PP1γ2 LEVELS ARE HIGHLY REGULATED IN TESTIS TO ENSURE NORMAL SPERMATOGENESIS AND MALE FERTILITY

A thesis submitted to the
Kent State University Honors College
in partial fulfillment of the requirements
for General Honors

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May, 2013
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ACKNOWLEDGEMENTS

I would first like to thank my parents, for supporting me all my life and especially through my undergraduate years at Kent State University. I also thank my defense committee: Dr. Douglas Kline, Dr. S. Vijayaraghavan, Dr. Sara Newman, Dr. Susan Roxburgh, the Honors College of Kent State University for providing this opportunity, and National Institutes of Health for funding this project. I would especially like to thank my advisor, Dr. S. Vijayaraghavan for guiding me through this project and for being a supporting mentor through my undergraduate studies and beyond. I would like to thank Dr. Nilam Sinha, Sabyasachi Sen and Tejasvi Dudiki for their guidance, support and encouragement and also Nidaa Awaja, Suranjana Goswami and Rahul Bhattacharjee for helping me put together my thesis.
CHAPTER I
INTRODUCTION

The mammalian male reproductive system is comprised of the testis, encased in connective tissue Tunica albuginea and an intricate system of interconnected ducts. Both exocrine and endocrine in nature, the primary functions of the testis are to synthesize and secrete male sex hormones and generate sperms by the process of spermatogenesis. Sperm develops in the environment of the seminiferous tubules, which converges on the rete testis and leads out to a collective duct system called Epididymis [fig 1]. The Sertolli or Sustentacular cells lining the Epididymis sustain the proper environment required for spermatozoa development. One of the major roles of the Sertolli cells is to define two physiologically distinct compartments in the seminiferous tubule.

Spermatogenesis is a continuous process by which primary male germ cells in the seminiferous tubules produce mature male gametes, the spermatozoa. It begins with diploid spermatogonial cells dividing by the process of mitosis to produce the primary spermatocytes. The primary spermatocytes generated by the process of spermatocytogenesis move into the tubule, where it’s DNA is duplicated. The primary spermatocytes then undergo meiosis I to produce haploid secondary spermatocytes. These haploid spermatocytes rapidly enter meiosis II to produce spermatids by the process of spermatidogenesis. Each spermatid condenses the DNA contained within it, develops a tail and an acrosome giving rise to an immotile spermatozoa [fig 2]. These are then transported to the epididymis where they become motile [1].
Fig 1. A cross section of the mammalian testis
Fig 2. Development of the haploid spermatozoa from a primordial germ cell in the mammalian testis.
Phospho protein phosphatases, abbreviated as PPP, are enzymes that dephosphorylates certain phosphorylated proteins. Proteins are phosphorylated on nine amino acids, for example serine, threonine, tyrosine, cystine, etc. Of these, serine, threonine and tyrosine phosphorylation is predominant in eukaryotic cells and plays a role in key regulatory mechanisms. PPP1 belongs to protein serine/threonine phosphatases. Protein serine/threonine phosphatase is a big family of proteins found ubiquitously in eukaryotes. Protein phosphatase and protein kinase dynamically control the process of phosphorylation of structural and regulatory proteins in eukaryotes. Some of the physiological activities they are involved in include glycogen metabolism, calcium transport, muscle contraction and mitosis [2,11]. PPP 1 has four catalytic subunits PP1α, PP1β, PP1γ1 and PP1γ2 [fig 3]. PP1γ1 and PP1γ2 are obtained by alternate splicing of the PP1γ gene [fig 4]. The main structural difference between them lies in the C-terminus tail region [11]. PP1γ1 has seven exons while in PP1γ2, exon seven is internally spliced and translation of the mRNA adds an extra twenty one amino acid chain towards the 3’ UTR [13,14]. In terms of expression, PP1γ1 is expressed in Sertolli cells and PP1γ2 is expressed in developing germ cells. PP1γ2 is the most abundant isoform in the testis and an important regulator of sperm motility and function. PP1γ2 is also the only isoform detected in spermatozoa [5,12]. Previous research on mice has shown that PP1γ1 by itself is able to restore partial spermiogenesis but is unable to bring about motility and normal morphology, rendering those mice infertile. However, PP1γ2 by itself, in the absence of the γ1 isoform, is able to restore complete fertility [3].

