PROPOSAL

A. Background

What is the biological problem?
Propagation of a species depends on successful transmission of the male genome. The process of germ cell differentiation into mature sperm capable of successful egg fertilization is very complex. Genetic mouse models provide an excellent mechanism to characterize mammalian spermatogenesis and investigate causes of infertility. The goal of this project is to characterize repro27 mice, a model of male infertility. Our sequencing data indicates that the repro27 phenotype is caused by a point mutation in the Golga3 gene. Golga3 encodes a golgi autoantigen that is a member of the golgin protein family.

Infertility is defined as the inability to conceive after one year of regular unprotected intercourse. Roughly fifteen percent of couples in the United States experience problems conceiving. The financial burden to infertile couples seeking assisted reproductive technologies is estimated to exceed $50,000 per live birth (reviewed by Katz et al., 2002). The causes of infertility include chromosomal aberrations, hormonal imbalances, age, obesity, infectious diseases, psychological disorders, as well as genetic and environmental conditions that cause abnormal gametes or reproductive organ development (Egozcue et al., 2000; Shah et al., 2003). Male infertility accounts for about half of all cases. 25-40% have unknown origins. It is hypothesized that genetic defects leading to abnormalities in sperm number, motility, and morphology may account for a significant percentage of idiopathic male infertility. Advancement in contraceptive technologies provides additional incentives: "All couples and individuals have the right to decide freely and responsibly the number and spacing of their children and to have access to the information and means to do so" (United Nations Population Fund). This will become increasingly more important in the coming decades. It is estimated that over 1 billion people will be entering their childbearing years by 2020 (Holden, 2002). While females have multiple choices for contraception, development of a reversible male contraception method has lagged. Understanding the genetic and molecular basis of male reproductive physiology, and in particular sperm cell differentiation, will increase our ability to successfully diagnose and treat male infertility and may provide novel targets for male contraceptive agents.

How are sperm produced?
Male gamete formation (spermatogenesis) occurs continuously throughout the reproductive life of an animal. Spermatogenesis occurs in the seminiferous epithelium (tubules) of the testes and requires complicated patterns of cell differentiation that can be characterized by three phases. The first phase involves proliferation and renewal of spermatogonial stem cells. Spermatogonia undergo successive mitotic divisions eventually giving rise to spermatocytes. During the second meiotic phase, spermatocytes undergo two maturation divisions reducing cells from the diploid to the haploid state found in spermatids. During the third phase, spermiogenesis, round spermatids undergo a series of cytological transformations leading to the elongated complex structure of a spermatozoa. Progression from spermatogonia to the release of sperm into the lumen of the testis for transport to the epididymis takes 33 days in the mouse. In seminiferous tubules, developing stages of germ cells are seen at successively higher levels within the epithelium (Hess, 1999). Somatic Sertoli cells extend from the basal laminal surface to the lumen and interact physically and biochemically with all germ cell types (Hecht, 1993). Sertoli cells directly influence and support the various mitotic, meiotic, and differentiation events occurring within the developing germ cells. Germ cells, in turn, modulate Sertoli cell function.

Spermatogenesis is choreographed by the expression of thousands of genes encoding proteins essential for specific phases of development. While most cells in an animal contain the same genomic information, expression of genes is cell-type specific. Genes are turned on or off at specific stages of development and specific cell-types display differential patterns of expression as evidenced by recent microarray data (Ellis et al., 2004; Schultz et al., 2003; Shima et al., 2004; Small et al., 2005, Yu et al., 2003). In adults, development of sperm is asynchronous; seminiferous tubules are populated with germ cells at every stage of development making analysis of cell-specific gene expression problematic. However, prior to puberty and continuing through the first wave of spermatogenesis, germ cells multiply and differentiate synchronously (Nebe et al., 1961). The mouse testes at birth contain only undifferentiated type A1 spermatogonia. Differentiation and mitotic divisions begin about 3 days post partum (dpp) and early stages of meiosis (proleptotene...
spermatocytes) are first observed by 8 to 10 dpp. Meiosis is extends over many days and is classified according to the shape of the germ cell nucleus and the degree of chromatin compaction. Pachytene spermatocytes occur by 17–19 dpp and post-meiotic haploid round spermatids appear between 20-22 dpp. Differentiation into elongated spermatids to be released into the lumen occurs over the next 13 days. As germ cells differentiate, they migrate from the basal lamina surface to the lumen of the seminiferous tubule creating distinct layers and specific cellular associations. The cycle of the seminiferous epithelium is 6.5 days and consists of 12 stages characterized by both the degree of germ cell differentiation and germ cell specific cellular associations (Hess, 1996; Russell, et al., 1990). Thus, a detailed analysis of the mammalian spermatogenesis can be accomplished using an animal model during the postnatal period and early adolescence.

