12-7-2010

# Spermatogenesis: Laser Microdissection Optimization

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# Spermatogenesis: Laser Microdissection Optimization

by

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A dissertation submitted in partial fulfillment

of the requirements for the degree of Doctor of Philosophy

in Nursing

University of Missouri - St. Louis MO

2010

December 18, 2010

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# Dedication

For my father, a kind, gentle, and gifted man who survived the unspeakable at Pearl Harbor, Okinawa, and Nagasaki Japan. He was never able to speak of the cost of survival, but he wrote to convey his experiences to his family and in his search for peace. The closing statement from his eyewitness account entitled *Incident at Pearl Harbor* gave voice to his love of country and provided the following cautionary and visionary statement.

> May we pray that God and the United States may never again become so complacent that another Pearl Harbor could happen, for the next Pearl Harbor will be your town, or my town; and the thousands who die will be your neighbors and mine. May God grant us the strength and wisdom to protect ourselves wisely.

## Acknowledgements

The inspiration for this research began with a commitment to deployment related health care. The research design and ideas emerged as a NIH fellow at the 2007 National Institute of Nursing Research (NINR) Summer Genetics Institute. The University of Missouri-St. Louis then opened the door to inter-disciplinary and inter-institutional collaboration with Washington University and the St. Louis VA Medical Center. I would like to extend my sincere thanks to:

1) Roberta K. Lee, DrPH, RN, FAAN**,** University of Missouri-St. Louis, for serving as academic adviser, and dissertation chair. Dr. Lee coordinated and managed the administrative and academic concerns, and challenged me to improve my work. It was Dr. Lee's resourcefulness and leadership that opened the door to the inter-disciplinary collaboration with Washington University. Dr. Lee made the impossible possible. This research would not have been possible without her time and commitment.
2) Kelle Moley, MD, Professor and Vice Chair OB/GYN and division director of research at Washington University, for embracing collaboration with the University of Missouri-St. Louis and the St. Louis VA. Dr. Moley graciously agreed to act as my senior scientific mentor and committee member. Dr. Moley is a visionary leader who shaped and challenged my thinking, generously offered her time and scientific expertise, and extended the considerable resources of her lab. Dr. Moley was instrumental in achieving research funding through the VA.
3) Prabagaran Esakky, PhD, research associate in the Department of Obstetrics and Gynecology at Washington University, and the senior scientist of the Paternal Environmental Exposures research team. Dr. Esakky is an experienced creative research scientist who demanded precision in all laboratory assays. It was Dr. Esakky's expertise

in biochemistry and male reproductive physiology that were the cornerstones of our success.

4) Christine Kasper, PhD, PhD, RN, FAAN, Department of Veterans Affairs and Uniformed Services University for the Health Sciences, fulfilled the role of VA research mentor and committee member. Dr. Kasper facilitated and helped navigate the VA research process and was instrumental in bridging national and local VA research policies.
5) Nathan Ravi, MD former Chief of Staff and Patricia Hendrickson, RN Chief of Quality Management at the St. Louis VA Medical Center, for their leadership, flexibility, and support of this continued partnership and research endeavor.
6) Lori L Paul, PhD, University of Missouri-St. Louis, and M. Christina Hines, PhD, RN, University of Missouri-Kansas City for generously giving their time and expertise as committee members.

Finally, this research would not have been possible without the opportunities that were provided by the Department of Veterans' Affairs and the St. Louis VA Medical Center. Funding for this research included the Veterans Integrated Service Network (VISN) 15 2008 research award, and grant funding through the VA Health Services Research and Development (HSRD) NRI 09-107.

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## ABSTRACT

Studies link paternal environmental exposures to pesticides, herbicides, and radiation to adverse reproductive outcomes such as developmental delays, malignancies, and structural birth defects. To date, 900 children of Vietnam veterans with presumed Agent Orange exposure receive VA presumptive service connected benefits because these children have Spina Bifida. To date, there are limited data to explain the underlying causal mechanisms whereby paternal environmental exposures result in adverse reproductive events. Laser microdissection (LMD) is the only technology that allows the isolation of cell subpopulations from complex tissues such as the testes without disturbing the cell's bimolecular signature. LMD is essential in the comparison of the molecular signatures of diseased and non diseased cells. As a result, LMD will translate to toxicology protocols that seek to elucidate gene expression data from exposed and non exposed populations.

The purpose of this study was to a) demonstrate the reliability of the sequential methods of LMD and the selected downstream applications through the establishment of qualitative and quantitative checkpoints upon which each subsequent step was based, b) ensure that the expected differences in gene expression during spermatogenesis can be characterized with LMD, and c) validate the LMD methods prior to proceeding to animal exposures.

This study used the Leica LMD 6000 combined with qRT-PCR to generate stage specific gene expression data during spermatogenesis. Cluster LMD was used to maximize RNA yield and obtain pure stage specific cell populations. qRT-PCR was used to validate stage specific LMD success. With the exception of non specific *mMagea4* amplification across all cell stages, the 10 fold rise in *HspA2* in spermatocytes, and the 12 fold increase in *Tnp1* and the 10 fold increase in *prm2* in elongated spermatids supported stage specific LMD success. Cyp1A1 was found in spermatogonia, but was absent in spermatocytes and spermatids. These expression patterns paralleled the published data and coincided with the physiologic functions of these genes and the blood testes barrier.

# CHAPTER ONE

## Introduction and Statement of the Problem

The Institute of Medicine (IOM, 1995) report stated that developmental end points such as miscarriage, birth defects, growth retardation, and cancer are generally unrelated to the exposure of the human male to chemicals and ionizing radiation. In the time since this statement, a growing body of knowledge recognizes the role of paternal environmental exposures and adverse reproductive events (Hansen & Schriner, 2005). Population based studies in environmental, occupational, and military settings linked paternal exposures to pesticides, herbicides, and radiation to a plethora of adverse reproductive outcomes such as developmental delays, malignancies, and structural birth defects. One of the more commonly reported birth defects associated with paternal environmental exposures was neural tube defects (NTD). The Ranch Hand Study, the CDC Birth Defect Study, and the CDC Vietnam Experience Study reported suggestive evidence implicating paternal 2, 3, 7, 8-tetrachlorinated-p-dioxin (TCDD) exposure in NTD (IOM 2001). In addition, a variety of adverse reproductive outcomes related to paternal exposures were documented in animal studies. In a recent study of TCDD treated male mice that were mated with untreated females, there were decreased rates of implantation and increased rates of respiratory distress syndrome and a variety of congenital malformations (Nomura, 2008). To date, there are limited data to explain the underlying causal mechanisms whereby paternal environmental exposures result in adverse reproductive events.

## Statement of the Problem

The biological mechanisms whereby paternal preconception exposure to environmental agents act to influence reproductive outcomes requires further elucidation. There are four potential mechanisms whereby paternal exposure to drugs and chemicals can impact reproductive outcomes: a) chemicals or medications may enter the maternal circulation after absorption of the seminal fluid from the vagina, b) chemicals bound to the sperm may be delivered to the egg at conception, c) toxins adversely impact male germ cell development, and d) dioxin is known to accelerate epididymis transit time (Perrault, Klinefelter, & Clegg, 2008) thereby compromising sperm maturation and subsequent fertilization.

The Institute of Medicine Update on Herbicide Exposure, *Veterans and Agent Orange Update 2006*, noted that it is unlikely that additional population based studies of reproductive outcomes of TCDD exposed Vietnam veterans will be generated. At the same time, the committee acknowledged that most etiologic research reviewed during the semi-annual update focused on maternal and fetal exposure with some work that addressed paternal risk, and viewed the increased numbers of epidemiologic studies related to paternal exposures as particularly relevant to a largely male service population (IOM 2007). Because it is not possible to obtain human data from single chemical exposures, protocols that are designed to evaluate male reproductive risk must be able to be translated from animals to human populations. While no animals or human subjects will be exposed to environmental agents for this study, the design must assure that the methods are valid so that subsequent studies yield valid, reproducible observations.

*Current Approach- Artificial Insemination (AI)*

There are several existing laboratory approaches which explore the impact of male exposures on reproductive parameters. Klinefelter and colleagues (1994) recovered a fixed volume of sperm from the cauda epididymis of ethane dimethanesulfonate (EDS) exposed male rats to inseminate non exposed females via surgical AI. The study demonstrated diminished fertilization capacity of male germ cells in the absence of seminal fluid contact. A second approach to identifying the influence of toxins delivered via seminal fluid on reproductive outcomes, would be to design a study in which sperm were extracted from TCDD exposed and non exposed males, washed, suspended in TCDD exposed versus non exposed seminal plasma, and introduced via artificial insemination (AI) into proestrus/estrus positive or super ovulated females via non surgical or surgical AI. While AI is done in larger animals including humans, it is rarely done in laboratory mice and typically it is only performed in rare instances in which males and females from different Mus species refuse to mate, or when confronted with a valuable transgenic male that refuses to mate (Nagy, Gertenstein, Vintersten, & Behringer, 2003). Nagy and colleagues (2003) provided a protocol for both non surgical and surgical AI in mice. In each case sperm are obtained from the cauda epididymis of sacrificed males. While the protocols for sperm preparation differ somewhat for non surgical and surgical AI, in each case the sperm are incubated in human tubal factor (HTF) to allow for capacitation. Capacitation results in several biochemical changes which enable the sperm to bind to the zona pellucida to undergo the acrosome reaction which is essential for fertilization (Hafez, Hafez, & Hafez, 2005).

*Current Approach The Dominant Lethal Assay*

The Dominant Lethal Assay (DLA) was developed to assess the reproductive consequences of preconception exposure of male laboratory animals to chemical mutagens (Epstein, Arnold, Andrea, Bass, & Bishop, 1972). In 1998, the Environmental Protection Agency (EPA) published the rodent DLA (EPA, 1998). The rodent DLA is conducted by exposing male mice to the selected environmental agent as a single acute dose, an intermittent sub-acute dose, or by continuous dosing throughout the period of spermatogenesis. Exposed males are then mated with one to two non exposed females at weekly intervals for the duration of spermatogenesis. Through back extrapolation, coupled with the timed sequential mating, this assay determines the test substance's effects on male reproductive fitness during the various stages of germ cell development. Females are sacrificed at 14 days post coitum (dpc) to determine pre and post implantation loss.

While the DLA considers early pregnancy loss as the most significant indicator of a dominant lethal mutation, other investigators have broadened the outcomes to include structural malformations, litter size, growth, and development (Aitken & De lullis, 2010; Perreault, Klinefelter & Clegg, 2008). However genetic or genomic integrity is not assured by a structurally normal, viable offspring that is generated by a toxin exposed male. Phenotypic changes may present as subsequent cancers, developmental delays, and complex polygeneic neurologic diseases that may appear later in life or in subsequent generations (Aitken & De lullis, 2010; Friedler, 1996). In addition, errors in germ cell imprinting and methylation which occur in the absence of DNA damage can result in changes in gene expression or silencing with far reaching consequences that are heritable

across subsequent generations (Miller, Brinkworth & Iles, 2010; Schaefer, Ooi, Bestor, & Bourc'his, 2007).

### *Current Approach Protocols for Toxicity of the Epididymis*

The epididymis plays a crucial role in the maturation of sperm and successful fertilization. Although the epididymis provides a very limited amount of fluid to the volume of the total ejaculate, one additional consideration to further segregate male germ cell factors from epididymis factors is the protocol for toxicity in the epididymis as described by Perreault and associates (2008). Male environmental exposures would occur based upon the species specific transit of sperm through the epididymis. The epididymis sperm transit time in mice is one week (Adler, 1996). Thus, in vivo TCDD exposure for male mice would occur 7 days before in vitro fertilization or natural mating, thereby allowing fertilization to occur with sperm that were in the epididymis, while sperm that were in the testes at the time of exposure would not be capable of fertilization. One additional factor requires consideration. Some substances, including TCDD, are known to accelerate epididymal transit time. Thus timelines may have to be modified based on this information (Perreault, et al., 2008). This fact also raises the possibility that the accelerated epididymal transit time adversely impacts sperm maturation and subsequent successful fertilization.

### *A Novel Approach: Laser Microdissection and Gene Expression during Spermatogenesis*

Since the development of LMD in 1996 at the National Institutes of Health (NIH), LMD has become an important tool in the study of RNA, DNA, and proteins. Using the Acturus PIXEL LMD, Sluka, O'Donnell, and Stanton (2002) captured entire cross

sections of the rat seminiferous tubule to result in aggregate cellular signals from the complex cell population. Cells captured included leydig cells, myoid cells, sertoli cells, and male germ cells in various stages of development. In a subsequent work, the Zeiss PALM laser catapulting LMD system allowed the microdissection of three zones of the rat seminiferous epithelium: a) the basal zone which contained the sertoli cell nuclei, early germ cells, and spermatogonia; b) the mid section which captured the round spermatids; and c) the luminal region which contained the elongated spermatids (Sluka, O'Donnell, McLachlan, & Stanton, 2008). Real time quantitative polymerase chain reaction (qRT-PCR) was used to raise the signal of cell specific markers from the background, as a measure of the target cells' presence.

*Existing limitations*

Ethical considerations preclude the known exposure of humans to environmental hazards and prevent access to sufficient quantities of human testicular tissue for the study of gene expression during spermatogenesis. Donor tissue collected 1 to 2 hours after death provided a novel approach to studying this problem. He, Kokkinaki, Jiang, Dobrinski, and Dym (2009) reported using immunohistochemistry (IHC) to characterize human spermatogonial stem cells. However autolytic changes distorted the seminiferous tubule morphology, and the prolonged hypoxia and ischemia would be expected to result in gene expression changes. Therefore animal models are essential for the study of the harmful effects of toxins on the male reproductive system.

One challenge presented by the DLA is the inability to segregate the effects of toxins delivered via the seminal fluid from male germ cell effects. As an alternative,

investigators report surgical and non surgical approaches to murine AI segregate sperm from seminal fluid contact. While AI in the absence of in vitro fertilization (IVF) allows blastocysts to be retrieved at 3.5 dpc, earlier developmental data would be lost because of the inability to assess the qualitative and quantitative measures such as fertilization rates and blastocyst development. A second consideration is that AI creates synchronized pro-estrus females through super-ovulation. While super-ovulation is also used with assisted reproduction (AR) via IVF, the numbers of oocytes retrieved, fertilized, and implanted are strictly controlled in IVF. In contrast, AI results in an uncontrolled number of oocytes and embryos resulting in potential intrauterine over-crowding and fetal growth retardation (K. Moley, personal communication, June 16 2010).

While it is relatively simple to obtain large amounts of mRNA for expression analysis from rodent seminiferous tubule cross sections, it is much more labor intensive to obtain single cell populations by LMD (P. Stanton, personal communication, August 6 2009). Based upon 100% efficiency in RNA isolation from LMD cells, approximately 0.01 ng RNA can be isolated from a single cell. Therefore, approximately 2000 cells are needed to obtain 20 ng RNA. However LMD is a meticulous endeavor that can require up to 20,000 LMD shots to yield nanograms of total RNA (Espina, et al., 2006). Yet, LMD is the only technology that allows the isolation of cell subpopulations from complex tissues without disturbing the cell's bimolecular signature. As a result, LMD will be informative in toxicology protocols that seek to elucidate gene expression data from exposed and non exposed populations.

*Toward a Research Framework*

Our 2008 VISN 15 funded protocol *Paternal Environmental Exposures and Gene Expression during Spermatogenesis* and the companion HSRD (NRI 09-107) funded protocol *Paternal Environmental Exposures and Reproductive Outcomes: A Comparison of in Vitro and in Vivo Fertilization* combine and expand upon selected elements of the AI techniques and the DLA. Similar to AI, our funded toxicology protocols segregate male germ cell effects from seminal fluid effects. However the use of IVF in place of AI allows for the assessment of blastocyst numbers, viability, and biochemical assays; and controls the number of embryos for implantation. This in turn avoids variables that may influence embryo size and development.

Similar to the DLA, our protocols will assess 14.5 dpc early pregnancy loss and successful implantations. Our protocols will also use a sequential mating scheme (as noted by the DLA) corresponding to the stages of murine spermatogenesis. This allows the effects of exposure to be determined by the stage of male germ cell development (Environmental Protection Agency [EPA], 1998). However our protocols expand upon the DLA in two important ways. First the analysis has been expanded to include: a) sperm evaluation parameters, b) 14.5 dpc crown rump length, fetal weight, and litter size; c) term litter size, birth weight, size, and gross and microscopic examination for malformations, and d) 5 week development. This approach shares characteristics of the extended DLA which examines the pups 1 day before term for congenital malformations (Aitken & De lullis, 2010). Second, TCDD toxicokinetics such as the prolonged half life, the irreversible ligand activation, and the absence of reactive metabolites to covalently

bind to DNA and proteins, allows the impact of acute dosing, sub-acute dosing, and chronic dosing to be determined via a single TCDD intra-peritoneal (IP) injection. Moreover, the exposed male mice and their controls can be used across the spectrum for natural mating, IVF, and gene expression assays. In addition, our protocols incorporate the analysis of those genes which are differentially expressed during spermatogenesis following the exposure of male mice to TCDD (see Figure 1). This design will not only provide invaluable data and suggest future avenues for investigation; but will also correspond to the animal welfare principles of refinement, reduction, and replacement by reducing the number of TCDD exposed males. Prior to the planned exposure to TCDD all protocols must be optimized and validated.

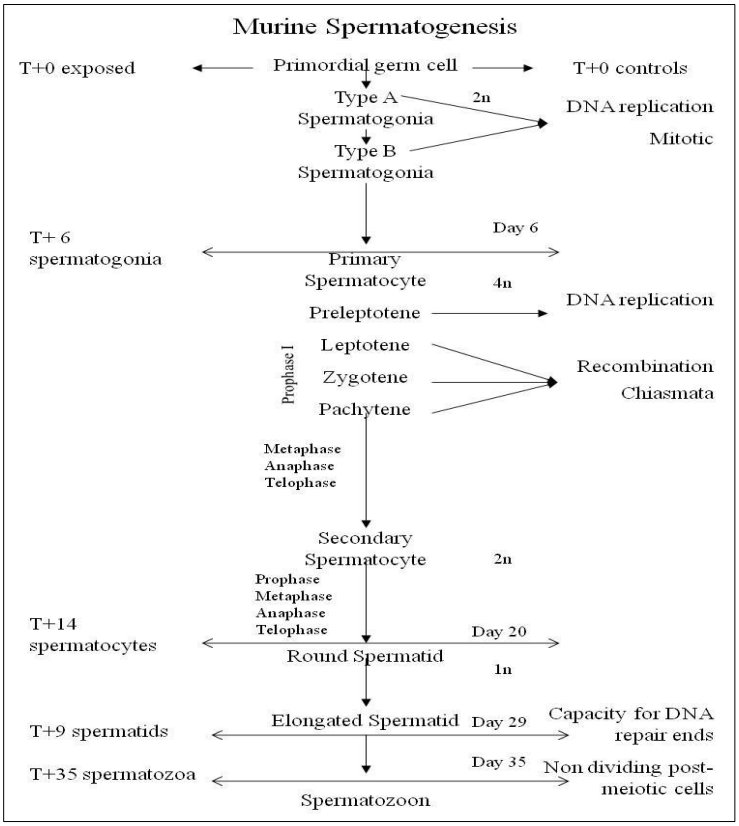


*Figure 1.* A graphic representation of the stages of murine spermatogenesis that informed *Laser Microdissection: Optimization of Methods;* and the sampling T+ timeline that will be used for and *Paternal Environmental Exposures and Gene Expression during Spermatogenesis*.

## Purpose of this Study

Central to this research plan is the use of LMD to isolate stage specific male germ cells for subsequent downstream applications such as qRT-PCR and gene expression microarray. LMD is the only technology that allows the isolation of cell subpopulations from complex tissues such as the testes without disturbing the cell's bimolecular signature. It is an essential tool in the comparison of the molecular signatures of diseased and non diseased cells (Espina, et al., 2006). As a result, LMD will successfully translate to toxicology protocols that seek to characterize gene expression data from exposed and non exposed populations. Since the development of LMD in 1996 at NIH, there has been 1998 articles published using LMD; with 23 of these articles using LMD for testes. This is then reduced to 14 articles when searching for gene expression of the testes with LMD. Finally there are two articles using LMD to obtain stage specific gene expression of the seminiferous epithelium in the rat (Sluka et al., 2002; Sluka et al., 2008). To date there are no published papers or protocols specific to the optimization and validation of the use of LMD combined with qRT-PCR to generate transcript specific gene expression measurements from stage specific male germ cell transcripts, nor are there any published papers using LMD in toxicology protocols seeking to characterize gene expression during spermatogenesis in exposed and non exposed animals.

Therefore, the purpose of this study is to: a) ensure the reliability of the sequential methods of LMD and the selected downstream applications through the establishment of qualitative and quantitative checkpoints upon which each subsequent step will be based, b) ensure that the expected differences in gene expression during spermatogenesis can be elicited with LMD, and c) preserve precious limited sample (TCDD exposed males) until the larger study is undertaken. Thus this initial study will lay the foundation upon which the planned VISN 15 and HSRD toxicology protocols will be based.

## Research Questions and Specific Aims

The varied approaches to the practice of LMD and the preceding and the subsequent downstream applications introduce methodological challenges, with each having the potential to enhance or compromise all subsequent downstream applications. The methodological challenges will be organized into three distinct research questions followed by specific aims.

### *Research Question 1*

What is the effect of tissue sampling methods, sectioning, and staining on the histology of the seminiferous epithelium, and on the morphology of stage specific male germ cells?

*Aim 1*

Selection of the best approach to sample processing, tissue sectioning and staining, slide preparation and sectioning, and LMD techniques that are consistent with maintaining the integrity of the seminiferous epithelium and cell morphology.

*Aim 2*

Identification of the histological characteristics of the seminiferous epithelium, and the cytological features of the stage specific male germ cells.

*Research Question 2*

What factors influence the quality, the quantity, and the amplification of nucleic acids from LMD tissue?

*Aim 1*

Selection of the assays that maximize the quantity and quality of nucleic acid recovery.

*Research Question 3*

What are the optimal conditions for qRT-PCR from LMD samples of stage specific male germ cells and of the seminiferous epithelium?

*Aim 1*

Validate the primer and probe sets to ensure that they amplify the target genes of interest.

