NOVEL INSIGHTS ON THE ROLE OF SELENOPROTEIN P IN SPERM VIABILITY

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ABSTRACT

Selenium (Se) is a micronutrient essential for life in many organisms. Selenium is incorporated into selenoproteins as the twenty-first amino acid, selenocysteine (Sec), and has antioxidant properties. One member of the family of twenty-five selenoproteins in humans is Selenoprotein P (Sepp1). This protein is synthesized primarily in liver and is proposed to transport selenium throughout the body, particularly to the brain and testes. Sepp1 knockout (KO) mice on (normal) diets without selenium supplementation have decreased selenoprotein expression in brain and testes. Previous studies have suggested that Sepp1 male KO mice are infertile due to kinks in the flagellum of spermatozoa, greatly reducing sperm motility, therefore leading to dramatically decreased fertility.

In this two-part study, our first objective was to further understand the role of Sepp1 on sperm viability. We hypothesize that Sepp1 plays a critical role in sperm DNA viability independent of motility, potentially through modulating glutathione peroxidase 4 (GPx4) biosynthesis. GPx4 is another selenoprotein known to protect cells from membrane lipid peroxidation and has been implicated in development and fertility [71]. The second objective of this study was to introduce a novel application to rescue Sepp1 global expression in knockout animals (Sepp1\textsuperscript{fr} CMV+) using Cre recombinase transgenic mice.

We addressed the role of Sepp1 in sperm DNA viability with intracytoplasmic spermatozoa injections (ICSI) of Sepp1 KO sperm into wild type oocytes. Surrogate female mice carrying embryos resulting from injection of Sepp1\textsuperscript{fr} sperm resulted in a 72.3% reduction in live pups born compared to Sepp1 heterozygous control sperm. Our results from the ICSI experiments, in which sperm were directly injected into oocytes without flagella, suggest that Sepp1 is critical for sperm DNA viability independent of motility. We show through western blot analysis that GPx4 levels are significantly decreased in the testes and epididymides of Sepp1 KO mice, whereas Sepp1\textsuperscript{fr} CMV+ rescue mice had restored expression levels comparable to the Sepp1 wild type (Sepp1 WT). Immunohistochemistry studies using an antibody against GPx4 further confirmed that GPx4 levels were undetectable in fresh Sepp1 KO mouse sperm, while GPx4 levels in Sepp1\textsuperscript{fr} CMV+ rescue mice were similar to those of wild type controls.
Cre-Lox recombination is a commonly used genetic tool for site-specific gene deletion. However, we demonstrate that this system can be used to rescue gene expression as well, restoring the expression of Sepp1 in KO mice. We show that this approach produced viable progeny of the systemic Sepp1<sup>r/r</sup> CMV+ (rescue) mice that express the CMV-Cre driven Sepp1 gene in all tissues. We confirmed through the Morris Water Maze (MWM) and other behavior assays that in contrast to Sepp1 KO mice, Sepp1<sup>r/r</sup> CMV+ mice had normal neuromotor function and memory compared to Sepp1<sup>+/+</sup>. Successful implementation of this method can further be utilized to restrict gene expression to specific cells.

Our study presents new data showing that Sepp1 is crucial for viability of sperm DNA, potentially through regulation of GPx4 levels. Furthermore, we demonstrate an innovative method for restoration of gene expression using the Cre recombinase transgenic system, which can be applied to restrict gene expression to specific cells.
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>AP-1</td>
<td>activator protein-1</td>
</tr>
<tr>
<td>ApoER2</td>
<td>apolipoprotein E Receptor 2 (aka LRPR8)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD-1</td>
<td>general multipurpose mouse strain</td>
</tr>
<tr>
<td>CZB</td>
<td>modified BMOC-2 (Brinster's medium for ovum culture) medium named after Chatot, Ziomek, and Bavister</td>
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<tr>
<td>DIOs</td>
<td>deiodinases</td>
</tr>
<tr>
<td>Dio1</td>
<td>iodothyronine deiodinase 1</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
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<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>ICSI,</td>
<td>intracytoplasmic spermatozoa injection</td>
</tr>
<tr>
<td>NGS,</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RBC</td>
<td>red blood cells</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SBP2</td>
<td>selenocysteine insertion sequence binding protein 2</td>
</tr>
<tr>
<td>Se</td>
<td>selenium</td>
</tr>
<tr>
<td>Sec</td>
<td>selenocysteine</td>
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<tr>
<td>SECIS</td>
<td>selenocysteine insertion sequence</td>
</tr>
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<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>SecS</td>
<td>Selenocysteine tRNA Synthase</td>
</tr>
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<td>SelI</td>
<td>Selenoprotein I</td>
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<td>SelT</td>
<td>Selenoprotein T</td>
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<td>SelV</td>
<td>Selenoprotein V</td>
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<tr>
<td>SelW</td>
<td>Selenoprotein W</td>
</tr>
<tr>
<td>SeMet,</td>
<td>Selenomethionine;</td>
</tr>
<tr>
<td>Sepp1</td>
<td>Selenoprotein P</td>
</tr>
<tr>
<td>SPS2</td>
<td>Selenophosphate synthetase 2</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>tRNA(^{[\text{Ser}\text{Sec}]})</td>
<td>Selenocysteine tRNA</td>
</tr>
<tr>
<td>TRxR</td>
<td>Thioredoxin reductase</td>
</tr>
<tr>
<td>TRx</td>
<td>Thioredoxin</td>
</tr>
<tr>
<td>TYH</td>
<td>Toyoda, Yokojama and Hoshi (ICSI media)</td>
</tr>
<tr>
<td>U</td>
<td>Sec</td>
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Selenium is a nutritional trace mineral essential for human health that is acquired primarily through dietary intake. Selenium (Se) is primarily found in soil which provides nutrients for crops and livestock. Although soil and food in the United States have adequate selenium, soil in other parts of the world, such as parts of China and New Zealand are selenium deficient. Selenium is incorporated into selenoproteins as the 21\textsuperscript{st} amino acid, selenocysteine. Many of the twenty-five known selenoproteins in humans, of which 24 are also found in rodents [19], have antioxidant properties. Selenium deficiency has been implicated in many diseases and conditions including neurodegeneration [88], infertility [12,61], cardiovascular disease, immune disorders, and cancer [25,37,43] in humans, and white muscle disease in livestock [64]. While Se is a vital micronutrient, excessive Se intake can lead to toxicity, a condition known as selenosis. In certain Se deficient areas of China, Keshan’s disease is a prevalent but preventable cardiomyopathy that can be treated with Se supplementation [99,101]. Despite much progress in describing members of this unique family of proteins, the properties and mechanisms of many of the selenoproteins are still not clearly understood. Vertebrate selenoproteins are highly conserved through evolution. Along with the fact that Se is required for life and implicated in a host of diseases, further elucidating the diverse functions and mechanisms of the family of selenoproteins may make critical contributions to treating many diseases and conditions. Here, I provide an overview of the current understanding of Se and members of the selenoprotein family, including the two that are central to this study, selenoprotein P (Sepp1) and the antioxidant enzyme glutathione peroxidase 4 (GPx4).

**Selenoproteins**

Selenium (Se) is a requisite micronutrient that is biochemically processed by selenoproteins that incorporate Se through the 21\textsuperscript{st} amino acid, selenocysteine (Sec) [11]. Sec has a structure that is nearly identical with that of cysteine, with the exception of a single atom, selenium, in place of sulfur, giving Sec a lower pKa (5.2 versus 8.3) and higher reactivity [9]. The general selenocysteine biosynthesis pathway has been...
conserved in archaea and eukaryotes that have selenoproteins [96]. However, not all kingdoms and phyla within these domains have the capacity for selenoprotein synthesis [47]. The bioavailable source of Se for most organisms occurs in the organic form of selenomethionine which has been implied to be more effectively absorbed and in the inorganic sodium selenate (i.e. supplements and animal food) [36]. The selenoprotein family exhibits diverse tissue allocation, from globally to tissue-specific expression. Subcellular localization also varies greatly, with some selenoproteins solely expressed in certain organelles or as transmembrane proteins, while others are secreted to extracellular spaces or plasma [9,74]. Several selenoproteins have been characterized as antioxidant enzymes that alleviate damage caused by reactive oxygen species (ROS) [4,22,80]. Selenoproteins may have potential roles as modulators of redox-regulated signal transduction [4].  