What wrong with repro27 mutant mice?
C3FeB6-repro27 mice (abbreviated hereafter as repro27) were produced by the ReproGenomics Program at the Jackson Laboratory (TJL), Bar Harbor, ME using a whole-genome, random ethynitrosourea (ENU) mutagenesis strategy (Fig. 1). C37BL/6J (B6) male mice were treated with ENU to produce mutations and subsequently mated to C3HeB/FeJ (C3Fe) female mice for two generations (G). G3 mice were screened for mice carrying homozygous recessive mutations that exhibit infertility as the sole phenotype. See Lessard et al., (2004) for a complete explanation of the program and description of the relative strengths of ENU mutagenesis as a means to assign function to genes. The mutant models are available to the research community. There are currently 21 infertile mouse models available through their website (reprogenomics.jax.org/index.html, May, 2006).

Interestingly, 19 of the 21 models show male specific defects. We chose to characterize repro27 mutant mice because spermatogenesis is abnormal. This model allows us to examine the specific defects and defective processes and aid our understanding of what gene products and processes are essential for spermatogenesis.

Male mice homozygous for the repro27 mutation (m/m, mutant) exhibit gender specific infertility. Initial characterization of repro27 mutant mice at 10 wk of age indicated very low testis weight, very low epididymal sperm concentration, abnormally shaped sperm heads without tails, very low motility, and no success with in vitro fertilization; the phenotype was characterized as post-meiotic (unpublished data, Handel ME, TJL). Preliminary developmental profiling from 2 to 10 wk of age was conducted using repro27 mutant (n=2/time pt) and control (m/+; n=2/time pt) mice produced from a heterozygous breeding colony established at NMHU in the Hewett Hall Animal Facility in July, 2005. The testis/body weight ratio was similar at 2 wk (14 dpp) but significantly lower in mutants by 3 wk (Table 1). The defect appears to be limited to the male germ cell. There are no differences between mutants and control in accessory glands like the epididymis or seminal vesicle. Histological analysis of cross-sections of testes reveals substantial cell loss (Table 2; Fig. 2). The timing of germ cell loss corresponds with a defect in late rather than post meiosis. Germ cell differentiation appears normal to the pachytene stage. Interestingly, while there is substantial germ cell loss, some germ cells do complete meiosis to form round spermatids. However, germ cell development in mutant mice is delayed and the specific pattern of germ cell associations, described as the cycle of the seminiferous epithelium, is disrupted. Spermiogenesis leads to defective sperm. These results suggest two possibilities: a) the mutant gene encodes a partially functional protein, or b) the compensatory proteins are upregulated allowing some germ cells to complete meiosis.
Traditional mapping strategies in collaboration with Dr. Handel at TJL placed the mutation between 107,760,207 bp and 109,702,800 bp on Chr 5. There were 45 known genes in this region and numerous novel genes (www.ensembl.org, December 2005). Because ENU generally creates point mutations, identification of the mutated reproto27 gene required DNA sequencing of candidate genes to identify differences between mutant and control mice. Data mining of known genes within the region of the mutation identified one very strong candidate. The Golga3 gene is located within our candidate region and gene disruption is known to cause defects in spermatogenesis that appear similar to reproto27 (Banu et al., 2002, Matsukuma et al., 1999). Golga3 (common names, male-enhanced antigen two, golgin-160) encodes a protein that is important for the redistribution of the Golgi apparatus during mitosis and appears to play a role in apoptosis (Cha et al., 2004, Maag et al., 2005, Mancini et al., 2000, Nakamura et al., 1995). Golga3 is located on Chr 5 between 109,232,563 and 109,275,245 bp (www.ensembl.org). The gene is almost 50 kb and contains 25 exons, the transcript is 4,815 bp and encodes a 1,447 amino acid polypeptide. We sequenced most of the expressed transcript in reproto27 mutant and B6 control DNA and identified a point mutation in exon 19 (117 bp) that converts a cytosine to a thymine (www.ensembl.org, May 2006). When translated, this mutation replaces a glutamine (Q) with a stop codon leading to a truncated protein with unknown functionality (Fig. 3).