*Aim 2*

Determine the optimal protocols which will generate reliable gene expression measurements from the transcripts of LMD male germ cells**.**

## Significance of the Study

At this writing, 900 children with Spina Bifida of Agent Orange exposed Vietnam veterans receive Department of Veterans Affairs (VA) presumptive service connected benefits. With appropriate medical and surgical interventions, the majority of children with Spina Bifida survive to experience significant physical and developmental disabilities (Wallingford, 2005). This underscores the pressing need to explore the intersection of paternal environmental exposures and birth outcomes via molecular and developmental paths and corresponds to the expectations of the *Agent Orange Act* of 1991 (Public Law 102-4 as cited in IOM, 2001) that new scientific information related to the exposures will be generated. Because LMD is the only technology that will provide gene expression data without disturbing the cell's biomolecular state, it promises to be an important tool in male reproductive toxicology assays seeking to identify the mechanisms whereby paternal exposures result in adverse reproductive events. However, significant methodological challenges arise when using LMD in complex tissues such as the testes where there are somatic cells and differentiating gametes. If successful, the optimization

of LMD will represent a significant breakthrough in male reproductive toxicology assays. Through the identification of potential biologic mechanisms and the identification of relevant candidate genes and pathways for analysis, progress can be made in the prevention of future adverse reproductive outcomes that result from male preconception exposure to environmental agents.

# CHAPTER TWO

## Review of the Literature

Developmental endpoints such as structural birth defects, failed pregnancy, low birth weight, increased childhood cancers; and developmental, endocrine, and biochemical abnormalities have been reported in filial 1 and filial 2 (F1 and F2) generations following paternal exposures to pesticides, herbicides, and radiation. There are multiple mechanisms whereby paternal exposure to drugs and chemicals can impact reproductive outcomes: a) chemicals or medications may enter the maternal circulation after absorption of the seminal fluid from the vagina, b) chemicals bound to the sperm may be delivered to the egg at conception, c) toxins have the potential to adversely impact male germ cell development, d) endocrine disrupting chemicals can disrupt fertility via interaction with the neuroendocrine axis, and e) TCDD is known to accelerate epididymis transit time thereby compromising sperm maturation and subsequent fertilization (Klemmt & Scialli, 2006; Perreault, et al. 2008; Stine & Brown, 2006). It is known that germ line mutations occur in animal models following exposure to chemotherapeutic agents or ionizing radiation. Yet, there has been little success in identifying human chromosomal abnormalities or genetic mutations (Friedler, 1996). There are also multiple studies documenting the presence of drugs or other compounds in the seminal fluid of the human male (Schecter, et al., 1996; Teo, et al., 2001). To date, there has been little success in identifying specific mechanisms of action of male mediated developmental effects.

*Male Mediated Developmental Effects*

Multiple studies document the presence of drugs or chemicals in the seminal fluid of exposed animals and the human male. Following the administration of thalidomide to male rabbits before mating, there were high levels of spontaneous abortions, increased malformations, decreased litter size, and low birth weights (Laffitte, 2007). Hales, Smith, and Robaire (1986) not only demonstrated that radio labeled cyclophosphamide was present in the seminal fluid of adult male Sprague-dawley rats, but also that the drug was present in non exposed females post mating. The authors found significantly lower rates of embryo implantations and live births in the offspring of exposed males. In a subsequent experiment the authors then attempted to segregate seminal fluid factors by first mating non exposed females with non exposed males. Within 2 hours the females were mated again with vasectomized exposed males. The authors found a statistically significant increase in preimplantation loss in the females that were mated with vasectomized exposed males. However the approach by Hales and colleagues (1986) cannot discern the potential impact of male germ cell effects. It is also unclear how a second mating with the vasectomized male was accomplished in the presence of an intact copulatory plug from the first mating. The copulatory plug in mice typically persists for 8 to 24 hours after mating (Hedrich, 2004).

There are additional studies documenting the presence of drugs or other compounds in the seminal fluid of the human male. Thalidomide was found to be present in human semen after dosing for 4, 8, and 12 weeks in HIV positive patients. Blood and semen samples were taken at baseline and again at 4 and 8 weeks in two of four patients

who were able to provide semen samples. Plasma thalidomide levels ranged from 192 to 347ng/ml at week 4, and 7 to 29 ng/ml at week 8. Thalidomide concentration in semen samples ranged from 58 to 247 ng/g at week 4, and 8 to 45 ng/g at week 8 (Teo et al., 2001). In reviewing the specifics about thalidomide in Lexi-comp drug information, all males (to include vasectomized males) must use latex condoms during any sexual contact with women of childbearing age. Males are required to acknowledge these risks in writing (Lexi-drugs December 15, 2008).

*Paternal Exposures and Vietnam Veterans*

The exposure of Vietnam veterans to Agent Orange occurred between the years of 1962 to 1970 (Schecter et al, 1996). Subsequently, the Agent Orange Act of 1991 (Public Law 102-4) required that a committee supported by the IOM review and update the scientific findings related to herbicide exposure every two years. In 1996, the committee found that the Ranch Hand Study, the CDC Birth Defect Study, and the CDC Vietnam Experience Study provided suggestive evidence that paternal TCDD exposure led to NTD. Suggestive evidence requires that at least one high quality study demonstrate a positive correlation, while acknowledging that chance; bias, or confounding variables may exist (IOM, 2001). Conversely, a review of paternal serum TCDD and reproductive outcomes among Operation Ranch Hand Veterans concluded that there was little or no support for the hypothesis that exposure to Agent Orange resulted in birth defects (Wolfe et al., 1995). In 1997, Public law 104-204 provided presumptive service connected status to children with Spina Bifida if their parent was an Agent Orange exposed Vietnam Veteran (US House of Representatives. 38 USC. Chapter 18, 2003). In the Veterans and

Agent Orange Update 2006, the committee acknowledged that the most etiologic research focused on maternal and fetal exposure, with a limited body of work that addressed paternal risk. Consequently, studies related to paternal exposures were viewed as especially salient to a largely male service population (IOM 2007).

*Agent Orange and Vietnam Veterans.* While Agent Orange was not the only herbicide used in Vietnam, it was the most widely used, and it is the one that is most often mentioned. Throughout the course of the war, more than 12 million gallons of Agent Orange were sprayed from aircraft over South Vietnam. Additional spraying occurred from boats, helicopters, and back packs (Schecter et al., 1995). Agent Orange refers to an herbicide mixture containing a 1:1 mixture of 2, 4-dichlorophenoxyacetic acid (2, 4–D) and 2, 4, 5-trichlorophrnoxyacetic acid (2, 4, 5-T). Dioxin, 2, 3, 7, 8-tetrachlorodibenzo-p-dioxin (subsequently referred to as TCDD), was produced as contaminant during the processing of 4, 5-T (IOM, 2005). Dioxins include 75 polychlorinated dibenzodioxins (PCDDs), 135 polychlorinated dibenzofurane (PCDFs), nine polychlorinated biphenyls (PCBs), and 2, 3, 7, 8-tetrachlorinated-p-dioxin ([TCDD] Grassman et al. 1998). Dioxin refers to any of the compounds within this class.

*TCDD and seminal fluid.* A recent study of TCDD treated male mice that were mated with untreated females provides evidence of genomic instability in the $F_1$ generation. There were lower rates of implantation, fewer living fetuses, and higher rates of a wide variety of congenital anomalies following paternal TCDD treatment (Nomura, 2008). In addition Schecter and colleagues (1996) documented the presence of TCDD and dioxin like compounds in the semen of Vietnam veterans 19 to 29 years after their

exposure to Agent Orange ended. Study inclusion criteria included Michigan Vietnam Veterans with self reports of Agent Orange exposure and/or a history of having subsequent cancer or fathering children with birth defects. Military record review and interviews were used to select those veterans with the most convincing history of Agent Orange exposure during military service coupled with no history of other likely TCDD exposure outside of the military setting. Fifty men were selected for study inclusion. No women met the selection parameters. This study was the first to document TCDD and dioxin like compounds in the semen of US males. However, the authors' decision to randomly pool semen samples from 17 individual specimens resulted in the inability to correlate the presence of TCDD like compounds in the seminal fluid with serum levels or the reported histories of birth defects (A. Schecter, personal communication, October 24, 2008). A study to determine such effects is clearly indicated.

*Paternal Exposures and Gulf War I Veterans*

A large VA population based study compared self reports of birth defects among deployed and non deployed Gulf War I veterans and found that male and female deployed Gulf War I veterans reported higher rates of moderate to severe structural birth defects. In addition higher rates of miscarriage were reported among the partners of deployed male Gulf War I veterans (IOM, 2005a). Using military records linked to birth certificates, a six state study of the prevalence of birth defects among Gulf War I veterans reported a higher prevalence of tricuspid valve insufficiency, aortic valve stenosis, and renal agenesis or hypoplasia among postwar infants conceived by deployed male Gulf War I Veterans. The numbers continue to reflect the male predominance of active duty

Gulf War personnel (i.e. 10% of postwar infants in this study were born to Gulf War I females). Because the study used Department of Defense (DOD) records, it did not capture the births that occurred after military separation nor the births to non active duty deployed Gulf War I service members such as National Guard personnel or reservists (Araneta et al., 2003; Araneta, Edmonds, Merz, & Flood, 2004). Potential Gulf War exposures included carbon monoxide, sulfur oxides, hydrocarbons, particulate matter, nitrogen oxides, heavy metals, and depleted uranium, either alone or in combination (Veterans Health Administration, 2007). The US General Accounting Office noted that 21 reproductive toxins and teratogens were present in the Gulf War I setting (United States General accounting Office 1994). Gulf War and Health Volume 3: Fuels, Combustion Products and Propellants reported that among the reproductive studies which were reviewed by the committee there were no studies that addressed the effects of specific biologic or chemical agents (IOM 2005). This statement, coupled with the Institute of Medicine Update on Herbicide Exposure, *Veterans and Agent Orange Update 2006*, recommendation to form an ad hoc committee to review mechanistic studies that could advance the knowledge of paternally mediated adverse reproductive events (IOM 2007) highlighted the ongoing urgent need for research to discover the mechanisms of action related to male mediated developmental effects.

### *Polyaromatic Hydrocarbons*

Dioxin like compounds, also known as poly aromatic hydrocarbons (PAH), represent a class of highly toxic and widely dispersed environmental hazards which are formed as the unintentional byproducts of many industrial processes such as incineration,

burning treated wood, the incomplete combustion of fossil fuels, and diesel truck exhaust which may then be absorbed as particulate matter (Grassman, et al., 1998; IOM, 2005a). As such, the exposure of Gulf War I Veterans to fossil fuels, combustibles, and propellants is particularly relevant to studies that seek to characterize the effects of exposure to dioxin like compounds and subsequent adverse reproductive events.

*TCDD Toxicokinetics*

TCDD is stable, lipophilic, and resistant to biologic or environmental degradation. Once formed, TCDD disperses throughout the water, soil, and air. Significant systemic absorption occurs through ingestion and inhalation. Approximately 80% of human TCDD exposure results from consuming fat containing foods; such as meat, milk, and fish. Liver and fat deposits represent the major sites of TCDD stores, and the body burden of dioxin like compounds bioaccumulates (IOM, 1999). Although background lipid adjusted serum levels are typically low at 3 to 6 parts per trillion ([ppt] Schecter et al., 1995), the initial exposures of Operation Ranch Hand Veterans, the unit responsible for aerial spraying, are placed between 11.5 and 423 ppt (Michalek et al., 1995). Half-life ranges from 5.8 to 14.1 years in humans and rises significantly with increasing body fat (Grassman, et a., 1998; Michalek et al., 1995).

In mice, the half-life of TCDD ranges between 10 to 15 days. With six half lives required to achieve steady state, TCDD achieves steady state in 60 to 90 days. Repeated administration of low dose TCDD in mice found an 11 fold and 41-fold increase in hepatic ethoxyresorufin – *O* – deethylase (EROD) activity at weeks 4 and 13 respectively. EROD is a marker for CYP1A1 induction that is the hallmark of TCDD

toxicity. While measures at 4 and 13 weeks demonstrated significant EROD activity, the 30-fold rise by week 13 is an exemplar of TCDD pharmacokinetics. These findings emphasize the need for study designs that incorporate a compound's pharmacokinetics (DeVito & Birnbaum, 1995).

On the other hand, TCDD toxicology is noted for significant inter and intra species variability. For example, the DBA mouse is at least 10 fold less sensitive than the C57 mouse to TCDD toxicity. DBA mice carry two aryl hydrocarbon receptor (AHR, *AHR, Ahr*) polymorphisms that are not found in C57 mice, which results in a low affinity for TCDD binding and reduced AHR bound ligand effects. Interestingly humans share these *AHR* polymorphisms. When a human-mouse chimera was created by inserting human *AHR* into a C57 knockout mouse, a similar reduction in TCDD responsiveness occurred (Moriguchi, et al., 2003). It was also estimated that the human genome carries 40% fewer dioxin responsive genes. Accordingly, evaluation of existing data suggests that humans are neither sensitive nor are they resistant to TCDD's effects. These facts raise the question of the appropriateness of animal research to model human TCDD toxicity (Aylward, Lamb, & Lewis, 2005).

However, the Institute of Medicine Update on Herbicide Exposure, *Veterans and Agent Orange Update 2006,* discussion of the physiologically based pharmacokinetic model (PBPK) model related to TCDD toxicokinetics found that peak human exposures may be more similar to doses used in animal toxicity studies than previously estimated (IOM 2007).The PBPK model holds that CYP1A1 induction accelerates the elimination of high dose TCDD to result in a shortened half life of 1 to 3.6 years, while at low dose

exposure; diffusion from adipose tissue is the rate limiting step (IOM 2007). Thus the retrospective dose extrapolation used by Schecter et al (1996) in which one compartment first order kinetics was used (with a TCDD half life of 5 or 10 years) to determine the initial TCDD exposures of Vietnam veterans from their serum TCDD levels more that 20 years after their exposure to TCDD ended, may have significantly underestimated the exposure of the study participants.

*Dose response.* The challenge in TCDD risk assessment is to determine whether a non-linear threshold level exists. The non-linear threshold level is the cornerstone of toxicology and holds that at low exposure levels no significant health effects exist (Okey, 2007). Yet the findings of statistically significant malformations in the offspring of male mice after sub acute TCDD exposure (IOM 1999) challenged conventional thinking to raise the possibility of a linear dose response at low levels, which implied that any dose is capable of producing toxic effects (Popp, Crouch, & McConnell, 2005). AHR down regulation is thought to be protective by preventing excessive stimulation in the presence of a potent AHR ligand. However, in vivo studies found *Ahr* down regulation to be transient in the presence of persistent low dose TCDD exposure indicating that most tissues will not be desensitized to TCDD's effects (Okey, 2007).

### *TCDD as the Surrogate Environmental Agent.*

While other PAHs cause AHR activation, these are readily biotransformed into reactive metabolites that covalently bind to DNA and proteins to act as carcinogens, mutagens, and teratogens thereby effectively obscuring the consequences of exogenous AHR activation. As such TCDD has many desirable properties that make it the ideal

surrogate environmental agent when seeking to understand the mechanism of action of a single biologic or chemical agent. Because of its ubiquitous environmental presence, TCDD continues to hold significant biologic relevance (A. Okey, personal communication, September 12, 2008).

### *TCDD /AHR/ARNT Complex*

Prior to binding, AHR resides in the cytoplasm where it binds to heat shock protein 90 and other chaperone proteins that stabilize AHR in the cytoplasm. TCDD and dioxin like compounds bind with high specificity and affinity to AHR. Once bound to AHR, TCDD is transported to the nucleus and the chaperone proteins dissociate. AHR then binds with aryl hydrocarbon receptor nuclear translocator (ARNT) resulting in the TCDD /AHR/ARNT complex. The complex then combines with the Aryl hydrocarbon response element (AHRE) that flanks the 5' region of many genes and modulates gene expression (Thackaberry, Jiang, Johnson, Ramos, & Walker, 2005). TCDD is a persistent AHR ligand leading to increased gene expression, which results in TCDD's toxic effects. There are also reports that AHR inhibits cell proliferation via interaction with the retinoblastoma tumor suppressor protein (Barouki, Coumoul, & Fernandez-Salguero, 2007). The *Ahr* allele is located on chromosome 12 in mice. In humans, *AHR* maps to 7p15 (OMIM 2007).

### *AHR Ligands*

AHR regulated genes are best known for the induction of *CYP1A1/ Cyp1a1*and *CYP1A2/ Cyp1a2* (members of the cytochrome P450 super family here to fore

abbreviated as CYP) which are essential to detoxification of endogenous and exogenous ligands (Puga et al., 2002). Conversion of proto-toxicants to toxic substances occurs via CYP metabolic activity (Goldstone & Stegeman, 2006). Bilirubin activates AHR to eliminate the toxic byproducts of heme degradation. Ultraviolet irradiation photo converts tryptophan into a potent AHR ligand (Okey, 2007). The toxic effects of cigarette smoke (Kitamura & Kasai, 2007) and PAHs (Izawa, Kohara, Wantabe, Taya, & Sagai, 2007a) are also mediated by the AHR/ARNT complex. TCDD dependent expression of *CYP1A1*/*Cyp1a1* can be found in lymphocytes, the liver, the kidneys, the lungs, and the small intestines (Puga et al., 2002).

*AHR health effects*. TCDD and cigarette smoke are known to cause a variety of health problems. TCDD is associated with chloracne, cancers, altered levels of sex hormones, type II diabetes, adverse reproductive outcomes, developmental defects, impaired thyroid function, thymic atrophy, immune toxicity, liver toxicity, and altered cellular growth and differentiation (Fujiyoshi, Michalek, & Matsumura, 2006; Roman, Pollenz, & Peterson, 1998). Cigarette smokers share similar health consequences as evidenced by increased rates of cancers, developmental and reproductive abnormalities, cardiovascular and cerebrovascular pathology, and obstructive pulmonary disease (Kasai, et al., 2006). The ubiquitous presence of AHR and ARNT across tissues and across species provides evidence of the importance of AHR/ARNT in developmental and metabolic processes and offers one explanation for TCDD's and cigarette smoke's often diverse and contradictory effects (Kasai, et al., 2006; Puga et al., 2002). AHR is associated with cell growth, cell death, and physiologic B cell activation (Allen & Sherr, 2005).

*The AHR/ARNT Complex in Development*

The loss of *Ahr* function through genetic deletion as well as sustained *Ahr* activation has detrimental developmental effects. Cleft palate, hydronephrosis, and cardiovascular defects result from sustained *Ahr* activation (Thackaberry et al., 2005). *AHR/Ahr* and *AHRE/Ahre* are a subgroup of the highly conserved basic helix loop helix (bHLH/PAS) super family of transcription factors that bind as dimers to specific DNA sequences to control essential physiologic processes such as metabolism, organ development, metabolism, and neurogenesis (Barouki et al., 2007; Grassman et al., 1998; Okey, 2007). Microarray analysis of murine cardiovascular development during fetal development found significant alterations in a variety of genes following maternal TCDD administration. Genes involved in xenobiotic metabolism, cardiac homeostasis, extracellular matrix production, and cell cycle regulation exhibited significant dysregulation (Thackaberry et al., 2007).

A recent study of TCDD treated male mice that were mated with untreated females provides evidence of genomic instability in the $F_1$ generation. There were lower rates of implantation, fewer living fetuses, and higher rates of a wide variety of congenital anomalies following paternal TCDD treatment (Nomura, et al., 2008). Another study of mouse embryos found that *Arnt* expression correlated with embryonic development. Maximal expression of *Arnt* occurred at days 10-11 in the neuroepithelial cells of the neural tube, the visceral arches, the heart, the optic and otic placodes, and the preganglionic complexes. After day 11, *Arnt* expression declined in the brain and the heart. Days 15 and 16 coincided with *Arnt* expression in the adrenal glands, liver,

submandibular glands, ectoderm, tongue, bone, and muscle. The authors concluded that the role of *Arnt* in the embryo is independent of *Ahr* expression (IOM, 1999). Yet studies highlight the importance of *Ahr* in the physiological and developmental processes of invertebrates underscoring the importance of *Ahr* in neuronal differentiation and early development (Barouki et al., 2007). The cellular processes involving growth, development, and differentiation are the most sensitive to TCDD's toxic effects (Grassman et al., 1998).

*The AHR/ARNT Complex in Spermatogenesis*

A wide distribution of AHR and ARNT was identified in rat and human testes. Using northern blot analysis, transcripts specific for *Ahr* mRNA were found in all stages of the rat seminiferous epithelium. Stronger mRNA signals for *Arnt* were found in the tubular and interstitial cells of the rat testes. *AhR* and *Arnt* are present in the testes, epididymis, vas deferens, prostate, and the seminal vesicles of male rats (Roman et al., 1998). A recent study of male mice exposed to diesel exhaust particles (Izawa, Kohara, Wantabe, Taya, & Sagai, 2007b) found that increased hepatic EROD was strongly correlated with increased morphologically abnormal sperm and decreased daily sperm counts. In humans *AHR* and *ARNT* were found in pre and post meiotic cells in the seminiferous epithelium, leading to the speculation that the activation of the AHR/ARNT has far reaching consequences for spermatogenesis. The wide and abundant distribution of AHR and ARNT in the human testis provides one explanation for TCDD's impact on spermatogenesis and fertility. The testes have been reported to be among the most sensitive organs to TCDD's effects. (Schultz et al., 2003)

*Spermatogenesis and Spermiogenesis*

Undifferentiated stem cells, known as prospermatogonia, occur before germ cell activation. At puberty, type A spermatogonia replenish cell lines, while type B spermatogonia become primary spermatocytes through rapid mitosis and proliferation (Costanzo, 1998). Transcriptional activity is low in type A spermatogonia, while type B spermatogonia are characterized by increased gene expression. Nearly 30% (62 genes) of the 260 genes that were detected during mouse spermatogenesis were upregulated with marked increases in cyclins, growth factors, transcription factors, and oncogenes (Yu et al., 2003). Spermatogonial differentiation requires 6 days in mice and 16 days in men (Adler,1996).

There is increased expression of 38 genes during preleptotene (Yu et al., 2003). Preleptotene corresponds with the final DNA replication of spermatogenesis (Adler 1996). During the remainder of prophase I (leptotene, zygotene, and pachytene), homologous chromosomes intertwine to exchange genetic material and create tetrads, which are also referred to as bivalents (Jorde et al., 2000). As primary spermatocytes transition from preleptotene to pachytene, 35 genes are down regulated, two transcription repressors are upregulated, and aryl hydrocarbon receptor (AHR) expression is increased (Pang et al., 2006; Yu et al., 2003). Homologous chromosomes then separate to create secondary haploid spermatocytes (Jorde et al., 2000). After 14 days in mice and 25 days in men, spermatocyte development is complete (Adler, 1996).