Three classes of selenoproteins, the GPxs (glutathione peroxidases), TRxRs (thioredoxin reductases) and DIOs (deiodinases) were among the first eukaryotic selenoproteins discovered and are the most extensively studied [9]. The GPxs, the largest selenoprotein family in vertebrates [54], are integral to antioxidant glutathione pathways, providing protection from reactive oxygen species (ROS). Five of the GPxs in humans (four in mice) are selenoenzymes [3]. GPxs are hydroperoxidases that use glutathione as a cofactor. The TRxRs use NADPH for reduction of TRx (thioredoxin) in cellular redox pathways [89]. One member of the GPxs which will be further discussed in this study is the enzyme GPx4. The three known isoforms of GPx4 are cytosolic, mitochondrial (mGPx4), and nuclear (nGPx4). The last two have been implicated in recent studies to be involved in spermatogenesis and male fertility. Glutathione peroxidase reduces hydrogen peroxide and alkyl hydroperoxides at the expense of glutathione. These selenoenzymes contribute to the antioxidant defense system in mammalian cells [15,56].  

Other selenoproteins include SelW which is highly expressed in skeletal muscle and found to be involved in white muscle disease in livestock [92]. It is similar to the GPx family in that it shares the redox motif and binds glutathione [7]. SelH (selenoprotein H) is a nuclear-localized DNA-binding protein that may act as a transcription factor that is involved in oxidative cellular stress response [9,63]. SelI (selenoprotein I) was found to be the mammalian form of the phospholipid-synthesizing enzyme ethanolamine phosphotransferase [9,40]. SelR (selenoprotein R)/SelX (selenoprotein X) is a member
of the methionine sulfoxide reductase family, important for reduction of sulfoxymethyl groups [9]. SelN (selenoprotein N) is localized to the ER (endoplasmic reticulum) membrane and may be necessary for proper muscle development [67]. SelS (selenoprotein S) is also ER-localized and is important for removal of misfolded proteins from the ER membrane [5]. The 15 kDa selenoprotein, Sep15, and SelK, SelM and SelT (selenoproteins K, M and T respectively) are small ER proteins with largely unknown functions [74]. SelM was recently reported to have neuroprotective functions [73]. SelO and SelV (selenoproteins O and V, respectively) are two of the least known in this family. SelO is widely distributed, whereas SelV expression is limited to testes [32].

Selenoprotein P (Sepp1 or SelP) is one of the more well studied selenoproteins, and has been implicated in having a protective function in conditions including neurodegeneration in Alzheimer’s disease [8,41,88]. Sepp1 is a secretory protein theorized to be the delivery system that transports Se from liver to other tissues and hence enables selenoprotein synthesis. Sepp1 has also been suggested to play a critical role during development and normal male fertility [61]. Numerous studies have shown that selenium plays an important role in reproductive health [12,20,30,31]. Selenium deficiency has been reported to be involved in several reproductive and obstetric complications including male and female infertility, miscarriage, preeclampsia, fetal growth restriction, preterm labor, and gestational diabetes [57]. As this study focuses on reproductive biology, we will further discuss our findings involving Sepp1 in male fertility and its interaction with GPx4 later in this study.

Further investigation is important for elucidating the mechanisms of selenoproteins to understand how altering levels of Se and different selenoproteins may affect biological functions and human health.

**Synthesis of Selenoproteins**

Selenocysteine (Sec) biosynthesis is unique in that it takes place on its tRNA that recognizes the UGA codon, typically a stop codon [72]. Thus, recoding of the UGA codon, located in the coding region of selenoprotein mRNAs, from a stop codon to a selenocysteine-insertion codon is required. During the unique translational sequence of selenoproteins, *cis-* and *trans-*acting factors work synchronously to redirect translational machinery to insert selenocysteine at UGA codons instead of terminating polypeptide synthesis. These factors include an element in the 3′-UTR (untranslated region) of
eukaryotic selenoprotein mRNAs, termed the Sec insertion sequence (SECIS), which was discovered following the cloning of another selenoprotein, iodothyronine deiodinase 1 (Dio1) [9,10]. All eukaryotic selenoproteins require a form of the SECIS element for recoding UGA to the Sec codon [53]. The translational machinery within the cell typically identifies the UGA codon as a termination signal, thereby releasing the nascent polypeptide from the ribosome [33]. This biosynthesis of selenocysteine on tRNA^{Ser}Sec is catalyzed by selenocysteine synthase [51,95] and the tRNA^{Ser}Sec-modifying enzyme phosphoseryl-tRNA^{Ser}Sec kinase [21]. The translation process also requires a SECIS-RNA binding protein (SBP2) [26,44] which recruits a specialized elongation factor [27,91], that delivers selenocysteyl-tRNA^{Ser}Sec to the A-site of the ribosome. Another Sec-tRNA^{Ser}Sec binding protein, SecP43, is required for methylation of the 2'-hydroxyl-ribosyl moiety in the wobble position of the selenocysteyl-tRNA^{Ser}Sec. This may regulate the shuttling of the selenocysteine synthase-selenocysteyl-tRNA^{Ser}Sec complex between the nucleus and cytoplasm [83,97].

Under low dietary selenium conditions, selenocysteine incorporation is inefficient, resulting in some selenoprotein mRNAs being degraded via nonsense-mediated decay [58]. Se remains high in testes and brain even in low Se conditions [34], in which Sepp1 may potentially be broken down to recycle Se or remain circulating in blood while gradually being taken up in brain and testes. Nonsense-mediated decay is a pathway that targets mRNAs containing premature termination codons for degradation [44]. The presence of both a UGA codon and an RNA element downstream of the UGA were shown to be necessary for selenium-dependent regulation of mRNA turnover [58]. Degradation of selenoprotein mRNAs under conditions of low Se is not uniform, with some transcripts being more sensitive to nonsense-mediated decay than others [86,94]. Several factors may contribute to the sensitivity of selenoprotein mRNAs to nonsense-mediated decay [84] at different steps of the translation process [21]. This highly conserved and unique process that is energetically demanding on the cell suggests and underscores the importance of selenoproteins.
**Selenoprotein P**

Selenoprotein P was first identified through biochemical studies in 1982 [60]. Sepp1 is an unusual selenoprotein, containing ten Sec residues in humans, 16–18 in amphibians and fish, and 28 in sea urchins [9]. With the exception of selenoprotein P, most selenoproteins typically have only one selenocysteine residue per polypeptide chain. Sepp1 is primarily secreted from liver cells to deliver selenium to other tissues and organs in the body (Fig. 1) [17]. Selenium cycling through Sepp1 in plasma occurs at a high rate as indicated by the 3-4hr half life in plasma [34]. Roughly 25% of whole-body selenium passes through rat plasma daily [17]. Plasma Sepp1 most likely supports homeostatic expression of GPxs, TRxRs and other selenoenzymes [9] through its role in supplying selenium to cells throughout the body [18]. In addition, albeit at the cost of oxidizing TRx [9,87], Sepp1 also reduces peroxynitrite-induced protein oxidation and nitration, as well as lipid and low-density lipoprotein (LDL) peroxidation, indicating a potential role in oxidant defense [2]. Sepp1 is also synthesized in smaller amounts in the brain. Sepp1 has been found to be highly expressed in the liver, testes and kidneys. Under selenium deficient conditions, selenium is better retained in the testes and brain relative to the other organs, which is likely due to Sepp1 uptake through its known receptors, ApoER2 and Megalin. When selenium is limited, Sepp1 synthesis has priority over glutathione peroxidase synthesis [17] and [39,100].

Sepp1 consists of two domains with the smaller C-terminal domain containing nine selenocysteine residues, while the larger N-terminal domain has only one Sec residue in a redox motif [34]. Sepp1 binds to at least two receptors of the lipoprotein receptor family, ApoER2 and Megalin. ApoER2 facilitates the uptake of Sepp1 into the testis and allows retention in the brain. Megalin facilitates uptake of filtered Sepp1 into the proximal tubule cells of the kidney [34]. Evidence shows that mutant mice absent of the Sec-rich C-terminus exhibit severe deficiencies in brain Se if not supplemented in the diet and have greater susceptibility to infections and morbidity [18,72]. Previous work from Bellinger et al [8] have also demonstrated that Sepp1 is associated with amyloid plaques and neurofibrillary tangles in Alzheimer’s disease (AD) in a potentially neuroprotective capacity and may have neuroprotective properties [88].

