RESTORING SPERMATOGENESIS: LENTIVIRAL GENE THERAPY FOR MALE INFERTILITY IN MICE

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ABSTRACT

Background: Male infertility of genetic origin affects nearly 1 in 40 men. Yet 80% of men with low sperm production are considered idiopathic due to negative genetic testing. Based on mouse studies there are several hundred possible candidate genes for causing isolated idiopathic male infertility due to their involvement in spermatogenesis and male germline-specific expression. Although little is known about their pathophysiology and epidemiology in human males, these genes represent vast potential for diagnosing and treating infertility. Lentiviral vector gene therapy has recently been shown to be effective in restoring gene expression, and has the potential to serve as treatment in male infertility caused by gene defects.

Methods: This project proposed to use a lentiviral vector to restore spermatogenesis in infertile, but viable male mice with a single-gene defect in Sertoli cells as a proof of concept project. Candidate genes were identified utilizing the Mouse Genome Informatics (MGI) database and literature review. One of these candidate genes, *Dnaja1*, was selected for experimental lentiviral vector gene therapy in mice.

Results: Several candidate genes expressed primarily in Sertoli cells known to cause isolated azoospermia were identified. Viral vector preparation was successful, but *Dana1*$^{-/-}$ mice were not produced, possibly indicating embryonic lethality.

Conclusion: Because the development of the vector was successful, and with the list of candidate genes identified, future experimentation using lentiviral gene therapy to restore spermatogenesis caused by single-gene defects is now ready to be performed. Selecting genes expressed in somatic cells, rather than germ cells, maintains the possibility of translating this research into clinical treatment for infertility by avoiding ethical implications of genetically altering the germ line. In the future, we hope to perform similar therapy on genes identified through the study of men with unexplained infertility. Of significance to public health, if successful this work may represent a novel treatment option for men who are born with single gene defects preventing sperm production and natural conception.

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PREFACE

I owe thanks to Dr. Alex Yatsenko and members of his laboratory for assisting me with the logistics and protocols necessary for the completion of this project. Archana Kishore, Ph.D, was an invaluable mentor and supporter throughout this project. She not only helped manage the mouse colonies and design and complete protocols, but she also facilitated my growth and development as an independent researcher. Her help was crucial to the success of this project. I would also like to thank Phillip Sweet, and Ashton Basar, two ambitious undergraduate researchers building themselves a strong foundation for a career in biomedical research, who provided valuable assistance by performing required genetics laboratory protocols throughout the project's completion. Finally, I would like to thank Dr. Kyle Orwig and Dr. Yi Sheng for helping develop the viral vector, and performing the GFP expression studies.

1.0 INTRODUCTION

Infertility is defined as the failure to conceive after twelve months of unprotected intercourse. It is a health burden with many psychological, economical, and social sequelae that can significantly decrease quality of life, and that is often associated with serious medical disorders. Male infertility of genetic origin affects nearly 1 in 40 adult men, yet little is known about the genetics of isolated infertility, and up to 80% of men with azoospermia or oligozoospermia (absent or low sperm production) are considered idiopathic due to negative genetic testing[1; 2]. There is also no direct treatment to restore sperm production in men with idiopathic infertility. Treatment options are limited to highly expensive *in vitro* fertilization which is rarely covered by insurance providers, and is ineffective in cases of severe azoospermia or complete absence of spermatogenesis[3].

There are several experimental gene therapies that have shown promise in treating genetic disorders, including male infertility. One of these, which has been demonstrated to be effective in treating male infertility as well as a number of other genetic diseases is lentiviral vector gene therapy[4]. Researchers were able to restore spermatogenesis in male mice with a gene mutation in the steel factor gene by transfecting the Sertoli cells with a working copy of the gene using a lentiviral vector. However, there is no equivalent to the steel factor gene in human males, and mice knockouts were unable to reproduce naturally[5].

This project aimed to identify candidate genes for experimental gene therapy in male mice with isolated infertility, develop a viral vector that can be used to express in Sertoli cells a functioning copy of a knocked out gene, and to use this vector to restore spermatogenesis in mice with azoospermia. I identified several candidate genes for experimentation, and we selected a gene known to cause isolated azoospermia in mice, *Dnaja1*, to test the utility of the lentiviral therapy[6]. We are now prepared to perform similar trials using genes identified in infertile men through whole exome sequencing, RNA expression, and array comparative genomic hybridization studies.

1.1 BACKGROUND AND SIGNIFICANCE

1.1.1 Male Infertility

Infertility is defined as the failure to conceive after twelve months of unprotected intercourse. It is a health burden with many psychological, economical, and social sequelae that can significantly decrease quality of life, and that is often associated with serious medical disorders (in about 1 –10% of cases). Infertility affects ten to fifteen percent of American couples, of which half is due to a male factor, meaning the couple's inability to reproduce is caused by a health condition or defect in the male partner[7; 8]. Among infertile men, twenty percent have azoospermia or oligozoospermia (AZ/OZ), with nearly a third of that due to genetic defects. Thus up to one in forty adult men may be affected by genetic infertility[1]. The cost of diagnosing and treating infertility and its complications has been estimated to exceed roughly five billion

dollars per year [9; 10]. These expenses that are most often placed directly on patients themselves due to lack of insurance coverage of infertility diagnosis and treatment in most states [11].

Genetics of Male Infertility

Reproductive success requires thousands of necessary gene products. The foundation of reproduction begins years before the interaction of the sperm and the oocycte, and depends on careful coordination and development of multiple systems, including the germline lineage, formation of future gonads, genital tracts, etc. [12].

Up to 50% of male infertility may be of genetic origin. These include chromosome aneuploidies and translocations, Y chromosome microdeletions, X chromosome abnormalities, single gene mutations, and DNA mismatch repair and chromosomal recombination defects. The most common chromosome aneuploidies and translocations are Klinefelter syndrome (XXY), when a male has two X and one Y chromosome, as opposed to the normal amount of one X and one Y. These also include XYY syndrome, XX male syndrome, mixed gonadal dysgenesis, and Robertsonian translocations. Known Y chromosome microdeletions are located on the AZF region, also known as the azoospermic factor region. These microdeletions account for nearly 10% of azoospermia and oligozoospermia. The X chromosome also contains genes important for male reproduction. Alterations such as translocations, deletions, and inversions on the X chromosome can result in infertility and azoospermia [13]. Knowledge of X Chromosome abnormalities causing male infertility, however, is limited to mouse models. Human subject research in this area is needed, but it is postulated that these problems could also be seen in human males.

There are several single gene mutations known to cause male infertility. These include, but are not limited to, Kallmann syndrome (X-linked and familial), Cystic Fibrosis and *CFTR* mutations, generalized Wolffian duct anomalies, persistent müllerian duct syndrome, insulin – like growth receptor mutations, androgen receptor gene mutations, Xq11-11.2, and SRY mutations. Single gene mutations such as these typically occur in less than 1: 20,000 live deliveries. However, mutations in the *CFTR* gene cause the most common autosomal recessive disease in Caucasians, cystic fibrosis, with an incidence of 1 in 2,500 live births. Non-obstructive azoospermia involves very low or absent sperm in the ejaculate without any structural defects present. *CFTR* mutations also account for a large portion of men with non – obstructive azoospermia, which represents about 50% of azoospermic men [14 12].

Much research is required in the area of male infertility, but genomic medicine may one day explain a significant portion of idiopathic male infertility. The majority of research on the genetics of male infertility have focused on AZ/OZ [15]. The two types of azoospermia are obstructive and nonobstructive. Obstructive azoospermia is caused by a post – testicular defect that results in the failure of sperm to be transported from the testicles to the ejaculate. This condition affects between 7 to 51 percent of men with azoospermia [14], and up to 50% of men with obstructive azoospermia carry a Cystic Fibrosis mutation[15]. Among men with nonobstructive azoospermia, meaning no sperm is being produced by the testes, 10% have Y chromosome micro deletions in one of three regions: AZFa, AZFb, and AZFc, and about 4 % have Klinefelter syndrome (47, XXY) or other numeric chromosome abnormalities. Overall, about 80% of men with azoospermia or oligozoospermia are considered idiopathic due to negative genetic testing.

Some work has been done to identify additional candidate genes via animal model approaches, but there have been several limitations to identifying novel genes associated with male infertility. These limitations include inappropriate controls, single gene analysis, lack of family pedigrees to corroborate significance of findings, and lack of functional studies. Over 1,500 genes may be involved in spermatogenesis. Several hundred of these are expressed exclusively in male germ cells, which indicates that they could be candidates for causing isolated infertility[1]. However, no additional genetic causes are recognized to be important for patient care, mainly because results have either not been replicated, explain small effects, or have only been seen in mouse models[16]. There are over 150 single gene mutations that have been found to result in male infertility in mice, most of which affect specific cell types and stages of spermatogenesis, including germ cells as well as Somatic cells. These single gene defects represent vast potential for targeted therapy if they are observed in men with infertility[17].

1.1.2 Male Infertility Treatment

Treatment options available to achieve conception for men with little or absent sperm is very limited. For those with azoospermia or oligozoospermia due to environmental exposures, such as gonadotoxin exposure, reduction of these exposures may be useful for reversing damage. For those with endocrine disorders, hormone therapy may allow the revival of sperm production. In cases of incorrect coital timing, counseling the patient is necessary to correct these issues. However, for all others, men with idiopathic infertility, there is no direct treatment available to restore or increase spermatogenesis. Conception is only made possible for these men by therapy involving in vitro fertilization (IVF) with intracytoplasmic sperm injection (ICSI) coupled with testicular sperm extraction (TESE). Although this therapy has made it possible for millions of

previously infertile men to reproduce, there are several limitations. For men with severe azoospermia, or complete absence of sperm in the testes, also known as Sertoli – cell only syndrome, these methods will not be useful for successful reproduction. IVF, which now accounts for nearly 1% of all U.S. births, is also associated with increased birth defect rates including hypospadias, cleft lip and palate, septal heart defects, esophageal atresia, Beckwith – Weidemann syndrome, and others. Furthermore, with prices reaching 12,000 – 15,000 dollars, IVF is very costly, and rarely covered by insurance[3; 18]. The shortcomings of available treatment for azoospermia and oligozoospermia highlight the need for a better understanding of the genetics of male infertility, as well as the importance of alternative, more direct treatments. Understanding the genetic pathology underlying male infertility is imperative for appropriate genetic counseling, understanding higher birth defect rates in infertile couples, as well as developing safe, alternative, and effective primary treatment of reduced or absent spermatogenesis[7].

1.1.3 Experimental Treatment for Male Infertility

There are several potential prospective treatments with promise for restoring spermatogenesis in men with low or absent sperm production; some of which are now in pre-clinical, or clinical trial phases, while others remain limited to *in vitro* and/or animal models. These include stem cell, micro RNA, electroporation, artificial chromosome, circular auto replicating DNA episodes, and viral vector therapies. First, stem cell therapy involves the development of sperm by differentiation from pluripotent cells. Medrano and colleagues at Stanford University recently reviewed the current state of research regarding germ cell differentiation from stem cells[19]. In their research they use induced pluripotent stem cell (iPSC) lines from dermal fibroblasts of

infertile men with Sertoli cell only azoospermia. These stem cells are implanted into the testes of mice where they have been used to produce precursors to sperm. However, there are currently several considerable challenges to translating this type of therapy to clinical trials. Notably, achieving fully functional *in vitro*-derived germ cells has been impeded by issues with epigenetic reprogramming of germ cells *in vivo*, and large differences between species. These authors conclude that future translational applications of stem cell therapy requires an enormous amount of work. Nonetheless, they envision success provided that proper efforts are undertaken[19].

Another experimental form of gene therapy that has been investigated is the use of Micro RNA's. MiRNAs are non-coding RNAs that suppress gene expression following transcription. Demonstrated to be important to cancer, metabolic disease, and even viral infection, they may represent potential drug targets in several human diseases[20]. There is reason to suspect that aberrant expression of specific miRNAs may be associated with certain types of male infertility. First, single nucleotide polymorphisms (SNPs) at miRNA binding sites have been reported in association with idiopathic male infertility. Relationships have also been demonstrated between male infertility and SNPs in genes essential to miRNA biogenesis. Additional research of miRNAs offers potential in finding novel molecular genetic causes, as well as future targets for gene therapy[21].