**Fig 3. PPP1 and its four main isoforms**

**Fig 4.** Alternate splicing of PP1γ gene producing the two isoforms PP1γ 1 and PP1γ2
Previous research, found PP1γ2 to be the most abundant isoform in testis and an important regulator of sperm motility and function. Targeted disruption of PP1γ gene eliminated both spliced variants PP1γ1 and PP1γ2 and resulted in infertility in males due to impaired spermatogenesis and immotile sperms. However, females are unaffected, which implies that PP1γ gene has a crucial role to play in the testis [4].

In order to determine which isoform of PP1γ gene was the more important one, PP1γ2 was expressed as a transgene in mice lacking the PP1γ gene, using a testis specific promoter called PGK2. PGK2, which stands for phospho glycerate kinase 2, is a promoter which directs expression in testis [15]. Analysis of the results in terms of sperm count, morphology and motility, found that transgenic PP1γ2 males were fertile. This means that the absence of PP1γ2 is the main cause for impaired spermatogenesis [3].

Previous research has also shown that the amount of PP1 activity affects sperm motility. Too much activity renders them immotile but low activity levels initiate motility, as seen in caudal sperms [5]. The goal of the present work is to determine if over expressing PP1γ2 gene in a wild type (++) testis has any effect on spermatogenesis.

To go about doing that, wild type male mice were mated with heterozygous transgenic (+−/Tg+) females. This mating scheme was set up to produce mice with three copies, one transgenic and two endogenous copies of PP1γ2. Efforts have also been made to produce mice with four copies, two endogenous and two transgenic copies of PP1γ2. This is to see whether four copies further enhance the expression of PP1γ2 protein in the testis, that is, increase the production of sperms and their motility.
### Table 1: Reference table for genotype symbols

<table>
<thead>
<tr>
<th>Genotype Symbol</th>
<th>Meaning</th>
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<tbody>
<tr>
<td>++</td>
<td><strong>Wild type</strong>: mice have two endogenous copies of PP1γ2.</td>
</tr>
<tr>
<td>++/Tg+</td>
<td><strong>Heterozygous transgene positive</strong>: mice have one transgenic copy of PP1γ2 in addition to the two endogenous copies. Thus a total of three copies. [‘Tg’ stands for PP1γ2 Transgene]</td>
</tr>
<tr>
<td>++/Tg+Tg+</td>
<td><strong>Homozygous transgene positive</strong>: mice have two transgenic copies of PP1γ2 in addition to the two endogenous copies. Thus a total of four copies.</td>
</tr>
<tr>
<td>R (−−/Tg+)</td>
<td>R stands for <strong>Rescue</strong>. It is the genotypic condition when the mice lacks the endogenous copies of PP1γ2. The PP1γ2 has been transgenically introduced and rescued.</td>
</tr>
<tr>
<td>HT (+−)</td>
<td>HT stands for <strong>Heterozygous</strong>. It is the genotype of a mouse with only one copy of PP1γ2.</td>
</tr>
</tbody>
</table>
CHAPTER II

AIMS

1) To see if the presence of four copies of PP1γ2 gene increases the expression of the PP1γ2 protein in the testis.

2) To determine if four copies of PP1γ2 gene in the testis increased the amount of this protein in sperms and if it adversely affected sperm morphology.

3) To determine if PP1γ2 transcript levels are altered in transgenic compared to wild type mice?
Design of the transgene construct used to develop the PP1γ2 transgenic mice


**Fig 5. Design of transgenic contract**

The PP1γ2 transgene is driven by the PGK2 (phosphoglycerate kinase 2) promoter.

Adjacent to the PGK2 promoter sequence, lies the PP1γ2 gene sequence, which is
flanked by the SV40 ply adenylate sequence. Mice containing this specific transgene were created by injecting this transgene construct into the pronucleus of a fertilized mouse egg. The zygote is allowed to divide by mitosis to form a two cell embryo and it is then implanted in the uterus of a pseudo pregnant foster female mouse. These female mice were crossed with PP1γ2 containing wild type males to produce the desired mice over expressing the γ2.

Fig 6. Mating scheme for obtaining a single copy of the transgene, a total of three copies of PP1γ2.
In order to obtain mice with a single copy of the transgene, male PP1γ2 wild type mice were crossed with female PP1γ2 mice containing one transgenic copy of PP1γ2. The cross produced litters with four different genotypes, as shown in the figure 6 above. The male mice which had three copies of PP1γ2, that is two endogenous copies and one transgenic copy was picked for further analysis and designing mating schemes. All the litters obtained from this cross were genotyped to identify the wild type-transgenic male mice.