The role of Se in fertility has been increasingly studied. Sepp1 has important implications in normal male reproductive function. Studies have shown that mRNA that encodes Sepp1 in rats is selectively expressed in Leydig cells, the testosterone
producing cells in the testes [61]. It is unknown whether Sepp1 is produced by the epididymis or whether it may be transported to the tubule lumen to act directly with sperm. Thus the absence of Sepp1 may contribute to the defective sperm phenotype. Furthermore, Sepp1 is also suggested to function in oxidant protection [18,59,61] and has been shown to promote the survival of cells in culture [61,80]. Increased oxidative stress may occur if epididymal selenoprotein synthesis is compromised [49,61]. Maintaining Sepp1 homeostasis is critical for overall Se balance. Researchers investigating the role of Se in spermatogenesis have identified a remarkable increase in Se specifically in late spermatids by X-ray fluorescence microscopy [45]. Se was primarily observed in the midpiece of the sperm. The study suggests that the enrichment was due to elevated levels of the mitochondrial form of GPx4 which was completely reliant on the Se supplied by Sepp1 [45]. Impaired GPx4 biosynthesis, due to selenium deficiency or to genetic defects in GPx4 or in Sepp1, has been implicated in causing male infertility [30]. In 2001, the nuclear GPx4 isoform was identified to be exclusively in the late spermatids and involved in DNA reorganization [68,81]. The mitochondrial isoform of GPx4 has been suggested as one of the most relevant involved in spermiogenesis [30]. However, evidence from a recent study demonstrated that absence of nGPx4 leads to sperm nuclear matrix/chromatin instability that may negatively affect the embryo development [71]. These and other studies implicate an important relationship between Sepp1 and GPx4 in reproductive function and other dysfunctions in human diseases, thus meriting further investigation.
Figure 1. Selenoprotein P biosynthesis pathway. Sepp1 is principally synthesized in the liver, and secondarily in smaller amounts in the brain. Sepp1 is thought to transport selenium to other tissues in the body. Sepp1 binds to the low-density lipoprotein receptor ApoER2 in brain and testes and Megalin in kidneys.
Selenoprotein P Knockout Mice

Sepp1<sup>−/−</sup> mice were generated by introducing a construct containing a neo<sup>′</sup> cassette flanked by LoxP sites inserted into the second exon, 9 bases downstream from the Sepp1 start codon by electroporation into 129S9/SvEvH-derived embryonic stem (ES) cells [34]. These ES cells were subsequently injected into C57BL/6 blastocysts. The resulting chimeric males were bred with C57BL/6J females. The stop codons present in both LoxP sites prevent translation of SEPP1.

In brief, the LoxP sites are part of the Cre-LoxP site-specific recombination system. LoxP sites consist of two 13 bp inverted repeats separated by an 8 bp asymmetric spacer region. The Cre-LoxP system was discovered in the 1981 in which Cre is a recombinase protein that originates from the P1 bacteriophage [85]. The fundamentals of the system is based on one Cre molecule that binds per 13 bp inverted repeat or two Cre molecules binding at one LoxP site. Recombination occurs in the asymmetric spacer region in which directionality of the recombination site is dependent on this 8bp region. Two arrangements of the LoxP sites can be designed. When two LoxP sites are in opposite orientation to each other, inversion of the DNA flanked by the sites occurs. Alternatively, if the two LoxP sites are oriented in the same direction as each other, this dictates an excision of the flanked DNA, leaving only one remaining LoxP site. The Sepp1<sup>−/−</sup> mice were constructed by the latter of the two described LoxP arrangements. The site-specific excision of a particular piece of DNA can be used to eliminate or inactive the endogenous gene or a transgene, or activate a transgene [76]. The Cre-LoxP system is typically used to generate conditional knockout animals. In some cases, researchers may choose to generate a conditional knockout due to an embryonic lethality of a complete whole body knockout of a gene of interest. In other cases, researchers may use this method to study the function of a gene when it is absent or knocked out in a specific organ or cell population.

In this study, we will demonstrate the novel application of the Cre-LoxP system to restore the Sepp1 gene in the Sepp1<sup>−/−</sup> mice using the LoxP site’s own start codon. As the first objective of this study, based on sequence analysis of the Sepp1<sup>−/−</sup> construct, we predicted that introduction of a Cre recombinase transgene to the Sepp1<sup>−/−</sup> mice would excise the floxed neo<sup>′</sup> containing construct, however preserving a start codon in the LoxP site in frame with the Sepp1 gene. Further details regarding the genetic restoration
of Sepp1 gene, using this unique application of the Cre-Lox P system, will be discussed as this is one of the two objectives in this study.

Sepp1 KO mice fed a selenium inadequate diet of 0.1 mg/kg, originally generated by the Burk lab, exhibited an abundance of deficiencies compared to their wild type counterparts including reduced weight, smaller body size, poor motor coordination development, and strikingly reduced fertility [34]. Sepp1 KO mice have very low selenium concentrations in the brain, the testis, and the fetus, with severe pathophysiological consequences in each tissue [17]. A study by the Burk lab showed genetic deletion of Sepp1 did not alter liver selenium levels apart from when Sepp1 KO mice were on a less than adequate selenium supplemented diet, below 0.1 mg/kg, liver Se levels were then increased [34]. Selenium levels in brains of mice on the same diet were reduced by 19% and did not change with selenium supplementation [34]. Kidney selenium levels were reduced to 76% of normal levels and did increase to normal levels upon a selenium adequate diet of 0.25 mg/kg diet [34]. Deletion of Sepp1 causes increased excretion of selenium in the urine and, as a result, decreases whole-body selenium [16,18]. Sepp1−/− mice on a 0.10 mg/kg selenium diet developed spasticity and abnormal movements, performing poorly on motor coordination tests such as the rotorod and pole climb, whereas this diet served as sufficient selenium requirements for wild type mice [35]. The results of the study implicate the absence of Sepp1 contributing towards irreversible brain damage. ApoER2 is one of the known receptors in which Sepp1 binds. In other studies, ApoER2−/− mice on selenium deficient diets, showed similar neurological deficits to that of the Sepp1−/− [18]. The study suggests that interruption of selenium supply evidenced by decreased selenium uptake to the brain leading to neurological deficits were due to impairment to the Sepp1-ApoER2 pathway [18].
A. Sepp1 KO design

Hill, K.E., et al, 2003
Figure 2. Sepp1 KO rescue strategy. (A) Selenoprotein P knockout construct design [34] (B) Sepp1 mice were screened by genotyping and PCR of genomic DNA with primers spanning the Neo\textsuperscript{r} construct and exon 2 (C) Sequence comparison between Sepp1 wild type and newly recombined Sepp1\textsuperscript{fr} CMV+: LoxP sequence, signal peptide sequence of Sepp1 KO after recombination with CMV-Cre gene to produce Sepp1\textsuperscript{fr} CMV+ mice.
The Role of Selenoprotein P in Male Fertility

Studies have demonstrated that the Sepp1 gene is essential for development of functional spermatozoa. The testis exports selenium as sperm selenoproteins whereas the brain is not known to export the element. Severe selenium deficiency has been found to produce defective spermatozoa resulting in reduced fertility [61]. Sepp1 is suggested to be an indispensible component of the selenium delivery pathway for developing germ cells [61].

A previous study found that Sepp1 and GPx4 gene expression were down-regulated in cadmium-induced testicular pathophysiology in rats [55]. Cadmium is a toxic heavy metal that is associated with severe testosterone depression and sperm motility defects, resulting in infertility. Infertile men with oligoasthenozoospermia exhibit decreased sperm motility and sperm counts. In an early study of infertile men with this condition, it was revealed that total mitochondrial GPx expression levels were significantly decreased in sperm of these subjects [42].