The next three classes of experimental gene therapy are artificial chromosomes, circular auto replicating DNA episodes, and electroporation. Of these three, artificial chromosomes are not technically feasible at this time for infertility therapy. Electroporation, however, has been successful in transfecting Sertoli cells; it involves an electric field being applied to cells in order to increase permeability in the cell membrane, which allows DNA to be introduced into the cell. In 2002, Yomogida et al.[22] used this technique to rescue spermatogenesis in infertile mice due to

damaged Sertoli cells with a mutated stem cell factor (SCF) cytoplasmic domain resulting from a splicing defect. Although the Yomogida and colleagues conclude that this technique shows promise as a somatic gene therapy for male infertility, they recognize that a major obstacle to its utility in infertility treatment is the reduced viability of treated germ cells and the instability of transfected genes[22]. The final of the three, circular auto replicating DNA episodes, has been used in mice to obtain stable transgene expression without genomic integration. This technology involves episomally transfecting spermatogonia stem cells *in vitro*, allowing clonal expansion, and then transfecting stem cells back into the host. This is a promising approach for gene therapy, but germ cell transfection has shown limited success[17].

The final type of prospective gene therapy for male infertility, which was chosen for this project, is lentiviral gene therapy. Lentiviral vectors have been successfully utilized to re-establish gene function in mouse models and clinical trials for multiple human diseases. It has also been successfully used to restore fertility in male mice. Although the cause of spermatogenetic failure in most cases of male infertility remains idiopathic, and no treatment to improve spermatogenesis is available to these men, more investigations on the genetics of spermatogenesis and male infertility along with the development of gene therapy may offer significant advances for treatment[23].

1.1.4 Lentiviral Gene Therapy

Lentiviral vector gene therapy has been shown to be an effective tool for *ex vivo* and *in vivo* transfer with several research groups reporting that it can be used to treat or even cure diseases. [4; 24]. The first clinical trial using lentiviral vector gene therapy was begun in June 2012, and since that time there are more than 300 ongoing or approved trials[25]. These include treatment of

single gene diseases such as X-linked cerebral adrenoleukodystrophy, Sickle cell anemia, Wiskott-Aldrich syndrome, metachromatic leukodystrophy, X-linked chronic granulomatous disease, and several others. There are also trials to test the utility in certain cancers, Parkinson's disease, and Stargardt macular degeneration[4; 24].

One group of researchers even reports restoring spermatogenesis in infertile male mice. Their work involved c-kit ligand (steel factor) deficient mice[5]. This group injected a lentivirus into the seminiferous tubules to transfect Sertoli cells with a functional c-kit ligand. Spermatogenesis was restored (low levels), and the viral vector was not seen in germ cells. They were also able to use the sperm of these mice for successful intracytoplasmic sperm injection, and offspring did not inherit the transgene. Although the vector therapy appeared to be successful, there were limitations to this study. First, the treated male mice could not generate pups by normal mating. The group speculates on several plausible explanations. These include: the number of Sertoli cells transduced by the lentiviral vector might not have been high enough, the mating period might not have been long enough, and the fact that the CMV promoter was used in the study, which may not accurately model natural c-kit ligand expression. Also, there is unfortunately no steel factor homologue known in humans. There are, however, functionally similar genes important to Sertoli cell function in humans such as claudin-11, protein C inhibitor, and tyro-3 families that are necessary for spermatogenesis[5].

Much research is required to achieve the full potential of lentiviral gene therapy. The primary concerns at this point regarding therapy in humans involve safety. The major safety concerns are the formation of replication competent retroviral vectors, disruption of normal gene function upon integration of the virus into the host genome (such as cancer development caused by insertion of the vector into an oncogene), and that most lentiviruses are derived from a known

human pathogen, HIV. Safety efforts made so far have involved splitting the vector by expression cassettes, the use of self-inactivating vectors, using low volumes of viral vectors, and homology reduction between viral elements[4].

Beyond the demonstrable success in treating disease in mouse models, there are numerous advantages in using lentiviruses as a medium for gene therapy. First, one advantage to lentiviruses over another commonly used vector, the adenovirus, is that the adenovirus cannot integrate into the host DNA. Therefore, the gene of interest is not replicated upon cell division[17]; meaning the patient may need numerous viral injections to achieve a lasting effect. Furthermore, adenoviruses cannot infect germ cells, while lentiviral vectors are not affected by epigenetic silencing during development, and have the capacity to infect dividing and non-dividing cells[26]. Compared to other viral vectors the lentivirus enables long-term stable expression of the transgene with low immunogenicity[27]. Lastly, integration into the host genome once caused a great concern for the development of cancer as a side effect of lentiviral therapy. However, although lentiviral vectors target active transcription units, for the most part they are integrated randomly throughout the genome. On the other hand, γ-retroviral vectors interact preferentially with active host cell promoters and enhancer-regions that are enriched in transcription factor binding sites (TFBS) due to their integrase and LTRs. These regions are often rich in cancer – related genes. Therefore triggering cancer development is of less concern for lentiviral gene therapy, and this in part might explain the difference in safety profiles of the two vector types[28]. Although lentiviral vector therapy presents a promising means for gene therapy, additional results from patients and animal models treated with lentiviral vector gene therapy are needed before solid conclusions can be drawn concerning the safety of the vector system[28].

1.1.5 Yatsenko Lab Research Efforts

Dr. Yatsenko of Magee-Womens Research Institute and Foundation (MWRI) is working together with researchers and physicians at Magee-Womens Hospital to identify novel genetic causes of male factor infertility. The focus of the research is on idiopathic azoospermia and oligozoospermia. At this point, three primary methods of gene discovery are utilized in the affected population to identify novel variants that may be associated with infertility. These include array comparative genomic hybridization (aCGH) (genome wide as well as an X chromosome array designed to identify variants associated with male infertility), RNA expression studies, and familial-based whole exome sequencing studies. These projects involve recruiting appropriate research subjects, performing RNA expression studies, aCGH, or whole exome sequencing on their DNA samples, identifying potential causative variants of infertility, and eventually developing mouse models that can be used to validate and study these candidate genes. My role in these research efforts has been in recruitment, performing essential laboratory bench work, identifying candidate genes for a gene therapy pilot project, and helping manage the mouse colony for this gene therapy pilot project.

I recruit subjects primarily through the reproductive endocrinology and infertility (REI) clinic, and urology clinic at Magee-Womens Hospital of UPMC, but I have also made efforts to expand the recruitment base for the male infertility research study. The main subjects of interest are men diagnosed with idiopathic, nonobstructive azoospermia or oligozoospermia. To ascertain information about the patient's medical and family history, I ask them to fill out a questionnaire, and take a pedigree. Results of their semen analysis are also reviewed.

To expand the recruitment base of the study, I advertised the research through online media, social networks, and other infertility clinics in the greater Pittsburgh area. These efforts

of expansion also required the institutional review board's approval. First, I wrote a section describing the study, and how to contact the group if interested in participating. This section, posted through online mediums, required IRB approval to determine whether it was appropriately advertising the study, free of coercion, and that it would not be biased toward a specific ethnic or socioeconomic group. Areas the advertisement was posted online included Facebook groups for infertility and male infertility. I also posted it onto other forums meant for couples struggling through infertility, as well as forums that were specific to male infertility. Lastly, the advertisement was posted and distributed by infertility support groups, hotlines, and other reproductive and infertility clinics in the Pittsburgh area. Through this expansion, several individuals were recruited outside of the clinics within Magee-Womens Hospital, and we received interest in the study from men throughout the United States.

Recently, myself, Alex Yatsenko, MD, PhD, and Kyle Orwig, PhD, began efforts to perform an experimental gene therapy on infertile male mice as proof-of-concept that this may serve as a possible treatment for restoring spermatogenesis and infertile men. The goal of this project was to restore spermatogenesis in viable male mice with low or absent spermatogenesis due to a gene defect in Sertoli cells. At this point, although there are likely numerous targets for gene therapy in germ cells, the intention of the project is to focus on somatic cells, e.g. Sertoli cells, to avoid ethical concerns of altering the germ line, which would be unacceptable for human therapy. In the long term, if the gene therapy is successful, we hope to repeat the therapy on a mouse line with a single gene knockout of a candidate gene identified in his study population of infertile males. This would have great potential for translation in the clinical setting, as it would show that spermatogenesis may be restored in men previously incapable of reproducing even with available treatments for infertility.

1.2 SPECIFIC AIMS

This project consists of three specific aims. The first aim involved the identification of candidate genes for the gene therapy via bioinformatics databases and literature review, along with data mining of our study population. The second aim was to characterize the Dnaja1 gene, and to replicate data found in the available literature. The final aim was to develop the viral vector and perform gene therapy to restore spermatogenesis in viable, infertile male mice.

2.0 METHODS

2.1 IDENTIFYING CANDIDATE GENES

2.1.1 Candidate Gene Selection Criteria

The purpose of this research was to identify potential gene therapy targets for *Mus musculus* knockouts. Figure 1 shows the search and filtration process I used to choose candidate genes. At the outset of the project, it was decided that ideal candidate genes would have known mouse phenotypes exclusively related to male infertility, including one or more of the following: azoospermia, asthenozoospermia, teratazoospermia, and/or oligozoospermia. This would model isolated, idiopathic infertility in human males. Candidate genes must also have expression profiles limited to the testis and reproductive organs. This criterion was chosen in order to avoid the possibility of the gene knockouts causing severe phenotypes other than infertility, as well as the possibility of the gene therapy having effects beyond the reproductive system. Furthermore, because the goal was to target somatic cells, infertility in male mice must be caused by a primary defect in Sertoli cells due to a mutated gene that is expressed in Sertoli cells, rather than germ cells. Because the gene therapy would be administered at approximately day 15 of the mouse's life cycle, the infertility phenotype must also be due to an error in spermatogenesis, rather than a developmental defect. Realistically, severe developmental abnormalities such as testicular

agenesis, absence of the vas deferens, etc. could not be repaired by a gene therapy given to a mature mouse. On the other hand, gene therapy may be more likely to repair an error at a single step in spermatogenesis or aberrant interaction between Sertoli cells and germ cells. Lastly, due to the laboratory's limited capacity to develop mouse lines, as well as time constraints of the project, the ideal candidate gene would have an available live mouse line ready for order through a mouse repository, such as Jackson Laboratories, and antibodies available through commercial laboratories. In the long term, however, if this pilot project is successful, we may perform the clustered regularly interspaced short palindromic repeats (CRISPR) technique ourselves to knockout future candidate genes in mice of their own. The criterion of known testis – specific expression was later relaxed in order to accommodate the gene selected for the first experimental gene therapy.

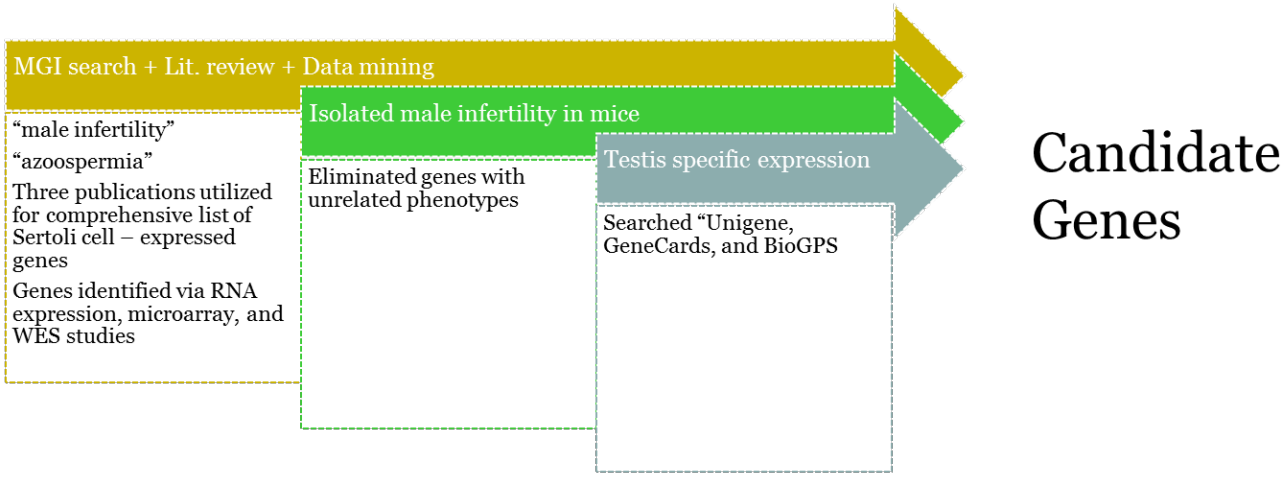


Figure 1. Candidate gene selection

2.1.2 Bioinformatics Databases and Literature Review

I took three primary approaches to identify and filter candidate genes. First I performed a raw search in the Mouse Genome Informatics Database (MGI) using terms related to male infertility, particularly "azoospermia" and then cross referenced this list with expression profiles provided by NCBI's UniGene, BioGPS, and GeneCards. The next approach involved beginning with genes known to be expressed in Sertoli cells, and then performing searches on MGI and the available expression profiles. Lastly, I performed similar searches for genes with variants identified in Dr. Yatsenko's study population of infertile males discovered through microarray, exome, and RNA expression studies.