Fig 7. Mating scheme to obtain two copies of the transgene, thus a total of four copies of PP1γ2.
Our next aim was to obtain male mice with four copies of the PP1γ2 gene. To go about doing that, we used the male and female mice with three copies of PP1γ2 obtained from the previous cross, and bred them. The cross produced litters with four different genotypes as shown in the above figure. All the pups produced from this cross were genotyped and the males containing four copies of PP1γ2, two endogenous copies and two transgenic copies were used for further analysis. The blank line indicates that the second copy of transgene is absent [fig 6].

Once the transgenic male mice were identified, we had to determine whether they contained one or two copies of the PP1γ2 transgene. Test crosses were put up to identify those mice with a total of four copies of the γ2 gene.

**Test cross mating scheme to test for homozygosity**

For this particular cross, both male and female transgene positive mice obtained from the previous cross were assumed to be potentially homozygous for PP1γ2 that is, they contained two transgenic copies of PP1γ2. Those mice were bred with a wild type male or female. If the transgene positive mice contained only one copy of the γ2 gene, at least one of the pups produced as a result of this cross would be a wild type. However, if the transgene positive mice contained two copies of the γ2 gene, all the pups produced by that breeding pair would contain the PP1γ2 transgene. This indicates that the transgene positive parent of this particular breeding pair is homozygous for the transgene.
Fig 8. Test cross mating scheme to identify the genotype of the male parent

If the male contains only one copy of the transgene, when crossed with a wild type female, 50% of the pups produced will be wild type. Therefore, the male is not homozygous for the transgene.
Fig 9. Test cross mating scheme to identify the genotype of the male parent

If the male contains two copies of the $\gamma 2$ transgene, as assumed, all the pups produced by this breeding pair will be transgene positive. Therefore, the male is homozygous for the transgene.
Genotyping and PCR

Ear punch samples from each mouse were immersed in Lysis Buffer (10 N NaOH, 0.5 N EDTA and distilled water) to extract DNA using a heat block machine. Samples were placed in the machine for an hour. 40mM Tris HCL was added to each sample after one hour. This was followed by PCR to identify the genotype of each animal.

List of chemicals used for PCR:

- Taq Polymerase Buffer
- Primers I and II
- dNTPs
- Taq Polymerase enzyme
- Double distilled water

Table 2: List of primers used:

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tr>
<td>Exon 6 Forward</td>
<td>5’ GTG GTT GAA GAT GGC TAT GA 3’</td>
</tr>
<tr>
<td>SV 40 Reverse</td>
<td>5’ AAG CTG CAA TAA ACA AGT TGG 3’</td>
</tr>
<tr>
<td>Intron IV</td>
<td>5’ CTC AGG CCA ATG CTG TCT 3’</td>
</tr>
<tr>
<td>Intron VI</td>
<td>5’ ACT CAT AGC CAT CTT CAA CCA 3’</td>
</tr>
</tbody>
</table>
Primers Exon 6 Forward and SV40 Reverse were used to detect the presence of the transgene, primers Intron IV and Intron VI were used to detect wild type from heterozygous.

PCR was followed by Gel Electrophoresis on agarose gel. 1% agarose gel was made using 1x TAE buffer and agarose powder.

The desired animals were dissected to harness the testis and sperm for western blot, northern blot and motility analysis. Homogenized testis extracts were made in HB+ buffer (Benzamidine, PMSF, TPCK, Mercaptoethanol). Extracts were centrifuged and the supernatant obtained was used for protein estimation and western blotting.

Sperm was squeezed out from the caput and vas deferens of the dissected mice and centrifuged. The pellet obtained was re-suspended in 1% SDS solution and further centrifuged. 6x sample buffer (bromophenol blue, SDS, DTT, 4X Tris, Glycerol) was added to the supernatant and each tube was boiled and stored in -20 degree Celsius for western blotting.
Bradford Protein Assay

Protein estimation and serial dilution was performed with the supernatant testis extracts. The extracts were left for overnight precipitation with 20% TCA solution. Protein estimation assay was performed the following day. The estimated protein values were used to plot an absorbance graph, and the linear absorbance equation was used to calculate the concentration of protein in each testis extract sample. These values were used for calculating the amount in volume of the extracts to be used for running the western blot gels.