Numerous studies in mice have shown that mature spermatozoa of Sepp1−/− males have specific structural defects in sperm flagella that develop sequentially during spermiogenesis and after testicular maturation in the epididymis [61]. The study found development of a truncated mitochondrial sheath, an extrusion of a specific set of axonemal microtubules and outer dense fibers from the principal piece, and an acute bend present at the midpiece-principal piece junction [61]. The mitochondrial capsule, containing the selenoprotein GPx4 as a structural element [52,61], was also implicated in at least some of the sperm defects [61,69]. GPx4, as a member of the glutathione peroxidase selenoprotein family, has well-characterized antioxidant activity. This study determined that these sperm defects found in Sepp1−/− males were similar to those observed in wild-type (Sepp1+/+) males fed a low selenium diet (<0.25 ppm) [19,61]. Sepp1 has also been reported to be present in vesicle-like structures in the basal region of Sertoli cells [62], which have been referred to as “nurse” cells since they nourish the developing sperm cells through the stages of spermatogenesis. Sertoli cells phagocytose residual cytoplasm during spermatogenesis [62]. Studies in ApoER2−/− mice suggest that Sertoli cells aid in the uptake of Sepp1 from the interstitial fluid by hydrolyzing Sepp1 within the lysosomes potentially using a receptor-mediated pathway [62].
ApoER2 has been suggested to mediate endocytosis by which Sepp1 supplies selenium for spermatogenesis. Researchers have found evidence that Sepp1 functions in conjunction with ApoER2 and absence of either may interrupt selenium supply, which could also contribute to the production of defective spermatozoa and some abnormalities in sperm function. This may be correlated to the signaling function of ApoER2 specifically in the testis, involving Sepp1 in various capacities including selenium trafficking [62]. The complex interaction of Sepp1 with GPx4 and the ApoER2 receptor in spermiogenesis and ultimately sperm fertility is an important issue that necessitates greater understanding in developing effective therapies for male infertility.
CHAPTER II

MATERIALS AND METHODS

Animals

Animals were provided food and water as needed per University of Hawaii veterinary protocol. All animals in this study were maintained on diets containing adequate Se (~0.25 ppm). Animals were on kept on a 12-h light cycle and group housed during breeding and rearing. Prior to and during behavioral testing, animals were individually housed in polycarbonate cages. Each cage was provided food, water, and a layer of bed-o-cob (corn cob) bedding (Newco Distributers). All animal protocols were approved by the University of Hawaii Institutional Animal Care and Use Committee.

Generation of Sepp1−/− and Sepp1+/− CMV+

Sepp1+/− mice were obtained from the laboratory of Dr. Raymond Burk at Vanderbilt University. Mutant mice were backcrossed to C57BL/6J for at least 10 generations before arriving in our lab and were bred with our C57BL/6J colony to ensure congenic strains [38]. As male Sepp1 mice are infertile [34], Sepp1+/− mice were used for breeding resulting in littermate Sepp1−/− and Sepp1+/+ pups. Sepp1 whole body genetic rescue mice (Sepp1+/− CMV+) were generated by breeding Sepp1+/+ mice to C57BL/6N-Hprttm1(CMV-cre)Brd/Mmucd (Jackson Labs). Confirmation of the expected recombination of Sepp1 was carried by polymerase chain reaction (PCR) of extracted genomic DNA from mouse tails using specific primers to amplify a 151-bp product in the targeted region present in the wild type (forward-ACCTCAGCAATGTGGAGAAGCC, reverse-TGCCCTCTGAGTTAGCA), and a 472-bp and a 224-bp product specific for the knockout allele and floxed gene, respectively (forward-ACCTCAGCAATGTGGAGAAGCC, reverse-GATGATCTGGACGAAGAGCATCA). Products were run on a 1.5% DNA agarose gel, and SYBR® Safe DNA Gel Stain (InVitrogen) was detected by UV imaging to confirm genotype.

Animal Behavior

Adult Sepp1+/+, Sepp1+/− CMV+, or Sepp1−/− mice 12 to 20 weeks of age (age matched for each assay) were evaluated for neuromotor and neurobehavior effects using typical behavior paradigms that included Morris water maze [66], and open field test [70,93].
**Morris water maze**

Hippocampal-dependent spatial learning and memory was assessed using a Morris Water Maze (MWM) assay [66]. Mice were placed in a large circular pool of opaque 24°C heated water (nontoxic, water based paint was added to achieve opacity). Visual cues were placed on walls to provide mice with spatial orientation and reference points. The time required to escape from water onto a hidden platform is measured. The total time required for mice to swim to a visual platform (60 s maximum) was determined prior to the training days. Each mouse was given 60 s to find the visible platform or after this period, the mouse would be removed from water and placed on the platform for 15 s. During the 8 days of training of 4 trials per day, the platform was submerged and the total time (60 s maximum) for mice to find the hidden platform was recorded. On the ninth day, the platform was removed for the probe trial. The total time spent in each quadrant and the number of platform crossings was monitored over a 60 s period.

**Pole Test**

Locomotor function was evaluated using a pole test that examines rodent locomotor function involving the cerebellum, motor cortex, and basal ganglia [29]. Mice were placed on the top of a pole with heads oriented upward and parallel to the pole. After 2 days of training of 4 trials per day, the time taken to invert to facing downward and the total time to descend were recorded in 4 trials on the third day. Mice were given 60 s to perform this task.

**Stride test**

A stride test, modified from Fernagut et. al, 2002 [28], was used to measure deficiencies in gait and motor ability. Paw prints were obtained by applying ink to the hind limb paws of the mice before placing them on graph paper in a narrow runway. Bright lighting was used to encourage the mice to walk toward a dark enclosure at the opposite end of the runway. Length of stride for each paw and width of strides were measured from the resulting footprints.

**Sperm morphology**

Sperm morphology studies were performed by isolating fresh epididymal sperm from 4-6 month old male Sepp1 WT, Sepp1 KO, and Sepp1<sup>+/−</sup> Cre+ mice. Mice were
euthanized by CO\textsubscript{2} prior to harvesting epididymides. In a glass bottomed culture dish, cauda epididymal sperm were extracted by applying pressure to the cauda epididymis in Toyoda, Yokojama and Hoshi (TYH) media. Sperm were visualized using an Olympus IX-71 microscope with DIC optics under 40X and 63X oil objectives and Pictureframe imaging software.

**Oocyte and sperm collection**

Mature oocytes at metaphase II were obtained from B6D2F1 females at 2-3 months old. Equine chorionic gonadotropin (CG) (5 IU) and human CG (hCG) (5 IU) were administered 48 h apart. About 15-16 h after the injection of hCG, cumulus-oocyte complexes were collected from the oviducts and treated with 0.1% hyaluronidase to remove cumulus cells. Sperm were collected from Sepp1\textsuperscript{+/-} and \textsuperscript{-/-} adult males at 4-6 months old, euthanized by cervical dislocation. Cauda epididymal sperm were suspended in HEPES-CZB medium (20mM HEPES-HCl, 5mM NaHCO\textsubscript{3}, 0.1 mg/ml polyvinyl alcohol) [98], for 20 min at 37°C before use [79].

**Intracytoplasmic spermatozoa injections (ICSI)**

ICSI was performed according to Kimura and Yanagimachi [48] with minor modifications [98]. A drop of sperm suspension was mixed with 12% (w/v) polyvinylpyrrolidone (PVP) in HEPES-CZB. The head of a single sperm was isolated from the sperm flagella and injected into each oocyte with a piezo-driven micromanipulator. We observed pronuclear formation under an inverted Olympus microscope at 6 h after sperm injection. Fertilized oocytes with two pronuclei were cultured for 24 h in CZB medium [23] at 37°C under 5% CO\textsubscript{2} in air. The next day we observed 2-cell stage embryo development.