Meiosis II proceeds at a quickened pace. During anaphase II, centromeres split to create four haploid spermatids (Eddy, 2002). The spermatids are no longer dividing and

the number of expressed genes and the expression levels decline precipitously. At this point protamines replace histones and the capacity for DNA repair ceases. The morphological changes that attend nuclear condensation now take center stage (Yu et al., 2003). Golgi vesicles form the acrosome, which stores the enzymes that facilitate fertilization. The formation of a flagellum is accompanied by cytoplasmic reduction, the release of the spermatozoa from the sertoli cells, and the rupture of the cytoplasmic bridges. Sperm now migrate to the seminiferous tubules and pass to the epididymis where they mature for an additional one week in mice, and 8 to 17 days in men (Adler, 1996). All cells within a mature sperm population are in $G_0$; therefore DNA replication and RNA transcription cease (Goodrich, Johnson, & Krawetz, 2007; Sharman, Yamauchi, Ward, 2007). Altogether, spermatogenesis spans 35 days in mice, and 64 days in men (Adler, 1996). Two million spermatogonia give rise to 128 million sperm per day (Costanzo, 1998). Successive overlapping cycles of spermatogenesis begin every 8.7 to 8.9 days in mice and 16 days in men (Adler, 1996).

*Spermiogenesis*

During the process of spermiogenesis, post meiotic haploid round spermatids transform to progressively motile spermatozoon that are capable of fertilization. Spermiogenesis requires cytoplasmic reduction, extensive restructuring and remodeling, and the degradation of organelles and proteins. During this transformation, the numbers of mitochondria are typically reduced by 50%, with the migration of the remaining mitochondria to the sperm middle piece. The mitochondria are a) reinforced with an outer mitochondrial membrane, b) adapted for sperm motility, and c) rearranged to form a

helical sheath. At the same time, nuclear hyper-condensation and sperm head shaping is proceeding. Protamines replace histones to allow DNA packaging to be reduced to a volume that is less than 5% the size of somatic cells thereby rendering the highly compact DNA resistant to oxidative stress. However spontaneous DNA damage in sperm occurs more frequently in male germ cells than in somatic cells, with DNA damaged sperm retaining their capacity for fertilization. This provides one explanation for the male mediated toxicology that has been associated with paternal environmental exposures and advancing age (Aitken & Baker, 2006; Sutovsky & Manandhar, 2008).

*Structure and Function of the Seminiferous Epithelium*

The wave of the seminiferous epithelium can be described as the unique arrangement of haploid and diploid cells at the successive stages of the cycle along the length of the seminiferous epithelium. The highest stage (XIV in the rat, XII in the mouse, and VI in the human), can be found at either end of the tubule, with the two descending waves meeting somewhere in the middle. Each seminiferous tubule is a loop with each end opening into the rete testes (Sutovsky & Manandhar, 2006). In non human species, a single stage occupies a significant length of the tubule. As such, a single cross section of the seminiferous tubule will reveal only one stage of the seminiferous epithelium. In comparison, human cellular associations appear as irregularly shaped non-sequential patches, with as many as six stages of the seminiferous epithelium appearing in a single cross section of the seminiferous tubule (Ross & Palina, 2006). Seminiferous tubules make up 80% of the adult testes (Seli, Mahutte & Arici, 2002).

*Structure and Function of the Sertoli Cells*

Throughout the process of spermatogenesis, the developing germ cells maintain cell to cell contact with the sertoli cells and with each other via connecting cytoplasmic bridges. The cytoplasmic projections of the sertoli cells extend from the basal lamina to the lumen to surround and support the developing germ cells. This serves to a) protect the developing spermatids through the tight junctions of the blood testes barrier, b) provide direct and interactive contact with three to four layers of cells that are in various stages of development, and c) assure uniform development of primary spermatocytes by connecting cytoplasmic bridges (Seli et al., 2002; Sutovsky & Manandhar, 2006). Thus the structure of the sertoli cells presents distinctive challenges when either seeking to isolate the pure sertoli cell populations or microdissect stage specific developing germ cells from the seminiferous epithelium (P. Stanton, personal communication, August 6 2009).

*Blood testes barrier.* The tight junctions of the Sertoli cells create the two compartments of the blood testes barrier (BTB). The BTB controls the passage of molecules such as nutrients and cellular wastes, provides a specialized environment for movement and development, and provides immunologic protection to a) prevent an auto-immune response to male germ cell specific antigens, and b) secrete interferons to provide an anti-viral defense. Spermatogonia, preleptotene and leptotene spermatocytes, comprise the basal compartment of the mature male BTB. The basal compartment of the BTB is in contact with the testicular blood and lymphatic systems. In contrast, the adluminal compartment of the BTB contains differentiating cells at various stages which

include pachytene spermatocytes, round and elongating spermatids. The adluminal compartment does not have direct contact with testicular blood or lymphatics (Mruk & Cheng, 2004). Based upon this information, spermatogonia and the premeiotic spermatocytes would be expected to have increased vulnerability to xenobiotic exposures, while the post meiotic cells would be protected by the BTB. Yet Aguilar-Mahecha, Hales, and Robaire (2001) noted that cyclohphosamide caused increased damage in round spermatids. Here it is important to understand that sertoli cells extend from the basal lamina to the lumen of the seminiferous tubules and maintain direct contact with male germ cells throughout differentiation. Sertoli cells synthesize, secrete, and deliver products that are required for growth and differentiation. As a result, receptors found in the basal compartment of the sertoli cell membrane have the potential to affect sertoli cell processes and ultimately germ cell development. *Ahr* expression is increased in sertoli cells, and type A and B spermatogonia. In contrast *Ahr* expression is decreased in pachytene spermatocytes and round spermatids (Mammalian Reproductive Genetics ([MRG] University of Washington Seattle, 2009). This pattern of expression is consistent the concept of the BTB and the physiologic role of *Ahr* in detoxifying endogenous and exogenous agents. While no information can be found regarding Sertoli cell *AHR/Ahr* localization, *AHR/Ahr* function suggests that it will localize to basal compartment of the BTB of the sertoli cells' plasma membrane where there is contact with testicular blood and lymphatics, thereby suggesting another avenue for future exploration.

*Structure and Function of the Epididymis*

Several proteins and enzymes within the lumen of the epididymis play a role in maturation and the capacity for successful fertilization. The epididymis is divided into three regions, the proximal caput, the corpus, and the distal cauda. There are dramatic differences in cellular structure within each region. Significant absorption of water takes place in the caput, and the cells are high columnar epithelium. The low columnar epithelial and clear cells of the cauda epididymis are specialized for the removal of cellular debris and the storage of mature spermatozoa until ejaculation. Most sperm achieve full fertilization capacity in the cauda epididymis, where the sperm are stored until ejaculation (American Society of Andrology, 2005; Hafez, Hafez & Hafez, 2005). During the transit, maturation, absorption, and storage, immunologic privilege and protection from xenobiotics and free radicals is provided to the spermatozoa by the blood epididymis barrier (American Society of Andrology, 2005; Kandeel, 2005). There is a debate regarding the effectiveness of the blood epididymis barrier, since it is believed that chemicals and toxins in semen arrive primarily via fluid from the seminal vesicles or the prostate (Klemmt & Scialli, 2005).

*Anatomy, Structure, and Function of the Accessory Glands*

Anatomically the ducts of the seminal vesicles, prostate gland, and bulbourethral glands lie in close approximation. Along with the prostatic lobes, the seminal vesicles open into the seminal collicle (the forepart of the urethral crest). The excretory ducts of the bulbourethral glands open directly into the urethra (Hedrich, 2004).

*Seminal vesicles.* The seminal vesicles in mice are large paired glands which are located to the back and the lateral side of the urinary bladder. The walls of the seminal vesicles are composed of smooth muscle and tall columnar epithelium which forms branching mucosal folds (Hedrich, 2004). Seminal vesicle fluid is rich in fructose and prostaglandins (Hafez, Hafez, & Hafez, 2005).

*Prostate.* The prostate consists of the anterior, dorsal, and ventral lobes. In mice the anterior lobes are attached to the seminal vesicles and are known as the coagulating gland and are responsible for the copulatory plug that forms in the vagina after mating which promotes sperm transport into the uterus. In comparison, human semen forms a loose gel rather than the fibrous plug that is found in rodents. It is interesting to note that the human seminal gel is degraded within one hour by prostate specific antigen ([PSA] Suarez & Pacey 2005), whereas the copulatory plug in mice typically persists for 8 to 24 hours after mating (Hedrich, 2004). Biochemical markers of prostatic fluid include acid phosphatase, citrate, calcium, zinc, spermine, and vesiculase.

*Bulbourethral glands.* The Cowper's glands (also known as the paired bulbourethral glands) are located at the base of the penis. Secretions contain IgA and mucoproteins (Hafez, et al., 2005; Hedrich 2004).

*Components of the Seminal Fluid*

Over 95% of the ejaculate originates in the sexual accessory glands (American Society of Andrology, 2005; Kandeel, 2007). The ejaculate of the human male ranges between 2 ml and 6 ml, with an average of 3 ml per ejaculation. Of this amount, 0.2 mls

originate from the bulbourethral glands, 0.5 mls from the prostate, with 2 mls secreted from the seminal vesicles (American Society of Andrology 2005). By subtraction, approximately 0.3 mls is secreted from the epididymis and testes.

*Seminal fluid function.* Seminal fluid contains sperm and seminal plasma. Seminal fluid acts as a diluent and vehicle for sperm transport that protects sperm viability through nutritive and protective factors (Robertson, 2007). Functionally, seminal plasma: a) facilitates sperm transport, b) provides a coagulating system, c) provides an energy source, and d) neutralizes the vaginal acidic pH. Seminal plasma also has potent immunosuppressive effects to inhibit the immune response to spermatozoa. In mice the inflammatory response is initiated when seminal proteins interact with the estrogen primed uterus and cervix to activate the synthesis of proinflammatory cells. In response to pro-inflammatory cytokines, inflammatory leukocytes migrate to the area. It is believed that these inflammatory events serve to: a) clear unneeded sperm and microorganisms, b) activate the female immune response specific to seminal antigens and proteins, c) remodel the tissue in preparation for endometrial receptivity, and d) activate the expression of cytokines and growth factors associated with preimplantation embryo development. It has been suggested that seminal fluid contact greatly enhances successful pregnancy by protecting the sperm from neutrophil attacks. Thus female seminal fluid contact greatly increases the chances of pregnancy success (Robertson, 2006).

### *Stress Response Genes during Spermatogenesis.*

Aguilar-Mahecha, Hales, and Robaire (2001) noted that the vulnerability of male germ cells varied by the type of exposure and the stage of development. For example,

spermatogonia were more vulnerable to damage induced by radiation, while round spermatids displayed increased sensitivity to damage caused by cyclohphosamide or other alkylating agents. In the same manner, spermatocytes and spermatids displayed increased rates of damage when exposed to elevated temperatures; whereas spermatogonia and mature spermatozoa were resistant to heat stress. To better understand the preferential and differential patterns of sensitivity and resistance, the authors determined the expression profiles of 216 stress response genes during rat spermatogenesis. Expression patterns were further categorized according to function and include a) heat shock proteins (HSPs), b) oxidative stress, and c) DNA repair.

*Heat Shock Proteins*

Heat shock proteins: a) assist in the folding of maturing proteins, b) prevent non specific protein-protein interactions, c) repair damaged proteins by either refolding or degradation, and d) act as molecular chaperones. They represent a ubiquitous and highly conserved family of proteins with constitutive, developmental, and stress induced patterns of expression. Any sudden changes in the cellular environment such as (but not limited to) heat, inflammation, and toxins, can trigger HSP expression (Csermely et al., 2008; Soti, et al., 2005). Molecular weight is used to distinguish among the members of this class (Powers & Workman, 2007).

*HSP90*. It is precisely these patterns of expression that generate interest in HSPs when pursuing gene expression toxicology protocols within the context of spermatogenesis. The majority of HSP expression to include *Hsp90α* (also known as *Hsp90aa1*inducible and *Hsp86*), and *Hsp90 β* (also known as *Hspab1, Hsp90 β*, and

*Hsp84*) decreased as the cells transitioned from pachytene spermatocytes to elongating spermatids. While *Hsp90α,* and *Hsp90 β* expression patterns were similar, *Hsp90 β* intensity was more than three times higher than the expression of *Hsp90α* as cells transitioned from pachytene spermatocytes to elongating spermatids (Aguilar-Mahecha et al., 2001). While *Hsp90 β* predominates in the peripheral organs, and is constitutively expressed, *Hsp90α* expression predominates in the testes and is highly inducible in under stress (Mayer, Promdromou & Frydman, 2009; Vamvakopoulos, 1993). HSP90 client proteins include the AHR, glucocorticoid, estrogen, and progesterone receptors; and many client proteins linked to carcinogenesis (Hughes, et al., 2008; Zhao & Houry, 2005).

It was concluded that decreased *Hsp90* expression during spermatogenesis may compromise the male germ cells' capacity to respond to stress (Aguilar-Mahecha et al., 2001). Yet evidence suggests that HSP90 is required for the efficient binding of AHR ligands; and in the absence of HSP90, it was not possible to form a stable AHR/TCDD complex (Hughes et al., 2008; Whitelaw et al., 1995). This raises the competing theory that decreased *Hsp90* expression may be protective, while increased *Hsp90* expression enhances TCDD's toxic effects. Several studies targeting cancer chemo-prevention indicate that HSP90 inhibitors induce rapid AHR degradation (Hughes, et al., 2008).

*Oxidative Stress in the Testes*

Speculation holds that the low oxygen tension in the testes protects the developing gametes and the steroid generating Leydig cells from oxidative stress. Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS), and

the antioxidant defense systems which include antioxidant enzymes and antioxidants. The antioxidant enzymes consist of the somatic homologues glutathione-s-transferase, glutathione perioxidase (GPx), and superoxide dismutase (SOD). SOD includes cytosolic SOD1 (zinc/copper) and mitochondrial SOD2 (iron/magnesium). SOD2 displays maximal expression in the post meiotic male germ cells and is developmentally expressed. At the same time, testes specific phospholipid hydroperoxide GPx (PHGPx) is one of the most important GPx isoforms is the testes and is highly expressed in the leydig cells and the male gametes. ROS typically begin with the production of a superoxide free radical ($O_2^-$) with the rapid conversion to hydrogen peroxide ($H_2O_2$) in the presence of SOD. $H_2O_2$ is then eliminated by glutathione perioxidase (GPx) thereby preventing $H_2O_2$ from creating the powerful membrane permeating hydroxyl radicals which can result in oxidative damage to DNA, lipids, and proteins (Aitken & De luliis, 2010).

*Mitochondrial ROS.* The generation of ROS occurs in the mitochondria and from the activity of enzymes such as NADPH oxidases, leukocyte infiltration, and cytochrome P450 activation. Oxidative stress can result from clinical conditions such as infections, cryptorchidism, testicular torsion, varicocele, hyperthyroidism, diabetes, exertion, steroid imbalance, and the exposure to redox cycling compounds such as polyaromatic hydrocarbons ([PAHs] Aitken & Roman, 2008). The generation of ROS occurs in the mitochondria, from the activity of enzymes such as NADPH oxidase (NOX), leukocyte infiltration, and cytochrome P450 activation. Because P450 (CYP1A1) is a recognized marker of AHR activation, there is a distinct probability that AHR activation will therefore increase oxidative stress. This promises to be an important area of future exploration.

*Oxidative stress*. Oxidative stress is believed to play a significant role in sub-fertility and DNA damage in sperm with the potential to impact subsequent viability and to result in developmental abnormalities. On the surface, it seems inconsistent that damaged sperm can result in fertilization. Yet robust evidence supports the fact that damaged sperm are not only capable of fertilization, but also have the potential to create adverse reproductive outcomes in subsequent generations. Mouse sperm damaged by repeated freeze-thaw cycles used for IVF resulted in delayed post natal growth, decreased longevity, and increased cancers (Aitken, De luliis, & McLachian, 2008). Likewise cigarette smoking has been linked to an elevated protamine 1/ protamine 2 ratio and oxidative damage in sperm (Hammadeh, Hamad, Montenar, & Fischer-Hammadeh, 2010). Despite the fact that cigarette smoke is a complex mixture of various substances, many of these compounds (volatile hydrocarbons, aromatic amines, polycyclic aromatic hydrocarbons, and benzene) are known AHR ligands (Grassman, 1995; IOM, 2005).

*Seminal fluid and antioxidant protection*. There is loss of cytosolic volume as male germ cells transit the stages of development, thereby creating cells that are deficient in antioxidant protection. Consequently these cells are dependent upon the male reproductive tract antioxidant defenses. Despite the fact that the epididymis contributes a limited amount of fluid to the seminal plasma, several days are spent in the epididymis during post testicular maturation and the epididymis plays a key role in antioxidant defenses. The epididymis secretes free radical scavengers such as Vitamin C and uric acid, and specialized forms of extracellular SOD (SODex) and glutathione perioxidase (GPx). Extracellular glutathione perioxidase 5 (GPx5) is under androgen control, is only

expressed in the caput epididymis, and aligns with the cell membrane to protect the sperm from free radical attack (Aitken & De luliis, 2010).

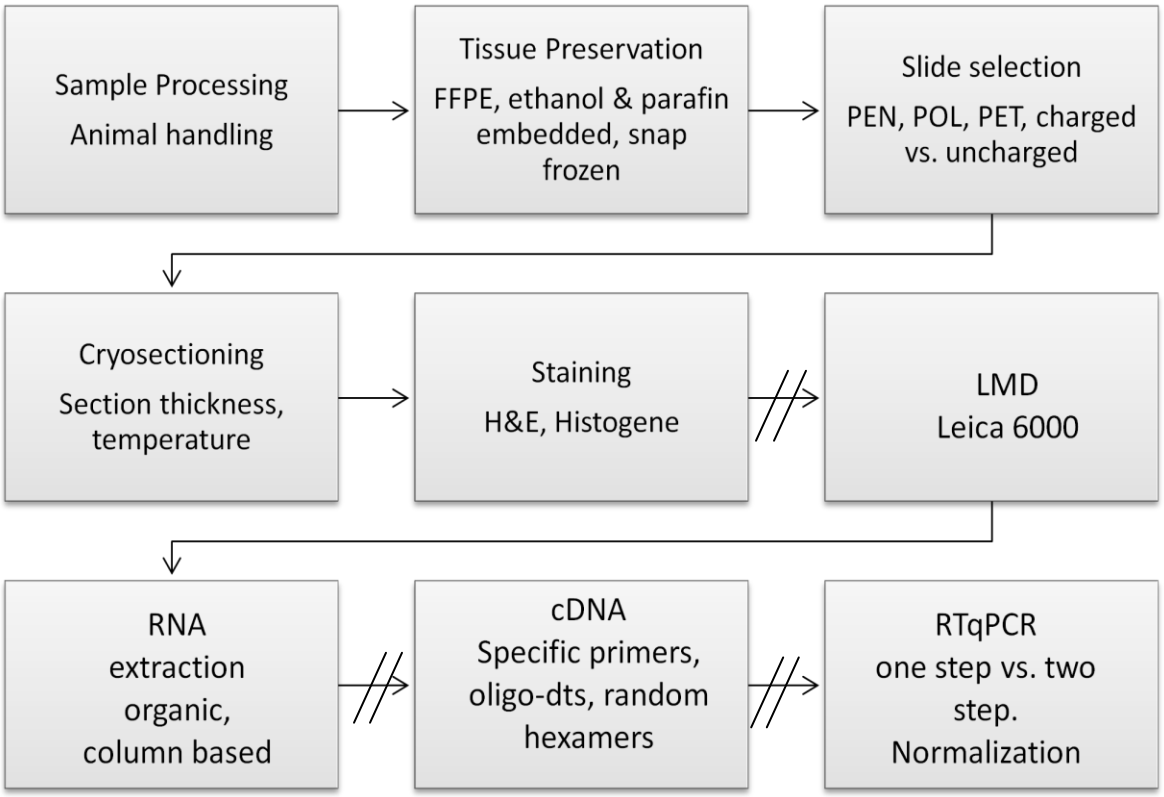
*The Question of Apoptosis*

Current understanding posits that controlled apoptosis participates in normal spermatogenic functions such as the removal of abnormal sperm and the control of the proliferating germ cell population. In fact nearly 100% of cells display DNA damage as evidenced by strand nicks as they transit from round spermatids to elongated spermatids during the process of protamination. The nicks are then absent after condensation (Shaman, et al., 2007). Consequently the presence of pro-apoptotic markers and positive TUNEL assays in mature sperm raise several questions. Do these findings merely represent residual makers of cytoplasmic reduction? Or do these findings indicate that immature, poorly remodeled cells are evading apoptosis as they transit through spermatogenesis? Are the positive assays in mature sperm the result of ROS? It should be noted that markers of DNA fragmentation increased in the presence of a glutathione perioxidase inhibitors which supports the hypothesis that DNA damage in mature sperm may occur as a result of oxidative stress (Shaman, et al., 2007).

# CHAPTER THREE

## Research Design and Methodology

Chapter three provides an overview of the research design and the proposed methods that will be used in this study. As shown in Figure 2, the research design is a graphic representation of the sequential steps in the use of LMD when LMD is paired with qRT-PCR as the end target downstream application. The methods and procedures are organized into the following sections: a) sample processing, b) tissue preservation, c) slide selection, d) cryosectioning, e) staining, f) LMD, g) RNA extraction and purification, h) cDNA generation, and i) qRT-PCR.




Legend: // = quantitative and qualitative measures

*Figure 2.* A graphic illustration of the design of the LMD and qRT-PCR workflow

### *Animal Welfare*

Optimum breeding aged male mice (age 8 to 12 weeks) were allowed a minimum of 5 days for physiological, psychological, and nutritional stabilization before tissue harvest. Unless raised together from birth, adult male mice may display aggressive behavior that can be detrimental to cage mates (Collaborative Institutional Training Initiative [CITI], 2010). Therefore the mice were housed in single cages when displaying aggressive behavior. However, individually housed animals still obtain, visual, olfactory, and auditory cues from adjacent animal cages. No antemortem procedures were performed. After euthanasia and recovery of the testes, the techniques included: a) sample processing, b) tissue preservation, c) slide selection, d) cryosectioning, e) staining, f) LMD, g) RNA extraction and purification, h) cDNA generation, and i) qRT-PCR. Animal protocol approval was received from a) the University of Missouri-St. Louis Institutional Animal Care and Use Committee (IACUC) protocol number 09-11-10, b) Washington University Animal Studies Committee 20080290, and c) St. Louis VA IACUC approval number 0910-242 (see Appendix A).