**What is the significance of this project?**

It is hypothesized that genetic defects underlie many unexplained cases of infertility. Identification of reproductive defects in well over 200 genetic mouse models support this prediction (Matzuk and Lamb, 2002). Identification of genes that are critical to sperm cell development will increase our understanding of cellular differentiation. Between 14 and 21 dpp reproto27 mutant mice show large decreases in the testis/body weight
ratio and germ cell numbers (Tables 1 and 2; Fig 2). Surviving round spermatids exhibit defects in spermiogenesis leading to abnormally shaped spermatozoa. We hypothesize that the identified mutation in GoIga3 is responsible for the mutant phenotype. GOLGA3 protein is required for normal spermatogenesis; Dr. Matsukuma and colleagues demonstrated in a transgenic mouse model that elimination of exons 1 – 7 leads to defects in pachytene spermatocytes (Banu et al., 2002; Matsukuma et al., 1999). However, repro27 suggests a novel and unique mutation in GoIga3 eliminating exons 19 through 25. The role of GOLGA3 in spermatogenesis and the observed germ cell loss has not been fully investigated. In vitro results in HeLa and Chinese hamster ovary cells implicate GOLGA3 in regulating apoptosis (Cha et al., 2004; Maag et al., 2005). Confirmation and analysis of GOLGA3 function in an in vivo model will increase our understanding of spermatogenesis and cellular differentiation. We hypothesize that programmed cell death, apoptosis, is responsible for degradation of germ cells in repro27. Programmed cell death, unlike necrosis, is a tightly regulated process orchestrated by specific signaling pathways. Apoptosis is triggered by specific proteins (e.g. p53) that tell the cell to commit suicide rather than continuing to divide. During apoptosis, a dying cell produces proteins necessary for self-destruction. Death by apoptosis avoids the release of potentially toxic cell constituents. Programmed cell death is important to the proper development and regulation of the cell cycle. The testis is one of the few tissues in the body that rely on the continual renewal of cell types via differentiation of spermatogonial stem cells. Understanding the underlying mechanisms responsible for the repro27 phenotype will provide important clues into the regulation of apoptosis and germ cell development.

Spermatogenesis is an ideal system to further our understanding of normal biological function, development, and cell differentiation: (1) it is essential for the propagation of life; (2) it requires a large number of morphologically and biochemically distinct cell types; (3) it proceeds through both mitotic and meiotic cell divisions; and (4) the entire process is coordinated by the expression of a large number of genes regulated in response to signals coming from outside the testes, from neighboring cells, and from the germ cells themselves.

Relevant Research Experience
My postdoctoral research used rats and mice as model organisms to analyze (1) the stage-dependent expression of mRNA important in spermatogenesis and the role of germ cells in the regulation of Sertoli cell mRNA within the seminiferous epithelium (Linder et al., 1991; Linder and Griswold, 1993; Trezise et al., 1993; McGuiness et al., 1994; Law et al., 1997); and (2) stage and tissue-specific regulation of expression of the follicle stimulating hormone receptor in Sertoli cells (Linder et al., 1994; Griswold et al., 1995). Molecular techniques and expertise acquired included RNA/DNA isolation, northern, Southern, and western blot analysis, DNA amplification, RT-PCR, cDNA cloning, histology, cell culture, and microscopy. In addition, I have 10 yr of professional experience in mammalian genetics and the use of mouse models and scientific information systems from my tenure at the Jackson Laboratory (Simpson et al., 1997; Smith et al., 1997; Linder, 2001, 2002, 2006, Berry and Linder, 2006).