Filtering Genes with male infertility phenotype

I began the search for candidate genes using the Mouse Genome Informatics (MGI) database hosted by The Jackson Laboratory. This database provides open access to searchable data on the genetics of laboratory mice to facilitate the study of human health and disease[29]. By entering the search term "azoospermia" in the Mammalian Phenotype Browser query 242 genes associated with azoospermia in mice were identified. Of these genes, I eliminated those that included phenotypes not related to fertility, e.g. more severe phenotypes such as embryonic lethality, heart defects, etc. I then filtered genes with phenotypes exclusively related to infertility based on their expressed sequence tag profiles in *Mus musculus* on NCBI's UniGene. I included genes with expression limited to the testis, or with high testis expression and relatively low expression in other tissue as candidate genes.

Due to time constraints for this project, our laboratory was not able to develop mouse lines with these genes nullified. Therefore, I searched The Jackson Laboratory's JAX Mice Database for available null and knockout mouse lines[30].

For the final step in identifying candidate genes, I searched for available antibodies that would be reactive in *Mus musculus* and could be used for immunohistochemistry assays. I used the Antibody Resource website to identify vendors of the necessary antibodies. All of the genes identified through the MGI/UniGene/JAX search had available antibodies corresponding to the proteins they transcribed.

Filtering genes expressed in Sertoli cells

To avoid ethical concerns of altering the genetic makeup of sperm cells, one goal of this project was to perform gene therapy on targets in somatic cells. Because Sertoli cells can strongly influence sperm production, but are not considered part of the germline, another method of identifying candidate genes was to begin by searching for genes known to be expressed primarily in these cells.

I utilized three primary publications to identify genes differentially expressed in sertoli cells in the presence of gonadotropins. These articles included: "Oligonucleotide Microarray Analysis of Gene Expression in Follicle – Stimulating Hormone – Treated Rat sertoli Cells," "The Biology of infertility: research advances and clinical challenges," and "RiboTag Analysis of Actively Translated mRNAs in Sertoli and Leydig Cell In Vivo," the last of which claimed to have a comprehensive list of differentially expressed genes in sertoli cells comprised of several thousand genes[15; 31; 32]. This was developed utilizing next – generation sequencing coupled with

a RiboTag approach. The list of genes expressed specifically in Sertoli cells in both prepubertal and adult mice was coined the "SC translome".

I first entered these genes into the MGI mouse phenotype query, and then searched those genes that were associated with male infertility phenotypes using UniGene's expressed sequence tag profiles, as well as BioGPS and GeneCard's expression profiles. I include genes that were associated with male infertility, and with expression restricted to the testis as candidate genes.

2.1.3 Yatsenko Lab Data Mining

The final method of searching for candidate genes I used involved mining previous data produced by RNAseq, whole exome, and aCGG performed on Dr. Yatsenko's study population of male infertility patients. From these studies, several variants had been discovered in genes that may be related to infertility. Unfortunately, all of these genes were expressed in germ cells. One gene, *Cib1*, did appear to be a promising candidate. *Cib1* has been found to be essential in mouse spermatogenesis. And although it is expressed in germ cells as well as Sertoli cells, the expression is much higher in Sertoli cells. Therefore the primary defect causing azoospermia in otherwise healthy mice may be due to aberrant differentiation of Sertoli cells. *Cib1* homozygous male knockout mice are sterile due to disruption at the haploid phase of spermatogenesis. Expression of *Cib1* is not testes specific according to NCBI's expressed sequence tag profile, but the only phenotypes known in *Cib1* knockouts are related to reduced spermatogenesis[33].

2.2 DNAJA1 CHARACTERIZATION

2.2.1 *Dnaja1* Expression Profile

I utilized RT-PCR to confirm the expression profile of the *Dnaja1* gene in mice. First, a cDNA library from mRNA in multiple mouse tissues was developed using the epicentre kit from illumina®. Tissues included the fetal brain, fetal liver, cerebellum, thymus, spleen, placenta, lung, uterus, heart, liver, kidney, ovary, skeletal muscle, small intestine, epididymis cauda, corpus, caput, and whole epididymis, interstitial cells, seminiferous tubules, and testes. A portion of this cDNA panel had been developed previously, but there were several tissues for which I needed to generate new cDNA. I designed *Dnaja1* PCR primers using the University of California Santa Cruz genome browser and optimized at various temperatures using mouse testes tissues and a gene known to have ubiquitous expression in mice as a control, *Gapdh*. Following primer optimization, I selected one pair of primers for the RT-PCR expression panel. The primers were manufactured by Integrated DNA Technologies. *Dnaja1* primer sequences were: Dnaja1_RT2-2-F 5'-AGTCATCACCTCTCATCCAGGT-3' and Dnaja1_RT2-2-R 5'-GTCCACCAGTTCTACCTGATCC-3'. The PCR program was as follows: 95^{o}C for five minutes [98^{o}C for 25 seconds, 61^{o}C for 25 seconds, 72^{o}C for 25 seconds] X 30. *Gapdh* was also used as a positive control. I ran the PCR products on gel electrophoresis and photographed them under ultra violet light.

2.2.2 Immunohistochemistry

I performed the immunohistochemistry with the assistance of Kayla Golnoski, MS and Archana Kishore, PhD. The testes of wildtype mice were fixed in Bouin's fixative at 4^{o}C for 12-16 hours, dehydrated, and embedded in paraffin block. Sections (5 μm thick) were cut and mounted onto poly-lysine-coated slides. Microwave antigen retrieval was employed as described previously[34]. After blocking, an aliquot of 100 μl primary antibody diluted at 1:200 was applied to each section and incubated at 4^{o}C overnight. Incubation with secondary antibody and visualization of positive cells were performed using Vectastain Elite-kit (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions. Preimmune serum was used in control sections. A monoclonal anti – *Dnaja1* antibody was used. Biotin conjugated anti – mouse was used as a secondary antibody. Images were captured by Zeiss microscope equipped with AxioCam MRc5 camera system. *Dnaja1* antibody was developed by abcam® using fusion protein corresponding to N terminal amino acids 1 – 179 of Human HDJ2.

2.2.3 Sequence Conservation Analysis

I analyzed the conservation of genomic and protein sequence between human and mouse *Dnaja1*/DNAJA1 utilizing the Multiple Sequence Alignment tool by CLUSTALW, which is available for use online. First, genomic and protein sequences for *Homo sapiens* and *Mus musculus* Dnaja1 were obtained from NCBI's gene database and saved into a Word Document. I then entered these files into the CLUSTALW tool which read and analyzed the sequences conservation of sequence between species.

2.3 LENTIVIRAL THERAPY

2.3.1 Breeding Strategy and *Dnaja1* Knockout Mice Generation

Archana Kishore, PhD, and I developed and managed the *Dnaja1* mouse knockout line. Breeding to obtain two to three male *Dnaja1* homozygous knockout mice began with the ordering of 2 *Dnaja1* heterozygous knockout males, and one heterozygous knockout female. The knockout mutant had been generated at Jackson Laboratories by the Knockout Mouse Phenotyping Program (KOMP2). To produce the mutation, a beta-galactosidase-containing cassette disrupts the *Dnaja1* gene in this strain. The insertion of Velocigene cassette ZEN-Ub1 created a deletion of size 9258bp between positions 40670749-40680006 of chromosome 4 (Genome Build 37) and subsequent *Cre* expression excised the neomycin selection cassette. The expected litter size was about eight mice. Therefore each round of breeding should have produce roughly 2 wildtype mice, 4 heterozygotes, and 2 homozygous knockouts. Wildtype mice were sacrificed, while heterozygous mice were either sacrificed or used for maintaining the *Dnaja1* knockout line. Homozygous knockout males were to be used for the lentiviral vector injection. The breeding strategy is shown in Figure 2 below.

For the knockout mice, the strain of origin is C57BL/6NTac, as previously used by Terada and colleagues[6]. They generated their *Dnaja1* knockout mice using an embryonic stem cell line generated with a positive/negative-targeting vector. They then used neomycin phosphotransferase gene to create a deletion of the J domain and the G/F-rich domain (exons 2-3).

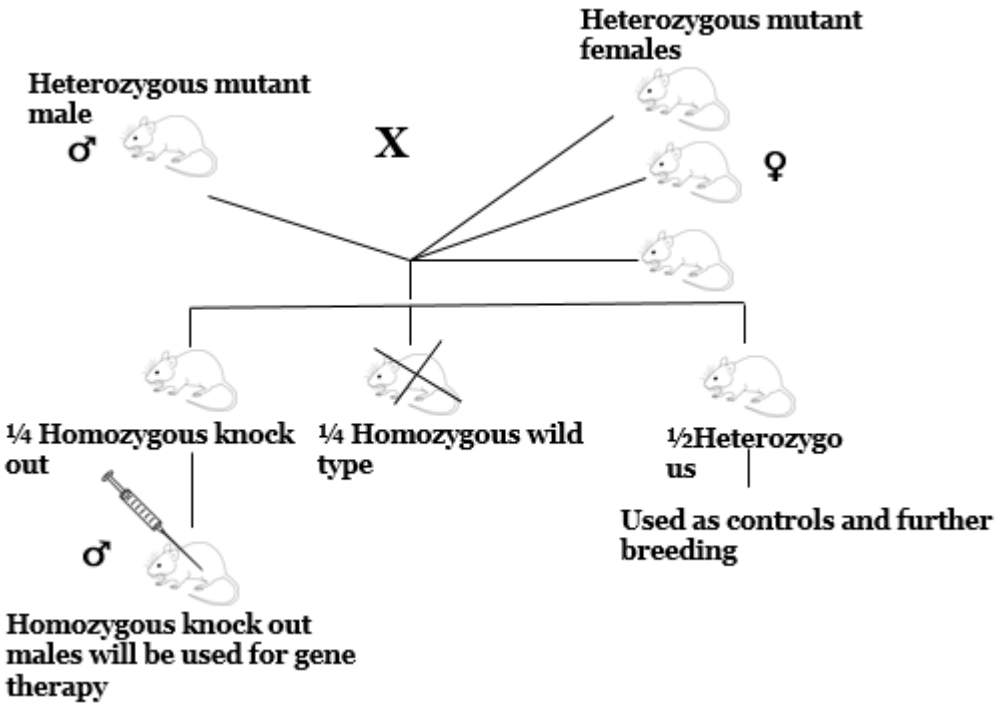


Figure 2. Mouse breeding strategy

2.3.2 Mouse Genotyping

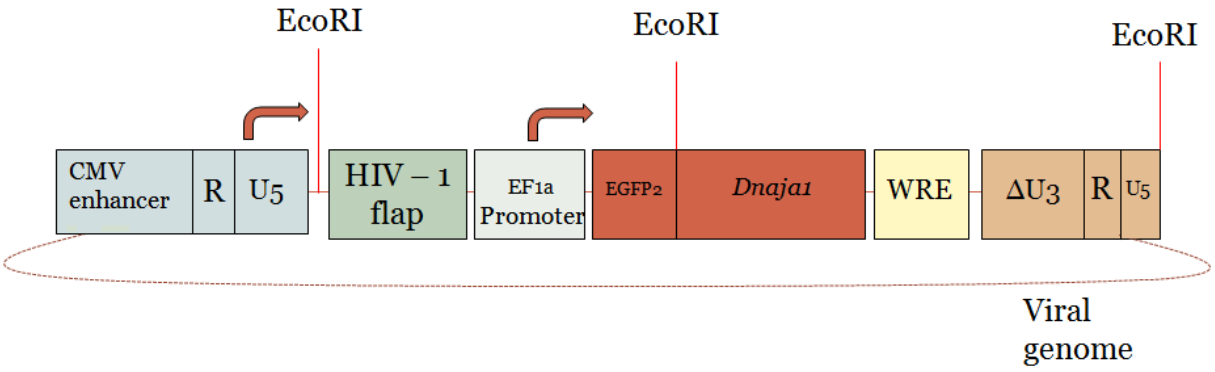
I genotyped mice using the following PCR primers designed by Jackson Laboratories: Wildtype Forward-5'-TTCTTCTGGTGAACTTTGGAG-3', Wildtype Reverse ACCATTCCTGGT CCAATCTG-3', Mutant reverse-5'-CCCATAGCTTAACAATGAAGGG – 3', Mutant Forward-5'-CGG TCG CTACCATTACCAGT-3'. These primers were designed to produce one PCR product for each allele. Thus, wildtype and homozygous knockout mice would show one unique band on gel electrophoresis, while heterozygotes would show two bands. PCR was performed on mouse tail DNA using the amfisure® kit. To isolate DNA from the mouse tail, tail clippings were first placed in 1.2 ml microfuge tubes. 0.5 ml of 50 mM NaOH was added to each tube. Tubes were heated at 100^{o}C for 30 minutes. Following incubation tubes were shaken three to

four times, and then placed at 4°C for 10 minutes. 1M Tris-HCL, pH 8.0 was added and tubes were vortexed, then centrifuged at 21, 000 rcf for 10 minutes. Supernatant was transferred to fresh tube following centrifugation. The PCR program was set at 93°C for 3 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 25 seconds, and 72°C for 30 seconds. Product was run on gel electrophoresis and viewed under ultraviolet light.