**Embryo transfer**

Embryos at 2-cell stage were transferred into the oviduct of CD-1 surrogate mothers (day 0.5) that had been mated with vasectomized CD-1 males the previous night. Surrogate mothers were euthanized on Day 19.5 after embryo transfer to obtain newborn pups.
**Immunofluorescence assay**

Sperm preparation for immunofluorescence was performed according to Paul et al [65]. Sperm were harvested from Sepp1\(^{/+/}\), \(^{{-/-}}\), and \(^{n/r}\) CMV+ 4-6 month old male mice epididymides in ice cold 1X phosphate buffered saline (PBS). Epididymides were minced and sperm strained in 70-µm nylon strainer (VWR International), red blood cells (RBCs) were lysed with 0.45% saline, washed. Epididymis/sperm lysate were sonicated twice on ice for 30 min in 20 mM phosphate buffer, pH 6 with 1mM EDTA. Samples centrifuged at 500 x g for 5 min at room temperature (RT) and resuspended in 50mM Tris-HCl, pH7.4 containing 1% SDS, incubated for 10 min at RT. After centrifugation (10,000 x g, 1 min) supernatant was removed, pellet rewashed with 50mM Tris-HCl, pH 7.4. Sperm resuspended in 25mM Tris-HCl with 0.25M NH\(_4\)SO\(_4\) and 40mM DTT, and incubated at RT for 10 min. 30 µl aliquots were placed on glass slides (Fisher Scientific) on ice, incubated for 40 min. washed in PBS (2 min), fixed in 4% paraformaldehyde (PFA) for 30min at RT. Slides were washed (PBS), air dried and stored at -20°C. Frozen slides were thawed in PBS for 5 min the following day. Blocking was in 1:4 normal goat serum (NGS) in 3% bovine serum albumin/tris-buffered saline (BSA/TBS) for 30 min. Samples were incubated at 4°C with (1:500) rabbit GPx4 monoclonal primary antibody overnight (AbFrontier). The next day, slides were placed in glass staining containers and gently rinsed three times with PBS. Alexafluor 546 anti rabbit (Invitrogen) secondary antibody was applied to samples and incubated for 45 min. at RT in a light protective container. Sperm samples were washed three times with 1X PBS and mounted using Vectashield Hardset Mounting Media containing the nuclear stain DAPI (Vector Laboratories). Samples were viewed with the Zeiss Axioskop Plus 2 and images collected with the AxioCam MR3 digital camera.

**Western Blot analysis**

GPx4 protein expression in testes and epididymides of age matched Sepp1 WT, KO, and rescue mice were measured by western blot. Tissues were lysed with CelLytic MT (Sigma Aldrich Co) according to the manufacturer’s instructions. Protein lysates were resolved by SDS-PAGE separated on a 10–20% gradient Tris-HCl Criterion Precast gel (Bio-Rad Laboratories) and transferred to polyvinylidene difluoride (PVDF). For detection of GPx4 expression in testes, membranes were incubated in rabbit GPx4 polyclonal antibody (AbFrontier) diluted 1:2000, and for epididymides, GPx4 polyclonal antibody
diluted 1:5000 (Epitomics) in 1:4 Odyssey blocking solution in PBS (LiCore Biosciences) for 90 min at RT. Following washes in PBS (5 X 5 min), primary antibodies were detected with LiCor near-infrared fluorescent secondary antibodies in 1:4 blocking solution in PBS for 45 min followed by washes as described above. Subsequently blots were developed using β-actin (Sigma Aldrich Co.) as a loading control. Protein was detected using the Odyssey® Infrared Imaging System (Li-core Biosciences). Scanning and analysis were performed with Licor Odyssey software.

**Statistical analysis.**

Statistical analysis was performed with GraphPad Prism software. Interaction between genotypes and sex was ascertained by Student’s t-test and two-way analysis of variance (ANOVA) with Bonferroni’s posthoc test for multiple comparisons. To determine genotype differences between experimental groups in the water maze training and quadrant entries in the probe trial, repeated-measures ANOVA with Bonferroni’s posthoc test were used. Statistical significance was defined as having p<0.05 for all statistical tests.
Chapter III

RESULTS

Selenoprotein P expressing Cre-recombinase mediated genetic rescue. Sepp1<sup>r/r</sup> CMV+ rescue mice were generated by breeding Sepp1<sup>r/r</sup> mice to CMV-Cre expressing mice, C57BL/6N-Hprt<sup>tm1(CMV-cre)Brd</sup>/Mmucd purchased from Jackson Labs. Sepp1 KO mice, originally generated by the Burk lab, were designed with a reverse-orientation neo<sup>r</sup> cassette flanked by LoxP sites, inserted into the second exon, 9 bases downstream of the start codon (Fig 1). The neo<sup>r</sup> containing construct effectively disrupts Sepp1 expression. The genetic deletion of Sepp1 results in a host of impairments including male infertility [61]. Sepp1<sup>r/r</sup> littermates were used for generating the rescue mice due to the infertility of Sepp1<sup>−/−</sup>. Breeding with the CMV-Cre resulted in recombinase expression in all cells, and the resulting recombination excised the neo<sup>r</sup> construct in the Sepp1<sup>−/−</sup> mice. A start codon in the remaining single LoxP site was left in frame with the Sepp1 gene, thus resulting in translation of a form of Sepp1 with a minor mutation in the Sepp1<sup>r/r</sup> CMV+ mice (Fig. 3B-C). Sepp1 genetic recombination in the Sepp1<sup>r/r</sup> CMV+ was confirmed with PCR genotyping, by identifying an amplified 224-bp product specific for the recombined gene in comparison to the 151-bp product in the targeted region present in the wild type and a 472-bp for the knockout allele (Fig. 3A). The ability of the resulting Sepp1<sup>r/r</sup> CMV+ progeny to sire pups was indicative that the Sepp1 gene was restored and functional.

Neuromotor behavior and gait impairments are recovered in Sepp1<sup>r/r</sup> CMV+ mice. Sepp1 KO mice have severe motor impairments encompassing irregular gait patterns described to include dragging of the limbs and uneven strides as well as motor coordination deficits [35,78]. We used the pole and stride tests to determine whether the Sepp1<sup>r/r</sup> CMV+ had complete genetic restoration. Motor coordination and general locomotor function of Sepp1<sup>r/r</sup> CMV+ mice, assessed with the pole test, were indistinguishable from those of the wild type group. The rescue mice in comparison to the wild type group exhibited no significant differences in the total time to descend the pole, whereas the Sepp1 KO mice had significantly decreased coordination and locomotion as shown by the greater total amount of time Sepp1 KO mice took to
descend to the bottom of the pole (Fig. 4). One-way ANOVA showed an effect of genotype on the time taken to descend (One-way ANOVA, **P = 0.0086 for Sepp1\textsuperscript{r/r} CMV+, Bonferoni’s post hoc test **P < 0.01). For the turn time, One-way ANOVA also showed effect of genotype (**P = 0.0024), with Bonferoni’s posthoc test (**P < 0.01 for Sepp1\textsuperscript{r/r} CMV+ and *P < 0.05 for Sepp1\textsuperscript{+/-}).

We subjected the mice to a stride test to determine whether the ataxia seen in Sepp1\textsuperscript{-/-} mice was recovered in the Sepp1\textsuperscript{r/r} CMV+. The rescue mice had restored gait patterns similar to wild type mice. Stride length and width were measured for four sets of paw prints of the mice hind paws. Both Sepp1\textsuperscript{r/r} CMV+ and wild type mice had significantly different stride length patterns compared to the ataxic irregular stride lengths of the Sepp1\textsuperscript{-/-} mice. Two-way ANOVA determined there was a significant effect of genotype (**P = 0.0034) with Bonferroni’s Post hoc analysis (*P < 0.05).

**Restoration of spatial learning and memory.** The Morris Water Maze (MWM) test was administered to assess whether spatial learning and memory was restored in the Sepp1\textsuperscript{r/r} CMV+ mice. The visual platform test was given on the first day to introduce mice to learning to escape the water by climbing onto the platform, prior to beginning the training period. There were no significant differences between groups in the time (60 sec maximum) they took to find the visible platform (2-way ANOVA). During the training period, rate of learning was assessed by daily changes in escape latency times, which are measured as the time taken to climb onto the hidden platform during each trial (60 sec maximum). Sepp1 rescue mice learned at the same rate as the wild type group, as shown by lack of differences in escape times between the groups. 2-way repeated measures ANOVA confirmed that there was no significant difference in escape latency during the 8 training days between Sepp1\textsuperscript{r/r} CMV+ and Sepp1\textsuperscript{+/-} mice. Escape latency times of both wild type and rescue mice were markedly less than those of Sepp1\textsuperscript{-/-} mice.