Current Available Resources
My research group at NMHU currently includes two master’s level graduate students, four undergraduate research assistants, and a part-time technician. Since July 2005, we have established a mouse breeding facility, optimized PCR-based genotyping protocols to identify mice for research and breeding purposes, and expanded the colony to produce enough mutant and control mice for the proposed research within the two yr of the project. My laboratory is equipped to conduct almost all the proposed research at NMHU except for those services that are routinely performed by core facilities or outsourced. We are successfully conducting (histological and morphological analysis, RNA analysis, RT-PCR) or are optimizing (immunohistochemistry, germ cell apoptosis assessment) the methodologies described in this proposal. We have purchased a three yr license for the GeneSpring software (Agilent Technologies, Redwood City) to analyze the raw microarray data at NMHU. I also the New Mexico Bioinformatics Symposium: Microarray Technologies, Applications and Methods Santa Fe, NM March 23-24, 2006.

I have established research collaborations with two key research institutions. repro27 mutant mice were initially identified and characterized within the ReproGenomics Program at The Jackson Laboratory. Dr. Mary Ann Handel, principal investigator of the ReproGenomics program, serves as my NM-INBRE mentor (NIH RR-03-
010; letter of collaboration included). Dr. Handel’s laboratory performed the mapping crosses while we focused on producing sufficient mice for characterization of the spermatogenic defects. Histological services will be provided by TJJL because their current prices are more competitive than service suppliers available in Albuquerque, NM (e.g. University of New Mexico uses Tricore Reference Laboratory). We will continue to monitor costs and convenience in the future and adjust as necessary. We will be analyzing gene expression patterns to explore changes in gene expression patterns as a result of the repro27 mutation. This work will be done in collaboration with Dr. Michael Griswold, Washington State University Pullman WA using gene-chip microarrays (letter of collaboration included). My graduate student, Larry Agbor, and I visited the Griswold laboratory in March, 2005 working with laboratory personnel to learn techniques and computer software analysis of raw data. We successfully performed preliminary microarray analysis on 18 dpp mice in December, 2005 with approximately a two week turn around time. Dr. Griswold is providing microarray processing to numerous small and large laboratories through his core facility (http://www.wsu.edu/%7Egriswold/microarray/sample_form.pdf).

B. RATIONALE/PURPOSE

The goal of this research project is to characterize and explore the underlying mechanisms responsible for the spermatogenesis defects in repro27 mutant mice, a novel mouse model of male infertility in humans. Our findings will increase our understanding of germ cell differentiation and provide clues to mechanisms regulating cell death. The mouse is an ideal model organism to accomplish this goal; approximately 99% of the roughly 40,000 genes within the two genomes are conserved between mice and humans and thus, genes important for regulation of spermatogenesis are likely conserved between the two species. The identification of the underlying pathways in which Golga3 functions will increase our understanding of male reproduction in mammals and further define the etiology of infertility.

There are some perceived limitations to performing the proposed research project at New Mexico Highlands University. Positional cloning of mouse mutations historically takes substantial resources and years to accomplish. In the case of repro27, we were fortunate to find a good candidate gene in Golga3 and to identify a point mutation in the mRNA transcript that causes premature termination of protein synthesis. This mutation has been confirmed by sequencing genomic DNA. While we are a small laboratory with limited funding, identification of a mutation in our candidate gene allows us to characterize a novel mouse model for infertility and explore the specific role of GOLGA3 in spermatogenesis.

C. OBJECTIVES

Specific Aim 1. Characterize the spermatogenic defects in repro27 mutant mice.
1a. Morphological and histological analysis of mutant and control mice from 12 to 44 days of age.
1b. Analysis of the mechanisms responsible for the germ cell loss. Is apoptosis, programmed cell death, occurring?