2.3.3 Lentiviral vector development

2.3.3.1 Vector development

The vector, shown in figure 3 below, used was designed and developed by Dr. Yi Sheng working in Dr. Kyle Orwig's laboratory. The vector was constructed with the pC – FUW lentiviral backbone using methods previously described [35; 36]. The three important functional elements in the vector were the EF1a promotor (a ubiquitous promotor), enhanced green fluorescent protein (*eGfp*) to tag expression of the vector, and the *Dnaja1* gene. The *eGfp* and *Dnaja1* gene were inserted as a single construct in order to avoid the possibility of EGFP2 expression with a lack of DNAJA1 expression. The *Dnaja1* cDNA construct was manufactured by Transgene. First, *Dnaja1* cDNA was amplified by PCR with the primers: Dnaja1-F 5'-GGTACCGTCGACATGGTGAAAGAAACCACTTACTAC-3' and Dnaja1-R 5'–GAATTCGGATCCTTAAGAGGTCTGACACTGAACG-3'. The EGFP-T2A fragment was isolated from pEGFP-T2A-mCherry via Nhel/SaII digestion and gel purification. These two fragments were inserted into the SpeI/EcoRV sites of pEF-ENTR A (addgene 174527), followed by transfer of EF1 promoter and EGFP2-Dnaja1 into pC-FW-DEST via Gateway LR recombination (Life technologies). The lentiviral vector was produced according to Virapower Lentiviral Expression System (Life technologies).



The replication – deficient lentiviral vector contains a self-inactivating 3'UTR and a CMV enhancer replacing the U3 region of the 5' UTR. WRE: woodchuck hepatitis posttranscriptional regulatory element. HIV-1 flap is a viral element that initiates transduction. The universal promotor EF1a was used. Δ3 represents a deletion rendering this region inactive. The vector was constructed with the pC - FUW lentiviral backbone using methods previously described [35; 36].

Figure 3. Lentiviral vector

2.3.3.2 Confirmation of Dnaja1 cDNA insertion

Yi Sheng, MD, PhD, confirmed that the *Dnaja1* cDNA was successful via PCR and gel electrophoresis. Eight bacterial colonies were transfected with the EF1a-EGFP-*Dnaja1* lentiviral vector construct. The vector was then linearized with an EcoRI restriction digest followed by amplification by PCR. This product was run on gel electrophoresis and photographed under ultraviolet light.

2.3.3.3 Confirmation of GFP and vector expression in testes

Yi Sheng, MD, PhD, confirmed vector expression in the testes via GFP studies. Mice were anesthetized and testes were exposed. The lentiviral vector, mixed with one tenth of 0.4%

trypsin blue dye was injected into seminiferous tubules through the efferent duct of one side of the testes, while the vehicle (DMEM media) was injected into the other side of the testes. The testes were removed 72 hours after injection, and observed under epifluorescent microscopy at 10x and 20x views.

2.3.4 Vector Injection Strategy

Kyle Orwig, PhD, had previously developed a methodology for lentiviral vector injection. The lentiviral EF1a-EGFP construct is injected into the homozygous knockout male mouse testes at day 15-18 of the life cycle. The mice are then allowed to mate with the females to determine if they are fertile or infertile. After the mice are given sufficient time for breeding the experimental male mouse is removed and the testes will be used for semen analysis, histological analysis, and to determine whether the vector is being expressed using the GFP expression tag.

3.0 RESULTS

3.1 SPECIFIC AIM 1

Filtering genes with azoospermia mouse phenotype

I identified several genes by searching “azoospermia” and “male infertility” on MGI, eliminating genes with phenotypes unrelated to male infertility, and by cross-referencing these genes with expression profiles on NCBI’s Unigene, BioGPS, and Genecards, that were expressed exclusively in mouse testes, and that were known to cause isolated male infertility in mice. However, most of the genes were expressed in the germ line, rather than Sertoli or other somatic cells. Because these genes show germline-specific expression, they were not considered as candidates for experimentation at this point. This project aimed to avoid altering the germline due to ethical concerns, and it was not anticipated that germ cells would be transfected by this virus vector. However, these genes may be used in future experimentation since they do represent potential causes of isolated infertility in men, and may be acceptable targets of gene therapy if germline gene therapy is ever considered accepftable for use in the clinical setting. A brief description of selected genes identified via this method is provided. The following genes are summarized in Table 1 below.

Table 1. Known male mouse infertility phenotype in germ cell expressed genes

Gene	Isolated Male infertility MP	Testis – Limited Expression
Catsper1	•	•
Mei1	•	
Mov10l1	•	•
Piwil1	•	•
Spo11	•	•
Stag3	•	
Stra8	•	•
Sycp1	•	•

Catsper1

Catsper1 transcribes a Ca_2+ cation channel located specifically in the principle piece of the sperm tail. Targeted disruption caused male infertility in otherwise unaffected mice, with markedly decreased sperm motility[37]. JAX has a cryopreserved – ready for recovery null mouse line for *Catsper1* and there are available antibodies for the transcribed protein (Cation channel sperm – associated protein 3 antibody).

Mei1

The phenotype of *Mei1* mouse knockouts is reminiscent of Sertoli-cell-only syndrome in humans [38]. The EST profile for this gene, which encodes meiosis inhibitor protein 1, shows high

testis expression, and low expression in the brain, eye, and bladder. There is a knockout mouse line that is cryopreserved and ready for recovery through JAX.

Mov10l1

Mov10l1 is necessary for a group of noncoding RNAs that function as an endogenous defense mechanism against transposable elements to function properly. *Mov10l1*, which is exclusively expressed in germ cells, is has been found to play a role in silencing retrotransposons in mouse germ cells. Mouse knockouts were essentially healthy, and displayed Sertoli-cell-only phenotypes [39]. The EST profile for this gene shows high testis expression, and low expression in the heart and ovaries. There is a JAX knockout mouse line available.

Piwil1

Piwil1 initiates transposon silencing via DNA methylation in the embryonic germline of male mice and is expressed in postnatal germ cells [40]. Phenotypes in mouse knockouts include: arrest of spermatogenesis, male infertility, decreased testis weight, azoospermia, abnormal male germ cell morphology, and arrest of male meiosis. There is a JAX knockout line available, and *Piwil1* was also in our RNAseq dataset.

Spo11

Knockouts of *Spo11*, which encodes meiotic recombination protein SPO11, cause meiotic arrest at the spermatocyte stage. Smagulova et al. 2013 found that the SPO11 protein causes double stranded DNA breaks in "recombination hotspots" in order to elicit recombination[41]. All phenotypes shown in mice through MGI and literature review are related to fertility, and include

azoospermia. The EST profile for this transcript shows high testis expression, and low thymus expression, and there is a JAX mouse line available.

Stag3

Stag3 codes for a cohesin that is specifically involved in germ cell meiosis. Spermatogonia, Sertoli, and leydig cells were all apparently normal in mouse knockouts. The only phenotypes identified in these knockouts were absent oocytes, and azoospermia. The EST profiles shows high expression in the testis, and low expression in a limited number of other organs. There is a JAX knockout line available.

Stra8

Stra8 homozygous knockouts were generally healthy, with phenotypes similar to Sertoli-cell-only syndrome in humans. *Stra8* does not show expression in Sertoli cells, but the EST profile shows expression specific to the testis. There is a JAX mouse line available. STRA8 protein may play a role in chromosome pairing in spermatocytes[42].

Sycp1

Sycp1 mouse knockouts were generally healthy with azoospermia. The EST profile shows expression in the testis and ovaries only, and a JAX knockout line is available. SYCP1 protein is involved in the construction of transverse filaments, which are responsible for connecting the axial elements that which assemble into synaptonemal complexes during meiosis.

Filtering genes expressed in Sertoli cells

Using this search method finding genes with phenotypes restricted to male infertility, as well as genes with expression limited to the testis, was more difficult and less prolific. I first identified genes expressed in Sertoli cells via literature review. I then cross – referenced that list with MGI and expression databases (Unigene, Genecards, and BioGPSS) to identify candidates with isolated male infertility phenotypes in mice and testes-specific expression. The genes discovered will be discussed here, with focus given to the *Dnaja1* gene, which was eventually the gene selected for experimentation due to its known phenotype, and the availability of live mouse knockouts available for order through the Jackson Laboratory's mouse repository. Some of these genes had available frozen mouse embryonic stem cell knockout lines through JAX, however, developing a live mouse knockout line from these cells (in an experienced laboratory, and assuming each part of the process is successful) would take at least six months, which was beyond the time I had available to complete the project. The following genes are summarized in Table 2.

Table 2. Sertoli cell expressed genes with known male infertility phenotype in mice

Gene	Male infertility MP	Testis – Limited Expression
L1cam	•	
Egr1	•	
SSeCKS	•	
Cyp17a1	•	•
Dhh	•	•
Dmrt1	•	•
Lhcgr	•	•
Serpina 5	•	•
Dnaja1	•	
Sox8	•	
Slc9a2	•	•
Wt1	•	•
Nr0b1	•	•
Inha	•	•
Prnd	•	
Amhr2	•	•
Pcyt1b	•	•
Amh	•	
Dab1	•	•

Of the genes that were shown to have mouse knockouts with male infertility and expression relatively limited to the testis, only three were associated with azoospermia in mice. These were *Dhh, Wt1,* and *Amhr2*. Unfortunately, each of these genes has shown deficiencies in several systems other than the male reproductive system in mouse knockouts, and their expression is not limited to the testis. Three genes, *Lhcgr, Dnaja1,* and *Prn*, displayed oligozoospermia in null mice. *Lhcg* and *Dnaja1* knockout mice also displayed other phenotypes such as postnatal growth retardation and decreased body weight. *Lhcg* mice showed enlarged kidneys as well. *Prnd* mouse knockouts only displayed deficiencies related to the male reproductive system, including oligozoospermia, asthenozoospermia, and teratozoospermia. According to the EST profile for mice on NCBI's UniGene, *Prnd* is ubiquitously expressed in mice. However, the accuracy of this profile may be questioned since the human expression profile shows high expression in the testis, and low expression in only the eye, heart, bone marrow, and prostate, and because the only phenotypes shown in mouse knockouts on MGI are related to male infertility. There is no available *Prnd* mouse line from JAX.

Four other genes displayed asthenozoospermia in mouse knockouts. These include *Cyp17a1*, *Serpina5, Dnaja1,* and *Sox8*. *Cyp17a1*, *Dnaja1,* and *Sox8* included severe phenotypes other than male infertility, including embryonic lethality, postnatal growth retardation, and sex reversal. *Serpina5*, on the other hand, is only expressed in the testis and ovaries, and only phenotypes involved in the reproductive system were observed in mice. Unfortunately, there is no JAX line available for *Serpina5*. *Dnaja1* is a chaperone primarily expressed in sertoli cells. The two phenotypes of concern shown in *Dnaja1* mouse knockouts were postnatal growth retardation, and decreased body weight. There is an available mouse line through JAX as well.

Dmrt1 is an ancient, zinc finger-like DNA-binding motif that is gonad specific and sexually dimorphic expression pattern. It is expressed in Sertoli cells, and is necessary for maintaining male germ cells, but there is no mouse line available from JAX.

Because the search criteria for Sertoli-cell expressed genes was too restrictive, our criteria was relaxed in order to select at least three to four genes to study. We chose to include genes expressed in Sertoli cells whose mouse knockouts showed male infertility related to sperm maturation or function, including azoospermia, oligospermia, morphology, motility, and oocyte binding. Also, rather than maintaining the requirement for these genes to have testis specific expression based on NCBI's EST profile, genes were included provided that their knockout lines produced viable, infertile males. Other known mouse phenotypes in knockouts were disregarded assuming they did not severely decrease the viability of the mice. Furthermore, at this point there is no interest in attempting gene therapy on the genes expressed in germ cells. The reasoning for this is twofold. First, there are several ethical concerns involved with gene therapy on germ cells due to the potential of affecting future generations. Because of this, performing gene therapy on germ cells in mice does not translate well to clinical utility, since gene therapy on human germ cells is not feasible/ethical/legal/etc. Secondly, it is possible that the virus vectors will not matriculate into germ cells, since their phagocytic potential is much lower than Sertoli cells.

