,83,86].

that besides the mitochondrial sheath Gpx4 immunoreactivity can also be found in the fibrous sheath and the acrosome [95].

Despite these intriguing insights, there are still a number of other unsolved questions. For instance, the source of ROS for inducing the oxidation events in the mitochondrial sheath (and other parts of spermatozoa) is still unknown, although the mitochondrial chain might be one possibility. Additionally, it has remained a mystery why mGpx4 probably first enters the mitochondrial matrix due to its cognate targeting sequence, and then forms the mitochondrial capsule that actually surrounds and embeds sperm mitochondria. In an elegant study by Liang and colleagues it was unequivocally and directly proven that it is cGpx4 that is essential for embryo survival and somatic tissues. Mice transgenic for cGpx4 crossed on the *Gpx4*$^{-/-}$ mouse background rescued embryonic lethality of mice [96], but failed to rescue male infertility as seen in *mGpx4*$^{-/-}$ mice. In contrast, mice transgenic for mGpx4 and crossed on the *Gpx4*$^{-/-}$ background did not rescue embryonic lethality, which is in line with data that overexpression of mGpx4 in tamoxifen-inducible *Gpx4* knockout cells [97] does not rescue cell death induced by Gpx4 deletion (Conrad M., unpublished observation) [98], whereas ectopic expression of cGpx4 in these cells fully rescued cell death induced by endogenous Gpx4-deficiency [97].

In a follow-up study it was subsequently demonstrated that overexpression of cGpx4, where the Sec residue was mutated to Cys, also prevented cell death induced by Gpx4 deletion, indicating that at least in vitro Sec can be functionally replaced by Cys [99]. In light of the previously questioned role of Gxp5 to act as a classic Gpx due to the presence of Cys instead of Sec in its active site and lack of peroxidase activity in biochemical assays a similar characteristic might apply for the Sec/Cys-cGpx4: also completely lacks measureable Gpx4-specific activity but still rescues cell death when expressed in knockout cells [99], as was also demonstrated for Gpx5 previously [100]. Furthermore, even a cGpx4 mutant devoid of all remaining Cys residues except the Sec or a Cys in the active site was able to prevent cell death induced by Gpx4 deletion [99], suggesting that none of the remaining Cys acts as a resolving Cys as usually found in Cys containing glutathione peroxidases and peroxiredoxins [101]. This implies that these non-peroxidatic Cys residues might be only important for cross-linking with other sperm capsular proteins in vivo.

Besides the oxidizing and stabilizing function of mGpx4 in sperm midpiece, another morphological defect provoked by severe selenium deficiency in rodents, so-called sperm with giant heads, can be explained on the basis of the thiol peroxidase function of Gpx4. In 2001, a sperm-nucleus specific selenoprotein was purified from rats, sequenced by Edman degradation and eventually identified as a novel and nuclear form of Gpx4 [89]. Cloning of the gene identified an N-terminal extension of Gpx4 that harbors clusters of arginines and lysines reminiscent of protamines, small, highly basic and cysteine-rich nuclear proteins that replace the majority of histones during the final steps of spermiogenesis. In addition, this alternative exon contains a nuclear localization signal enabling the efficient transport of nGpx4 into nuclei [89]. As the nuclear form is expressed from its own promoter, it was possible to generate mice that specifically lack the nuclear form without impinging on the expression of the mGpx4 and cGpx4 variants [94]. Although *nGpx4*$^{-/-}$ males are fully fertile, a clear increase in the ratio of free thiols/disulfides could be detected in cauda epididymis. Furthermore, a delay in sperm chromatin compaction could be unmasked, which was, however, fully resumed when the spermatozoa reached the cauda epididymis. From this it was concluded that Gpx4 indeed confers thiol peroxidase activity by introducing disulfides into protamines (Fig. 3). The impaired sulfoxidation of nuclear proteins was recently shown also to impact on decondensation upon fertilization as it clearly accelerates the decondensation process of sperm already 1 h after oocyte fertilization [102].

Yet further studies are needed to answer whether the decreased sperm compaction in *nGpx4*$^{-/-}$ mice might also have a functional consequence in progenies of males exposed to environmental stressors such as DNA damaging agents including irradiation and genotoxic agents. As for mGpx4, the oxidizing source for nGpx4 remains elusive up until now. But as the reduction of free thiols and thus oxidation of sperm proteins mainly takes place during the epididymal transit, higher ROS levels in the caput lumen might be a source, which was also