Specific Aim 2. Characterize the role of GOLGA3 protein in spermatogenesis.
2b. Analysis of genome wide differential gene expression patterns in repro27 mutant and control mice to define disrupted biochemical pathways.

D. METHODS

Specific Aim 1. Characterize the spermatogenic defects in repro27 mutant mice.
1a. Morphological and histological analysis of mutant and control mice from 12 to 44 days of age.

Animals

Three heterozygous breeder pair of C3FeB6-repro27 were originally obtained from the ReproGenomics Program at the Jackson Laboratory, Bar Harbor, ME. We established a breeding colony of repro27 in the Hewlett Hall Animal Facility. Mice are housed in a temperature and humidity controlled room in duplex cages (12.13" x 12.13" x 5.53"), Thoren Caging Systems, Hazleton, PA with aspen shavings (Harlan-Teklad, Indianapolis, IN), wire tops, water bottles, and a filter cage cover. Mice are allowed free access to food (2018 Teklad Global 18% Protein Rodent Diet) and tap water. Cage enrichments provided to all mice include Shepherd shacks (Shepherd Specialty Papers, Watertown TN) and nesting material (10279-140 VWR, Bristol CT). Cages are changed weekly and water changed twice a week.
repro27 is a recessively-inherited mutation induced by ENU (Fig. 1). Male homozygous mutants are infertile. Female mice, both heterozygotes and homozygotes, breed normally (Linder, unpublished observations). We currently have 7 pair and trio mating units producing mice. All pups must be genotyped, usually at 10 dpp, using single sequence length polymorphisms (SSLPs) DNA markers that flank the repro27 genetic mutation to identify homozygotes. SSLP markers are polymorphic between the two background strains, C57BL/6J and C3HeB/FeJ (D5Mit338 and D5Mit317). Genotyping is performed using DNA isolated from the tip of the tail (Linder NMHU Animal Assurance No. 03-16-06a, March 2006), followed by the polymerase chain reaction (PCR) using the SSLP primer sets. Custom DNA primers are purchased from Operon Technologies (Alameda, CA), a division of Fisher Scientific. PCR is performed on a Pernod Mastercycler Gradient using Pernod Taq DNA Polymerase (E0032003419) using standard procedures (Pernod North America, Westbury, NY). The SSLP markers yield different product lengths depending on the allele’s strain origin and are identified by running PCR products on a 3% Metaphor agarose gel (Fisher Scientific, Pittsburgh, PA). Genotyping identifies mice that are repro27 homozygotes (m/m), heterozygotes (m/+), and wildtype (+/+). Each mouse is identified using a unique ID Number and a hindlimb toe clip number to help with identification within the cage. The JAX Colony Management System database (free software licensed by TJL, Bar Harbor ME) is used to track of breeding, genotyping, and experimental use of mice.

Tissue Collection
Mice will be euthanized by CO2 anesthesia followed by cervical dislocation according to guidelines approved by the NMHU IACUC committee (Linder Animal Care Protocol No. 03-16-06a, approved March 2006). Mice will be weighed and the testes surgically removed from 4 male mice per genotype (m/m, m/+, and +/+ on days 12, 15, 18, 21, 35, and 44 dpp for a total of 72 mice. These time points will allow characterization of germ cell loss during first meiosis (12 – 21 dpp), completion of meiosis and formation of the haploid round spermatid (21 dpp), completion of spermiogenesis to form elongated sperm (35 dpp), and second spermiogenesis (44 dpp). Testes and seminal vesicle weights will be recorded for each animal. Bilateral testes samples will be immersed in 4% paraformaldehyde (Fisher Scientific, Pittsburgh, PA) for fixation. Testes samples will be incubated overnight before sectioning. Testes samples will be mounted in 3X in 1X phosphate buffered saline pH 7.4. Samples will then be stored at 4°C. Samples will be shipped overnight with freezer packs to the Histology and Microscopic Sciences Inc (managing Lesley Bechtold) at the Jackson Laboratory for paraffin embedding and sectioning. Slides and blocks will be shipped back to NMHU for analysis.