Originally, the genes selected for experimentation were *Akap9* (however no mouse line was available through any searched repositories) *Prnd, Dnaja1*, *Dmrt1*, *Dhh,* and *Serpina5*. A brief summary of these genes, and potential alternative candidate genes are given below. *Dnaja1* was ultimately chosen for experimentation because of the phenotype described by Terada and

colleagues in 2005[6] and due to the availability of a live knockout line being maintained by The Jackson Laboratories. This enabled the project to be initiated with optimal time efficiency.

Dhh

Dhh is a Sertoli cell-expressed gene that regulates early and late stages of spermatogenesis. Male *Dhh* knockout mice are viable, yet infertile due to lack of mature sperm. Females displayed no phenotype[43].

Dmrt1

Dmrt1 is expressed in pre-meiotic germ cells and in Sertoli cells in mice. Serving important functions in these cells, it is necessary for the radial migration of germ cells to the periphery of seminiferous tubules, and for postnatal differentiation in Sertoli cells[44].

Prnd

Prnd is a gene that codes for the prion protein duplet (DPL) protein. *Prnd* knockouts in mice resulted in male infertility. DPL protein is expressed in late stages of spermiogenesis. Behrens and colleagues found that "spermatids of *Dpl* mutants were reduced in numbers, immobile, malformed and unable to fertilize oocytes *in vitro*." They concluded that "*Dpl* regulates male fertility by controlling several aspects of male gametogenesis and sperm-egg interaction"[45].

Serpina5

Serpina5 mouse knockouts appear to be healthy, but males are sterile. Uhrin and colleagues found that male infertility may be a result of damage to the Sertoli cell barrier causing aberrant spermatogenesis[46].

Akap9

Akap9 has been found to play an important role in coordinating regulation of Sertoli cells in the testis in a tissue-specific manner. In *Akap9* knockout mice, gap and tight junctions essential for blood-testis barrier (BTB) organization are disrupted. Connexin43 and zona occludens-1 are also improperly localized in *Akap9* mutant testis. Male knockout mice are viable, but infertile. Phenotypes include azoospermia, oligozoospermia, globozoospermia, and decreased testis weight. The expressed sequence tag profile shows that expression is nearly ubiquitous, but phenotypes shown by MGI contradict this[47].

Pvrl2

Although *Pvrl2* homozygous knockout male mice produce normal amounts of motile spermatozoa, scanning electron microscopy performed on these mice showed deformities of the spermatozoan head and midpiece. This gene is expressed exclusively in Sertoli cells, but unfortunately there is no line available on JAX. Mouse production is planned according to IMPC search results. EST does not show testis-specific expression. This might be a priority for experimentation if there were an available mouse line[48].

Sox8

Sox8 null mice were seen to have male infertility that progressed with age. Null males occasionally produced small litters at young ages. SOX8 protein is produced by Sertoli cells. Spermatogenesis abnormalities that have been observed in null males include loss of spermatocytes and round spermatids, disorganized spermatogenesis, and absence of spermiation. O'Bryan and colleagues also observed abnormal motility of sperm within the epididymides, and that SOX8 regulates Sertoli cell function. The protein is also required for the cytoarchitectural and paracrine interactions between Sertoli cells and germ cells[49].

Evt5

Etv5 is a potential future candidate gene discovered via literature review following the initial search methods. In 2013, O'Bryan et al. reported male infertility in male mice with a missense mutation in ETV5[50]. The mutation in this transcription factor led to embryonic and perinatal lethality, poor growth, renal asymmetry, and polydactyly. In 2005, Chen et al. showed that this gene displays Sertoli cell-specific expression[51]. They also demonstrated that ETV5 is necessary for regeneration of spermatogonial stem cells. Mice with targeted disruption display progressive germ cell depletion and Sertoli cell-only syndrome by age 8 weeks. Sertoli cells showed alterations in secreted factors known to regulate the hematopoietic stem cell niche. The mutation generated by O'Bryan and colleagues was located in exon 12, and it caused a premature

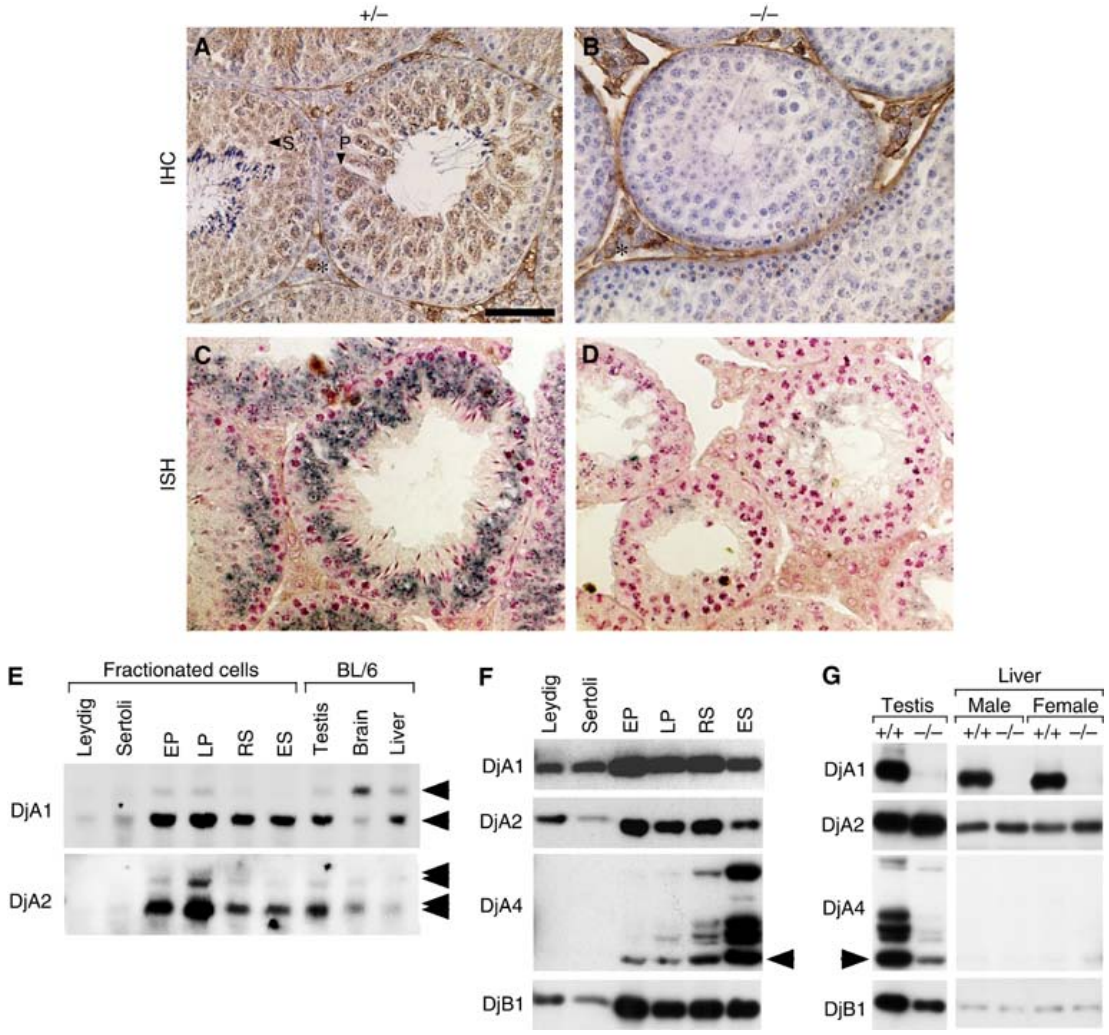
stop codon at position 412 in the ETS DNA binding domain. ETV5 therefore appears to play a role in several aspects of development beginning in the embryonic state.[50; 51].

Dnaja1

Of the above genes, ***Dnaja1*** was determined to be a viable candidate gene based on a study performed by Terada et al. in 2005, and was selected for the pilot gene therapy because it was the only one of these candidates for which there was an available live mouse maintained by the Jackson Laboratories[6]. Other genes were unavailable through JAX, or only available as frozen stem cells. Generating a live mouse line using these cells would require several months of labor. Alternate genes mentioned may be used for future projects pending the success of the ***Dnaja1*** effort. As mentioned previously, ***Dnaja1*** is a chaperone primarily expressed in Sertoli cells. Although growth retardation and decreased body weight have been observed in ***Dnaja1*** mouse knockouts, the mice are otherwise viable, which was expected to allow the attempt of viral vector therapy.

Terada and colleagues reported that "loss of *Dnaja1* in mice led to severe defects in spermatogenesis that involved aberrant androgen signaling." Also "transplantation experiments with green fluorescent protein-labeled spermatogonia in *Dnaja1*$^{-/-}$ mice revealed a primary defect of Sertoli cells in maintaining spermiogenesis at steps 8 and 9"[6]. They also noted accumulation of the androgen receptor and increased transcription of androgen-responsive genes, such as *Pem* and *Testin* in Sertoli cells of *Dnaja1*$^{-/-}$ mice. There was also evidence of disrupted Sertoli – germ cell adherens junctions in *Dnaja1*$^{-/-}$ mice[6]. Figure 4 shows the expression profile of *Dnaja1* in various tissues based on northern blot analysis. It also shows histology of the testes in *Dnaja1* hetero and homozygous knockout mice. Based on this histology, it is evident that homozygous

knockout males are producing some early germ cells, but fail to produce fully mature, elongated sperm. *Dnaja1*$^{-/-}$ mice also had no structural defects, and were completely healthy[6]. For these reasons *Dnaja1* was chosen for experimentation. It would serve as an excellent model of isolated idiopathic infertility in men, and gene therapy at day 15 may have a potential positive effect on these mice since there are no major developmental abnormalities.



High expression of type I DnaJs in testis and increased expression of DjA2 in DjA1−/− mice. (A, B) Sections of DjA1+/− and DjA1−/− testes were mounted on the same slide glass, and decorated with a mAb against DjA1. Peroxidase activity was visualized using 3,3′-diaminobenzidine as a chromogen. Sections were counterstained with hematoxylin. Signals of DjA1 in primary and secondary spermatocytes are indicated with P and S, respectively (A). Nonspecific signals in the interstitial area are indicated with asterisks (A, B). (C, D) In situ hybridization of DjA1 mRNA from DjA1+/− and DjA1−/− testes was carried out using digoxigenin-labeled DjA1 probe. Alkaline

phosphatase activity was visualized using 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium as a chromogen. Sections were counterstained with nuclear fast red. (E) Total RNA samples from fractionated testicular cells and mouse tissues (5 μg each) were subjected to Northern blot analysis, using digoxigenin-labeled DjA1 and DjA2 cRNAs as probes. Arrowheads indicate positions of 2.4 and 1.6 kb bands (DjA1) and 2.1, 2.2, 3.1 and 4.0 kb bands (DjA2). Protein samples of testicular cells (F) or tissues (G) were subjected to Western blot analysis using a monoclonal anti-DjA1, or polyclonal anti-DjA2 or anti-DjB1 antibodies (0.4 μg/ml each), or antiserum against DjA4 (1/1000 dilution). An arrowhead indicates position of expected size of DjA4.

* Reused with permission from The EMBO Journal[6]

Figure 4. High expression of type 1 DnaJ in testis

3.2 SPECIFIC AIM 2

3.2.1 *Dnaja1* Expression Profile

As expected, *Dnaja1* showed ubiquitous expression in mice. Based on the results of the RT-PCR, *Dnaja1* was amplified in all mouse tissues, with apparent reduced expression in the uterus. This reduced expression in the uterus may be a results of the methods used to generate cDNA, so it is possible that it does not accurately represent the expression of Dnaja1 in mice. The results of the RT-PCR for *Dnaja1* on the mouse cDNA tissue panel are shown in Figure 5. These results were compared to the BioGPS expression profile of Dnaja1 (available at http://biogps.org/#goto=genereport&id=ENSMUSG00000028410), which are shown in Figure 6. The BioGPS profile also shows ubiquitous expression. The RT-PCR profile were concordant with the BioGPS data for *Dnaja1*, as well as the expressed sequence tag data presented compiled by NCBI.