### *Number of Animals Needed*

In order to optimize and validate the techniques and to obtain maximal RNA purification, the mice were sacrificed in accordance with the IACUC and animal welfare approved method, and then used to perfect the required procedures such as tissue isolation and preservation, LMD, cell segregation, staining, RNA extraction, and qRT-PCR. For the first year, we estimated that approximately five animals per week x 52 weeks (260 mice) were needed. However, these numbers cannot be informed by the

power analysis and are based upon the need to: a) maintain a constant supply of mice that are at the optimal reproductive age, b) optimize and validate methodology before TCDD exposure, c) preserve precious limited sample until the toxicology study is undertaken, and d) the expertise of Dr. Kelle Moley who has more than 20 years experience with mouse reproductive techniques.

### *Sample Processing*

Carbon dioxide ($CO_2$), inhaled anesthetics, and injected barbiturates cause prolonged periods of hypoxia and tissue ischemia with resulting changes in gene expression with the potential to introduce error into the interpretation of gene expression data (Pritchard, et al. 2001; Seta et al., 2002; Sutler et al., 2007). With the exception of cervical dislocation, each of the methods noted above will either directly, through the use of chemical agents, or indirectly, through extended time to death or altered physiological processes adversely impact gene expression. A second consideration is the fact that endogenous enzymes cause cell and tissue destruction after tissue sampling or death. Autolysis of the seminiferous tubules from endogenous enzymes can be expected to begin within 10 minutes of clinical death to result in significant tissue destruction and changes in appearance (J. Rodriguez-Canalas, personal communication, August 5, 2009). Autolytic changes include: a) vacuolation of the sertoli cells, b) undermining of the basement membrane, and c) germ cell sloughing (Foster, 2009).This point is particularly relevant to LMD which depends upon the ability of the researcher to identify the characteristics of the target cells. Autolysis of the seminiferous tubules was increased

with poor tissue handling and extended length of time between tissue sampling and tissue preservation (J. Rodriguez-Canalas, personal communication, August 5, 2009).

### *Tissue Preservation*

Formalin fixed and paraffin embedding (FFPE) is the gold standard of tissue preservation. It provides optimal morphology, easy storage, and permanent tissue preservation (Perimutter, et al., 2004). However, formalin results in chemical cross-linking between DNA and proteins and results in extensive RNA fragmentation (Espina, et al., 2006). Gene expression studies and proteomic analysis from FFPE tissues require extensive optimization. Ethanol fixation with paraffin embedding was explored as a second option for gene expression profiles of LMD benign prostatic hyperplasia and prostate tumor samples. However, this also resulted in a significant drop in the mRNA transcripts (Perimutter, et al. 2004). In comparison, snap frozen tissue specimens stored as tissue blocks in Optimal Cutting Temperature (OCT) or isopentane, at $-80^{0}$ C for less than 2 months were ideal for downstream gene expression assays. The presence of OCT in tissue sections has been shown to interfere with downstream applications such as qRT-PCR (Erickson, et al., 2009). Hence, the water dips were included in the staining protocols to remove the OCT from the slides before proceeding to LMD.

### *Slide Selection*

Infrared (IR) LMD systems transfer the laser energy at 810 nm wavelength to the thermostable polymer (polyethylene naptholate [PEN]) membrane on either the laser capture caps, or the LMD slides. The term laser capture microdissection (LCM) is only

applied to the Arcturus Pixel instrument in which the polymer distends downward from the cap to adhere to and "capture" the cells. In comparison, the slide membrane used with other LMD systems support the cellular matrix by conforming to the nooks and crannies of the tissue. The ultraviolet (UV) laser cuts or ablates the polymer membrane at 355nm wavelength. In both cases, the polymer membrane absorbs the laser's energy without disrupting the target cells' molecular state (Espina, et al. 2006). The polymer is inert, and does not interfere with downstream applications.

Plain uncharged glass slides can also be used with the Arcturus Pixel downwardly distensible LCM polymer cap. Because positively charged glass slides and poly lysine coated glass slides increase adhesion between the slide and the tissue, their use in LMD is restricted to those circumstances where there is inadequate cell to slide adhesion. Polyester (POL) and polyethylene terapthalate (PET) membranes are used with metal frame slides for tissue culture LMD.

### *Cryosectioning*

The ideal cutting temperature for most tissues was -24 $^{0}$C. However, adipose rich tissues required colder temperatures at -28 $^{0}$C to -35 $^{0}$C. Cutting sections at 8 µm usually provided adequate cell morphology and increased the RNA yield. But, 4 µm sections may enhance morphology and assist the researcher in identifying the cells of interest. Cryosectioning was limited to one tissue block so that no more than two slides were created at one time. Next the LMD researcher proceeded promptly to staining followed by a drying time of 10 minutes. Continued modifications were implemented so that the duration of time from staining to the completion of the LMD process did not exceed 45

minutes. In one instance, slides were stored at - 20 $^{0}$ C overnight when it was not possible to immediately advance from sectioning, to staining, to LMD. However the freezing, thawing, and refreezing of the tissue compromised the RNA integrity and allowed ice crystals to form from the water within the tissue. Our experience paralleled a published report which documented an increase in the size of the ice crystals associated with thawing and refreezing, and appeared as artifacts, or holes, in the microscope sections (Carson, 1996).

## *Staining*

The goals of pathology tissue staining were to maintain optimum tissue and cell morphology for subsequent identification and downstream applications. In comparison, the goals of staining for LMD were to maintain adequate cell morphology for identification, while maximizing the retrieval of RNA, DNA, and proteins.

### *Hematoxylin and Eosin*

Hematoxylin and eosin (H&E) is the gold standard for pathology and is viewed as an excellent choice for LMD (Erickson, et al., 2009). Hematoxylin is a water soluble, basic stain which stains nuclei blue. Eosin is ethanol soluble, acidic, and stains cytoplasm and collagen pink. Eosin is an intense stain which shows fluorescence, and therefore may interfere with downstream fluorescent applications such as TaqMan qRT-PCR. As a result, the eosin stain time was decreased to 5 seconds from the traditional H&E pathology stain eosin time of 1 minute. Modified H&E staining yielded satisfactory RNA

quality, and exceeded the published reports of RNA quantity obtained via LMD during spermatogenesis. It is also known to provide good quality of DNA and proteins.

*Toulidine Blue*

Toulidine Blue is a one step stain that is reported to provide excellent integrity of RNA, DNA, and proteins. The color depends upon the chemical properties of the tissue. Histogene (Molecular Devices, 2009) is a trademark staining process that optimized the use of toulidine blue for LMD. Histogene claims to meet the demand for acceptable LMD tissue morphology within the context of the recovery of maximal RNA. It is described as a fast penetrating stain which limits exposure to water, and differentially stains the cell nuclei purple and the cytoplasm light pink. Histogene literature reports that it has been used successfully with mouse liver, kidney, brain, salivary gland, thymus, small intestine, and human foreskin. The mRNA profiles obtained from Histogene samples are reported to be free of degradation (Molecular Devices, 2009).

*Quantitative assessment of staining efficacy.* The original plan called for the use of the whole slide scrape test in order to assess the impact of competing staining protocols on the amount and the integrity of RNA. However, Histogene (Molecular Devices, 2009) modified Toulidine Blue for LMD was found to produce monochromatic staining of the cell stage specific gametes, which compromised the identification of cell morphology and organelles. As such, Histogene was eliminated from consideration thereby precluding the need for the whole slide scrape test.

*Qualitative assessment of staining efficacy.* A regular H&E slide (with longer staining than for LMD) with a cover slip was prepared as an archive slide to serve as a

reference and to facilitate the identification of the target cells of interest. While the initial plan called for the review of the archive slide with a pathologist or scientist trained in the histological identification of the target cells, project staff members were able to clearly identify and label the stage specific target cells of interest.

## *Laser Microdissection*

The Leica LMD 6000, the system available at Washington University, was used for this study. The Leica 6000 and 7000 are the only upright LMD systems that provide specimen collection by gravity. This provides contact free and contamination free specimens. In comparison the Arcturus PIXEL utilizes a cap to touch the sample and lifts the cells from the slide, while the Zeiss PALM catapults the cells upward to the collecting cap. There are anecdotal reports from LMD users that the catapulting action adversely affects the subsequent recovery of RNA (J. Hanson, personal communication, August 4, 2009).

### *LMD Systems*

All LMD systems contain a laser, a microscope for visualization, a computer, a movable stage for mounting slides, and a collection device to secure the collecting tubes or caps. The LMD platforms are divided into:

a) The pure infrared (IR) laser systems transfer energy at 810 nm wavelength directly to the tissue and to the PEN, POL, or PET membrane. These include the Arcturus manual Pixel, the automated Arcturus AutoPix, and the Biorad Clonis. IR systems do not allow the capture of single cells.

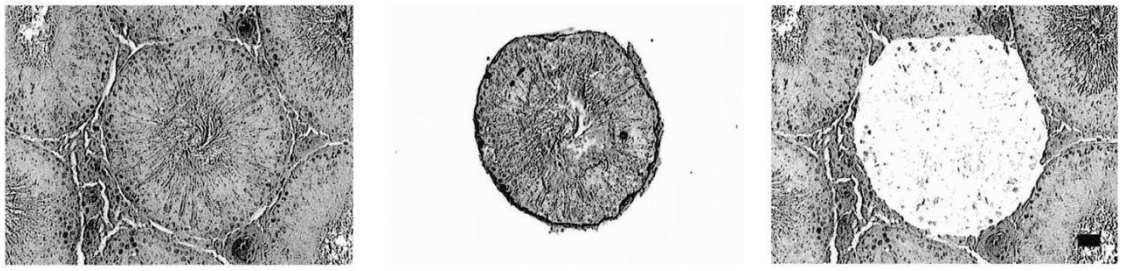
b) The pure ultraviolet (UV) laser systems transfer the laser energy to the membrane at 355 nm wavelength to cut around the tissue or cells of interest. The UV systems include the Leica LMD 6000 and 7000, the Zeiss PALM, and Molecular Machines CellCut. UV systems allow the dissection of single cells, which makes the UV system ideal for our applications.
c) The Arcturus VERITAS is a combined IR/UV system.

It is inevitable that the question will arise about the use of LMD for the pending toxicology protocols, which involve the in vivo exposure of male mice to TCDD. Because TCDD or other chemicals administered in vivo are embedded in the tissue, they will pose no unique LMD considerations. The recommendation is to use prudent laboratory practices which apply to the handling of all biologic tissues (J. Rodriguez-Canalas, personal communication. August 5, 2009)

*Quantitative assessment of LMD.* The LMD 6000 provided a running total $\mu m^2$ of LMD tissue. Therefore, existing recommendations called for the LMD researcher to measure the diameter of the target cells and divide the diameter into the total LMD area. In theory, this approach provided the approximate number of cells that were collected and allowed a rough estimate of the RNA yield. For example, if a typical cell contained 0.01 ng RNA, approximately 2000 cells would be needed to obtain 20ng total RNA. Thus, if the target cell was 3 µm in diameter, approximately 6000 µm of tissue would be needed (Espina, et al., 2006). This being said, these guidelines could inform a single cell population, but could not be directly translated into LMD isolation of stage specific male

gametes. For example pachytene spermatocytes contain tetrads, are characterized by their large size, and intense RNA synthesis.

*Qualitative assessment of LMD.* Figure 3 provided a graphic representation of LMD qualitative assessment. LMD qualitative assessment included: a) intact tissue before LMD, b) tissue after LMD, and c) tissue obtained by LMD. This provided visual confirmation that the LMD researcher captured the target cells. These images are expected in LMD publications.



| Intact testis cross section before LMD | Tubule cross section on LCM cap (Arcturus Pixel) | Remaining tissue section on slide after LMD |
|---|---|---|

*Figure 3.* The above images from Sluka and colleagues (2002) provided one example of a visual check point that is expected of the LMD researcher. Images are from the Arcturus PIXEL LCM.

### *RNA Extraction and Purification*

Any of the preceding steps from tissue harvesting to RNA extraction may lead to RNA fragmentation and degradation. Hence the challenge was to select the processes that would yield the maximal quantity and quality of RNA. There were various approaches to

RNA recovery. Phenol based extraction with ethanol precipitation is a standard approach for RNA isolation. However, when working with small amounts of RNA (< 5µg) as would be expected with LMD samples, it is necessary to add a co-precipitant to create a visible pellet (Barker, 2005). Next there are micro-column based RNA isolation and purification kits. Specifically the Arcturus PicoPure and Qiagen RNeasy micro kits were developed for applications such as LMD or fine needle aspirates which typically yield small amounts of cells or tissue.

*Quantitative Assessment of RNA*

The NanoDrop ND-1000 spectrophotometer provided the starting quantity of RNA ng per µl. It also provided the 260/280 ratio. The 260/280 ratio of absorbance at 260 and 280 nm was used to assess the purity of nucleic acids. A ratio of about 2 was indicative of pure RNA; with a ratio of about 1.8 accepted as evidence of the purity of DNA. Significantly lower ratios indicated the presence of proteins or other contaminants (Thermo Scientific, 2008). In addition, RNA quantity determined the appropriate Agilent 2100 E Bioanalyzer assay.

*Qualitative assessment of RNA*

Typically RNA quality is assessed by the presence of 18S and 28S ribosomal RNA (rRNA) bands during gel electrophoresis. A 28s/18s ratio $\geq$ 2 is considered evidence of high quality RNA. However the assessment was subjective, not comparable across labs, and did not generate digital data. In addition, LMD samples often surrendered limited quantities of RNA that were too low for use with conventional gel

electrophoresis. Therefore, the Agilent 2100 E was expected to be the preferred method of RNA qualitative assessment. The Agilent 2100 E Bioanalyzer assessed 18S and 28S rRNA, provided fragment length, and calculated the RNA integrity number (RIN). The RIN ranged from1 (totally degraded) to 10 (totally intact). A RIN $\geq$ 5 was indicative of acceptable RNA integrity and RIN $\geq$ 8 was considered excellent. Consistent with the recommendations of Erickson and colleagues (2009), the method of quantitative and qualitative assessment was consistent throughout the study for all tissue samples (Erickson, et al., 2009).

### *cDNA Synthesis*

Complimentary cDNA (cDNA) priming can occur either with random hexamers or oligo-dTs in the presence of reverse transcriptase. Oligo-dTs are used extensively in gene expression studies because it primes mRNA. While this presented obvious advantages for subsequent downstream gene expression assays, oligo-dTs required intact mRNA with a poly A (adenine) tail. Typically a string of 30 to 200 Adenine(s), is added to the 3' end of mRNA to protect the message from endonuclease activity. Thus, the length of the poly A tail determines how long the message will be translated (Passarge, 2007). Hence oligo-dTs are sequence specific, exhibit a 3' bias, and depend upon intact mRNA with a poly A tail.

However protocol specific circumstances argued against the use of oligo-dTs to either isolate mRNA or generate cDNA. First not all mature mRNA transcripts have a poly A tail. Furthermore ribosomal RNA does not contain a poly-A tail; and ribosomal 18s was the default endogenous control used in the ABI custom qRT-PCR plates. Next

oligo-dTs exhibited 3'bias and increased the chance of 5' secondary structures such as hairpin formations. This was attributed to reagent exhaustion, as cDNA was generated in a linear sequential fashion extending from the poly A tail. As a result, PCR primers and probes displayed decreased efficacy when they are designed to anneal to sequences which are located at distances greater than 415 base pairs (bp) from the poly A tail (Resuehr & Nikolai-Spiess, 2003). Finally the use of oligo-dTs for cDNA generation required high quality RNA and full length transcripts, and it was not the preferred choice for samples where there is limited quantities of RNA or if the RNA is fragmented (Bustin & Nolan 2004; Erickson et al., 2009).

In comparison, random hexamers removed the 3' bias, to non-preferentially generate cDNA throughout the transcript sequence. Hence random hexamers were the preferred reverse primers for fragmented RNA (Erickson et al., 2009). However, random primers also make cDNA from ribosomal and transfer RNA to result in a much more abundant and complex RNA population. It is estimated that 83% of total cellular RNA is ribosomal RNA, 15% is transfer RNA, with messenger RNA at 2%. This increased the risk of false priming in the subsequent PCR assay (Shipley, 2006).

There were two other approaches to cDNA synthesis that deserve to be mentioned. The first is assay specific primers that are specific to the target gene of interest. This approach required greater RNA abundance since each reverse transcriptase assay can only be used for one assay. In comparison oligo-dTs and random hexamers allowed the flexibility to generate non specific cDNA that can be used for multiple assays. A second approach was Quantiscript reverse transcriptase (Quiagen). This

approach generated cDNA by combining oligo-dTs with random hexamers. However this approach cannot be recommended due to the aforementioned oligo-dT limitations,

*Quantitative and qualitative assessment of cDNA*

This being said, the use of random hexamers over-estimated the quantity of cDNA generated from mRNA necessitating caution with the use of the NanoDrop ND-1000 to quantify cDNA. As a result, the NanoDrop ND-1000 spectrophotometer measurement focused on the 260:280 ratios as an indicator of cDNA purity. A 260/280 ratio of about 1.8 was accepted as pure DNA (Thermo-scientific, ND-1000 2008).

*One Step vs. Two step cDNA and qRT-PCR Assay*

The one step assay would combine reverse transcriptase (RT) for cDNA synthesis, with PCR in a single tube. If used, all reagents would be added at the same time. This approach offered to reduce time and potential contamination. However, the one step approach required more sample RNA and was limited to target specific primers, thereby restricting the generation of cDNA to the preselected genes of interest. It also had the potential to lead to extensive primer dimer formation to result in non specific product amplification which would exhaust the limited amount of reagents and compete with the target gene amplification. Finally, the one step approach did not provide the opportunity to segregate the reactions in order to optimize conditions.

*qRT-PCR*

Our future toxicology protocols called for the assessment of differential gene expression in exposed and non exposed samples. Because RNA quantity from microdissected samples was limited, it was imperative that primer and probe sets be tested to be sure that they were amplifying the genes of interest. While the original plan called for the testing of four to six primer pairs per target gene (Erickson, et al., 2009), ABI quality control processes assured 100% efficiency ($\pm$ 10%) and that the primer and probe sets amplified only the target genes of interest. All ABI TaqMan® Gene Expression Assays have been designed via validated bioinformatics to run with the same PCR conditions (ABI, 2010), thereby eliminating the need to optimize PCR conditions or to test four to six primer pairs per target gene. For the purpose of this study, primer sets were designed to amplify selected genes known to be differentially expressed during spermatogenesis. This served as verification of the successful capture of the cells of interest.

*Quantitative assessment.* The intent was to use qRT-PCR to determine relative quantification via $\Delta$ CT (crossing threshold) which was the most common approach to calculating fold change. This method required that the gene of interest be compared to an endogenous control. However, target qualitative RNA measures were not achieved in all cell stages necessitating a delay in qRT-PCR. Instead standard PCR was combined with gel electrophoresis for qualitative assessment of gene expression.

*Normalization for Relative Quantitative Assessment*

Because this study called for the use of relative quantification, it was necessary to begin with the determination of the most appropriate endogenous control (housekeeping gene). The choice of endogenous control required stable expression across tissues and that was not altered by experimental conditions. However housekeeping genes were known to be tissue specific, with suggestions that they were also developmentally expressed. Therefore the choice of endogenous control gene/s was validated prior to selecting the endogenous control for relative quantification. A starting point was the work of Radonic and colleagues (2004) who reported that RNA polymerase II (*RpII*) was stable across tissues, and that glucose 1-dehydrogenase (*G6pd*) did not vary by experimental conditions. Another avenue was glyceraldehyde-3-phosphate dehydrogenase (*Gapdh)* and beta actin (*β-actin*) which was used by Sluka and colleagues in their continued studies of the rat seminiferous epithelium (P. Stanton, personal communication, August 6 2009). *Gapdh* was normally considered to be a housekeeping gene with its expression used as an endogenous control for calibration. However *Gapdh* was inactivated during meiosis, and post meiotic glyceraldehyde-3-phosphate dehydrogenase, spermatogenic (*Gapds)* was expressed in the early post meiotic stages (Eddy, 2002). In contrast *β-actin* demonstrated stable expression across all stages of murine spermatogenesis (Wang, Page, & McCarrey, 2005).

*Number of Animals Needed*

A power analysis cannot inform the number of mice that were needed to optimize the sequential methods of LMD and the selected downstream applications. These

numbers were based upon the need to a) maintain a constant supply of mice that are at the optimal reproductive age, b) optimize and validate methodology before proceeding to the future exposure of mice to the environmental agent, c) preserve precious limited sample until the study is undertaken, and d) the expertise of Dr. Kelle Moley who has more than 20 years experience with mouse reproductive techniques. We estimated that we would need five male mice per week x 52 weeks (260 animals).

# CHAPTER FOUR

## Results

There were a wide variety of tools and approaches that were available to assess gene expression in biological samples. The choice depended not only upon the research questions and scientific aims but also on the knowledge of: a) the products, b) the equipment purposes and limitations, and c) the recognition that the wide variety of biomolecular techniques do not have universal applications. In addition, the varied LMD platforms and the preceding, and the subsequent downstream applications, each had the potential to either enhance or compromise all subsequent downstream applications. Moreover, the precious limited sample that resulted from LMD demanded due diligence in assay selection, development, and modifications. Each of these decisions was rooted in histology, molecular and developmental biology, anatomy, and the technology of the specific LMD system. In addition, the myriad of steps were implemented using stringent RNA technique to protect the precious limited sample, coupled with a constant awareness of time in order to protect the integrity of the RNA (see Appendix B for the protocol timeline). Assay validation was completed in the following manner, either alone or in combination.

a) Extensive review of existing literature and protocols.
b) Discussion and correspondence with experts.
c) NIH LMD training August 4$^{th}$ to 7$^{th}$ 2009.
d) Information gleaned from bioinformatics databases to include: a) Mouse Genome Database ([MGD] Bult, Eppig, Kadin, Richardson, & Blake, 2008), b)

Mammalian Reproductive Genetics Database ([MRG] University of Washington Seattle, 2009), and c) NIH NCBI DNA & RNA Nucleotide Database (National Library of Medicine, 2010).

e) The use of Applied Biosystems (ABI) bioinformatics (2010) to create custom designed protocol specific qRT-PCR plates.
f) The selection of optimized TaqMan primers, probes, and assays using ABI bioinformatics (ABI, 2010).
g) Assay development and modification in the presence of absent or conflicting information.
h) Collaboration with research team members.