Sperm Extract Preparation:

The sperm isolate was centrifuged at 610 rcf for ten minutes at 4 degrees Celsius. The supernatant was discarded. 1% SDS solution was added to the pellet and it was resuspended by flicking and light vortex. The pellet, was then boiled for five minutes followed by centrifugation at 12000 g for another five minutes. 6x sample buffer was added to the supernatant and the solution was boiled for four minutes. It was stored in -20 degree Celsius until used for loading in Western blot gels.
Western Blot

Proteins were electrophoretically transferred from the gel to polyvinylidene fluoride membranes (Millipore Corp.). The membranes were blocked with 5% non-fat dry milk in Tris buffered saline containing Tween (TTBS: 25 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20, pH 7.4). The blots were incubated with primary antibody in dilutions indicated by the manufacturer, over night at 4 degrees celcius. Actin and tubulin antibodies were obtained from GenScript and Epidomics. The PP1γ2 and sds22 antibodies were custom made in the laboratory, raised against the C-terminus tail. Antibodies were diluted in 5% milk/TTBS solution. Blots were washed three times in TTBS for 10 minutes. The blots were then incubated with anti-rabbit secondary antibody for one hour at room temperature. Blots were then developed with ECL chemiluminasance kit and Fuji dark box.
Table 3: List of Antibodies used

<table>
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<tr>
<th>Antibody</th>
<th>Manufacturer</th>
<th>Dilution</th>
<th>Secondary antibody</th>
<th>Detects</th>
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<tr>
<td>γ2</td>
<td>Custom made</td>
<td>1:5000</td>
<td>Anti-rabbit</td>
<td>γ2</td>
</tr>
<tr>
<td>Sds22</td>
<td>Custom made</td>
<td>1:5000</td>
<td>Anti-rabbit</td>
<td>Sds22</td>
</tr>
<tr>
<td>Actin</td>
<td>GenScript</td>
<td>1:5000</td>
<td>Anti-rabbit</td>
<td>Loading control</td>
</tr>
<tr>
<td>Tubulin</td>
<td>Epidomics</td>
<td>1:5000</td>
<td>Anti-rabbit</td>
<td>Loading control</td>
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Northern Blot

A 30-μl mixture containing 25 μg of total RNA, 2 μl of 10× 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, 4 μl of HCHO (37% solution; Amresco, Solon, OH), 10 μl of deionized formamide (Amresco), and 1 μl of ethidium bromide (200 μg/ml) was heated at 85°C for 10 min and chilled on ice for another 10 min. A 2-μl aliquot of 10× gel-loading buffer (50% glycerol, 10 mM edetic acid [EDTA] [pH 8.0], 0.25% w/v bromophenol blue, and 0.25% w/v xylene cyanol FF) was added to each sample, and samples were vortexed and briefly centrifuged. Samples were loaded
onto 1% agarose/MOPS gel (SeaKem GTG Agarose, Cambrex, NJ) and electrophoresed at 70 V. The gel was denatured in a solution of 0.05 M NaOH and 1.5 M NaCl for 30 min, neutralized in a solution of 0.5 M Tris-HCl (pH 7.4) and 1.5 M NaCl, and then equilibrated in 20× saline-sodium citrate (SSC) for 45 min. The gel was transferred to Hybond-XL nylon membrane (GE Healthcare, Piscataway, NJ) in 10× SSC for 16 h. The membrane containing RNA was then baked at 85°C for 2 h, after which it was prehybridized in 8 ml of modified Church buffer (1 mM EDTA [pH 8.0], 0.5 M NaHPO₄ [pH 7.2], and 5% SDS) for 1 h at 65°C in a water bath. Fresh Church buffer containing a probe of Ppp1cc2 cDNA between exon 5 and exon 7 and actin cDNA labeled with P³²-deoxycytidine triphosphate (MP Biomedicals, Solon, OH) was used for hybridization overnight at 65°C in a water bath. The membrane was then washed in 1× SSC/0.1% SDS 3 times for 2 min each at room temperature, followed by washing in 0.1× SSC/0.1% SDS twice for 5 min in a 65°C water bath. After washing, the membrane was dried at room temperature, covered with plastic wrap, exposed to film in a cassette with an intensifying screen overnight, and then developed in a Typhoon automated film developer (GE Healthcare).

**Densitometry**

Relative quantitation of data from the northern analysis was acquired by densitometric scanning of the film after development. This procedure was done by computer scanning of the film followed by computer analysis of the scanned image. The band obtained on the film for wild type RNA sample was taken as 100%, and the level of increase or
decrease of RNA in the wild type with transgene and heterozygous was measured against this 100%.

**Sperm DIC Images**

To study sperm morphology, the sperm extracted was mixed with 4% PFA (Paraformaldehyde powder, double distilled water, 1M NaOH, 1x PBS) and put on a slide. The slides were then observed under Olympus 70X under a magnification of 20X.
CHAPTER IV
RESULTS

Do extra copies of the PP1γ2 gene in the testis increase the amount of this protein in the sperm and does it adversely affect sperm morphology?