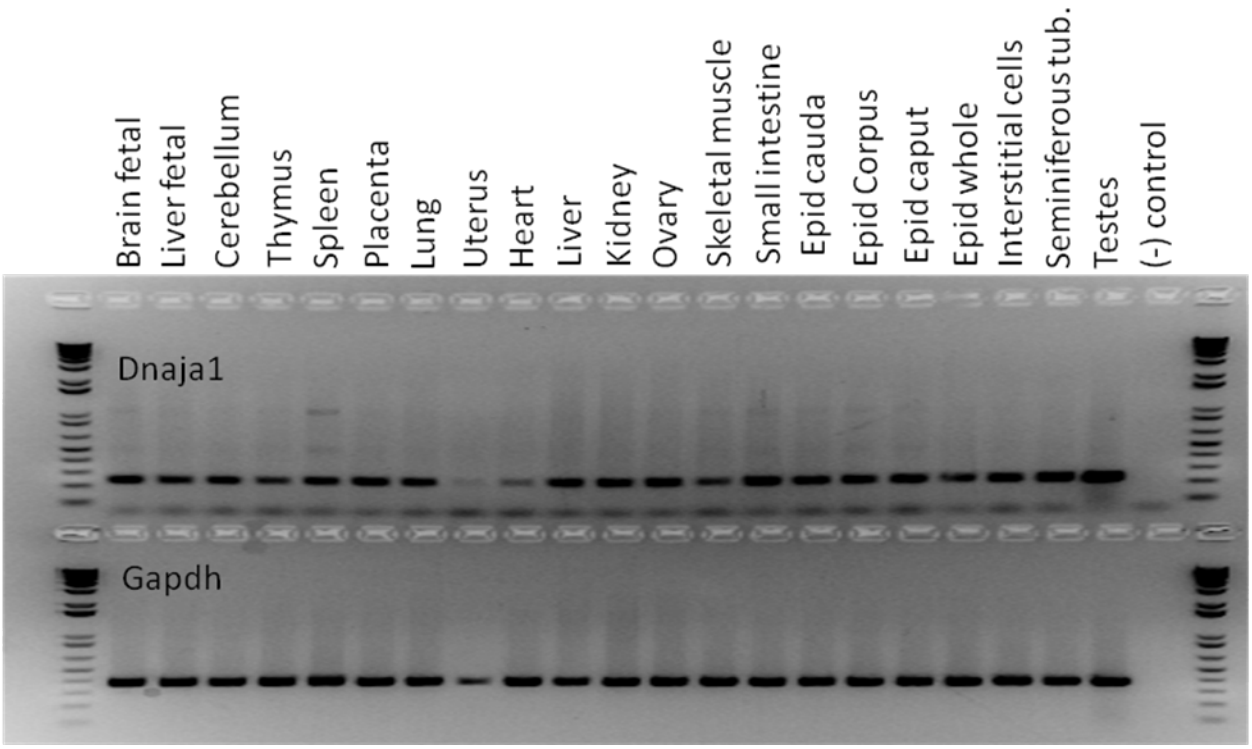


Figure 5. *Dnaja1* RT-PCR expression profile

Figure 6. BioGPS expression profile for *Dnaja1* in mice

3.2.2 Immunohistochemistry

The cross section of the seminiferous tubules stained with DAB shows *Dnaja1* expression in all cells in the seminiferous tubules. It is likely that the antibody concentration and DAB exposure was too high, making it difficult to determine if this is an accurate representation of *Dnaja1* expression, or if the pattern is due to the oversaturated antibody and DAB exposure. This IHC will need to be repeated for more accurate imaging. Figure 7 shows the image obtained via *Dnaja1* antibody staining in the testis.

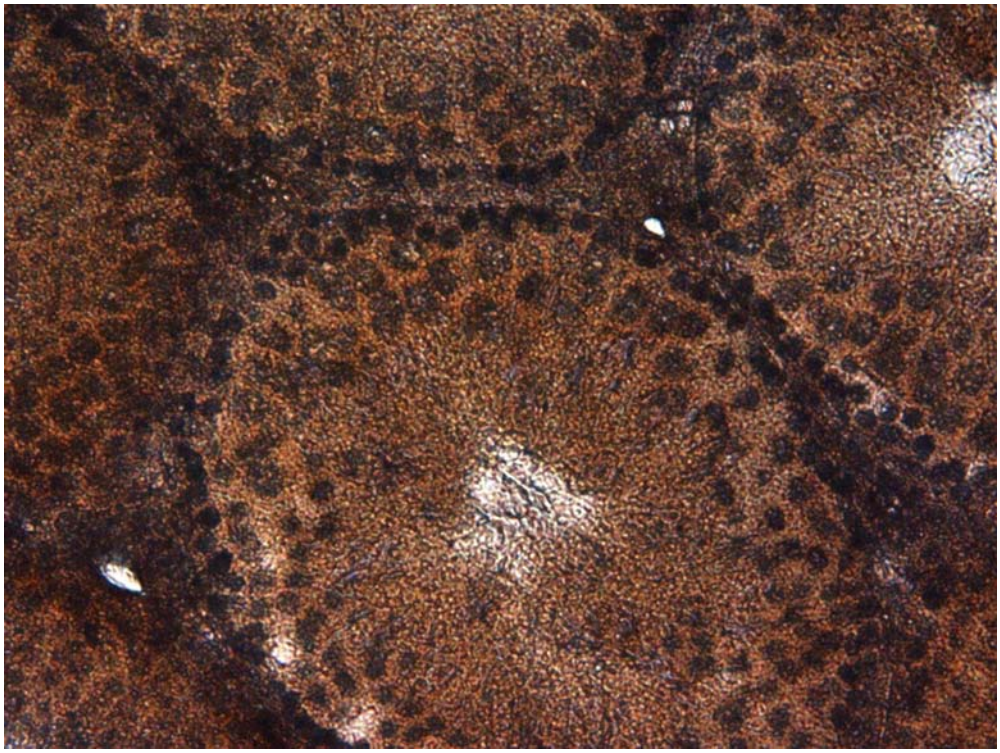


Figure 7. *Dnaja1* antibody staining of seminiferous tubules

3.2.3 *Dnaja1* sequence conservation analysis

Genomic sequence alignment of human and mouse *Dnaja1* shows 60% identity. The amino acid sequence showed 99.5% sequence identity. The primary functional domain of the DnaJ family is the J domain. Within the J domain is a conserved sequence motif (JPS) that accelerates ATPase activity of Hsp70. The ADP-bound state of Hsp70 binds polypeptide substrate. This ATPase cycle is essential to the function of Hsp70 (Dnaja1) as a chaperone[6]. Two other functional, conserved domains in the DnaJ family include a C-terminal substrate binding domain of DnaJ and Hsp40 that prevent aggregation of unfolded substrate, and a zinc finger domain as shown by the University of California Santa Cruz's genome browser (shown in Figure 11 of discussion).

3.3 SPECIFIC AIM 3

3.3.1 *Dnaja1* mouse breeding

The results of several months of mouse breeding are summarized in Table 3. No *Dnaja1*$^{-/-}$ pups were produced. 9 litters of pups have been born, ranging in size from 3 to 11, with an average size of about 6 mice. The number of homozygous knockout mice is significantly lower than the expected mendelian ratio, and indicates that this mutation may be causing embryonic lethality in these mice.

There is no abnormal phenotype seen in heterozygous mice, other than a possible unclassified pathogenesis in the male genitalia. This only occurred in two of the males, and it is likely to be injury sustained from aggressive female mice. Pictures of these two males are shown

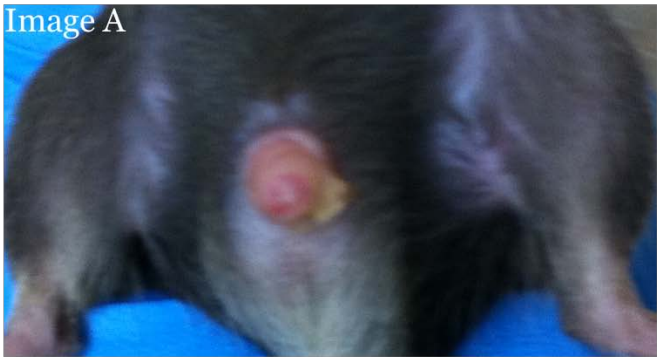
in Figure 8. *Dnaja1* heterozygous mothers also ate several offspring, which is not unusual as these are new mothers.

Table 3. *Dnaja1* mouse breeding results

Litter	Mouse Breeding *Dnaja1*$^{+/-}$ ♀ X *Dnaja1*$^{+/-}$ ♂			Total
	WT	+/-	-/-	
1	0	6	0	6
2	0	3	0	3
3	2	6	0	8
4	2	5	0	7
5	3	6	0	9
6	6	5	0	11
7 – 9	1	7	0	8
Total	**14**	**38**	**0**	**52**

Male to female ratio 1:1 (23 males:24 females)

Chi-square test p-value: 9.1 x 10-5



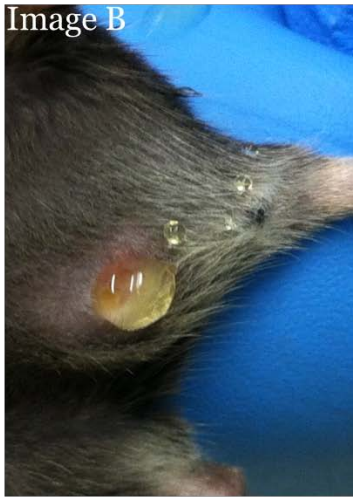
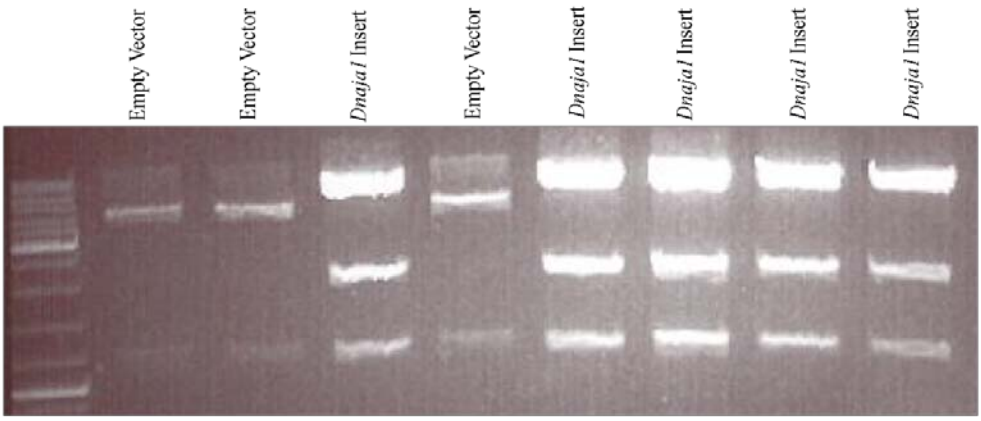


Image A and B show an unclassified pathogenesis and/or injury of the genitalia in male heterozygous *Dnaja1* knockout mice. Only two mice showed this and it appears to be the result of the males being attacked by the female

Figure 8. Possible pathogenesis of male *Dnaja1* heterozygotes

3.3.2 Confirmation of *Dnaja1* cDNA insertion

Figure 9 shows the PCR used to confirm the insertion of the *Dnaja1* cDNA into the lentiviral vector. Each band represents one of eight bacterial colonies, five of which show three bands with the expected digestion pattern after EF1a-EGFP-T2A-*Dnaja1* had been inserted into the lentiviral vector. The top band represents circular DNA, the middle band represents linearized DNA with *Dnaja1* insert, and the lower band shows linearized DNA without the *Dnaja1* insert (empty vector). The PCR was performed following an EcoRI restriction enzyme digest. The vector insertion and confirmation was performed by Dr. Yi Sheng, MD, PhD.



*Transfection performed by Yi Sheng, MD, PhD

Figure 9. EcoRI restriction and PCR confirmation for *Dnaja1* cDNA insertion

3.3.3 Confirmation of GFP and vector expression in testes

Lentiviral vector expression was confirmed via GFP expression observed under epifluorescent microscopy. At 10X and 20X magnification, GFP expression is clearly seen in the seminiferous tubules. Figure 10 shows the GFP Tag expression studies. Expression studies were performed by Yi Sheng, MD, PhD.