### *Research Questions and Specific Aims*

The stated purpose of this dissertation was to determine the capacity of LMD to isolate stage specific male gametes for the purpose of gene expression assays; initially via qRT-PCR and ultimately by gene expression microarray. The research questions and scientific aims provided the organizational template for presenting the data. Moreover, because LMD formed the foundation for the funded VISN 15 and HSRD toxicology protocols; reliability, reproducibility, and validity must be transparent.

#### *Research Question 1*

What was the effect of tissue sampling methods, sectioning, and staining on the histology of the seminiferous tubule, and on the morphology of stage specific male germ cells?

*Sample processing.* With the exception of cervical dislocation; $CO_2$, inhaled anesthetics, and injected barbiturates either directly, through the use of chemical agents, or indirectly, through extended time to death or altered physiological processes, adversely impact gene expression. In addition, cell and tissue destruction in the testes resulted from the actions of endogenous enzymes in the testes to compromise cell morphology and tissue histology within 10 minutes of either surgical resection or euthanasia. As a result, cervical dislocation was the preferred method of euthanasia. The University of Missouri – St. Louis, St. Louis VA Medical Center IACUC, and Washington University Animal Assurance approved cervical dislocation as the method of euthanasia for mice that will be utilized for LMD and gene expression assays (see Appendix A).

*Tissue preservation.* The evidence presented in chapter three was convincing. Neither FFPE nor ethanol fixed paraffin embedded tissues should be used with LMD samples when gene expression assays were the intended downstream application. FFPE resulted in extensive RNA fragmentation (Espina et al., 2006) and ethanol fixation with paraffin embedding resulted in a significant drop in mRNA transcripts (Perimutter, et al. 2004). As a result, snap freezing of the testes as tissue blocks embedded in OCT was the method of sample preservation. Tissue blocks were created by the immediate dissection and recovery of the testes following protocol approved euthanasia. Testes were then placed in a 25x20x5mm cryomold that was half filled with OCT, then covered with OCT, and immediately immersed in a beaker surrounded by dry ice and isopentane. The tissue blocks were covered with aluminum foil, transferred on dry ice, and subsequently stored at $-80^0$ C. Tissues remained frozen at all times until staining.

*Slide selection.* Leica LMD required the use of specially prepared slides in order to free the laser dissected tissue, from the seminiferous tubule cross sections. The UV absorbing PEN membrane was sealed to the edges of the glass slide resulting in a 1 µm space between the center of the slide and the PEN membrane. When the laser was fired, the membrane and the tissue in the cut line were ablated, thereby freeing the tissue which was supported by the PEN membrane to drop into the cap of a 0.5 ml microcentrifuge tube which was loaded with the PicoPure extraction buffer. Additional UV pre-treatment of the slides for 30 minutes was used to enhance adhesion of the tissue sections to the slide membrane (F. Schottler, personal communication, April 9 2010).The polymer was inert, and did not interfere with downstream applications.

*Cryosectioning and tissue alignment.* Typically cryosectioning for LMD recommended 3 to 5 µm cuts for ethanol or FFPE tissues. Consistent with the recommendations of Erikson and colleagues (2009), OCT embedded tissue blocks were cut at 8 µm. Excellent morphology was achieved with 8 µm cuts when combined with the optimized H&E staining. Spermatogonia, spermatocytes, and spermatids were clearly visible; and the sertoli cell bodies and nuclei were clearly evident at the basement membrane. Variances in the storage of frozen sections were explored through qualitative and quantitative RNA measurements. The storage of the unstained PEN membrane slide mounted frozen sections overnight at -80 C did not significantly impact RNA measures. However the overnight storage of frozen sections on PEN membrane mounted H&E stained slides, resulted in ice crystal formation with visible artifact attended by marked RNA degradation. These findings were consistent with the literature which reported that

freezing, thawing, and refreezing of tissue created ice crystals and compromised RNA integrity (Carson, 1996).

Each end of the convoluted loop that comprised the seminiferous tubule opens into the rete testis. The rete testis collects and concentrates sperm, and delivers sperm to the caput epididymis via the efferent ducts of the testis. The rete testis is located on the epididymal side of the testis and is parallel to the long axis of the testis. Therefore, the anatomy suggested that longitudinal alignment was the preferred approach for cryosectioning. This approach maximized the presence of intact seminiferous tubules and enhanced visualization. The cross sections contained clear cellular morphology and the seminiferous tubules appeared round and tightly compact. Yet at times the cross sections of the seminiferous tubules displayed an elongated, oval shape. While the convoluted pattern of the seminiferous tubules did result in seminiferous tubules being cut at oblique angles, the tubule distortion was intensified at the periphery of the testes where the seminiferous tubules were less densely packed. Therefore, the peripheral cross sections were discarded in favor of central cross sections. This differed from the recommendation to create archive slides from cross sections (cuts) one, five, and ten (Erickson et al 2009).

*Staining.* The goal of LMD staining was to retain acceptable morphology while maximizing the recovery of nucleic acids (J Rodriguez-Cannalas, personal communication, August 5 2010). As a result, the whole slide scrape test was proposed as a method to guide staining selection through the use of quantitative and qualitative RNA measures. However it was discovered that Histogene modified toulidine blue for LMD staining was monochromatic, which compromised the ability to identify cell morphology

and organelles. While Histogene may provide satisfactory visualization in uniform cell populations, it did not provide satisfactory discrimination in complex tissues such as the testes in which there were somatic cells and germ cells in various stages of development. Morphology was the deciding factor in LMD success.

As suggested by Sluka and associates (2008), 4′,6-diamidino-2-phenylindole, dihydrochloride (DAPI) nucleic acid stain (Invitrogen D1306) diluted in 70% ethanol was also used for staining. The use of a nucleic acid stain mixed with ethanol presented an attractive alternative for protocols seeking to maximize RNA recovery. This approach offered the chance to reduce, and potentially eliminate water contact, thereby minimizing RNA degradation. However, the morphology was again compromised, and there were concerns that the DAPI fluorescence may interfere with qRT-PCR. As a result neither Histogene nor DAPI was acceptable for the staining of the differentiating cell types found in the seminiferous epithelium.

Therefore we developed a modified H&E protocol for LMD staining (see Appendix C). This protocol: a) favored the use of a modified Hematoxylin stain, CAT Hematoxylin, which added an acid to enhance nuclear staining; b) eliminated the Scott's Bluing treatment proposed by Erickson and colleagues (2009) which was a mild alkaline treatment that was used to enhance nuclear staining; and c) reduced the two 30 second water baths to one quick water dip. The rationale for this approach resided in the fact that RNA was unstable in the presence of a Ph $\geq$ 7, and the exposure to water degraded RNA (P. Esakky, personal communication, September 23 2010). These modifications provided excellent morphology (see Figure 4) for LMD with improving RNA quantitative and

qualitative measures. The discovery that the morphology was unacceptable in the presence of Histogene or DAPI staining obviated the need for the whole slide scrape test.

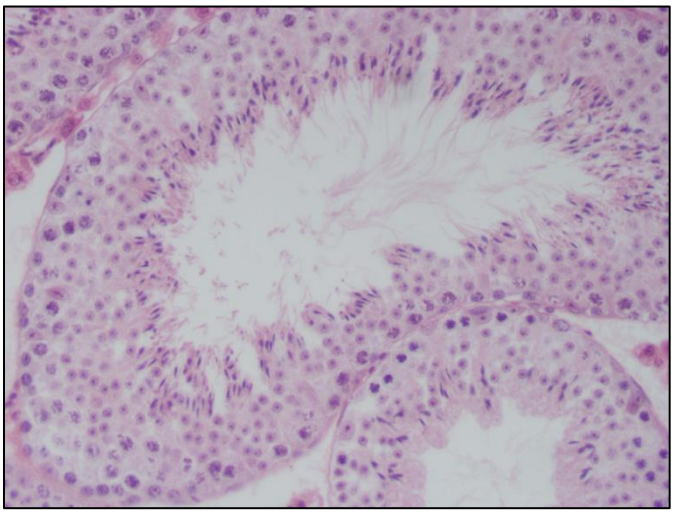


*Figure 4.* This 40x magnification image of a CAT modified H&E stained section demonstrated mouse seminiferous tubule cross sections. Photo courtesy of P. Esakky.

*Cell stage identification.* Here it was important to recall that the goal of LMD staining was to maintain adequate cell morphology within the context optimal RNA. As such, we were able to clearly identify the major cell stages (spermatogonia, spermatocytes, and spermatids) by common characteristics and location, but were not always able to differentiate cell stage subpopulations. This being said, type B spermatogonia were easily identified. They were round, with dark nuclear staining, and appeared clustered along the perimeter of the basement membrane. Conversely, the sertoli cell bodies were less distinct, asymmetrical and irregularly spaced; and appeared to support the clusters of spermatogonia along the basement membrane. In the same manner, the finger-like projections of the sertoli cells were not clearly visible, but were

implied by the alignment of the differentiating male gametes as they migrated towards the lumen of the seminiferous tubule.

The spermatocyte phase corresponded to meiosis I and II and was characterized by multiple cell substages. As a result, the spermatocytes varied in appearance and it was not always possible to differentiate spermatocyte subpopulations. Preleptotene spermatocytes contained a round spherical nucleus (similar to type B spermatogonia) and were located in the basal compartment of the seminiferous epithelium. Pachytene primary spermatocytes were characterized by their large shape and loss of contact with the basement membrane. As a result, size and location were paramount in spermatocyte identification. The elongated spermatids displayed extensive cell remodeling and differentiation and were easily identified in the adluminal region of the seminiferous epithelium. Pachytene spermatocytes, round and elongating spermatids were found in the adluminal compartment of the BTB. The alignment of the spermatogonia in relation to the location of the pachytene spermatocytes provided the location of the BTB (see Figure 5).

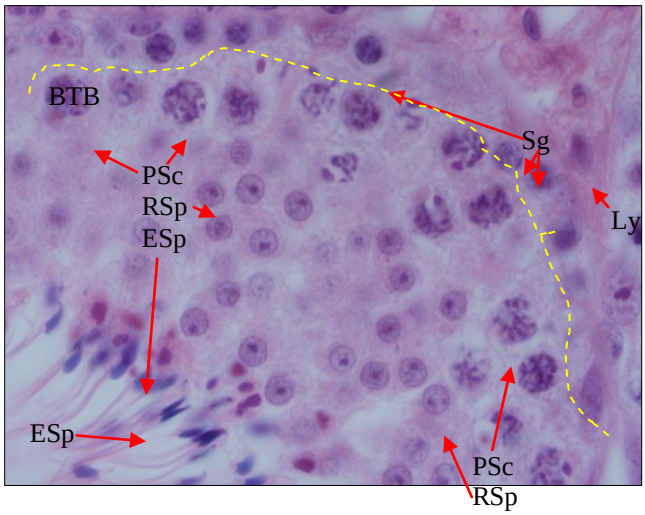



*Figure 5.* 100x FFPE H&E archive slide with cell identification. Photo courtesy of P. Esakky.

*Archive slide.* The LMD researcher must recognize the target cells of interest. To this end, H&E archive slides, reviewed and labeled by a pathologist or similar expert, can provide a valuable visual reference. This being said, the distinct cellular morphology of the male germ cells coupled with the germ cell specific locations within the seminiferous epithelium negated the need for an archived reference. Both members of the research team were able to independently identify and microdissect spermatogonia, spermatocytes, and spermatids.

*Leica LMD 6000.* Initially one slide was obtained from one testis, and two slides were utilized for one LMD session. The total time was 1 ½ hours from the end of staining to the end of the LMD session. The H&E stained PEN membrane mounted slides were transported in a slide box in the presence of Drierite indicating dessicant. Drierite absorbs ambient moisture thereby maintaining the integrity of the RNA. The slides were then placed, membrane mounted (and tissue section) side down into the Leica LMD 6000 slide

holding platform. Using the 4x objective, an overview image of the slides was created in order to facilitate image (and sample) navigation. The draw and cut feature was then used to dissect the cells using the 40x objective. Recalibration of the laser was performed at every objective change.

In addition to the draw and cut feature which allowed the LMD researcher to define the microdissected shape (see Figure 11), the LMD 6000 sample collection platform was ideal for cell stage segregation. The LMD 6000 sample collection platform held up to four separate tubes (caps) for sample collection. The 0.5 flat cap PCR tubes were preloaded with 50 µl Picopure RNA extraction buffer. Cell specific labels were then applied to the collecting tubes and the subsequent microdissected samples: a) spermatogonia (sg), b) spermatocytes (sc), and c) spermatids (sp). Thus, clicking on the designated collection cap activated the LMD draw and cut feature. The LMD cuts then corresponded to the cell specific labels. This feature also allowed all three cell stages to be dissected simultaneously. This not only saved time and protected the integrity of the RNA, but also reduced the number of animals needed from five mice per week to three mice per week which was in keeping with the animal welfare principals of refinement, reduction, and replacement.

However, the initial NanoDrop ND-1000 results were substantially less than target 260:280 ratio of 2 ($\pm$ 10%). To reduce time further, only one slide was prepared, and two cross sections per slide were used for each LMD session. The time was thereby reduced to 45 minutes from the completion of staining to the end of the LMD session. This approach was consistent with the recommendation of Erickson and colleagues

(2009) to reduce LMD time to 45 minutes as one approach to optimize RNA. As expected, the 260:280 ratios improved, but further optimization was needed (see Table 1).

Table 1.

*NanoDrop ND-1000 RNA quantity and 260:280 ratios.*

| Sample | Cell ID | RNA Quantity ng/µl | 260:280 |
|---|---|---|---|
| 1 | Sg | 20.33 | 0.89 |
| | Sc | 7.43 | 0.86 |
| | Sp | 5.78 | 1.02 |
| 2 | Sg | 5.26 | 1.5 |
| | Sc | 16.34 | 1.49 |
| | Sp | 10.8 | 1.41 |
| 3 | Sg | 3.37 | 1.65 |
| | Sc | 4.55 | 1.53 |
| | Sp | 2.68 | 1.25 |

*Note*. Spermatogonia (Sg), Spermatocytes (Sc), Spermatids (Sp). Data courtesy of P. Esakky.

In addition, the interwoven cell to cell contact between the sertoli cells and the developing gametes presented distinct LMD limitations. Past work that addressed the use of LMD for stage specific male germ cell gene expression assays either used the Arcturus PIXEL to dissect: a) entire cross sections of the seminiferous epithelium (Sluka et al., 2002) or b) used the Zeiss PALM to collect regional zones known to be populated by the

selected cell stages (Sluka et al., 2008). However both approaches were expected to result in somatic cell contamination with the potential to confound the ensuing gene expression assays. In response we used the cell stage specific morphology, the cells' location, and directional alignment within the seminiferous tubules to dissect clusters of spermatogonia circumferentially along the basement membrane. In comparison, clusters of spermatocytes and spermatids were dissected directionally as the cells progressed toward the lumen of the seminiferous tubule (see Figure 6).

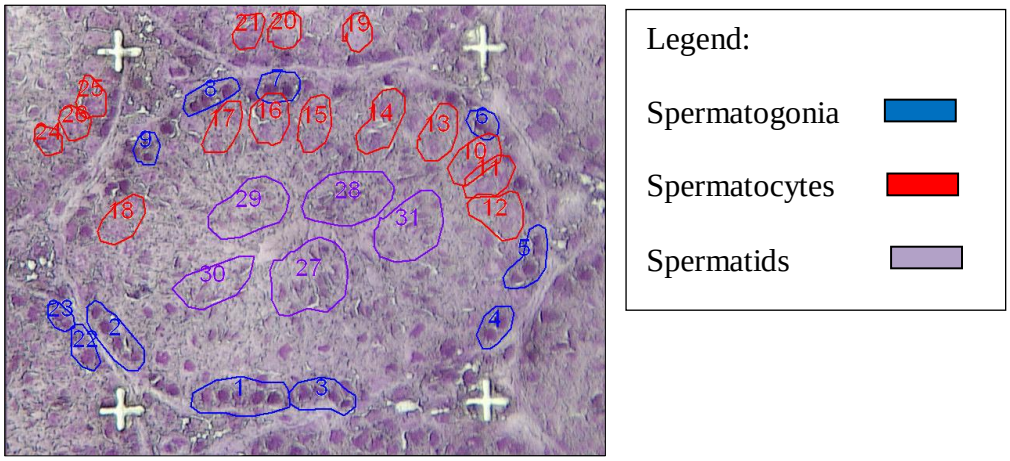



*Figure 6.* Cross section of modified H&E stained seminiferous tubules as seen with the Leica LMD 6000 40x before activating the LMD laser. Photo courtesy of P. Esakky.

This approach allowed: a) the maximal dissection of spermatogonia without sertoli cell contamination, b) reduced LMD time and protected the integrity of the RNA, c) increased RNA yield, and d) utilized the recommendation to cut wide margins around the target cells to avoid cell damage when using a UV laser. Thus, the enhanced margins effectively ablated the finger-like projections of the sertoli cells that maintained contact with the spermatocytes and spermatids (see Figure 7). At this writing there were no

published articles that described either LMD cluster dissection of stage specific male gametes from the seminiferous epithelium, or the use of the LMD 6000 for gene expression during spermatogenesis.

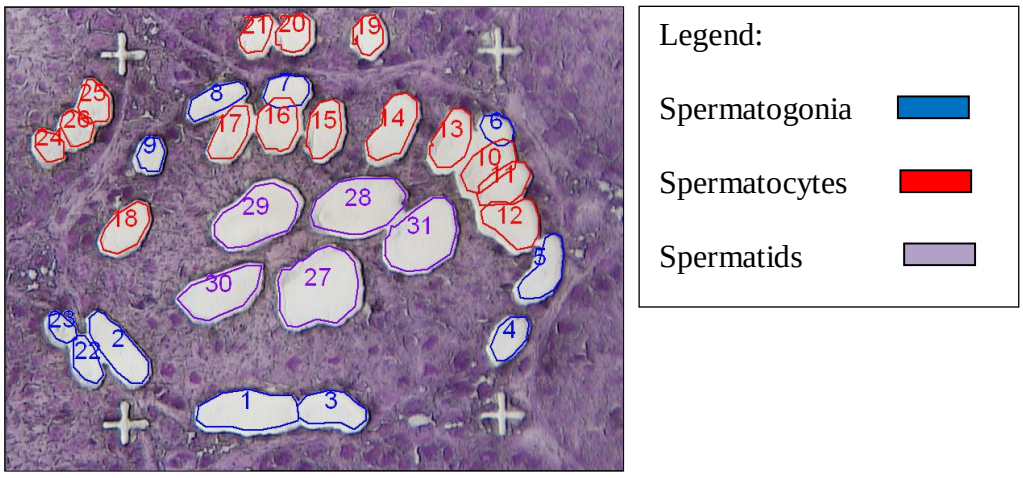



*Figure 7.* Cross section of the seminiferous tubules as seen with the Leica LMD 40x magnification after activating the laser. This served as validation of the successful capture of the target cells. Photo courtesy of P. Esakky.

While single cell LMD would certainly produce a pure germ cell population, it would also greatly increase the time spent in LMD with the potential to compromise the integrity of the RNA. Previous estimates suggested that approximately 2000 cells (assuming cells were 3 µm in diameter) would be required to obtain 20 ng/µl RNA, and that it was likely that many times more would be needed (Espina et al., 2006). It must be remembered that cell size and chromosome numbers (2n for spermatogonia, 4n for spermatocytes, and 1n for spermatids) varied as the developing male gametes transitioned through the various cell stages. As a result, estimated LMD RNA yield per cell stage could not be obtained by measuring the diameter of the target cells and then dividing the

cell diameter into the total LMD area. This being said, the following LMD area totals ($\mu m^2$) were obtained on September 11, 2010 during one LMD session: a) spermatogonia 24017 $\mu m^2$, b) spermatocytes 77498 $\mu m^2$, and c) spermatids 23216 $\mu m^2$. These numbers are consistent with the changes in cell size as the cells transition through spermatogenesis, but interestingly do not correlate with increased RNA yield (see Table 2).

Table 2

*The most recent NanoDrop ND-1000 RNA quantitative assessment with CAT modified H&E staining.*

| Cell ID | Quantity ng/µl | 260:280 |
|---|---|---|
| Sg | 15.7 | 1.5 |
| Sc | 2.82 | 1.75 |
| Sp | 23.5 | 1.5 |

*Note.* Spermatogonia (Sg), Spermatocytes (Sc), Spermatids (Sp). Data courtesy of P. Esakky.

While progress was made with the most recent 260:280 ratio at 1.5 for spermatogonia and spermatids, and 1.75 for spermatocytes, RNA quality did not achieve the target 260:280 ratio of 2 $\pm$ 10%. Modifications that were considered included:

a. Increased water dip time to eliminate the OCT. The water steps were necessary to remove OCT which interfered with downstream measures (Erikson et al. 2009).

b. The use of DAPI staining in the absence of OCT. The modified DAPI stain proposed by Sluka and colleagues (2008), mixed DAPI with ethanol, and eliminated any sample water contact.
c. The elimination of OCT for flash freezing in favor of isopentane. This eliminated the need for the water dips during staining.
d. Delay or eliminate the use of the Agilent Bioanlayzer in the presence of other indicators of high quality RNA.

*Research Question 2*

What factors influenced the quality, the quantity, and the amplification of nucleic acids from LMD tissue?

*RNA recovery.* The Qiagen RNeasy micro kit promoted the selective binding of RNA to the mini elute spin column which contained a silica based membrane. This technology preferentially selected mRNA by excluding RNA molecules that were less than 200 nucleotides (nt) in length. Thus the procedure selected for mRNA since molecules less than 200 nucleotides were excluded. This process was expected to result in decreased RNA quantity with enhanced RNA quality. However, the fragmented nature of RNA recovered from LMD samples (less than 200 nt) prevented RNA recovery by this method, and was expected to result in a further reduction in RNA yield. Therefore the RNeasy Micro kit was removed from consideration.