In figure 10 A, sperms extruded from cauda and vas deferens of wild type (++), PP1γ2 transgenic (+/+Tg+), R (––/Tg+) and HT (+–) mice were made into extracts using 1% SDS solution and 6x sample buffer. The SDS solution can extract soluble and insoluble proteins including mitochondrial proteins. This western blot was run to compare PP1γ2 protein expression levels between wild type (++) mice with two PP1γ2 transgenic mice (+/+Tg+) and (+/+Tg+Tg+). These extracts were subjected to gel electrophoresis and then analyzed with PP1γ2 antibody. Bands visible in all the six lanes correspond to the molecular weight of PP1γ2 (39 kDA). The visible bands in the western blot appear to be of equal intensity. This is further confirmed by figure 10B, where the same blot (from figure 10A) was re-probed with Tubulin, showing equal loading in each lane. Overall, it appears that there is no difference in expression of PP1γ2 between wild type (++), PP1γ2 transgenic mice (+/+Tg+) and (+/+Tg+Tg+).
RESULTS FOR WESTERN BLOT FOR SPERM SAMPLES

Fig 10 A. Western blot of mice sperm samples, comparing PP1γ2 levels in wild type and transgene positive with a PP1γ2 Rescue and Heterozygous controls. Lanes 1 to 3 were loaded with 2x10^6 sperms and lanes 4 through 6 were loaded with 1x10^6 sperms.

Lane 1: Wild type (++)
Lane 2: Transgene positive (++/Tg+)
Lane 3: Transgene positive (++/Tg+Tg+)
Lane 4: Wild type (+ +)
Lane 5: Transgene positive (++/Tg+)
Lane 6: Transgene positive (++/Tg+Tg+)

Fig 10 B. Same blot was re-probed with loading control Tubulin to show equal amount of sperm extracts were loaded in each lane.
Our next western blot (Figure 11 A) was run to see if there was any difference in the amount of PP1γ2 protein expressed in sperms between mice with two and mice with four copies of PP1γ2. For this, sperm was extruded from the cauda and vas deference of wild type mice (++) and PP1γ2 transgenic (+/Tg+Tg+) mice. Sperm extract solutions was prepared in the same way using 1% SDS and 6x sample buffer. These extracts were subjected to gel electrophoresis and analyzed with PP1γ2 antibody. Four bands in each of the lanes visible correspond to molecular weight of PP1γ2 (39 kDa). The band intensities of lanes loaded with equal amount of sperm extracts are comparable. There is no difference in band intensity between lanes 1 and 2. Correspondingly, the bands visible in lanes 3 and 4 are of equal intensity. This is further confirmed by figure 11B, in which the same blot (from figure 11A) was re probed with Tubulin, shows that equal amount of samples were loaded in each lane. Overall it appears that there is no difference in expression level of PP1γ2 in sperm samples of wild type (++) and PP1γ2 transgenic (+/Tg+Tg+) mice.
RESULTS FOR WESTERN BLOT FOR SPERM SAMPLES

Fig 11 A. Western blot of sperm samples comparing wild type mice with transgene positive mice. Lanes 1 and 2 were loaded with $1 \times 10^6$ sperms. Lanes 3 and 4 were loaded with $2 \times 10^6$ sperms.

Lane 1: Wild type sperm (++)
Lane 2: Transgene sperm (++/TgTg+)
Lane 3: Wild type sperm (++)
Lane 4: Transgene sperm (++/Tg+Tg+)

Fig 11 B. Same blot re-probed with loading control Tubulin showing equal amount of sperm protein extract was loaded in each lane.
Our next objective was to see if there was any sperm phenotype associated with the presence of extra copies of PP1γ gene. We examined the morphology of the sperms obtained from wild type (++) and PP1γ2 transgenic (++/Tg+) mice (figures 12A and 12B). Sperm extruded from the cauda and vas deferens of these mice was fixed in 4% PFA, 10 µL of the fixed sperm was put on a slide. 15 randomly selected fields were observed under light microscopy using DIC optics. The DIC images, in figures 12 A and 12 B, show that there is no detectable difference in morphology of sperm of transgenic mice compared to wild type sperm. Sperm from transgenic mice have normal morphology.
Fig 12 A. Sperms obtained from a wild type (++) mouse.
Fig 12 B. Sperms obtained from a transgene positive (++/Tg+) mouse.
Does the presence of four copies of PP1γ2 gene increase the expression of this protein in the testis?