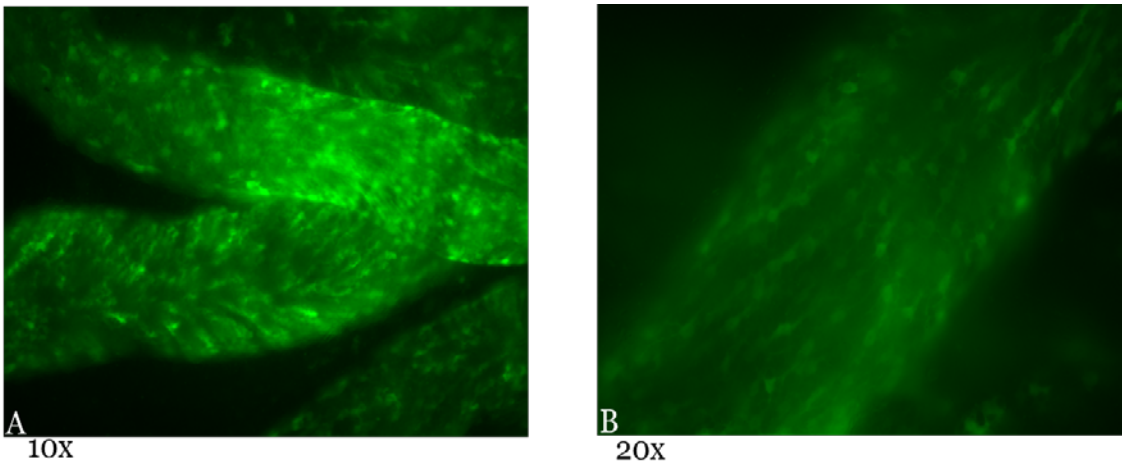


Figure 10. GFP Tag expression

4.0 DISCUSSION

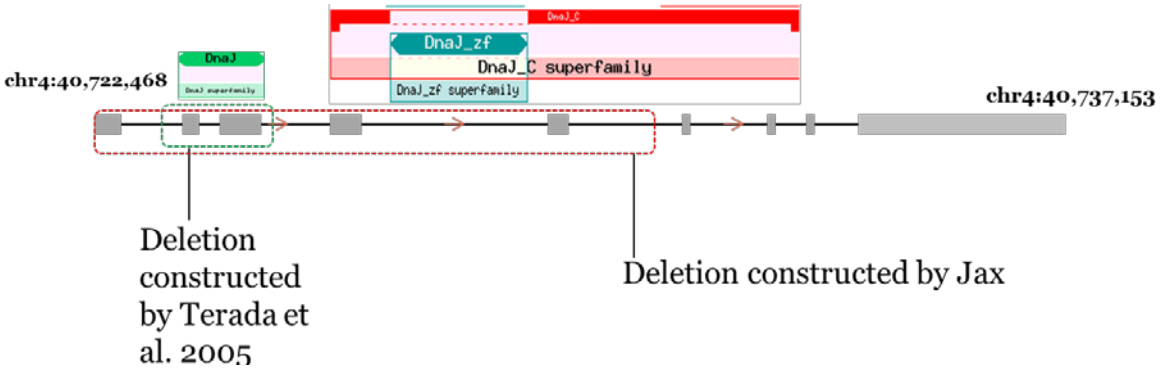
4.1 DNAJA1 KNOCKOUT BREEDING

Although it was expected that *Dnaja1* homozygous knockout mice would be viable, with male infertility as the only damaging phenotype, these mice were unable to be produced through breeding of heterozygotes. We suspect that this particular mutation of *Dnaja1* is causing embryonic lethality. Based on the assumption that one of every four mouse pups would have the homozygous knockout genotype, with 52 total pups approximately 13 homozygous knockout pups should have been produced. It is highly unlikely that having zero of fifty – two pups being double knockouts is due to random chance. A Chi – square test to examine this hypothesis gives a p-value of 9.1×10^{-5}.

To ensure that the genotyping results were not due to protocol errors, I used the UCSC genome browser to determine whether the wildtype primers might produce a PCR product seen on gel electrophoresis even in the absence of the wildtype allele. According to the browser there is only one possible PCR product using this primer pair. Therefore it is not suspected that the lack of homozygous recessive mice is a product of genotyping error.

I performed further analysis of the *Dnaja1* mutations produced by Terada et al. and the mutation produced by Jackson Laboratories and found that the embryonic lethality may be due to the size of the deletion in the Jackson Laboratories mutation. Terada and colleagues deleted only

the J functional domain of *Dnaja1*, which included exons 2 and 3. The mutation that Jackson Laboratories produced included exons 1-5. As shown in Figure 11, this region includes two additional conserved domains of the DNAJA1 protein. Domains not included in the Terada et al. knockout that are included in the Jackson Labs knockout include the C-terminal substrate binding domain of the DnaJ family as well as a Zinc finger domain. The C-terminal region mediates oligomerization and binding to denatured polypeptide substrate, prevents the aggregation of unfolded substrate, and forms a ternary complex with both substrate and DNAJA1. The Zinc finger domains of the DnaJ family are highly conserved and perform essential roles in protein translation, folding, unfolding, and degradation. Their primary function is stimulating the ATPase activity of heat shock proteins. The differences in the mutation offers a plausible explanation as to why the phenotype in the Jackson Mouse line would be more severe.



Dnaja1 contains 9 exons and is located between positions chr4:40,722,468 and chr4:40,737,153. The deletions constructed by Terada et al. only included exons 2 and 3, which encode the J domain of the protein product. Jax deleted exons 1-5. This regions encodes the J domain, a Zinc finger domain, and a c-terminal substrate binding domain.

Figure 11. DNAJA1 deletion

4.2 LENTIVIRAL VECTOR

Because no *Dnaja1*$^{-/-}$ mice have been produced, the efficacy of the lentiviral vector was unable to be tested. However, during the time that these mice were being bred, results did show that the *Dnaja1* cDNA insert was successfully transfected into the viral vector, and that the vector is expressed in the seminiferous tubules. Thus, preparations are in place to perform this experiment on future candidate genes. We are continuing this project, and are now ready to test the lentiviral vector therapy on alternative candidate genes.

4.3 STUDY LIMITATIONS

There are limitations to this study that may be assessed as the project continues. First, expression studies of the viral vector were performed only in the testes. It cannot be confirmed whether insertion of the vector will occur in other systems. Although the vector will be injected directly into the testes, it may be possible for the virus to travel to other tissue, which may have unpredictable side effects on the mice, especially with the consideration that a universal promotor is being used. Also, despite the viral vector expression studies using the GFP tag confirming expression of the vector in the seminiferous tubules, no cross – sectional view of the seminiferous tubules was taken. Therefore it is not certain exactly which cells in the seminiferous tubules had been infected by the virus, and germ cell expression cannot be ruled out. However, in prior experiments, Dr. Orwig's group had not observed lentiviral vector expression in the germline.

There are also limitations regarding clinical applications of lentiviral gene therapy. Although, as mentioned in the introduction, there are a number of clinical trials using lentiviral gene therapy that are either ongoing or approved, the therapy has not been approved for use in humans. This type of therapy has been shown to be effective in several studies, but safety concerns for human use remain despite years of effort and improvement. Lastly, at this time targets for lentiviral gene therapy to improve spermatogenesis in men are limited because there are very few known single-gene causes of infertility in human males. As more research is done, however, this number is predicted to increase significantly.

4.4 FUTURE CONSIDERATIONS

4.4.1 Alternative Candidate Genes

There are several other candidate genes identified through this research that may be used as alternatives to *Dnaja1*. For the next stage of the project, four genes will be used in the place of *Dnaja1*. These genes are *Cib1, Ar*, *Etv5,* and *Dhh*. *Cib1* is a gene that was identified with a likely pathogenic variant on whole exome analysis in a male with unexplained infertility in our study population. Mutations in this gene are known to cause isolated male infertility in mice, with the primary cause of azoospermia due to aberrant differentiation of Sertoli cells. Targeted mutations of the Androgen receptor gene (*Ar*), which encodes the receptor for the hormone that regulates development and maintenance of the male sexual phenotype, have been shown to cause isolated azoospermia in male mice[52]. Male mice with targeted disruption of *Etv5* display Sertoli – cell only syndrome beginning at age 8 weeks due to aberrant spermatogonial stem cell regeneration. This gene is expressed exclusively in Sertoli cells[49; 51]. Desert hedgehog (*Dhh*) male knockout mice produce no mature sperm, but are otherwise viable. This gene, expressed in Sertoli cells, regulates both early and late stages of spermatogenesis[43].

Other plans for lentiviral gene therapy include genes discovered through our primary research on men with idiopathic azoospermia or oligozoospermia. These genes may be knocked out or mutated using clustered regularly interspaced short palindromic repeats, or CRISPRs. The CRISPR system enables the creation of mouse models and reduces the time required to weeks, rather than months, to develop mutated mouse lines. Methods of gene discovery are discussed below.

4.4.2 Future Candidate Gene Discovery

Our research group will also continue its work to discover novel single-gene causes of male infertility, particularly azoospermia and oligozoospermia. Methods of gene discovery include RNA expression, whole exome, and array comparative genomic hybridization studies at the genomic level. Subjects for these studies are recruited primarily through the Reproductive Endocrinology and Infertility Clinic, as well as the Urology clinic of Magee-Womens hospital. Men interested in the study have also been recruited through social media, online support groups, and other local clinics and infertility support groups in the greater Pittsburgh area.

4.4.3 Insights

This section focuses on what I would have done differently from the outset of this project given a similar allotment of time, and knowing now what I have learned from this research. Although several candidate genes that may be used for future experimentation were identified, in order to complete the project more efficiently I would have begun the search for candidate genes differently. Performing raw searches of MGI, and finding a complete list of genes expressed in Sertoli cells may be useful in the long run, but since we had limited capacity to create mouse knockout lines, and limited time to complete the project it may have been wise to begin the search for candidate genes starting at the end of the filtration pathway rather than the beginning. What could have been done was to search mouse repositories, such as the Jackson Laboratories, for available knockout mouse lines first and then review the literature to determine whether these

genes would be appropriate for experimentation. By searching mouse repositories first, the time spent finding genes appropriate for our purposes would have been significantly shorter.

Another approach that could have been more productive and resourceful would be to seek assistance from researchers with experience in male infertility and Sertoli cell research. As novices with regard to Sertoli cell genetics, beginning with the guidance of such an expert may have allowed us a more streamlined search for candidate genes. Someone with this expertise could have also guided us with regard to details of these genes that may not be readily apparent through literature review. Such individuals may have been found by searching for investigators within the Mage-Womens Research Institute with publications in Sertoli cell research, or that were currently investigating this subject. We could have also found investigators throughout the country, or even internationally, by reaching out to those that had recently published in this area of research. The search method I used was appropriate for identifying numerous candidate genes that can be used for a long term project, but this alternative process would have allowed for a more focused search and for a more efficient use of my time.

5.0 CONCLUSION

This project offers insight for future development of lentiviral vector gene therapy to treat isolated male infertility. Although few single-gene causes of male infertility have been identified in humans, hundreds of these genes are known to cause isolated infertility in mice, which may represent undiscovered causes of unexplained infertility in men. If these single-gene defects can be corrected to restore spermatogenesis in mice with lentiviral gene therapy it is entirely possible that this therapy could be translated into the clinical setting. This research has compiled several candidate genes to test the efficacy of lentiviral vector gene therapy for male infertility in mice, has shown that the viral vector is expressed in the target cells of the seminiferous tubules, and prepared an avenue for attempting this therapy on genes discovered in human males with unexplained azoospermia or oligozoospermia. With continued efforts, the project serves as a foundation to prove that spermatogenesis can be restored in men with unexplained infertility, and that lentiviral gene therapy may serve as a viable option for treating infertility.

APPENDIX A: INSTITUTIONAL REVIEW BOARD APPROVAL

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Studies > Genetic Basis of Male Infertility

1 / 143 Next >

Activity Details (Renewal Completed: Approved)

Indicates that a Renewal was closed for this item. This is automatically added by the Renewal sub process.

Author:	Melanie Holloway (U of Pgh)
Logged For (Study):	Genetic Basis of Male Infertility
Activity Date:	12/9/2014 6:11 PM EST

Property Changes | Documents | Notifications

Property	Old Value	New Value
name		Renewal Completed
author		Melanie Holloway
Study		PRO10030036
activityType		_Protocol_Continuing Report Completed
Approve: (Approval Letter)		fromString.html
Approve: (Approval Letter As Text)		

University of Pittsburgh
Institutional Review Board

3500 Fifth Avenue
Pittsburgh, PA 15213
(412) 383-1480
(412) 383-1508 (fax)
http://www.irb.pitt.edu

Memorandum

To: Alexander Yatsenko, MD, PhD
From: Judith Martin, MD, Vice Chair
Date: 12/9/2014
IRB#: REN14110079 / PRO10030036
Subject: Genetic Basis of Male Infertility

The Renewal for the above referenced research study was reviewed and approved by the Institutional Review Board, Committee D, which met on 12/4/2014.

Please note the following information:

In the renewal next year, it would be helpful if you include a note which explains how the subjects are broken down by subgroups: male patients, family members and controls.

The risk level designation is Greater Than Minimal.

Approval Date: 12/4/2014
Expiration Date: 12/3/2015

Please note that it is the investigator's responsibility to report to the IRB any unanticipated problems involving risks to subjects or others [see 45 CFR 46.103(b)(5) and 21 CFR 56.108(b)]. Refer to the IRB Policy and Procedure Manual regarding the reporting requirements for unanticipated problems which include, but are not limited to, adverse events. If you have any questions about this process, please contact the Adverse Events Coordinator at 412-383-1480.