The PicoPure RNA isolation kit was developed for applications such as LMD in which RNA recovery was expected to be on the picogram to nanogram scale. It was

developed specifically for use with the Arcturus PIXEL LCM Capsure system, but can be modified to meet other LMD specifications. As such, protocol C from the PicoPure RNA isolation kit was adapted for the Leica LMD 6000. The major modification included the loading of 50 µl of the extraction buffer (XB) into the cap of the 0.5 tube cap. The cap was then loaded into the Leica LMD 6000 collecting system so that the open cap faced upward (while the membrane mounted slides faced downward) to collect the LMD samples. It should be noted that the failure to understand the Leica LMD system could result in protocol failure through the use of PicoPure protocol A or B which called for the loading of 10 µl of extraction buffer which would lead to RNA degradation related to extraction buffer evaporation.

*cDNA synthesis*. Protocol specific circumstances argued against the use of oligo-dTs to synthesize cDNA. First, oligo-dTs required mRNA with a poly A tail. However not all mature mRNA transcripts have a poly A tail. It was interesting to note that four of the six genes selected to validate stage specific LMD of male germ cells lacked polyadenylation. This not only implied that the mRNA was vulnerable to rapid degradation via endonuclease and resulted in restricted protein production as a result of limited translation (Eddy, 2002); but informed the methods used in male germ cell gene expression assays. In the same manner ribosomal 18s was the default endogenous control used in the ABI custom qRT-PCR plates. By definition, ribosomal RNA lacks a poly-A tail. In addition, oligo-dTs required high quality RNA with full length transcripts, and LMD samples often contain limited RNA quantity with fragmentation (Bustin & Nolan, 2004; Erickson, et al., 2009). Consequently, the use of oligo-dTs for cDNA synthesis in

male germ cell gene expression assays would result in compromised data, and were therefore removed from consideration.

In comparison, random hexamers removed the 3' bias to non-preferentially generate cDNA throughout the transcript. Hence random hexamers were used as the reverse primers for LMD samples and male germ cell gene expression assays. Then again, it was also known that the use of random hexamers created a complex total RNA population which over-estimated the quantity of cDNA. This required caution when using the NanoDrop ND-1000 to quantify cDNA. As a result, the NanoDrop ND-1000 spectrophotometer measurement focused on the 260/280 ratio as an indicator of cDNA purity, with a 260/280 ratio of about 1.8 generally accepted as pure DNA (NanoDrop ND-1000 2008, p 5.2). The 260/230 ratio represented a secondary measure with values between 2.0 and 2.2 indicative of pure nucleic acids. Lower values indicated the presence of contaminants which included phenolic solutions or quanidine isothiocyanate which are found in both the Trizol and the RNeasy Micro kit.

Furthermore, RNA (either from LMD cell stage or from total testes RNA samples) should not undergo a collective RT reaction. Instead cDNA synthesis should occur via individual RT reactions (see appendix D for RT protocol with modifications). This approach not only prevented the loss of precious limited RNA as a result of error during cDNA synthesis, it also prevented repeated freezing and thawing of the sample with resultant degradation. Therefore, the prepared 100 µl volumes of total testes RNA serial dilutions (see normalization strategy serial dilutions), were subdivided into 20 µl aliquots. 4 µl of each aliquot was then used for NanoDrop ND-1000 and Agilent 2100 E

Bioanalyzer quantitative and qualitative measures (see Tables 3 through 5). This resulted in approximately 16 individual RT cDNA reactions with some loss due to pipetting error or evaporation.1 µl RNA was used per each 20 µl RT reaction. cDNA was then stored at $-20^0$ C until used as the positive controls for qRT-PCR.

Table 3

*NanoDrop ND-1000 results cDNA from mouse total testes RNA 50 ng/µl serial dilutions*

| Sample # | 260 abs | 260/280 | 260/230 | Ng/ µl |
|---|---|---|---|---|
| 1 | 31.185 | 1.75 | 2.11 | 1029.1 |
| 2 | 39.98 | 1.76 | 2.17 | 1319.1 |
| 3 | 26.068 | 1.66 | 1.94 | 860.2 |
| 4. | 24.862 | 1.66 | 1.84 | 820.5 |
| 5. | 53.75 | 1.78 | 2.20 | 1774.9 |
| 6 | 39.403 | 1.75 | 2.15 | 1300.3 |
| 7 | 31.165 | 1.75 | 2.12 | 1226.4 |
| 8 | 31.319 | 1.75 | 2.14 | 1033.5 |
| 9 | 39.269 | 1.76 | 2.14 | 1295.9 |
| 10 | 26.443 | 1.67 | 1.99 | 872.6 |
| 11 | 37.830 | 1.76 | 2.14 | 1248.4 |
| 12 | 0.952 | 0.66 | 0.34 | 31.4 |
| 13 | 27.447 | 1.67 | 1.77 | 905.7 |

Table 4

*NanoDrop ND-1000 results cDNA from mouse total testes RNA 20 ng/µl serial dilutions*

| | 260 abs | 260/280 | 260/230 | Ng/ µl |
|---|---|---|---|---|
| 1 | 19.456 | 1.78 | 1.81 | 642 |
| 2 | 18.964 | 1.76 | 1.77 | 625 |
| 3 | 19.101 | 1.75 | 1.77 | 630.3 |
| 4. | 20.390 | 1.76 | 1.79 | 672.9 |
| 5. | 19.449 | 1.76 | 1.77 | 641.8 |
| 6 | 19.80 | 1.77 | 1.91 | 653.4 |
| 7 sample loss | | | | |
| 8 | 20.341 | 1.76 | 1.84 | 671.3 |
| 9 | 18.878 | 1.76 | 1.77 | 623 |
| 10 no sample | | | | |
| 11 | 29.036 | 1.73 | 1.73 | 958.2 |
| 12 | 16.967 | 1.77 | 1.73 | 559.9 |
| 13 | 19.041 | 1.75 | 1.72 | 595.3 |
| 14 | 18.112 | 1.77 | 1.82 | 597.7 |
| 15 | 18.056 | 1.76 | 1.75 | 595.8 |
| 16 sample loss | | | | |

Table 5

*NanoDrop ND-1000 results cDNA from mouse total testes RNA 10 ng/µl serial dilutions*

| | 260 abs | 260/280 | 260/230 | Ng/ µl |
|---|---|---|---|---|
| 1 | 44.254 | 1.81 | 2.17 | 1308.4 |
| 2 | 38.607 | 1.79 | 2.14 | 1460.4 |
| 3 | 38.953 | 1.80 | 2.13 | 1285.4 |
| 4. | 39.909 | 1.80 | 2.15 | 1317 |
| 5. | 37.613 | 1.81 | 2.12 | 1241.2 |
| 6 | 43.109 | 1.80 | 2.20 | 1422.6 |
| 7 | 42.795 | 1.80 | 2.12 | 1412.2 |
| 8 no sample | | | | |
| 9 | 40.694 | 1.80 | 2.15 | 1342.9 |
| 10 | 40.685 | 1.79 | 2.11 | 1342.6 |
| 11 | 48.461 | 1.78 | 2.11 | 1599.2 |
| 12 | 40.898 | 1.79 | 2.14 | 1349.6 |
| 13 | 41.841 | 1.81 | 2.14 | 1380.7 |
| 14 | 38.701 | 1.79 | 2.07 | 1277.1 |
| 15 | 41.727 | 1.8 | 2.09 | 1377 |

When compared to Tables 3 and 4, the results in Table 5 indicated robust cDNA synthesis in the presence of reduced mRNA (10 ng/ul). However, it must be remembered that the NanoDrop ND-1000 measured all single strand nucleic acids which included any remaining RNA and dNTPs. Yet, the amount of dNTPs was consistent across assays, and SuperScript III RT required high temperature incubation and inactivation ($50^0$ C and $70^0$ C for 45 minutes and 15 minutes respectively). RNase out was used as an added precaution to clean up any remaining dNTPs and RNA. It was therefore unlikely that any residual RNA or dNTPs remained. However, it was likely that the increased cDNA synthesis was related to the reuse of the random hexamers. The reuse of the random hexamers resulted in one additional freeze/thaw cycle and evaporation, thereby increasing the random hexamer concentration. The above information also provided direct evidence of the importance of qRT-PCR normalization strategies that use equal amounts of starting cDNA (Erickson et al., 2009). Unequal cDNA amounts may result in differences in the qRT-PCR crossing threshold (Ct) to appear as biologic fold changes which could erroneously be interpreted as gene expression changes. Higher cDNA concentrations can result in more starting sample, which in turn can lead to a lower crossing threshold ($C_t$) due to earlier amplification. So what appeared to be $C_t$ differences and biologic fold changes may well be the result of differences in the quantity in the starting template. Tables 3 through 5 also provided guidance in the selection of the total testes cDNA samples for use as positive controls in the subsequent qRT-PCR assays.

*Research Question 3*

What were the optimal conditions for qRT-PCR from LMD samples of stage specific male germ cells and of the seminiferous epithelium? It was therefore necessary to select genes with known stage specific expression patterns in order to validate the successful LMD of the stage specific male gametes.

The stated purpose of this research was to determine the capacity of LMD to capture stage specific male gametes for the purpose of gene expression assays; initially via qRT-PCR and ultimately by gene expression microarray. The genes were selected by an extensive review of the literature. The expression patterns were subsequently confirmed using The MRG bioinformatics database (University of Washington Seattle, 2009). Much of the information in the MRG database originated with the gene expression microarray experiments of Shima, McLean McCarrey, and Griswold (2004) which characterized murine gene expression in the mouse testes. However there were times when the expression patterns in the literature and the MRG bioinformatics database differed, and the splice variants of genes were not always stated. Thus, genes were selected according to the following criteria to assure the utility of the animal data: a) the presence of conserved homology across species, b) differential expression by the stage of germ cell development, c) to the extent possible, male germ cell specific transcripts, and d) when needed, independent verification. Independent verification was undertaken when the established literature and the MRG database disagreed, or when the MRG data base and Shima, et al., (2004) were the sole sources of information.

*Spermatogonia.* When seeking to validate stage specific gene expression assays via LMD, the identification of premeiotic male germ cell specific transcripts required

thoughtful consideration. Because spermatogonia undergo rapid mitosis and proliferation, it was expected that spermatogonia would share many somatic transcripts. Delta-like 1 homolog (*Dlk1*) was involved in cell differentiation and transformation and was a known participant in several differentiation processes such as wound healing, adrenal gland development, and adipogenesis. In 2004, gene expression microarray characterized *Dlk1* expression during spermatogenesis (Shima, et al., 2004). *Dlk1* was highly expressed in type A spermatogonia, peaked in type B spermatogonia, and displayed a precipitous 25 fold decline in pachytene spermatocytes (University of Washington Seattle, 2009). However the role of *Dlk1* during spermatogenesis required an independent resource for verification since the work of Shima et al (2004) provided the foundation for the MRG database. No independent resource was found thereby eliminating *Dlk1* as a potential spermatogonia candidate. The following genes were selected to validate the dissection of spermatogonia via LMD.

1. *mMage*a4 - RefSeq NM 020280.1 (National Library of Medicine, 2010). Wang, Page, and McCarrey (2005) explored differential autosomal and sex linked gene expression during spermatogenesis and reported that 14 of the 22 autosomal gene transcripts did not decrease during meiosis, while germ cell specific sex linked genes undergo meiotic sex chromosome inactivation thus making sex linked germ cell specific transcripts the ideal candidates for spermatogonia validation. Human Melanoma antigen family MAGE A, B, and C (*hMAGE*) and mouse *Mage* (*mMage*) genes reside on the X chromosome, and are sex linked genes that were expressed in some tumor cells, but were completely silent in normal tissues with the exception of male germ cells and the placenta. Murine MAGE A and B

proteins (*mMage*) displayed similar expression patterns (Chomez, et al., 2001; He et al., 2010). In testes recovered from organ donors, *hMAGEA4* was expressed in A and B spermatogonia (and possibly preleptotene spermatocytes), but was not detected thereafter (He, et al., 2010). Similar expression patterns were noted for *mMage*a4 with reactivation in round spermatids (Wang, et al., 2005; University of Washington Seattle, 2009). The consistent findings across independent resources led to the selection of *mMage*a4 as a candidate gene for the verification of LMD spermatogonia validation. *mMage*a4 does not have a poly A tail.

2. *Lin28* RefSeq NM_145833.1 (National Library of Medicine, 2010). In a recent study, *Lin28* (formerly known as *Tex17)* expression was shown to be specific to undifferentiated spermatogonia during the process of spermatogenesis. *Lin28* was also expressed in a variety of mouse and human embryonic tissues which included: a) embryonic stem cells, b) neural stem cells, and c) carcinoma stem cells (Zheng, Wu, Kaestner, & Wang 2009). The decision to use *Lin28* as a spermatogonia specific transcript was confirmed with the MRG database (University of Washington Seattle, 2009). Expression levels decreased eight fold as the cells transitioned from type A and type B spermatogonia to pachytene spermatocytes and round spermatids. *Lin28* lacks a poly A tail and it is located on chromosome 4 66.5 in mice and 1p36.11 in man MGD (Bult, et al. 2008; National Library of Medicine, 2010).

*Spermatocytes.* Spermatocytes also presented a distinct challenge. Johnston and colleagues (2007) characterized stage specific gene expression of rat spermatogenic and sertoli cells using gene expression microarray. Pachytene spermatocytes displayed

maximal expression of cell cycle genes such as cyclin-dependent kinase 2 (*Cdk2*) and cyclin-dependent kinase 1 (*Cdc2a*); and the excision repair genes mutL homolog 3 (*Mlh3*), poly (ADP-ribose) polymerase family, member 2 (*Parp2*), excision repair cross-complementing rodent repair deficiency, complementation group 1 (*Ercc1*), and RAD50 homolog (*Rad50*). However, this information conflicted with the MRG database. Here it was important to note that the sample processing methods differed. Johnston and colleagues (2007) used centrifugal elutriation, while Shima, McLean, McCarrey and Griswold (2004) isolated enriched cell types from whole testes via gravity sedimentation. As previously noted, with the exception of LMD, all existing methods of isolating cell subpopulations from complex tissues such as the testes have the potential to disturb the cells' biomolecular state and result in distorted gene expression data (Erickson et al. 2009).

1. Heat shock protein 2 (*HspA2*) RefSeq NM_008301.4 (National Library of Medicine, 2010) is also known as *Hsp70-2* and is a member of the heat shock protein 70 kilodalton family. *HspA2* is a chaperone of transition proteins that acquires spermatogenic specific functions which contribute to the dramatic chromatid remodeling and spermatid reorganization. Disrupted HspA2 content was associated with poorly remodeled dysmature cells during spermiogenesis with increased vulnerability to oxidative stress (Aitken & De luliis, 2010). Known expression patterns revealed a 10 fold increase in expression as cells transitioned from type A spermatogonia to round spermatids (Shima, et al., 2004; University of Washington Seattle, 2009). Homozygous null mutation males: a) were sterile, b) displayed the absence of post meiotic germ cells, and c) exhibited a dramatic

increase in spermatocyte apoptosis (Eddy, 2002). *HspA2* is located on chromosome 1234 cM in the mouse and 14q24.1 in man (Bult, et al. 2008).

2. Testicular cytochrome c (*Cyct*) RefSeq NM_009989.2 (National Library of Medicine, 2010). Somatic cytochrome C (*S-Cc*) and *Cyct* were 86.5% identical, yet displayed vastly different expression patterns during spermatogenesis. *S-C*c gradually declined as germ cells entered meiosis, while *Cyct* increased. The MRG database confirmed this expression with spermatocyte *Cyct* expression levels four times higher than type B spermatogonia *Cyct* expression, and three times higher than the expression recorded in round spermatids, thereby making *Cyct* an excellent candidate for spermatocyte specific validation. *Cyct* catalyzes $H_2O_2$ three times faster than *S-Cc* and has greater resistance to $H_2O_2$ degradation making *Cyct* important in protecting male germ cells from ROS. Homozygous null mutations have decreased sperm counts, impaired fertilization, and testicular atrophy due to dysfunctional oxidative phosphorylation (Liu et al., 2006). *Cyct* is located on mouse chromosome 2 (44.94 cM) and human 2q31.2. There is no poly A tail (Bult, et al. 2008; National Library of Medicine, 2010).

*Spermatids.* There were several male germ cell specific transcripts that were present only during post meiotic reorganization

1. Protamine 1 (*Prm1*) RefSeq NM_013637.4 and Protamine 2 (*Prm2*) RefSeq NM_008933 (National Library of Medicine, 2010). Protamines replace nuclear histones during spermatozoa reorganization to allow chromatin to be re-packaged into a very small space. Protamines also contain numerous cysteine residues that stabilize the chromatin structure and protect the DNA from ROS (Aitken &

Baker, 2006). *Prm1* expression patterns were low in type A and B spermatogonia, doubled in pachytene spermatocytes, followed by a seven fold increase in expression in round spermatids. The mature *Prm1* mRNA transcript does not have a poly A tail (National Library of Medicine, 2010). It is located on chromosome 16 5.87cM in mice and 16p13.2 in man (Bult, et al. 2008).

2. Transition protein 1 (*Tnp1*) RefSeq NM_009407.2 (National Library of Medicine, 2010). Transition proteins 1 and 2 act as intermediate DNA binding proteins during the histone-protamine exchange (Sutovsky & Manandhar, 2006). *Tnp1* expression patterns in Spermatogonia A and B remained relatively low, increased slightly in pachytene spermatocytes, and displayed a 20 fold increase (when compared to spermatogonia) in round spermatids (Bult, et al. 2008).

*Custom spermatogenesis qRT-PCR 7500 Fast plate.* The above information was then used to design custom qRT-PCR 7500 fast plates. ABI TaqMan gene expression assays used advanced bioinformatics to a) avoid inappropriate binding sites such as polymorphisms or repeat sequences, b) optimize primer and probe parameters such as the % GC content, $T_m$ (melting temperature), and amplicon length; c) avoid secondary structures such as primer dimer or hairpin formations; and d) when possible span exon-exon junctions to remove contaminating genomic DNA. In addition, TaqMan gene expression assays run under identical PCR conditions, assure primer and probe target specific amplification, and amplify at 100% efficiency, $\pm$ 10% (ABI 2010). To validate the claims of amplification efficiency, qRT-PCR confirmed 96% to 100% efficiency of the TaqMan primers and probes for the above genes. Thus, the ABI claim of 100% efficiency $\pm$ 10% was confirmed, and direct evidence of high quality RNA and cDNA

was obtained. Thus the need to test four to six primer pairs per target gene to further optimize PCR conditions was eliminated. However the optimized ABI primer sequences were proprietary and ABI would not provide the primer sequences (P. O'Connor, personal communication, October 8, 2010). Because ABI used 18s as the endogenous control (ABI 2010), *β-actin* was also added as the endogenous control. The literature determined that *β-actin* was the preferred endogenous control for male germ cell gene expression assays. The plate design details appear in Figure 8.

| Plate Details | |
|---|---|
| Name | spermatogenesis |
| Description | actin, beta uba1, Lin 28, Hsp 70 2, Smc 1b, Prm1, Tnp1 |
| Format | Format 8 |
| Species Selected | Mus musculus |
| Assigned Wells | 7 |
| Unique Genes | 7 |
| Empty Wells | 0 |
| Control Assays | 18s rRNA |

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 18S | 18S | 18S | 18S | 18S | 18S | 18S | 18S | 18S | 18S | 18S | 18S |
| B | Actb | Actb | Actb | Actb | Actb | Actb | Actb | Actb | Actb | Actb | Actb | Actb |
| C | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 |
| D | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 |
| E | Hspa2 | Hspa2 | Hspa2 | Hspa2 | Hspa2 | Hspa2 | Hspa2 | Hspa2 | Hspa2 | Hspa2 | Hspa2 | Hspa2 |
| F | Cyct | Cyct | Cyct | Cyct | Cyct | Cyct | Cyct | Cyct | Cyct | Cyct | Cyct | Cyct |
| G | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 |
| H | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 |

| Gene Symbol | Assay ID | Location |
|---|---|---|
| Actb | Mm00607939_s1 | B01 |
| Lin28 | Mm00524077_m1 | C01 |
| Magea4 | Mm00522322_s1 | D01 |
| Hspa2 | Mm00434069_s1 | E01 |
| Cyct | Mm00438362_m1 | F01 |
| Tnp1 | Mm00437165_g1 | G01 |
| Prm1 | Mm00726976_s1 | H01 |

*Figure 8.* Spermatogenesis custom ABI TaqMan 7500 fast gene expression plate. Plates arrived with dried down primers and probes. Spermatogonia, spermatocyte, and spermatid cDNA samples were quantitatively matched to the total testis cDNA positive controls. DNase/RNase free water was the no template control (NTC [negative]).

*Expected results.* In order to identify technical problems during qRT-PCR technical replicates called for the plating of the same samples in triplicate. However, the limited samples generated by LMD precluded the use of triplicate technical replicates. As an alternative, samples from three individual mice were loaded vertically by cell stage (spermatogonia, spermatocytes, and spermatids) into the custom qRT-PCR plate which

allowed for both biologic and technical replication. Thus if two of the three samples provided consistent results, the outlier was eliminated. While the samples were loaded vertically, gene expression fold changes were considered horizontally, so the gene expression data spanned the stages of male germ cell development. Therefore expression patterns that corresponded to the table below validated LMD success (see Figure 9).

| Mouse sgenesis LMD validation qRT-PCR | | Spermatogonia | | | Spermatocytes | | | Spermatids | | | Controls | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 Total testes + control | 11 Total testes + control | **12** NTC |
| Endogenous controls | A | 18S | 18S | 18S | 18S | 18S | 18S | 18S | 18S | 18S | 18S | 18S | 18S |
| | B | Actb | Actb | Actb | Actb | Actb | Actb | Actb | Actb | Actb | Actb | Actb | Actb |
| Spermatogonia | C | Magea4 ↑ | Magea4 ↑ | Magea4 ↑ | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 | Magea4 |
| | D | Lin28 ↑ | Lin28 ↑ | Lin28 ↑ | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 | Lin28 |
| Spermatocytes | E | Hspa2 | Hspa2 | Hspa2 | Hspa2 ↑ | Hspa2 ↑ | Hspa2 ↑ | Hspa2 ↑ | Hspa2 ↑ | Hspa2 ↑ | Hspa2 | Hspa2 | Hspa2 |
| | F | Cyct | Cyct | Cyct | Cyct ↑ | Cyct ↑ | Cyct ↑ | Cyct | Cyct | Cyct | Cyct | Cyct | Cyct |
| Spermatids | G | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 | Prm1 ↑ | Prm1 ↑ | Prm1 ↑ | Prm1 | Prm1 | Prm1 |
| | H | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 | Tnp1 ↑ | Tnp1 ↑ | Tnp1 ↑ | Tnp1 | Tnp1 | Tnp1 |

*Figure 9.* An illustration of the spermatogenesis custom plate layout and expected expression patterns by cell stage.