Our second aim was to examine the effect of the presence of extra copies of PP1γ2 gene on testis PP1γ2 levels. We ran western blots of testis extracts comparing PP1γ2 expression levels in wild type (++) mice, heterozygous transgene positive (++/Tg+) and homozygous transgene positive (++/Tg+Tg+) mice. Testis extracts of these three genotypically different mice were made in HB+ buffer. These extracts were subjected to gel electrophoresis followed by analysis with PP1γ2 antibody. Bands visible in all six lanes correspond to the molecular weight of PP1γ2 (39 kDA), implying that PP1γ2 is detected and expressed in the testis of the transgenic mice. The presence of extra copies PP1γ2 gene does not affect protein expression in the testis. The western blot (figure 13A) shows that the band intensities of the first three lanes are equal and the band intensities of the last three lanes are equal. This is further confirmed by figure 13 B where the same blot (from figure 13A) was re-probed with βActin, showing that equal amount of testis extracts were loaded in each lane. Overall, the results are similar to the situation in sperms, the amount of PP1γ2 protein in testis of a PP1γ2 transgene ( ++$Tg+$ ) or ( ++/Tg+Tg+ ) is not different from that produced in wild type (++) mice.
RESULTS FOR WESTERN BLOT FOR TESTIS SAMPLES

Fig 13 A. Western blot comparing PP1γ2 levels in testis extracts of wild type mice with transgene positive (++) and (++/Tg+Tg+) mice. Lanes 1 through 3 were loaded with testis extracts serially diluted to 5 µg. Lanes 4 through 6 were loaded with testis extracts serially diluted to 2.5 µg.

Lane 1: Testis extract of wild type (++) mice
Lane 2: Testis extract of transgene positive (++/Tg+) mice
Lane 3: Testis extract of transgene positive (++/Tg+Tg+) mice
Lane 4: Testis extract of wild type (++) mice
Lane 5: Testis extract of transgene positive (++/Tg+) mice
Lane 6: Testis extract of transgene positive (++/Tg+Tg+) mice

Fig 13 B. Same blot re-probed with loading control β-Actin, shows that equal amount of protein was loaded in each lane.
To further confirm this observation, we ran western blots comparing protein expression levels of wild type (++) mice with PP1γ2 transgenic (++/Tg+Tg+) mice. As before, testis extracts were made in HB+ buffer and subject to gel electrophoresis and analyzed with PP1γ2 antibody. Bands visible in all four lanes correspond to the molecular weight of PP1γ2 (39 kDA). In the western blot (figure 14 A) bands visible in lanes 1 and 2 are of equal intensity and bands visible in lanes 3 and 4 are of equal intensity. This is further confirmed by figure 14 B, where the same blot (from figure 14A) was re-probed with Tubulin to show that equal amount of samples was loaded in each lane. Overall, the results of this western blot confirm that the presence of four copies of PP1γ2 gene does not enhance the amount of this protein in the testis.
RESULTS FOR WESTERN BLOT FOR TESTIS SAMPLES

Fig 14 A. Western blot comparing PP1γ2 levels in testis extracts of wild type mice with transgene positive mice. Lanes 1 and 2 were loaded with testis extract serially diluted to 5 µg and lanes 3 and 4 were loaded with testis extracts serially diluted to 2.5 µg.

Lane 1: Testis extract of wild type (++) mice
Lane 2: Testis extract of transgene positive (++/Tg+Tg+) mice
Lane 3: Testis extract of wild type (++) mice
Lane 4: Testis extract of transgene positive (++/Tg+Tg+) mice

Fig 14 B. The same blot was re-probed with loading control Tubulin, showing equal amount of protein was loaded in each lane.
Are PP1γ2 transcript levels altered in transgenic compared to wild type mice?

Because the extra copies of PP1γ2 in the transgenic mice did not result in increased PP1γ2 protein levels in testis and sperms, we next determined if PP1γ2 mRNA amounts were altered. We used northern blot analysis of testis RNA. Testis extracts were prepared in solution containing trizol, chloroform, isopropanol and TE Buffer. The samples were loaded into agarose gel and subjected to gel electrophoresis. This northern blot (figure 15) was run to compare testis mRNA levels of PP1γ2 heterozygous (+ −), wild type (++) and PP1γ2 transgenic (++/Tg+) mice. Two distinct bands are visible in lane 3; the second shorter band is a cropped segment of the PP1γ2 mRNA derived from the transgene, that shows up only for heterozygous (+ −) or null (− −) mice. The band visible in lane 2 is comparatively larger than the bands visible in lanes 1 and 3. Overall, it appears that there are increased mRNA levels corresponding to the increased copies of the PP1γ2 gene.
RESULT OF THE NORTHERN BLOT

Fig 15 A. Northern blot comparing mRNA levels in wild type, heterozygous and transgene positive mice.