Histology
Testes will be cut in half, embedded in paraffin, and 4-5-micron sections cut. One slide from every sample will be stained with periodic acid-Schiff reagent and counterstained with hematoxylin. Histological analysis will be used to verify the germ cell population and the developmental stage at each time point as described by Linder and Griswold (1983). Testes will be evaluated by light microscopy (x400) on a Nikon Eclipse 501 microscope (AG Heinzle Inc, Lake Forest CA). Briefly, germ cell number and type (spermatogonia, spermatocyte, round spermatids, elongated spermatids) will be counted in 30 tubules per cross section and two sections analyzed for each sample (n=4/genotype). The average number of total germ cells, the number of each germ cell type, and the cycle stage will be determined according to guidelines established by Russell (1990). Preliminary histological analysis indicates that the timing of germ cell development is delayed and that they cycle of the seminiferous epithelium is disrupted (i.e. specific germ cell associations that define a specific stage of the cycle are present). Additional unstained slides will be cut and used for assessment of germ cell loss (cleaved caspase 3 immunohistochemistry and TUNEL, see Specific Aim 1b).

1b. Analysis of the mechanisms responsible for the germ cell loss. Is apoptosis, programmed cell death, occurring?
We will use two assays to determine the mechanisms responsible for germ cell loss, a protein marker for apoptosis (cleaved caspase-3) and an assay to detect fragmentation (TUNEL). Programmed cell death, unlike necrosis, is a tightly regulated process orchestrated by specific signaling pathways. Apoptosis is triggered by specific proteins (e.g. p53) that tell the cell to commit suicide rather than continuing to divide. Death by apoptosis avoids the release of potentially toxic cell constituents seen with necrosis. We are interested in
determining whether there are specific cell signals within mutant mice causing the degradation of germ cells between 14 and 21 dpp.

**Immunodetection of Cleaved Caspase-3**

Caspases are a family of effector proteases that function specifically in apoptosis. They were first identified in *C. elegans* and are conserved in all vertebrates. There are 15 types in humans that are initially synthesized as procaspases and activated upon proteolytic cleavage. Initiator caspases, like caspase-8, activate caspase-3 by cleavage and amplify the cell death signal by activating other proteases. Detection of cleaved-caspase 3 in cells is a specific indicator of apoptosis (Lodish et al., 2004). We will use the SignalStain Cleaved Caspase-3 (Asp175) IHC detection kit (Cat #3120, Cell Signaling Technology, Danvers MA) to determine if apoptosis is occurring in mutant mice. We will follow the manufacturer’s guidelines using unstained cross sections of testis from the 4 male mice per genotype (m/m, M/+ , and +/+ ) on days 12, 15, 18, 21 dpp (n=48) described under Specific Aim 1a. Briefly, sections will be deparaffinized using xylene and rehydrated and a series of washes with decreasing rations of EtOH:H2O. Slides will then be subjected to a series of incubations designed to unmask the antigen, quench and block to prevent non-specific binding of antibody, deliver the primary cleaved caspase-3 (Asp175) polyclonal antibody, and then add biotinylated secondary antibody. Slides will then be counterstained with hematoxylin to visualize the germ cells. Slides are then dehydrated and coverslips mounted. Expression of cleaved caspase-3 will be analyzed in at least 30 tubules per cross section and 2 cross sections per sample (×400) on a Nikon 50i series microscope. We have the option to go with fluorescence detection of cleaved caspase 3 and will explore these techniques in the second year of the grant.