*Normalization strategies endogenous controls.* The housekeeping gene/s (HK) were also referred to as the endogenous control/s or the normalizing gene/s. HK gene/s must demonstrate constant expression in all sample types, regardless of treatment, or other experimental conditions. While standard HKs such as *Gapdh, Hprt* or ribosomal

18s are typically used, several sources reported that the expression of these genes varied across tissues (Radonic, et al. 2004). In the study of stage specific expression of genes associated with rat spermatogenesis, Sluka, O'Donnell, and Stanton (2002) used *Hprt* as the reference gene. However *Hprt* was differentially expressed by the stage of the seminiferous epithelium. *Gapdh* and *β – Actin* were also commonly used as endogenous controls. However *Gapdh* was inactivated during meiosis and post meiotic *Gapds* was expressed in the early post meiotic stages (Eddy, 2002). In comparison, Wang et al. (2005) reported that *β – Actin* demonstrated stable expression across all stages of murine spermatogenesis.

When we combined standard PCR with gel electrophoresis and ethidium bromide staining, stable *B-actin* bands were seen when 8 µl of each sample was loaded across all cell stages. *B-actin* bands were identified at 146 bp which corresponded to the expected *B-actin* PCR amplicon length and there was no indication of contamination (see Figure 10). This initially supported the use of *B-actin* as the endogenous control for qRT-PCR gene expression assays.

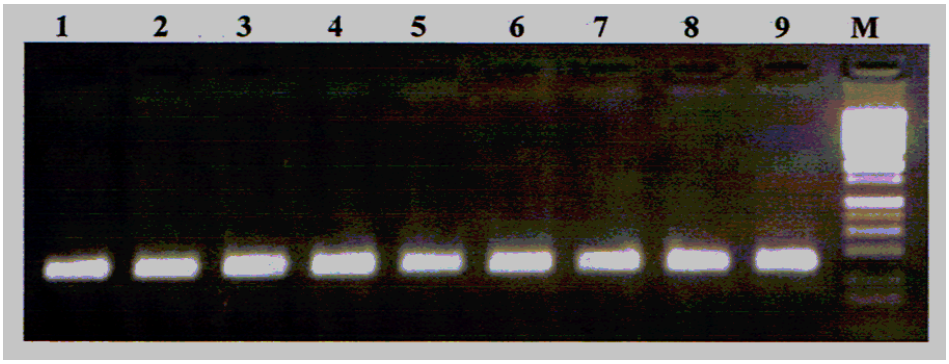
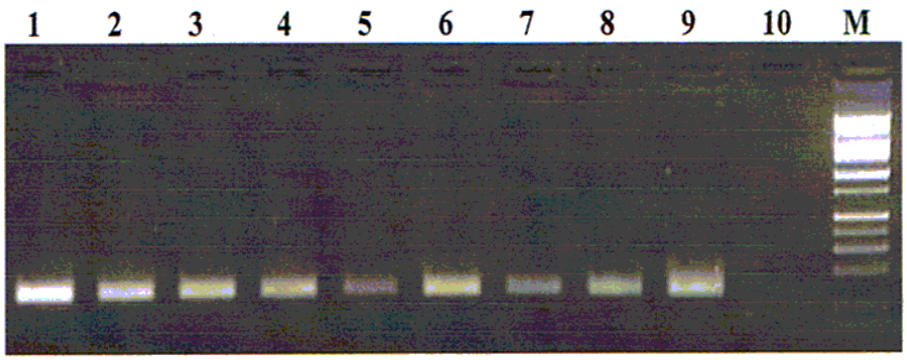



*Figure 10.* PCR with gel electrophoresis and ethidium bromide staining. DNA ladder was loaded in lane M. Photo courtesy of P. Esakky.

However, when 2 µl *B-actin* was loaded for gel electrophoresis, decreased band intensity was noted in lanes 5, 7, and 8 (see Figure 11). However, the variability in intensity was not confined to a single cell stage, but was noted among different samples and in different cell stages.




Legend
1. Spermatogonia
2. Spermatogonia
3. Spermatocytes
4. Spermatogonia
5. Spermatocytes
6. Spermatids
7. Spermatogonia
8. Spermatocytes
9. Spermatids
10. Negative control
M. Ladder

*Figure 11*. *B-actin* PCR with gel electrophoresis and ethidium bromide staining 2 µl. Photo courtesy of P. Esakky.

This information was then considered with the corresponding RNA NanoDrop results from the various samples. These results indicated that four different spermatogonia samples (I through IV) were loaded into lanes 1, 2, 4, and 7; and that three different samples (II through IV) were loaded for spermatocytes and spermatids. These results suggested that samples III and IV were suboptimal (see Table 6). However the gel images also represented a significant step forward and provided evidence of LMD success and the absence of sample contamination.

Table 6

*Sample ID, cell stage and NanoDrop values corresponding to Figures 15 and 16.*

| Starting RNA values for PCR and gel electrophoresis | | | | |
|---|---|---|---|---|
| Lane # | Sample ID | Cell stage | 260:280 | ng/rxn |
| 1 | I: | Sg | 1.5 | 15.7 |
| 2 | II | Sg | 1.75 | 2.82 |
| 4 | III | Sg | 1.55 | 4.99 |
| 5 | | Sc | 1.46 | 3.16 |
| 6 | | Sp | 1.48 | 20.04 |
| 7 | IV | Sg | 1.45 | 17.6 |
| 8 | | Sc | 1.45 | 12.28 |
| 9 | | Sp | 1.43 | 9.89 |

Because gel electrophoresis represented a qualitative assessment, qRT-PCR was also used to confirm the expression of *B-actin* during the discrete cell stages of spermatogenesis. The ideal endogenous control was expected to display less than one crossing threshold ($C_t$) difference across the cell stages or experimental conditions (L. Stewart, personal communication, April 1 2010). Our qRT-PCR revealed stable *B-actin* expression across all cell stages.

*Normalization strategies serial dilutions.* The ability to use the standard curve for normalization in qRT-PCR assays was not possible due to the limited amount of sample that resulted from LMD assays. Therefore mouse testes total RNA was purchased from Stratagene and was used as the qRT-PCR positive controls. Serial dilutions were generated from mouse testes total RNA 1000ng/µl (see Table 7). The samples were prepared on dry ice and stored as 20 µl aliquots in - 80C.

Table 7

*Total testes RNA serial dilutions used as positive controls*

| Dilution | RNA concentration | Recipe |
|---|---|---|
| 1:01 | 1000 ng/ µl | 100 µl of 1000 ng total RNA |
| 1:05 | 200 ng/ µl | 20 µl of 1:1 in 80 µl $H_2O$ |
| 1:10 | 100 ng/ µl | 10 µl of 1:1 in 90 µl $H_2O$ |
| 1:20 | 50 ng/ µl | 5 µl of 1:1 in 95 µl $H_2O$ |
| 1:50 | 20 ng/ µl | 10 µl of 1:5 in 90 µl $H_2O$ |
| 1:100 | 10 ng/ µl | 10 µl of 1:10 in 90 µl $H_2O$ |
| 1:200 | 5 ng/ µl | 10 µl of 1:20 in 90 µl $H_2O$ |
| 1:500 | 2 ng/ µl | 10 µl of 1:50 in 90 µl $H_2O$ |

*Note.* As described in (Erickson et al., 2009).

The Agilent RNA 6000 Nano Kit was subsequently used for RNA serial dilution qualitative assessment (see Table 8). A 28s/18s ratio $\geq$ 2 indicated high quality RNA. A RIN $\geq$ 5 indicated acceptable RNA integrity, while a RIN $\geq$ 8 was considered excellent.

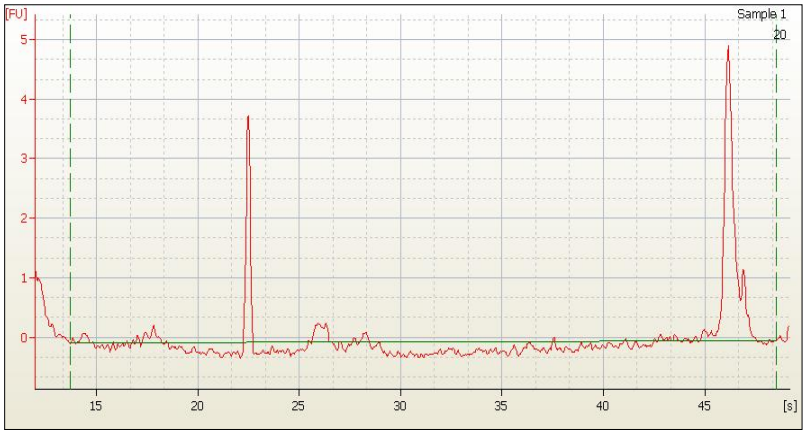
Table 8

*Quantitative and qualitative measures for serial dilutions.*

| Serial dilution ng/µl (expected) | NanoDrop ND-1000 Results | | | | | Agilent 2100 E Bioanalyzer Results | |
|---|---|---|---|---|---|---|---|
| | quant | 260 | 280 | 260:280 | 260:230 | RIN | 28s/18s |
| 20 | 19 | 0.474 | 0.247 | 1.92 | 2.05 | 0* | 0* |
| 50 | 53.2 | 1.329 | 0.698 | 1.9 | 2.17 | 2.6 | 0** |
| 100 | 100.26 | 2.507 | 1.261 | 1.99 | 1.94 | 8.8 | 1.4 |
| 200 | 204.2 | 5.105 | 2.523 | 2.02 | 2.07 | 8.9 | 1.3 |

*Note.* 260/280 ratio of ~2 indicated pure RNA. *Nano kit quantitative range was 25ng to 500ng. **See Figure 13. The sample was degraded.

The Nano Kit had a quantitative range of 25 to 500 ng/µl, and 5 to 500 ng/µl qualitative range for total RNA. This was evidenced by the failure of the Bioanalyzer to record the 20 ng/µl sample (see Figure 12). However it also did not provide the RIN, nor did it provide the 28s/18s ratio for the 20 ng/µl sample even though the sample was within the Nano Kit's reported qualitative range. The NanoDrop confirmed the expected RNA concentration of 19 ng/µl for the 1:50 serial dilution, with a 260:280 ratio 1.92 as evidence of high quality RNA. These facts pointed to sample degradation which occurred during the preparation of the Agilent 2100 assay.

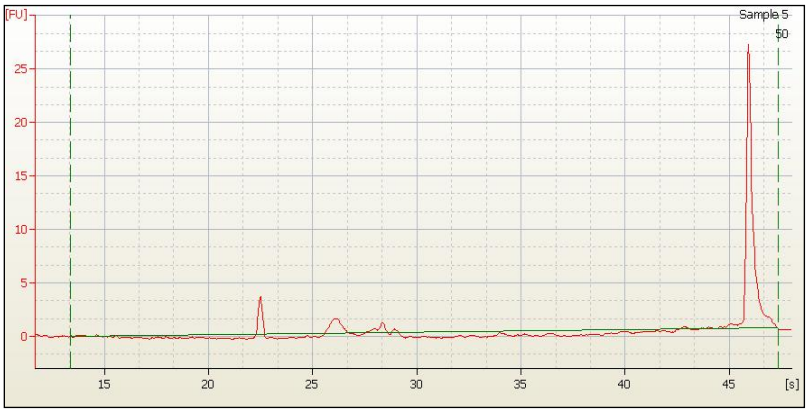



| Legend: Total testes RNA serial dilution 20ng/µl | |
|---|---|
| RNA concentration | 0 |
| 28s:18s | 0 |
| RIN | 0 |

*Figure 12.* Agilent 2100 Bioanalyzer results for total testes RNA 20 ng/ul. The expected ribosomal 18s and 28s ribosomal RNA were not identified and the unstable baseline suggested contamination.

In addition, it was reported that the Bioanalyzer quantitative measures displayed a variation of $\pm$ 20%. However, our results indicated a much broader range. Using the NanoDrop, the concentration of the 1:20 dilution was confirmed as 53.2 ng/µl. In comparison, the Bioanalyzer recorded 22 ng/µl as the RNA concentration. The RIN of 2.6 suggested marked sample degradation (see Figure 13). The large single peak almost

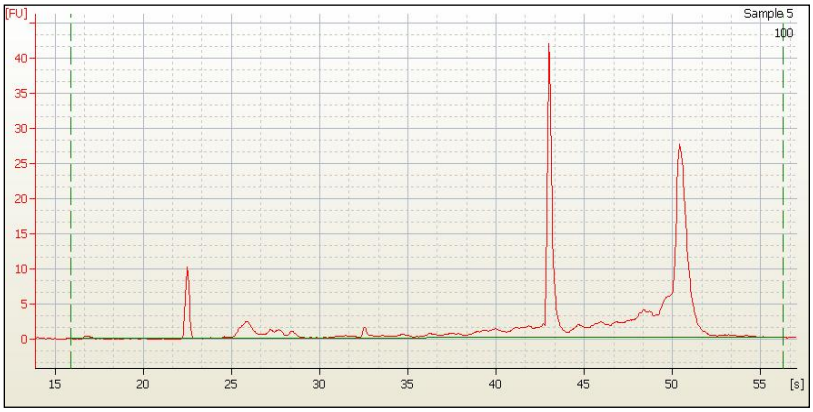
certainly represented extensive base pairing that occurred in the nicks or cleavages of compromised RNA. This resulted in large RNA fragments that migrated as a single unit.




| Legend: Total testes RNA serial dilution 50ng/µl | |
|---|---|
| RNA concentration | 22 |
| 28s:18s | 0 |
| RIN | 2.6 |

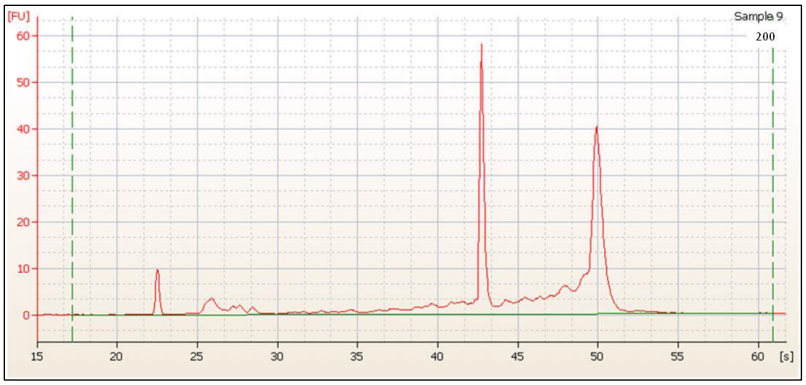
*Figure 13.* Agilent 2100 Bioanalyzer results for total testes RNA 50 ng/ul.

In response to the results in Figure 13, the RNA samples were heat denatured to separate the RNA molecules. As a result, Figures 14 and 15 displayed marked improvement as a result of the improved technique. Although the 28s band displayed some degradation, the 18s and 28s bands were clearly visible, and there were no well defined peaks between the 18s and 28s peaks. There was a + 52% variance in the Bioanlyzer quantitative measure.




| Legend: Total testes RNA serial dilution 100ng/µl | |
|---|---|
| RNA concentration | 148 |
| 28s:18s | 1.4 |
| RIN | 8.8 |

*Figure 14.* Agilent 2100 Bioanalyzer results for total testes RNA 100ng/ul.




| Legend: Total testes RNA serial dilution 200ng/µl | |
|---|---|
| RNA concentration | 225 |
| 28s:18s | 1.3 |
| RIN | 8.9 |

*Figure 15.* Agilent 2100 Bioanalyzer results for total testes RNA 200ng/ul.

In summary, the NanoDrop RNA quantitative measures closely matched those that were predicted by the serial dilutions, and the 260:280 ratios of 1.9 to 2.02 supported the presence of high quality RNA. In comparison, the Agilent bioanalyzer displayed a wide variance in sample quantity. The variance ranged from – 56% to + 52%. This exceeded the published reports of Bioanlyzer quantitative differences of $\pm$ 20%. When used, the Agilent 2100 Bioanalyzer must be combined with NanoDrop assessment.

*qRT-PCR data reporting.* Relative quantitation must be used to report qRT-PCR results when samples are normalized to HK genes or endogenous controls. The ABI 7500 fast software calculated the $\Delta C_t$ by subtracting the HK $C_t$ from the sample/s $C_t$. The software then calculated $\Delta\Delta C_t$, which was a measure of relative quantitation. $\Delta\Delta C_t$ defined over expression, under expression, or no change in expression. This was typically done to compare gene expression fold changes in diseased and non-diseased tissues, or to compare gene expression in treatment and un-treated samples (Erickson, et al., 2009). While comparative $C_t$ via $\beta$ – *Actin* normalization was used for this project, $\Delta\Delta$ $C_t$ was not adequately represented because experimental conditions were held constant among

the samples and candidate genes with known stage specific expression patterns were used to validate LMD success. Therefore the expression patterns for each gene were determined by: a) subtracting the HK $C_t$ from the sample $C_t$, which was also known as $\Delta C_t$; b) establishing untreated spermatogonia as the reference or calibrator sample, c) obtaining $\Delta\Delta C_t$ through the subtraction of spermatogonia $\Delta C_t$ from the target genes' $\Delta C_t$, and d) converting the results to log form to express fold changes. Fold change expression is interpreted in the following way:

Over expression was defined as:

a) 1+ (1.5 to 5 fold difference)
b) 2+ (5.1 to 10 fold difference)
c) 3+ (10.1 to 20 fold difference)
d) 4+ (> 20 fold difference)

Under expression is defined as:

a) 1- (1.5 to 5 fold difference)
b) 2- (5.1 to 10 fold difference)
c) 3- (10.1 to 20 fold difference)
d) 4- (> 20 fold difference)

*Stage specific validation.* The challenge laid forth in this dissertation, was to discover the optimal protocols which generated reliable gene expression measurements from the LMD stage specific male germ cell transcripts. Thus evidence of LMD success was dependent upon qRT-PCR gene expression patterns, which conformed to the existing

literature and the cell stage specific functions. The genes selected for LMD stage specific validation were: a) *mMage*a4 for spermatogonia, b) *HspA2* for spermatocytes, and c) *Tnp1* and *Prm2* for spermatids. $\Delta C_t$ qRT-PCR expression patterns were displayed for spermatogonia (see Figure 16), while relative expression ($\Delta\Delta C_t$) patterns were displayed for spermatocytes, round and elongating spermatids (see Figures 17 through 19).

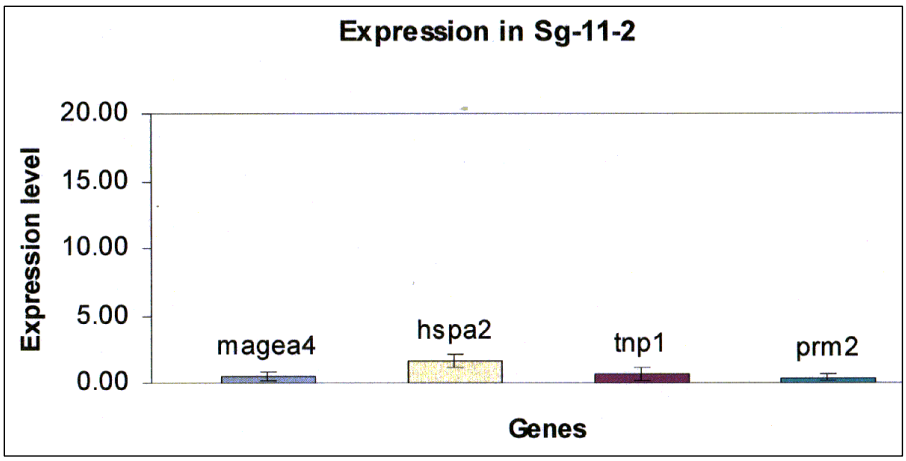



*Figure 16.* Baseline expression ($\Delta C_t$) of selected genes in spermatogonia normalized to *B-Actin*. All samples were untreated.

*mMage*a4 expression appeared at low levels throughout all cell stages. In contrast the increased expression of *HspA2* in spermatocytes (see Figure 17), with decreased expression in round and elongating spermatids (see Figures 18 and 19) was consistent with *HspA2* function as *Tnp1*.chaperone protein.

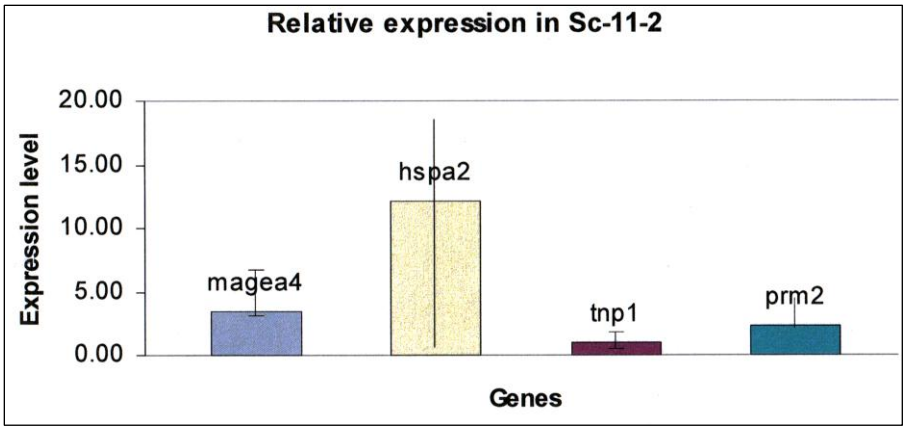



*Figure 17.* Relative expression ($\Delta\Delta C_t$) of selected genes in spermatocytes using spermatogonia as the calibrator or reference sample.

In the same manner, Transition proteins 1 and 2 act as intermediate DNA binding proteins during the histone-protamine exchange. The increased *Tnp1* in round and elongating spermatids (see Figures 18 and 19) paralleled the transition from histones to protamines and validated LMD success.