Lane 1: Wild type (++)
Lane 2: Transgene positive (++/Tg+)
Lane 3: Heterozygous (+ – )

Fig 15 B. βActin used as loading control showing equal loading in each lane.
To quantify and compare the intensities of these bands, Multi Gauge-ver3.X (Fujifilm Inc.) image analysis software was used for densitometry. Densitometry by image analysis of actin bands were used as control for equal loading. Statistical analyses were performed using the non-parametric Kruskal-Wallis one-way ANOVA by rank. Results were analyzed post-hoc for pairwise significant differences using Dunn’s procedure. All analyses were performed using XLSTAT-Pro software. In all cases, differences between samples were considered significant if p≤0.05. Simple linear correlation/regression analyses were performed for all data sets using steady state levels of PP1γ2 as the independent variable. Levels of correlation were determined with the on-line program, VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html). The densitometric values (figure 16) show that the relative expression of PP1γ2 mRNA is more in mice with three copies of the gene than in mice with two or fewer copies. The densitometric values shows that mRNA level in the transgene positive (++/Tg+) mice is about 35% more compared to the wild type (++), and the amount of mRNA in the wild type (++) is about 34% more in comparison to the heterozygous (+ –).
Fig 16. Densitometric analysis of the northern blot.
Our results so far, conclusively demonstrated that spermatogenesis and sperm
morphology is not affected by presence of extra copies of the PP1γ2 gene. In spite of the
extra gene copies being present and expressed in the system, the amount of protein being
translated was no different between a wild type (++) and a transgene positive (++/Tg+)
or (++/Tg+Tg+) mouse. However, the northern blot shows that the amount of mRNA
being transcribed from the PP1γ2 gene in the transgenic mice was significantly higher
compared to the wild type. Attempting to decipher a possible reason behind this, we
analyzed expression levels of Sds22 in the testis and sperm extracts if each of these mice.
Sds22 is a protein, originally identified in Yeast as a positive regulator of Protein
Phosphatase 1. It is an inhibitor of PP1γ2 in mammals. Any PP1γ2 bound to Sds22 is
rendered catalytically inactive. Research has shown that Sds22 – PP1γ2 exists as a single
complex in the testis. In sperms, however, the activity of Sds22 is region specific. Its
activity is significantly higher in caudal sperms compared to caput sperm. Higher amount
of Sds22 implies lower amount of free PP1γ2, since Sds22 binds to it and makes it
inactive. This is essential for caudal sperms to attain motility [8]. Previously run blots
(figure 11 A and figure 14A) were analyzed with Sds22 antibody to see if there was any
difference in the amount of Sds22 expressed in wild type (++) and PP1γ2 transgenic
(+/Tg+Tg+) testis and sperm.

In figure 17, the western blot previously run with testis extracts (figure 14A) was re-
probed with Sds22 antibody. Bands visible in all four lanes (figure 17) correspond to the
molecular weight of Sds22 (41 kDA). Bands visible in lanes 1 and 2 are comparable as
they were loaded with the same amount of extract. Similarly, bands visible in lanes 3 and 4 are comparable. The band visible in lane 2 is more intense than the band in lane 1, correspondingly the band in lane 4 is more intense than the band in lane 3. Overall, it appears that PP1γ2 transgenic (+/Tg+Tg+) mice have more Sds22 compared to wild type (++) mice; since the band intensities for the transgenic mice is more than that of the wild type mice.

![Western blot comparing Sds22 levels in wild type (++) mice with transgene positive (+/Tg+Tg+) mice. Lanes 1 and 2 were loaded with testis extract serially diluted to 5 µg. Lanes 3 and 4 were loaded with testis extract serially diluted to 2.5 µg.](image)

**Lane 1:** wild type (++)

**Lane 2:** transgene positive (+/Tg+Tg+)

**Lane 3:** wild type (++)

**Lane 4:** transgene positive (+/Tg+Tg+)

In figure 18, the blot previously run with sperm extracts (figure 11A) of wild type (++) and PP1γ2 transgenic (+/Tg+Tg+) mice was re-probed with Sds22 antibody. In figure 18, bands visible in all four lanes correspond to the molecular weight of Sds22 (41 kDa). Band intensities in all four lanes appear to be equal. Overall, this western blot shows that
there is no difference in the amount of Sds22 present in sperms of wild type and transgenic mice.