Sepp1\textsuperscript{-/-} mice MWM data were obtained in a different experiment in which all experimental procedures and parameters were the same, and kindly provided by Dr. Matthew Pitts. Sepp1\textsuperscript{-/-} mouse data were included only as an example for comparison of the behavioral deficits previously reported in Sepp1\textsuperscript{-/-} mice [66,72].

During the probe trial, the time spent in each quadrant was recorded to assess if mice recalled where the platform was during the training period. The number of platform crossings was monitored during a 60 second swim. We found no significant differences
between wild type and rescue genotypes for time in each quadrant, number of platform crossings, swim speed or distance (two-way ANOVA, P>0.05). Swim speed and total distance traveled were recorded to control for factors other than learning and memory differences that could affect the results, such as motor coordination.

**Sepp1<sup>tr</sup> CMV+ mice have normal sperm morphology and motility compared to Sepp1<sup>−/−</sup> mice.** Sepp1 KO mice have been shown to have abnormal sperm morphology. Structural differences in sperm have been implicated as being central to the infertility of Sepp1<sup>−/−</sup> mice [61]. As Sepp1<sup>tr</sup> CMV+ male mice were able to sire offspring, we investigated if they had restored normal sperm morphology. Sperm from cauda epididymides of Sepp1<sup>tr</sup> CMV+ mice as well as those of Sepp1<sup>+/−</sup> and Sepp1<sup>−/−</sup> mice were harvested for comparison. Microscopy imaging observations confirmed that the sperm from the rescue mice, Sepp1<sup>tr</sup> CMV+, had completely normal sperm morphology similar to Sepp1<sup>+/−</sup> absent of any kinks in the flagellum or narrowing at the posterior midpiece (Fig. 7). Additionally, Sepp1 rescue mice had normal sperm motility patterns and sperm numbers as compared to the controls.

**Sepp1 is required for viable sperm DNA, independent of sperm morphology.** Previous studies from other groups have implicated the role of Sepp1 in spermiogenesis and sperm morphology. Current literature shows that Sepp1<sup>−/−</sup> male mice have defective sperm morphology contributing to their infertility. In particular, the sharply bent flagella of Sepp1<sup>−/−</sup> sperm impede motility and prevent them from reaching oocytes and subsequent fertilization. However, the possible contribution of other abnormalities such as DNA aberrations to Sepp1<sup>−/−</sup> mice infertility has not been explored. Using intracytoplasmic spermatozoa injection (ICSI), we found that Sepp1 male sperm have reduced viability that is completely independent of motility. ICSI is a form of invitro fertilization (IVF) in which sperm heads are isolated and the flagella are discarded. Sperm heads are then directly injected into the oocyte, effectively excluding flagella morphology and motility as contributing factors to Sepp1<sup>−/−</sup> infertility. Our novel ICSI results strongly demonstrate that Sepp1 is required for sperm viability, independent of sperm flagella morphology (Table 1). The data show reductions in fertilization and development following injection of Sepp1<sup>−/−</sup> sperm into oocytes compared to Sepp1<sup>+/−</sup> sperm. Sepp1<sup>−/−</sup> mice sperm exhibited abnormalities reflected in the nearly 50% reduction in injected oocytes reaching the 2-cell pronuclei (PN) stage compared to heterozygous controls. Despite having a single
copy of the Sepp1 gene, Sepp1+/- sperm injected into oocytes resulted in normal fertilization and development at all stages observed. Only 45.3% of Sepp1-/- injected oocytes developed into 2-cell pronuclei zygotes, while 80.7% of oocytes injected with Sepp1+/- sperm progressed to 2-cell pronuclei zygotes. We also found that the rate of Sepp1-/- 2-cell pronuclei zygotes that survived (33.7%) until transfer to surrogate mothers was dramatically reduced compared to that of Sepp1+/- (76%). Finally, the rate of oocytes injected resulting in live pups born was also greatly reduced. Sepp1+/- control sperm injections resulted in 31 heterozygous pups, or 37.3% of the number of injected oocytes, whereas oocytes injected with Sepp1+/- sperm produced only 5 heterozygous pups, or 5.8% of the initially injected oocytes. Overall, Sepp1+/- sperm injected oocytes resulted in a more than 6-fold reduction in live born pups in comparison to controls.

**GPx4 protein expression is deficient in Sepp1 KO testes and epididymides.** Sepp1 maintains Se homeostasis by transporting Se from the liver to other organs. To further confirm our immunofluorescence microscopy results, we examined GPx4 expression by western blot. Protein was extracted from testes and epididymides were isolated from Sepp1+/-, Sepp1-/-, and Sepp1r/r CMV+. Western blot results show that GPx4 protein expression is dramatically decreased in both testes (one-way ANOVA, **P=0.0023) and epididymides of Sepp1-/- mice compared to Sepp1+/- as well as Sepp1r/r CMV+ (Fig. 8). As further evidence of the restoration of Sepp1 gene expression in Sepp1-/- mice by our unique application of the Cre-LoxP system, GPx4 protein expression in Sepp1r/r CMV+ testes and epididymides were similar to that of wild type.

**GPx4 immunoreactivity levels are decreased in Sepp1 KO epididymal sperm.** The importance of GPx4 in spermiogenesis is becoming increasingly clear. GPx4, a phospholipid hydroperoxidase, exists in three isoforms [71], two of which have been found to be important in spermiogenesis, sperm structure and function. Recent findings indicate that nuclear GPx4 (nGPx4) is central to sperm chromatin organization [12]. Using immunofluorescence imaging, we demonstrate that nuclear GPx4 immunoreactivity is significantly decreased in Sepp1-/- epididymal sperm compared to Sepp1+/- sperm, as shown by the decreased co-localization of GPx4 signal with the nuclear stain DAPI (Fig. 7). Furthermore, Sepp1r/r CMV+ rescue sperm GPx4 levels were restored and were similar to that of wild type controls.
Figure 3. Generation of Sepp1<sup>rr</sup> CMV+ rescue mice. (A) Polymerase chain reaction (PCR) amplification of mouse tail genomic DNA was used to confirm the genotypes of all animals. Specific primers were used to detect the 224-bp, 151-bp, and 472-bp product in the Sepp1<sup>rr</sup> CMV+ whole body rescue, Sepp1<sup>+/+</sup>, and Sepp1<sup>−/−</sup>, respectively. (B) Schematic of post recombination of Sepp1 KO with CMV-Cre mice in which the Neo<sup>+</sup> containing construct flanked by Lox P was excised, using the Lox P site start codon (C) that remained in frame with Sepp1 gene to restore Sepp1 translation.
Figure 4. Sepp1<sup>+/r</sup> CMV+ mice have normal motor coordination. (A) Total time taken for Sepp1<sup>+/r</sup> CMV+ rescue mice to descend the pole on the pole test was similar to wild type (WT) and significantly faster than Sepp1 KO mice as reflected in one way ANOVA (P=0.0086) results. (B) Time taken for mice to coordinate and turn on the pole before descending was significantly less for the Sepp1 rescue mice than Sepp1 KO mice, and similar to normal WT, one way ANOVA (P=0.0024) with Bonferroni's Post hoc analysis performed for both (*P<0.05, **P<0.01). Values are expressed as mean ± SEM.
Figure 5. Abnormal gait exhibited by Sepp1 KO mice are restored in Sepp1 Rescue mice. (A) Representative paw print showing gait patterns of Sepp1 wildtype, KO, and rescue mice. (B) Sepp1fr CMV+ mice exhibited more uniform stride length which were statistically significant compared to Sepp1 KO mice gait in the stride test. 2-way ANOVA of stride length (P=0.0034) with Bonferroni post hoc analysis showing the difference between genotype are significant (P<0.05). Length and width of hindpaw prints were measured from the center of each paw for each set of prints as indicated by the red lines.
A. Escape Latency

Mean Latency (sec)

Day of Training

Sepp1^{+/+} (n=6)
Sepp1^{-/-} (n=6) #
Sepp1^{+/r} CMV+ (n=6)