The protocol and consent forms, along with a brief progress report must be resubmitted at least **one month** prior to the renewal date noted above as required by FWA00006790 (University of Pittsburgh), FWA00006735 (University of Pittsburgh Medical Center), FWA00000600 (Children's Hospital of Pittsburgh), FWA00003567 (Magee-Womens Health Corporation), FWA00003338 (University of Pittsburgh Medical Center Cancer Institute).

Please be advised that your research study may be audited periodically by the University of

Pittsburgh Research Conduct and Compliance Office.

Approve: 528C54AB0D000 (name)	Approve
SYS_AgendaItem: (Letter To Study Team)	fromString.html
Renewal: (Approval Letter)	fromString.html
Set of Activity	Added elements: • 528C54AB0D000

APPENDIX B: IACUC APPROVAL

University of Pittsburgh
Institutional Animal Care and Use Committee
3500 Fifth Avenue
Suite 206
Pittsburgh, Pennsylvania 15213
Tel: 412-383-7000
Fax: 412 383-2020

IACUC APPROVAL

Protocol #:13031521

PHS Assurance Number: A3187-01

Principal Investigator: Alexander Yatsenko

Protocol Title: Mouse models of reproductive development

Additional Titles:

Funding Source(s): NIH HD058073

Approval Date: 2/3/2015

To Whom It May Concern:

The University of Pittsburgh's Institutional Animal Care and Use Committee has reviewed and approved the IACUC Renewal proposal referenced above.

The committee finds that the protocol meets the standards for humane animal care and use as set by the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

Sincerely,

Frank J. Jenkins, PhD
Institutional Animal Care and Use Committee

This letter is valid until 3/31/2016.

IS00001521

APPENDIX C: RECRUITMENT QUESTIONNAIRE

Male Infertility Questionnaire

For the purposes of the research study, I would appreciate it greatly if you could fill out the following questionnaire about your health. All of the information will be held in strict confidence as it becomes a part of your medical chart. Please bring this with you when you see me for your first visit.

Your Name: _____ Date ____/____/____

Home Tel. #: (___) _____

Work/Day # (___) _____ Your age: _____

Your email address: _____

Partner's Full Name_____ Partner's age: _____

REFERRING DOCTOR(S):

Yours: Dr._____ And Dr._____

_____ _____

_____ _____

Tel: _____ Tel:_____

Partner's Doctor:

Dr._____

_____

_____ Tel:_____

QUESTIONS FOR HIM (Fill in the blank or circle word):

1. If married, number of years ____
2. How many years trying to conceive? ____
3. Prior pregnancies between you and your partner? (number) ____
   - 3a. Number of pregnancies carried to term and delivered ____
   - 3b. Number of miscarriages ____
   - 3c. Number of planned abortions ____
4. Number of pregnancies between you and another partner ____

4a. Number of pregnancies carried to term and delivered _____
4b. Number of miscarriages _____
4c. Number of planned abortions _____
5. Method of birth control, if used in past _____
5a. How many times each week (on average) do you have intercourse? _____
6. Type of lubricant, if used_____
7. Have you had prior infertility treatments? Yes No
8. Do any of the following concern you?
8a. Your ability to get an erection Yes No
8b. Your ability to maintain an erection Yes No
8c. Ejaculating before your partner is ready Yes No

MEDICAL HISTORY

9. Have you ever been told (or know) that you have any of the following?
9a. Undescended testicles at birth? Yes No
9b. If you had undescended testes, which side(s)? L___R___
9c. Mumps after puberty with painful testes? Yes No
9d. Diabetes mellitus Yes No
9e. Cancer Yes No
9f. Multiple sclerosis Yes No
9g. Other neurological problems Yes No
9h. Infection of the urine Yes No
9i. Infection of the prostate (prostatitis) Yes No
9j. Infection of the epididymis (epididymititis) Yes No
9k. Veneral disease Yes No
9l. Green or yellow discharge from the penis Yes No
9m. Blood in your ejaculate Yes No
9n. Bothered by problems with urination Yes No
9o. Injury to the testicles that needed hospitalization Yes No
9p. Ulcers Yes No
9q. Kidney stones Yes No
9r. Pain in your scrotum or testes Yes No
9s. Lots of problems with bronchitis or pneumonias Yes No
9t. Any other medical problems (list below) Yes No

_____
_____
_____

9u. Please list the medications (doses too) that you take below:

_____
_____
_____

9v. Do you have **allergies or reactions** to medications you've taken Yes No

9w. If you have allergies to medications, please list these medications below:

_____

_____

SURGICAL HISTORY

10. Have you ever been told or remember any of the following?

10a. A hernia operation?	Yes	No
10b. If you had a hernia operation, which side(s)?	L___	R___
10c. Any bladder or penis operation as a child?	Yes	No
10d. Pelvic or back surgery	Yes	No
10e. Testis surgery	Yes	No
10f. Surgery for varicoceles	Yes	No
10g. Surgery for hydroceles	Yes	No
10h. Surgery for scrotal cysts	Yes	No
10i. Vasectomy	Yes	No
10j Other infertility surgery?	Yes	No

TURED	Yes	No
Sperm Aspiration	Yes	No
Vasectomy reversal	Yes	No
Epididymovasostomy	Yes	No
Electroejaculation	Yes	No

10j. Any other surgery in the past (list below) Yes No

_____

_____

_____

EXPOSURE HISTORY

11a. Do you smoke cigarettes (or cigars or pipes)?	Yes	No
11b. If/when you smoke(d), how long did you smoke? (years)	_____	
11c. If/when you smoke(d), how many cigs/cigars per day?	_____	
11d. If you quit smoking, how long has it been (years)?	_____	

11e. Do you use any of the following (circle one response)?

Alcohol	None	<2 drinks/day	>2 drinks/ day
Coffee	None	<2 cups/day	>2 cups/day
Soda	None	<2 cans/day	>2 cans/day
Marijuana	None	Infrequent	Frequent

11f. What do you do for work?_____

11g. Do you travel alot for work?	Yes	No
11h. Do you consider your job stressful?	Yes	No
11i. If stressful, can you rate it?	Low	

Moderate
High
Extreme

11j. Any radiation or harmful chemicals on the job? Yes No

11k. If yes to above, please list the exposure and when?

Agent	Year
_____	____
_____	____

11l. Any exposure to prolonged heat in work/hobbies? Yes No

11m. Any pesticide exposure? Yes No

11n. Do you use hot tubs, saunas or jacuzzi's? Yes No

How often? (circle one)
Every day
Every other day
Once a week
Occasionally

12. **REVIEW OF SYSTEMS**

Do you have, or have you ever had, any of the following?

	Yes	No
Constitutional		
Weight loss	___	___
Weight gain	___	___
Chronic fatigue	___	___
Difficulty sleeping	___	___
HEENT		
Migraines	___	___
Dizziness	___	___
Fainting	___	___
Cataracts	___	___
Glaucoma	___	___
Difficulty hearing	___	___
Vision loss	___	___
Need glasses	___	___
Wear dentures	___	___
RESPIRATORY		
Asthma	___	___
Emphysema	___	___
Bronchitis	___	___
Tuberculosis	___	___
Shortness of breath	___	___
Cough up blood	___	___

	Yes	No
GENITO-URINARY		
Difficulty urinating	___	___
Pain/burning w/ urinating	___	___
Blood in urine	___	___
Incontinence	___	___
Kidney stones	___	___
Urinary Tract Infection	___	___
STDs	___	___
MUSCULOSKELETAL		
Joint pain/stiffness	___	___
Difficulty walking	___	___
Muscle pain	___	___
Herniated disc	___	___
Back injury	___	___
NEUROLOGICAL		
Stroke	___	___
Seizures	___	___
Numbness	___	___
HEMATOLOGICAL		
Anemia	___	___

CARDIOVASCULAR
- Chest pain ____ ____
- Heart attack ____ ____
- Heart arrhythmia ____ ____
- Shortness of breath with exercise ____ ____
- High blood pressure ____ ____
- High cholesterol ____ ____
- Deep vein thrombosis ____ ____

GASTROINTESTINAL
- Poor appetite ____ ____
- Nausea ____ ____
- Vomiting ____ ____
- Diarrhea ____ ____
- Constipation ____ ____
- Ulcerative colitis ____ ____
- Crohn's ____ ____
- Irritable bowel disease ____ ____
- Bloody stools ____ ____

Bleeding problem ____ ____
- Blood transfusions ____ ____

IMMUNOLOGICAL
- Anaphylaxis ____ ____
- Autoimmune disorder ____ ____

ENDOCRINOLOGIC
- Thyroid disease ____ ____
- Adrenal disease ____ ____

SKIN/BREAST
- New moles ____ ____
- Breast mass/pain ____ ____
- Breast discharge ____ ____

PSYCHIATRIC
- Depression ____ ____
- Memory loss ____ ____
- Panic attacks/Anxiety ____ ____
- Suicidal thoughts ____ ____

13. In addition, do you have (or have you ever had) any of the following?
- 13a. Difficulty with smell? Yes No
- 13b. Difficulty with vision (besides needing glasses)? Yes No
- 13c. Changing skin color (not tanning related)? Yes No
- 13d. Problems with growth when you were young? Yes No
- 13e. Did your voice change later than your friends'? Yes No
- 13f. How often do you need to shave? _____Every day _____Every other day _____Twice a week or less
- 13g. Has your shaving pattern changed recently? Yes No
- 13h. Fevers in the last 3 months Yes No

FAMILY HISTORY

14. Concerning the rest of your family:
- 14a. How many blood related brothers do you have? _____
- 14b. How many blood related sisters do you have? _____
- 14c. Have any of your brothers or sisters had troubling having children? Yes No
  If yes: who?_____what problem?_____
- 14d. Are their any adopted children in your family Yes No
  If so, who has adopted children? _____
- 14e. Any miscarriages in the immediate family? Yes No
- 14f. Did your mother ever take DES (diethylstilbesterol)? Yes No
- 14g. Did your parents have troubling conceiving you or your brothers or sisters? Yes No

GENETIC INFERTILITY SCREENING

15. Do you or your partner have any of the following ancestry?

	You		Partner	
Asian/ Asian American	yes	no	yes	no
Caucasian	yes	no	yes	no
Southern European	yes	no	yes	no
Northern European	yes	no	yes	no
African/African American	yes	no	yes	no
Ashkenazi Jewish	yes	no	yes	no
Cajun/French Canadian	yes	no	yes	no
Hispanic/Caribbean	yes	no	yes	no

16. If you have children:

16a. Are they healthy?

Together		A previous partner		Your partner/ a previous partner	
yes	no	yes	no	yes	no

16b. Do they have birth defects, genetic condition or severe medical problems?

Together		A previous partner		Your partner/ a previous partner	
yes	no	yes	no	yes	no

If yes, please describe:_____

16c. Do they have developmental delay, learning disabilities or mental retardation?

Together		A previous partner		Your partner/ a previous partner	
yes	no	yes	no	yes	no

If yes, please describe:_____

17. Do you, your partner or blood relatives have one of the following disorders?

	You		Partner		Family		Who?
Never began menstruation	yes	no	yes	no	yes	no	_____
Early menopause	yes	no	yes	no	yes	no	_____
Recurrent miscarriages (3 or more)	yes	no	yes	no	yes	no	_____
Stillborn baby or baby died < 1year old	yes	no	yes	no	yes	no	_____
Cystic fibrosis	yes	no	yes	no	yes	no	_____

Tay-Sachs or Canavan disease	yes	no	yes	no	yes	no	_____
Muscular disorders	yes	no	yes	no	yes	no	_____
Neurological disorders	yes	no	yes	no	yes	no	_____
Bone/skeletal disorder (e.g.dwarfism)	yes	no	yes	no	yes	no	_____
Birth defect (e.g. cleft palate, hip dislocation)	yes	no	yes	no	yes	no	_____
Developmental delay, learning disability or mental retardation	yes	no	yes	no	yes	no	_____
Polycystic Kidney disease	yes	no	yes	no	yes	no	_____
Heart defect from birth	yes	no	yes	no	yes	no	_____
Down Syndrome	yes	no	yes	no	yes	no	_____
Sex chromosome abnormality (e.g. Klinefelter/Turner Sundrome)	yes	no	yes	no	yes	no	_____
Other chromosomal abnormalities	yes	no	yes	no	yes	no	_____
Marfan Syndrome	yes	no	yes	no	yes	no	_____
Bleeding disorders (e.g.hemophilia)	yes	no	yes	no	yes	no	_____
Sickle cell anemia, Thalassemia	yes	no	yes	no	yes	no	_____
Deafness /blindness (at birth or early onset)	yes	no	yes	no	yes	no	_____

18. Are you concerned someone in your or your partners' family has a genetic condition that put's you or your offspring at risk? _____

19. Did you have any problems in filling out this questionnaire? yes no.

Thank you very much for filling out this questionnaire.

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