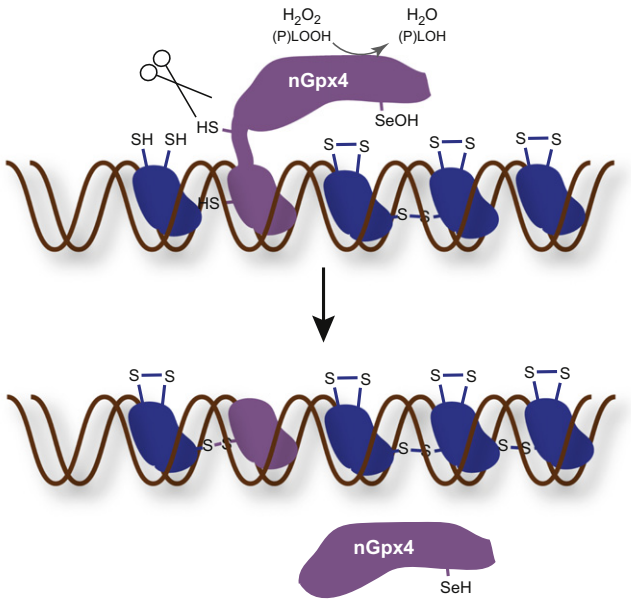

Fig. 3. Proposed mechanism of protamine cross-linking. Due to its N-terminal extension, which includes protamine-like clusters and a nuclear targeting signal, nGpx4 probably associates with DNA in a fashion similar to protamines (blue) that bind in the major groove of the DNA. A linker between the N-terminal extension and Gpx4 proper may allow sufficient flexibility to catalyze protamine sulfoxidation. Upon the epididymal transit of spermatozoa, nGpx4 is processed and truncated into smaller forms such a 24 kDa and a 22 kDa forms by a yet unknown mechanism [89]. nGpx4 may also become cross-linked via its Sec or its non-peroxidatic Cys residues to protamines.

suggested by Gpx5 knockout studies. *Gpx5*$^{-/-}$ mice presented higher levels of epididymal ROS and consequently a significantly higher level of sperm DNA condensation [82]. Yet additional enzymes contributing to disulfide isomerization in nuclear proteins still need to be uncovered that may work hand in hand with nGpx4.

For the increased DNA compaction seen in Gpx5 null sperm, compound mutant mice for Gxp5 and nGpx4 were generated (DKO) [103]. Although many of the condensation defects observed in *nGpx4*$^{-/-}$ sperm could be attributed to nGpx4-deficiency alone, there was an increased protein oxidation in caput spermatozoa of DKO mutants noticeable, suggesting that oxidases and isomerases other than nGpx4 may compensate under this condition. Furthermore, an increased overall peroxidase activity was noticeable in the caudal sperm along with decreased levels of malondialdehyde. Similar to the increased peroxidase activity, an increase in enzymes involved in protein isomerization could also be detected in DKO mice, probably to partially compensate for the loss of nGpx4 and Gpx5 [103].

In a broader, conditional knockout approach, Gpx4 was deleted in a sperm-specific manner [98]. Hereby, a loxP-flanked *Gpx4* allele including 5 kBp upstream of the Gpx4 gene was used to generate transgenic animals. These were subsequently crossed on the Gpx4 null background along with a Cre recombinase transgene expressed under the spermatocyte-specific phosphoglycerine kinase 2 (Pgk2) promoter [104]. This mouse expresses Cre in spermatocytes and spermatids but not in spermatogonia or in somatic cells of the testis. The spermatocyte-specific *Gpx4* knockout males presented a complex phenotype in male gametogenesis [98]. The animals showed marked testicular atrophy and consequently oligospermia. Isolated sperm had a strongly impaired forward motility, severe structural abnormalities in the sperm midpiece and lack of in vitro fertilizing capacity as reported for *mGpx4*$^{-/-}$ mice [95]. In addition to the defects observed in mGpx4 null mice, all spermatogenic cells except spermatogonia were strongly reduced in number as a result of increased cell death in the germinal epithelium, strongly suggesting that the loss of spermatocytes and thus spermatids is due to the absence of cGpx4 expression [98]. This is again supported by the study of Liang et al., who did not observe any histophathological defects in testicular or epididymal tissue despite the reduced number of total sperm and the sperm abnormalities as a consequence of mGpx4 loss [96].

5. Conclusion

During the last couple of years, great progress has been made in the molecular understanding of the role of the trace element selenium, a long known essential factor for male fertility, as an integral part of selenoproteins and in particular Gpx4. Unequivocal evidence has been provided on the basis of genetically modified animals that selenium in the form of Gpx4 confers crucial cell-protective functions already in early differentiating sperm cells, that acts as a thiol peroxidase and structural protein for shaping specific parts of spermatozoa including the sperm midpiece, and that ensures a highly condensed and tightly packed paternal genome by introducing disulfide bridges into protamines, thus rendering them extremely resistant to potentially deleterious mechanical and chemical impacts. This thiol peroxidase function of Gpx4 is reminiscent of the two Gpx4-related and novel members of the glutathione peroxidase family, the ER-resident Gpx7 and Gpx8, which are considered to be involved in oxidative protein folding in the ER [105,106], although their contribution to sperm development is unknown. As a more oxidized ratio of GSH/GSSG can also be found in the ER, the oxidative protein folding mechanism of Gpx7 and Gpx8 most likely shares many features with Gpx4 in the development of male gametes that are physiologically deprived of cellular GSH. Besides Gpx4, some intriguing insights into the role of other thiol-related enzymes, such as Gpx5, Prdx4 and Txndc2 and Txndc3 has been gained by the exploitation of loss of function mouse models, although at times the isolated and combined knockouts revealed more moderate and distinctive or age-dependent defects. Beyond these studies, still much needs to be done particularly in light of the high abundance of other (seleno)thiol-related redox enzymes in the male germ line to fully comprehend the thiol-mediated, oxidative steps that are necessary to produce fully competent sperm able to propagate the paternal genome and ultimately sustain life.

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