B. Probe Trial

% Time Spent in Quadrant

Target Opposite Left Right

Sepp1^{+/+} (n=6) Sepp1^{-/-} (n=6) Sepp1^{+/r} CMV+ (n=6)

C. Platform crossings

Crossings/60 sec

Sepp1^{+/+} Sepp1^{-/-} Sepp1^{+/r} CMV+
Figure 6. Sepp1<sup>+/r</sup> CMV+ mice have restored spatial learning and memory. (A) Latency to escape via the platform between wild-type and Sepp1<sup>+/r</sup> CMV+ mice was similar for the majority of the 8 training days, 4 trials per day, and both groups learned significantly faster than the Sepp1 KO mice. One-way ANOVA showed there was no significance difference between wild type and Sepp1<sup>+/r</sup> CMV+ mice. Escape latencies for both of these groups were faster than Sepp1 KO mice. (B) The probe trial recorded time spent in each quadrant and the number of platform crossings is monitored during a 60 second swim. All groups spent a similar amount of time in each quadrant. (C) Number of virtual platform crossing was recorded during the probe trial to determine if mice recalled where the platform was during training days. (D-E) Sepp1<sup>+/r</sup> CMV+ and wild-type mice performed equally in swim distance and speed. All analysis followed with Bonferroni’s post hoc analysis. (#) Sepp1<sup>+/r</sup> mice data were recorded on a different date of a MWM experiment under the same parameters and are present only to show inherent deficiencies of this genotype for relative comparison to normal behavior.
Figure 7. Sepp1<sup>−/−</sup> CMV+ (Rescue) mice have normal sperm morphology and motility. (A) Sepp1<sup>+/+</sup> normal cauda epididymal sperm. (B) Sepp1<sup>−/−</sup> have bent, extended flagellum, sharp bends and narrowing (red arrow) at the posterior midpiece, contributing to motility defects. (C) Sepp1<sup>−/−</sup> CMV+ exhibit normal flagella and restored motility.
Table 1. Comparison of Sepp KO and Sepp1<sup>+/−</sup> male fertility

<table>
<thead>
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<th></th>
<th>No. of oocytes injected</th>
<th>No. of 2 pronuclei (PN) oocyte (%)&lt;sup&gt;*&lt;/sup&gt;</th>
<th>No. of 2-cell PN embryos (%)&lt;sup&gt;**&lt;/sup&gt;</th>
<th>No. of 2-cell PN embryos transferred</th>
<th>No. of pups born (%)&lt;sup&gt;***&lt;/sup&gt;</th>
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<td>Sepp1&lt;sup&gt;+/−&lt;/sup&gt;</td>
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<td>67 (80.7)</td>
<td>63 (94)</td>
<td>63</td>
<td>31 (49.2)</td>
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<tr>
<td>Sepp1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>86</td>
<td>39 (45.3)</td>
<td>29 (74.4)</td>
<td>29</td>
<td>5 (17.2)</td>
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* % of oocytes injected  
** % of 2-PN oocyte formation  
*** % of 2-cell PN embryo transferred

Table 1. Comparison of Sepp1 KO and Sepp1<sup>+/−</sup> male fertility. Sepp1 is critical in sperm DNA viability, independent of flagella motility. Intracytoplasmic spermatozoa injection (ICSI) shows that Sepp1 is required for normal sperm DNA viability, independent of flagella morphology or motility. Sepp1<sup>+/−</sup> sperm injected oocytes resulted in a more than 6-fold reduction in sperm viability in comparison to controls. Sepp1<sup>−/−</sup> sperm injected oocytes resulted in 31 pups, 37.3% of the number of injected oocytes. Oocytes injected with Sepp1<sup>+/−</sup> sperm produced only 5 heterozygous pups, 5.8% of the initially injected oocytes.
Figure 8. GPx4 protein expression is significantly decreased in Sepp1<sup>-/-</sup> mice but restored in Sepp1<sup>+/r</sup> CMV+ mice. GPx4 protein levels are significantly decreased in Sepp1<sup>-/-</sup> mice testes (one-way ANOVA, **P= 0.0023) and epididymides. Sepp1<sup>+/r</sup> CMV+ testes and epididymides have similar GPx4 protein expression to Sepp1<sup>+/r</sup> confirming genetic restoration of Sepp1. These results show that Sepp1 is necessary for normal GPx4 protein expression and that Sepp1<sup>+/r</sup> CMV+ mice have normal GPx4 protein levels thus complete restoration of a functional Sepp1 gene.
Figure 9. GPx4 immunoreactivity is significantly decreased in epididymal sperm of Sepp1<sup>−/−</sup> mice. Representative images show DAPI (blue) nuclear staining and that GPx4 (red) staining is absent in Sepp1<sup>−/−</sup> epididymal sperm. Sepp1<sup>+/−</sup> CMV+ epididymal sperms have GPx4 immunoreactivity similar to Sepp1<sup>+/+</sup> confirming genetic restoration of Sepp1. These results correlate with western blot analysis that GPx4 levels are decreased to the point that GPx4 is not detectable compared to sperm from Sepp1 wild type and rescue mice. Images taken by Zeiss Axioskop Plus 2 40X objective.
CHAPTER IV

DISCUSSION and CONCLUSION

Sepp1 plays an important role in spermatogenesis and has been shown to be essential to normal sperm morphology. Surprisingly, the issue of sperm DNA viability due to the absence of Sepp1 has not been addressed. The results of this study demonstrate that the infertility of male Sepp1\(^{-/-}\) mice is partly due to sperm viability. Our ICSI data clearly demonstrates for the first time that sperm heads, consisting mostly of nuclei, are defective and result in impaired fertilization. Further, the developmental defects exhibited in early stages of the fertilization process ultimately lead to a 72.3% reduction in progeny number from oocytes injected with KO sperm compared to oocytes injected with heterozygous control sperm. In addition, we show that GPx4 protein levels in Sepp1 KO testes and epididymides are dramatically decreased overall and particularly in sperm nuclei. Western blot shows decreased GPx4 immunoreactivity in Sepp1\(^{-/-}\) epididymal sperm compared to both Sepp1\(^{+/+}\) and Sepp1\(^{+/}\) CMV+ mice, and immunofluorescence indicates that GPx4 colocalizes with the DAPI stained nucleus. We also show that our unique strategy of using the existing Cre-LoxP sites in the Sepp1\(^{-/-}\) mice to restore the Sepp1 gene was completely successful as the Sepp1\(^{+/}\) CMV+ mice had restored neuromotor function, spatial learning and memory, and fertility in males. Furthermore, GPx4 expression was restored to normal levels similar to those of Sepp1\(^{+/+}\) mice.

Sepp1 is a plasma protein thought to mainly function to transport Se from the liver to testes and other organs [18]. Studies show that Se is preferentially retained in brain and testes when dietary Se intake is low. Reduced Se levels and glutathione peroxidase (GPx) activity in reproductive organs of Se deficient male mice have been reported [81]. During spermatogenesis, Se content is noticeably increased in testes [82]. This requires increased uptake of Se to testes. As a transporter of Se, Sepp1 may be responsible for Se distribution during the reproductive process. Se deficiency is associated with abnormal testicular mass, sperm morphology, and is essential for biosynthesis of testosterone.

Sepp1 KO males are infertile and several studies have indicated that one of the main contributing factors to this condition is defective sperm morphology [61]. Sepp1 KO
males have acute kinks in their sperm flagella rendering them unable to swim to the oocyte, leading to infertility. Impairments from selenium deficiency can be restored in wild type animals by dietary supplementation; however this is not the case for Sepp1−/− mice. With dietary supplementation, neurological function of Sepp1−/− mice is improved, but other deficits remain. Sepp1−/− mice have abnormal sperm morphology regardless of supplementation. Sepp1 has direct antioxidant properties as well as facilitating synthesis of antioxidant selenoproteins such as GPx4.