**TUNEL**

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphatase (dUTP) nick end labeling (TUNEL) is a common technique for assessing apoptosis in tissue sections. It has been used to analyze cell death in both sperm and testis (Donnelly et al., 2000; Wang et al., 1998). TUNEL will be performed using unstained cross sections of testis from the 4 male mice per genotype (m/m, M/+ , and +/+ ) on days 12, 15, 18, 21 dpp (n=48) described under Specific Aim 1a. TUNEL measures cell death by detecting DNA strand breaks in individual cells by light microscopy. We will be using the In Situ Cell Death POD Kit (Cat. No. 1 684 817, Roche Diagnostics Corp, Indianapolis IN) to indirectly detect and quantify any DNA fragmentation in mutant germ cells (m/m) compared to controls (M/+ , and +/+ ) according to the manufacturer’s guidelines. Briefly, slides are permeabilized and exposed to the TUNEL reaction mixture containing TdT and fluorescein-dUTP. During this step, TdT catalyzes the attachment of fluorescein-dUTP to free 3’OH ends in the DNA. The signal is assessed by the addition of an anti-fluorescein antibody POD (peroxidase). Positive controls for apoptosis will be made by treating sample slides with increasing concentrations of recombinant DNAse I (Roche Diagnostics Corp, Indianapolis IN) to create a standard curve.

**Specific Aim 2. Characterize the role of GOLGA3 in spermatogenesis.**

We have identified a point mutation in the *Golga3* expressed transcript that inserts a stop codon in exon 19 (117bp). This would truncate the protein and eliminate ⅔ of exon 19 through exon 25. Dr. Matsukuma of the Kanagawa Cancer Center Research Institute in Nakao, Japan has most graciously sent us two GOLGA3 antibodies to explore the protein expression patterns in our mutant mice.

Preliminary analysis using Gene-Chip microarray from 2 mutants and 2 heterozygous controls at 18 dpp indicated that *Golga3* expression in mutants is approximately two-fold lower compared to controls. Genome wide, 417 transcripts were at least 2 fold higher in controls compared to mutants and 77 transcripts were 2 fold higher in mutants (Fig. 4). Subsequent histological analysis indicated that there is substantial germ cell loss already evident at 18 dpp (Fig. 5). Because we want to compare gene expression patterns in mutants and controls with the same germ cell population, gene expression analysis will be performed at 14 dpp. Expression profiles of genes related and within the *Golga3* pathway will be analyzed.
2a. Analysis of GOLGA3 protein expression patterns

Animals and Tissue Collection

*repro27* mice will be bred and genotyped as described under Specific Aim 1. No additional mice are needed. We will use histological tests samples collected at the various age groups and representing all three genotypes (m/m, m/+), and +/+.

Immunohistochemistry

Immunohistochemistry will be conducted as described in specific aim 1. An anti-Mea2 Ab (C-terminal 20 peptide: PRGDTKLNQNSVPRDGGLQ)(Banu et al., 2002) and an anti-human Mea2 Ab, affinity purified, 1/100 dilution for IF, 1/500 dilution for WB, which cross react with mouse Mea2. (Matsukuma, unpublished). The antigen is the recombinant peptide of human homolog (golgin 160), (138-268, STDSPNLPEKEEQRVRLQARKWILEEQL1KQYRVRQQERSSQPATKTRLFSTLDPELMNLNPENLPRASTLAMTKYESFLRTSVPRGPVGSGLGAHPREKKTSSKIRSLADYRTEDSNAGNSGGNVPAPDSTKGLKQNRSSAASVVSEIS). Immunohistochemistry will be carried essentially as described under Cleaved Caspase-3 detection using testes sections from mutant and control mice.

2b. Analysis of genome wide differential gene expression patterns in *repro27* mutant and control mice to define disrupted biochemical pathways.

Animals

*repro27* mice will be bred and genotyped as described under Specific Aim 1. Differential gene expression will be analyzed in 5 male mutants and 5 heterozygous controls at 14 days of age. We will also be using 5 C57BL/6J (JAX® Mice 000664, from existing NMHU breeding colony) and 5 C3HeB/FeJ (JAX® Mice 000658, from existing NMHU breeding colony) inbred mice to eliminate the possibility that differential expression patterns are the result of differences in expression between the two contributing strain backgrounds present in C3FeB6-repro27 mice. Total number of mice is 20.