Fig 18. Western blot of mice caudal sperm samples, comparing Sds22 levels in wild type and transgene positive mice. Lanes 1 and 2 were loaded with $1 \times 10^6$ sperms, lanes 3 and 4 were loaded with $2 \times 10^6$ sperms.

Lane 1: Wild type (++)
Lane 2: Transgene positive (++/Tg+Tg+)
Lane 3: Wild type (++)
Lane 4: Transgene positive (++/Tg+Tg+)
CHAPTER V
DISCUSSION

PP1γ2 plays an indispensable role in maintaining the fecundity of the male reproductive system. Region specific expression is tightly regulated and conserved for appropriate generation and maturation of sperms. Activity levels of PP1γ2 are vital for initiation of sperm motility. Its activity is inversely co related with motility, low activity in motile caudal sperm and high activity in immotile caput sperm. It is very likely that the higher activity of PP1γ2 in caput is because of the inability of its regulator Sds22 to bind to PP1γ2, because the binding ability of Sds22 to form a heterodimer complex with PP1γ2 develops during sperm maturation.

The western blot results show that there is no change in expression of PP1γ2 protein in between wild type (++), transgenic mice (++/Tg+) and (++/Tg+Tg+), in both testis and sperms. However Sds22 shows a slight increase in expression in testis, of the transgenic (++/Tg+Tg+) mice when compared to wild type (++) mice. The northern blot analysis showed that there is a significant amount of increase in the mRNA levels in transgene positive (++/Tg+) mice compared to wild type (++) and heterozygous (+ –) mice.

Densitometric data analyzing the northern blot statistically shows that there is about 35% increase in expression of mRNA levels in transgene positive (++/Tg+) mice compared to wild type (++) mice. There is about 34% decrease in mRNA levels in heterozygous (+ –) mice when compared to wild type (++) mice. The sperm DIC images prove that there is
no observable phenotypic change or morphological defect between mice containing varying copies of the PP1γ2 gene.

Our results conclusively show that presence of extra PP1γ2 gene copies does not negatively impact spermatogenesis or sperm morphology. There is no difference in the amount of protein translated from two, three and four copies of the PP1γ2 gene.

However, there is a significant increase in mRNA levels in the transgene positive mice (++/Tg+) compared to the wild type (++) mice, in both testis and sperms. Since increase in PP1γ2 mRNA levels is not followed by an increase in the amount of the PP1γ2 protein, this suggests the presence of some adeptly controlled regulatory mechanism determining the amount of protein being translated within the system. Considering Sds22 showed a slight increase in expression in testis of the transgenic (++/Tg+Tg+) mice when compared to wild type (++) mice, we speculate that the regulatory mechanism is operating at the translational or post translation levels. It would be expected that over expression of mRNA should be followed by over expression of the PP1γ2 protein. But this is not the case. However, the transgenic system is also producing a slight excess amount of the PP1γ2 regulatory protein Sds22, which is possibly binding to the excess protein in the system and could be causing degradation. This could bring down the protein levels in the transgenic mice to the levels equivalent in a wild type system. As a result, excess PP1γ2 protein is not detected in the testis samples in any of the transgenic mice (++/Tg+) or (++/Tg+Tg+). Any extra PP1γ2 protein getting restricted in the testicular cells with the surplus amount of Sds22, is another reason why Sds22 levels in
the sperms, unlike testis, of both transgenic (++/Tg+Tg+) and wild type (++) mice are the same and there is no over expression of the PP1γ protein in the sperms.

In summary, our work has conclusively established for the first time, that additional copies of the PP1γ2 gene do not adversely affect the process of sperm formation, generation and morphology. Increased amount of PP1γ2 message level is not accompanied by the corresponding amount of protein. The operating regulatory mechanism is most likely at the level of translation or post translation where any surplus PP1γ2 protein may be degraded by its possible binding to its regulatory protein such as Sds22. These initial studies will enable us to better understand regulatory mechanisms that keep the key protein PP1γ2 levels in tight control to ensure normal sperm production and male fertility. Further studies will determine the contribution of other PP1γ2 regulators I2 and I3 alongside Sds22 and give us a more mechanistic picture of how homeostatic levels of PP1γ2 are maintained in the testis.
REFERENCES