To test our first hypothesis whether other factors aside from defective sperm morphology were involved in the infertility of male Sepp1−/− animals, we investigated whether isolation of the sperm head from the defective flagella followed by ICSI would produce normal fertilized oocytes and resulting litter size. As evident by our ICSI results, genetic deletion of Sepp1 reduces viability of the sperm. We observed reduced fertilization rates and impairments of early cell division stages in oocytes injected with Sepp1−/− sperm compared to the oocytes injected with Sepp1+/− control sperm. Our data show that Sepp1−/− male infertility is not entirely due to morphological sperm flagella defects as suggested by other studies, as absence of flagella in the ICSI procedure did not result in restored litter size.

It is not surprising that deletion of the Se transporter results in reduction in the levels of other selenoproteins, such as GPx4. Previous studies have shown that GPx4 levels are reduced in Sepp1−/− [75]. A 2009 study suggests that of the three GPx4 isoforms (cytosolic GPx4, nGPx4, mGPx4), the mitochondrially expressed form of the GPx4 gene is the most relevant one in spermiogenesis [77] while the nuclear form, nGPx4, is not important for fertility [74,77]. However, a more recent study by Puglisi et. al, shows convincing evidence that nGPx4 is associated with the sperm nuclear matrix and is essential for sperm chromatin decondensation [71]. The nuclear isoform of GPx4 is reportedly expressed in male germ cells. GPX4 contains a selenocysteine as well as several cysteines with the ability to reduce protein thiols [71]. Our study supports previous findings that impaired GPx4 biosynthesis, due to selenium deficiency or to genetic defects in GPx4 itself or in proteins involved in Se distribution and selenoprotein biosynthesis, results in male infertility [80]. We examined the GPx4 levels in whole epididymis, epididymal sperm, and testes of the Sepp1 rescue and KO mice compared to wild type controls to confirm the complete restoration of Sepp1 in our rescue model and to confirm that the Sepp1−/- mice exhibited alterations in GPx4 levels as reported by
other groups. Our results confirmed reduced GPx4 protein expression in testes, where testosterone and spermatozoa are synthesized. Moreover, we also showed that GPx4 protein expression was reduced notably in the epididymis, where sperm undergo a maturation process and mature active sperm are stored in the cauda epididymis. To further confirm the correlation of GPx4 levels, we used immunofluorescence microscopy to assess GPx4 immunoreactivity. In support of our western blot data, we observed drastically diminished GPx4 signal in Sepp1−/− epididymal sperm and restored levels of GPx4 in our rescue mice. Our findings suggest that rendering the Sepp1−/− gene inactive leads to decreased levels of GPx4. Sperm viability is clearly dependent on Sepp1 to deliver Se and regulate biosynthesis of GPx4.

The latter part of our study was aimed to genetically restore Sepp1 gene expression in Sepp1 knockout mice. We describe a strategy that takes advantage of the Cre-LoxP system, using the start codon in the LoxP site, to restore gene expression in the Sepp1 KO mouse model. Previously the Schweizer lab generated a hepatically targeted Sepp1 transgene to rescue Sepp1−/− mice [75]. However, our approach is novel in that we use the start codon in the existing LoxP site to genetically restore Sepp1 function, and can therefore rescue Sepp1 in any tissues with the appropriate Cre and without additional transgenic manipulations. One of our primary interests in generating this Sepp1 rescue model is to apply this method to restrict gene expression to specific cells or tissues in order to study the gene’s function within those cells or tissue. This unique application can allow researchers to study any gene of interest in highly specific cell populations. A researcher interested in such an approach to elucidate a specific function of a gene can specifically design the knockout mouse to have LoxP sites in which the LoxP start codon will remain in frame with the gene of interest following Cre recombination. Subsequent mating of mice expressing Cre in specific cell types, such as a subset of cardiomyocytes or neurons, will excise the KO construct, leaving behind a LoxP site with an in frame start codon. Following the successful generation of the Sepp1−/− CMV+ mice, in which we restored global expression of Sepp1, we have used this unique strategy to generate a brain specific Sepp1 rescue mouse model. Using a Cre expressing mouse with a promoter that drives expression in forebrain neurons, this mouse model expresses Sepp1 restricted to neurons in the forebrain, while Sepp1 gene expression remains inactive in all other tissues. We confirmed the complete restoration of the Sepp1 gene by performing a battery of behavior tests which resulted in Sepp1−/− CMV+ mice.
having restored neuromotor function as observed in the pole and stride tests. Furthermore, Sepp\textsuperscript{r/r} CMV+ mice had normal spatial learning and memory and did not exhibit any of the deficits seen in Sepp1\textsuperscript{-/-} mice when administered the Morris Water Maze test. Ultimately, the ability of Sepp1 rescue mice to sire pups that were used in the behavior tests was indication that infertility seen in male KO mice was restored. Furthermore, the sperm morphology studies confirmed that the rescue mice had normal sperm morphology and motility.

Further research is essential to understanding the mechanisms involved in Se function and the roles of various selenoproteins, such as Sepp1 and GPx4, in male reproductive health. Some studies have suggested that Se deficient Balb/c mice show decreased mRNA and protein expression patterns for both cJun and cFos (components of transcription factor AP-1, activator protein-1) [82]. These factors regulate cellular growth and differentiation and also have regulatory roles in spermatogenesis and steroidogenesis [82]. Jun has been detected during specific stages of testes differentiation [1,82]. cFos activity has also been observed in premeiotic germ cells of mammalian testes during mouse spermatogenesis [46,82]. Studies show that Jun D knockout mice have impaired spermatogenesis as well as reduced reproductive aptitude [82,90]. cJun expression has been observed in Leydig cells, potentially enhancing testosterone secretion, whereas in Se deficient mice, these cells as well as the seminiferous tubules showed abnormal morphology [82]. Alteration in Se supply may lead to these altered cJun and cFos patterns in the testicular germ cells, which might be responsible for decreased germ cell number, differentiation and reduced fertility [82]. The study suggests that this may be an explanation for the mechanism of Se action in regulating spermatogenesis [82].

As previously mentioned, Sepp1 and especially GPx4 have been reported to have antioxidant properties protecting cells from oxidative stress and similar insults. Investigation in sperm damage suggests a link between DNA fragmentation and oxidative base damage. Lipid peroxidation also induces sperm damage [101]. There is evidence showing that a significant proportion of the free-radical induced DNA damage observed in human spermatozoa is due to oxidative processes [102]. Antioxidants such as Sepp1 and GPx4 may have beneficial effects in alleviating sperm damage and may be instrumental as a treatment. A study by Shalini et. al showed that sperm from dietary Se deficient mice exhibited incomplete chromatin decondensation and increased
incidence of DNA breaks [81]. Sperm chromatin condensation in spermatogenesis is a complex process that involves sequential replacement of the majority of histones by transition proteins and protamines in testis [13,14,24]. During spermatozoa transit through the epididymis from caput (head) to cauda (tail), protamine thiol oxidation is completed and intra- and intermolecular cross-links are formed. Subsequently, a transcriptionally inactive and tightly packed haploid genome results and renders sperm nuclei more resistant to mechanical and chemical insults and mature [24]. There are many potential molecular mechanisms underlying this sperm damage, but certainly taking all these findings into consideration is an important factor.

The exciting results of this study increase our knowledge of the role of Sepp1 in relation to GPx4 and male fertility. Significantly, our ICSI results highlight that Sepp1 KO male mice are infertile due to sperm viability issues other than abnormal flagella morphology. Our findings contribute to the further understanding of the function of Sepp1 in mammalian sperm infertility issues.
Concluding Remarks and Future Studies

Selenoprotein synthesis is dependent primarily on dietary intake of the trace element Se. Selenoproteins have a unique energetically costly expenditure for biosynthesis. The 25 human (24 in mice) selenoproteins have highly diverse tissue and subcellular localization with each exerting specific functions in the subcompartments in which they reside [9,74]. The unique aspect of Sepp1 having 10 Sec residues suggests its importance for synthesis of other selenoproteins and its role in preventing loss of Se levels in brain and testes during low dietary Se intake [6,32,50]. Future experiments that may help further elucidate the mechanism of Sepp1 in sperm and male infertility include examining sperm chromatin decondensation and sperm fragmentation in the Sepp1⁻/⁻ mouse model. Elucidating the functional roles and significance of individual selenoproteins, such as Sepp1 and GPx4, will eventually provide crucial insight into how dietary Se and selenoproteins affect human health.
REFERENCES