Tissue Collection and RNA Isolation

Mice will be euthanized by CO₂ anesthesia followed by cervical dislocation according to guidelines approved by the NMHU IACUC committee (Linder Animal Care Protocol, approved July 2005). Testes will be surgically removed as rapidly as possible and placed in Tri-reagent (Ambion Inc. Austin TX) to avoid RNA degradation.
Total RNA will be immediately isolated using Ambion's RiboPure RNA isolation kit (cat # 1924) according to the manufacturer's standard protocol. Samples will be stored at -70°C until total RNA is extracted. RNA quality will be determined by both electrophoretic methods using a denaturing agarose gel and absorption readings at 260 and 280nm (minimum of 1.8 OD$_{260/280}$ required). RNA samples will be shipped on dry ice to the laboratory of Dr. Griswold, Washington State University, for Gene-chip microarray processing. Microarray data will be sent to my laboratory via email.

Differential Gene Expression Analysis with Gene-Chip Microarray

Microarray processing requires ~10 µg of total RNA from each sample (4 m/m, 4 m+/4, C57BL/6J, 4 C3HeB/FeJ) to create the target for the microarray (i.e. biotinylated complementary RNA, cRNA). cRNA is generated using an oligo(dT) primer with reverse transcriptase with biotinylated cytosine and UTP. The labeled cRNA is fragmented, and hybridized to the GeneChip® Mouse Genome 430 2.0 Array (Affymetrix, Santa Clara CA) and stained in accordance with the manufacturer's standard protocol. This chip contains 39,000 transcripts and variants from over 34,000 well characterized mouse genes offering a comprehensive analysis of genome-wide expression. The arrays are stained and washed using the Affymetrix GeneChip Fluidics Station 400 and scanned using a Gene Array Scanner (2500A (Agilent, Palo Alto CA). The resulting data will viewed using Affymetrix's GeneChip Operating Software (GCOS) and expression patterns analyzed using GeneSpring 7.2 Software (Agilent Technologies, Redwood City, CA, NMHU license). This software provides analysis of overall expression patterns and also expression of each of the 39,000 transcripts on the gene-chip. There are links from each transcript to the numerous publicly available bioinformatics databases (GenBank, GEO Profiles, BLAST, Mouse and Human Genome Databases, etc.). Creating data sets of the different sample groups allows averaging of the expression signal. We will be looking at genome wide expression patterns in mutants (m/m) compared to the three control groups (m+/4, C57BL/6J, C3HeB/FeJ).

E. RELEVANCE

Characterization of genetic models of male infertility increases our understanding of germ cell development. Characterization of the gene responsible for the novel repro27 mouse model will ultimately aid in deciphering causes of male infertility. Despite advances in assisted reproductive techniques like in vitro fertilization and intracytoplasmic sperm injection, failure to conceive remains a major health concern worldwide. Childless couples endure tremendous psychological and financial burdens, often spending years with doctors trying to determine root causes and attempting to conceive using alternate methodologies. In many cases, these efforts are hampered by a lack of knowledge concerning the underlying causes of both female and male infertility.

In males, sufficient production of viable and motile sperm is a critical step in achieving conception. The highly complex and regulated process of germ cell development, called spermatogenesis, accomplishes this task. While it is fairly straightforward and noninvasive to assess the sperm morphology, quantity, and motility of human sperm, this limited analysis is not sufficient to determine the underlying mechanisms leading to infertility. Animal models provide this means. Phenotypic characterization, including histological analysis, evaluation of differential gene expression patterns and genetic mapping of individual mutant mice are necessary to determine the underlying genetic defect. Given the high homology between mice and humans, it is likely that gene function will be conserved across species. Identification of genetic pathways necessary for normal spermatogenesis will ultimately improve our ability to understand and treat human infertility. In addition, further characterization of gene expression patterns during spermatogenesis and identification of potentially novel mechanisms and physiological processes through the use of mutant mouse models will increase our understanding of normal development and cell differentiation.
F. LITERATURE REFERENCES