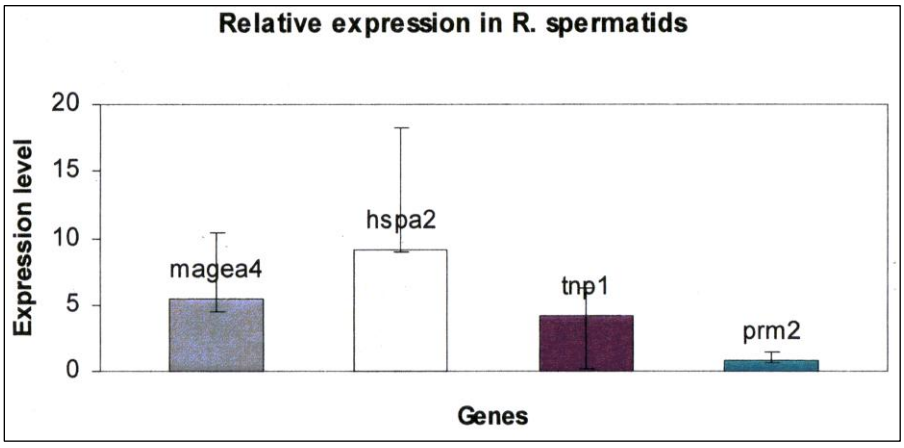



*Figure 18.* Relative expression ($\Delta\Delta C_t$) of selected genes in round spermatids using spermatogonia as the calibrator or reference sample.

Likewise, protamines replace nuclear histones during spermatozoa reorganization to allow chromatin to be re-packaged into a very small space. Therefore the increased expression of *Prm2* in elongating spermatids (see Figure 19) was evidence of LMD success and LMD stage specific validation.

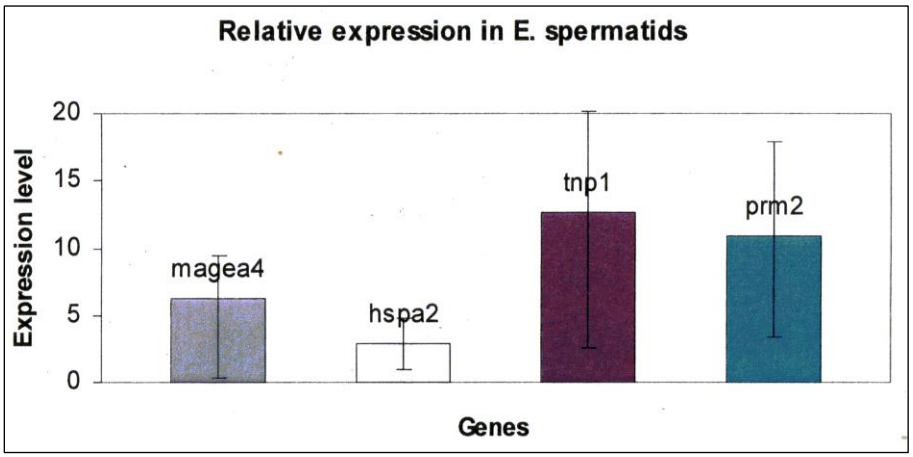
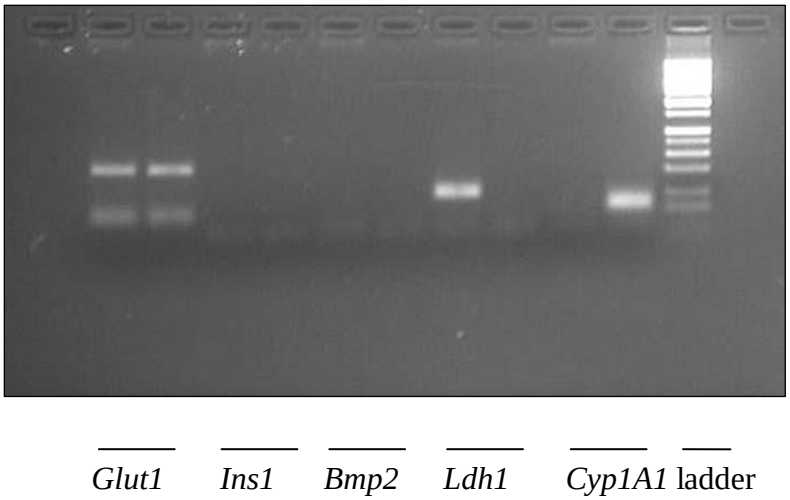



*Figure 19.* Relative expression ($\Delta\Delta C_t$) of selected genes in elongated spermatids using spermatogonia as the calibrator or reference sample.

Finally, standard PCR combined with gel electrophoresis provided an unexpected finding that not only supported stage specific LMD success, but conformed to the pattern of distribution that was predicted by the physiologic function of *Ahr* and the BTB. *Cyp1A1* was present in spermatogonia, but absent in spermatocytes and spermatids (see Figures 20 and 21).




Legend:
*Glut1* glucose transporter 1
*Ins1* insulin 1
*Bmp2* bone morphogenetic protein 2
*Ldh1* lacate dehydrogenase A
*Cyp1A1* cytochrome P450 1A1

*Figure 20*. Gel electrophoresis demonstrated the presence of *Cyp1A1* (at ~70 bp), *Ldh1*, and *Glut1* (at 136 bp) in spermatogonia isolated via LMD. There was no amplification of *Ins1* or *Bmp2*. The *Glut1* double band suggested primer dimer or non specific amplification related to high primer salt conditions.

*Cyp1a1* is known for its role in the detoxification of endogenous and exogenous ligands, and is a recognized marker of AHR activation. This distribution was consistent with the published findings of the MRG database (University of Washington Seattle, 2009) of increased *Ahr* expression in type A and B spermatogonia and sertoli cells; with decreased *Ahr* expression in pachytene spermatocytes and round spermatids. To date there are no studies which used IHC to localize Ahr to the sertoli cells or spermatogonia.

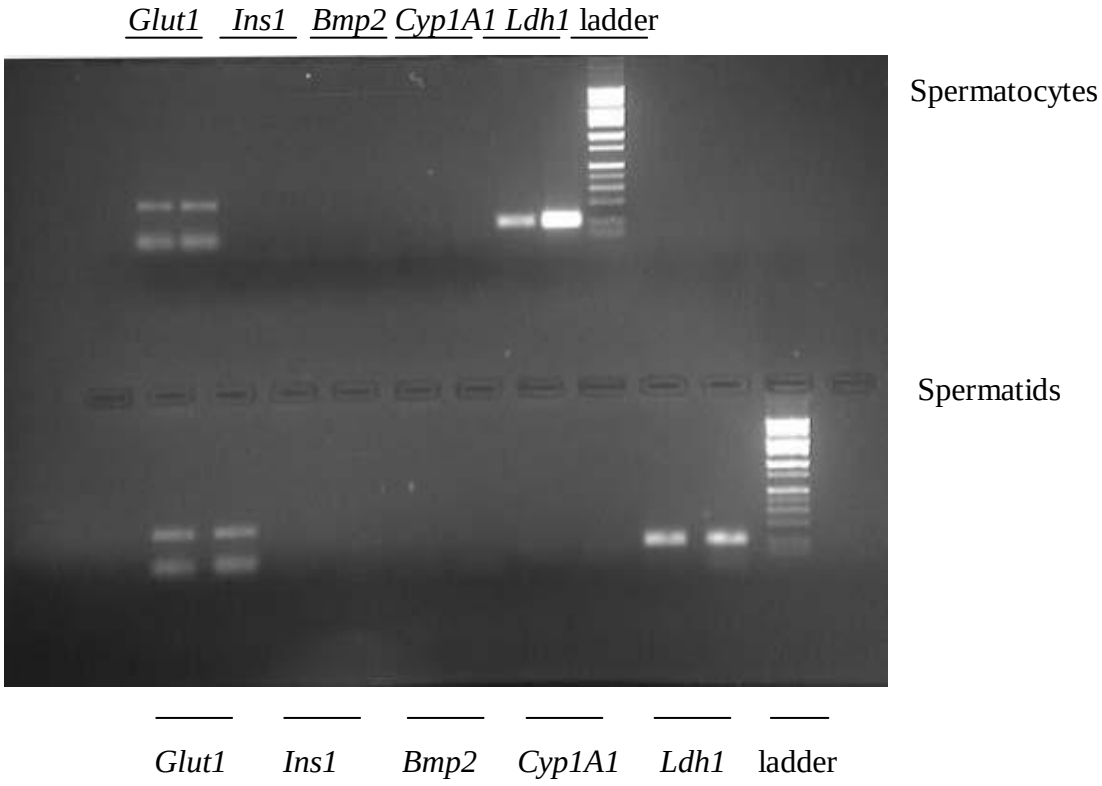



*Figure 21.* Gel electrophoresis demonstrated the presence of *Glut1* and *Ldh1* in spermatocytes and spermatids isolated via LMD. There was no amplification of *Cyp1A1*, *Ldh1*, or *Bmp2*. Photos courtesy of P. Esakky.

Glucose transporter 1 (*Glut1*) bands were clearly evident by gel electrophoresis in spermatogonia, with decreased band visibility in spermatocytes and spermatids. This was consistent with a recent review which described *Glut1* as a member of the solute carrier family 2 (SLC2A) with an affinity for erythrocytes, astrocytes, cardiac muscle; the plasma membranes of the proliferating cells of the embryo; and the cells that make up the blood tissue barriers (Carruthers, DeZutter, Gagnuly, & Devaskar, 2009). This description suggested that it was likely that *Glut1* expression would be highest in spermatogonia and in the sertoli cells that make up the BTB. The MRG database

confirmed increased *Glut1* expression in type A and B spermatogonia with decreased expression in spermatocytes and spermatids (University of Washington Seattle, 2009). Hence the above images were consistent with published data, but the *Glut1* primer required further validation.

# CHAPTER FIVE

## Discussion

LMD is the only technology that can isolate cell subpopulations without disturbing the molecular signature of the cells (Espina et al., 2006). However to date there has been limited success in the use of LMD for protocols which seek to characterize gene expression during spermatogenesis. In fact, many labs have tried and failed in their attempts to translate LMD to stage specific gene expression protocols during spermatogenesis. In all probability this resulted from the failure to control the numerous methodological approaches, with each variable having the potential to disturb all subsequent downstream applications. Therefore, *Spermatogenesis: Laser Microdissection Optimization* is essential to the success of our VISN 15 and HSRD funded protocols which seek to characterize the gene expression profiles of male germ cells and reproductive outcomes in exposed and non-exposed populations. It is the explicit long term goal of our research program to establish a versatile research framework upon which past, present, and future paternal preconception exposures can be quickly evaluated.

### *Results and Recommendations*

While LMD progress was made with the most recent RNA 260:280 ratio at 1.5 for spermatogonia and spermatids, and 1.75 for spermatocytes, RNA quality did not achieve the target 260:280 ratio of 2 $\pm$ 10%. However qRT-PCR results displayed target specific and stage specific amplification and provided evidence of the successful application of LMD to spermatogenesis.

*Stage Specific Gene Expression*

qRT-PCR of testis specific and stage specific transcripts were used to validate successful LMD application to spermatogenesis. Consistent with the design of the spermatogenesis custom qRT-PCR plate, the expression patterns of testes specific, and cell stage specific genes were followed via q RT-PCR across all cell stages. As such, expression patterns that conformed to published data and physiologic functions were key indicators of LMD success. *mMage*a4 was reported to be expressed solely in selected tumor cells, male germ cells, and the placenta which suggested the utility of *mMage*a4 as a marker of mitotic proliferation. *mMage*a4 was consistently found in type A and B spermatogonia, and variable expression in preleptotene spermatocytes (He, et al., 2010) and round spermatids (Wang, et al., 2005; University of Washington, Seattle, 2009). Our results indicated low baseline expression in spermatogonia and spermatocytes, with increased expression in round and elongating spermatids. Thus the shifting expression patterns did not provide convincing evidence of spermatogonia isolation. The results may also be tied to low transcript levels and the inability of LMD to discriminate between the meiotic cell stages. While it was possible for spermatogonia contamination to occur, the spatial alignment within the seminiferous tubules combined with the results that were achieved in the successive stages (spermatocytes and spermatids) made it very unlikely that contamination occurred across all cell stages.

When compared to the non specific *mMagea4* results, *HspA2, Tnp1*, and *Prm2* expression paralleled the published data and provided convincing evidence of stage specific LMD success. *HspA2* displayed a 10 fold rise as the cells evolved from

spermatogonia to spermatocytes, with decreased expression as the cells progressed to elongated spermatids. Conversely, *Tnp1* and *prm2* displayed an approximately 10 fold and 12 fold rise respectively in elongated spermatids. These expression patterns coincided with the physiologic function of: a) *HspA2* as a transition protein chaperone during chromatid remodeling and spermatid reorganization, b) *Tnp1* as an intermediate DNA binding protein during the histone protamine exchange and c) P*rm2* which allowed the chromatin to be repackaged into a highly condensed space.

Further evidence of LMD success was provided by the amplification of *Cyp1A1* in a cell stage specific manner by standard PCR and gel electrophoresis. While this did not directly assess the presence of *Ahr* in the developing gametes, *Cyp1A1* was a recognized marker of *Ahr* activation. Therefore, the presence of *Cyp1A1* in spermatogonia and the absence of *Cyp1A1* in spermatocytes and spermatids (see Figures 25 and 26 respectively) were supported by the physiologic function of the BTB and the role of *Ahr* in detoxifying endogenous and exogenous agents. Thus, the distribution of *Cyp1A1* suggested that: a) *Ahr* would be present at the basal lamina of the seminiferous epithelium where spermatogonia and sertoli cell plasma membranes were subject to xenobiotic exposure via their contact with testicular blood and lymphatics, and b) *Ahr* would be absent in spermatocytes and spermatids which were sequestered in the adluminal compartment of the BTB. However, no information was found regarding Sertoli cell *AHR/Ahr* localization. Therefore future plans include the use of IHC to localize AHR in the sertoli cells and the stage specific male gametes. In addition, the *Cyp1A1* pattern of expression was consistent with the published data (University of

Washington, 2009) and suggested *Cyp1A1*as an attractive candidate for LMD stage specific spermatogonia validation.

*LMD and Gene Expression during Spermatogenesis*

Existing technologies, such as flow cytometry with immunostaining, may be well suited to segregating cells in suspension. However, these technologies disrupt adherent cells and profoundly alter gene expression (Espina, et al., 2006). As an example, pachytene spermatocytes were reported to display maximal expression of cell cycle genes and the excision repair genes (Johnston, et al. 2007). However, this information conflicted with the MRG database. Here it was important to note that the sample processing methods differed. Johnston and colleagues (2007) used centrifugal elutriation, while Shima and associates (2004) isolated enriched cell types from whole testes via gravity sedimentation. As previously noted, LMD is the only technology that can isolate pure germ cell populations from the diverse interconnected cells of the testis without disrupting the molecular signal. The draw and cut function of the Leica LMD 6000 system allowed the dissection of three cell stages per slide, thereby limiting LMD time, protecting RNA integrity, and reducing the number of mice that were needed. The Leica LMD 6000 was the ideal LMD platform for dissecting stage specific male gametes from complex tissues such as the testes.

However, the promise of LMD was complicated by the cytoplasmic projections of the sertoli cells which extended from the basal lamina of the seminiferous epithelium to the lumen of the seminiferous tubule. Consequently, past work either dissected entire cross sections of the seminiferous tubules (Sluka et al. 2002), or zones of the

seminiferous epithelium (Sluka et al., 2008). However, both approaches would be expected to result in altered stage specific gene expression profiles as a result of somatic cell contamination (Fink & Bohle, 2002). In response, our laboratory utilized the cell stage specific morphology, in conjunction with cell location and directional alignment within the seminiferous epithelium, to accomplish cluster LMD of the stage specific gametes (see Figure 13). Cluster LMD facilitated the dissection of pure stage specific cell populations in the absence of somatic cell contamination, within the construct of maximal RNA quantity, reduced time, and RNA preservation. Thus far there were no published articles that described the use LMD cluster dissection of stage specific male germ cell within the seminiferous epithelium. This approach represented a noteworthy innovation which facilitated the translation of LMD to male reproductive gene expression assays.

*Polyadenylation*

The absence of polyadenylation in four of the six transcripts selected for LMD stage specific validation provided very important information. Specifically, oligo-dTs were sequential and required mRNA transcripts with a poly A tail for cDNA synthesis. Therefore the absence of a poly A tail precluded the use of oligo-dTs for cDNA synthesis because of the failure to amplify a subset of male germ cell specific sequences. While the LMD literature clearly favored the use of random hexamers for cDNA synthesis due to RNA fragmentation, no such recommendation was found for male germ cell gene expression assays. Therefore, it is the recommendation of this dissertation, that oligo-dTs should not be used for cDNA generation for protocols which target male germ cell transcripts. Instead random hexamers are required for cDNA synthesis for protocols

seeking to elucidate gene expression during spermatogenesis. There would also be great value in conducting a comprehensive review of published articles dealing with gene expression of male gametes in order to discover the method that was used for cDNA synthesis.

## Future Directions

This study also utilized LMD, standard PCR, and gel electrophoresis to document: a) *Cyp1A1* in a distribution that was consistent with the physiologic functions of *Ahr* and the BTB, and b) the presence of *Glut1* across all cell stages. When taken together these findings suggested several important avenues of exploration. A recent study of in vitro exposure of pluripotent embryonic stem cells to TCDD, reported that TCDD activated *Ahr*, increased *Cyp1A1*, and interfered with GLUT1 localization. In the presence of TCDD, GLUT1 was detected in the cell nucleus. In the absence of TCDD, GLUT1 remained at the plasma membrane (Tonack, et al, 2007). Taken together, these findings suggested that TCDD had the potential to alter GLUT1 function, and suggested the very real possibility that reduced glucose uptake may result in nutrient deficient cells and be a major cause of TCDD's toxic effects. In support of this suggestion was the fact that sperm concentration, motility, and fertilization rates were lower in diabetic mice when matched to controls (Kim & Moley, 2009); and studies in human sperm described severe structural defects and decreased motility in insulin dependent diabetics (Baccetti, et al., 2002). While the causes of these abnormalities were not known, there was growing evidence that diabetics have an increased vulnerability to oxidative stress.

*Two Step Model on the Origins of DNA Damage in Sperm as a Theoretical Framework*

While the intent of this dissertation was to validate the use of LMD as a method of stage specific male germ cell isolation, the dysregulation of a subset of these genes (*Hsp2A, Cyct, Prm1,* and *Prm2*) was also recognized as indicators of cellular stress. As such, the two step hypothesis on the origin of DNA damage in Sperm informed the foundation of our paired reproductive toxicology protocols and the ultimate formation of our proposed research framework.

A central tenet of this theory was that poorly remodeled cells retained residual mitochondria that generated increased ROS. This in turn led to the second step; DNA damage in sperm came from within, via excess mitochondria which generated excess ROS. These vulnerable dysmature cells were then subjected to DNA damage that resulted from: a) poor protamination, b) excess ROS, and c) the loss of motility that resulted from cell wall damage due to lipid perioxidation. As much as 50% of the fatty acids in the sperm plasma membrane are highly unsaturated fatty acids. While this superabundance of polyunsaturated fatty acids provides the membrane fluidity needed for fertilization, the six double hydrogen bonds per molecule facilitate the perioxidation cascade and leave male germ cells particularly vulnerable to oxidative stress (Aitken & De luliis, 2010; Aitken & Roman, 2008).

But what were the factors that created cellular dysmaturity to generate excess ROS? As previously noted, increased ROS occurred in the context of infection, a number of clinical conditions, and in the presence of xenobiotic exposures that activate cytochrome P450. However there was limited information on the role of xenobiotics on

free radical generation and oxidative DNA damage in sperm (Aitken & De luliis, 2010) nor was there information on the impact of xenobiotics on cellular dysmaturity or protamination. With this in mind, a custom qRT-PCR plate was designed to uncover the impact of TCDD exposure on spermatogenesis and spermiogenesis. This plate was entitled the two hit AHR toxicology plate and incorporated the markers of AHR and P450 activation (*Cyp1a1, Ahr, Hsp90aa1*) within the context of markers of cellular dysmaturity (*Hsp2A, Prm1, Prm2*) and oxidative stress (*Sod1, Sod2, Cyct, Gpx4*).

| 2 hit model | | | Exposed | | | | Non Exposed | | | | Testes total RNA Controls | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| | controls | A | 18S | Actb | 18S | Actb | 18S | Actb | 18S | Actb | 18S | Actb | 18S | Actb |
| HIT 1 | Immature cells | B | H2afx | Hspa2 | H2afx | Hspa2 | H2afx | Hspa2 | H2afx | Hspa2 | H2afx | Hspa2 | H2afx | Hspa2 |
| HIT 1 | protamine | C | Prm 1 | Prm 2 | Prm 1 | Prm 2 | Prm 1 | Prm 2 | Prm 1 | Prm 2 | Prm 1 | Prm 2 | Prm 1 | Prm 2 |
| HIT 2 | *DNA repair | D | Trp53 bp1 | Rad50 | Trp53b p1 | Rad50 | Trp53b p1 | Rad50 | Trp53b p1 | Rad50 | Trp53b p1 | Rad50 | Trp53b p1 | Rad50 |
| HIT 2 | Anti-ox/Ahr | E | Gpx4 | Hsp90 aa1 | Gpx4 | Hsp90 aa1 | Gpx4 | Hsp90 aa1 | Gpx4 | Hsp90 aa1 | Gpx4 | Hsp90 aa1 | Gpx4 | Hsp90 aa1 |
| HIT 2 | Ahr | F | Cyp1 a1 | Ahr | Cyp1a 1 | Ahr | Cyp1a 1 | Ahr | Cyp1a 1 | Ahr | Cyp1a 1 | Ahr | Cyp1a 1 | Ahr |
| HIT 2 | ROS | G | Sod1 | Sod2 | Sod1 | Sod2 | Sod1 | Sod2 | Sod1 | Sod2 | Sod1 | Sod2 | Sod1 | Sod2 |
| HIT 2 | Peri-ox apoptosis | H | Ucp2 | Cyct | Ucp2 | Cyct | Ucp2 | Cyct | Ucp2 | Cyct | Ucp2 | Cyct | Ucp2 | Cyct |

*Figure 27.* The proposed two hit AHR toxicology plate was designed to characterize the effects of TCDD on spermatogenesis and spermiogenesis and the role of TCDD in oxidative stress and is the companion to the custom spermatogenesis qRT-PCR plate.

This plate was designed to provide a window into the impact of male environmental exposures on the cellular processes during spermatogenesis and spermiogenesis that generate oxidative stress. However this plate can also be easily modified to elucidate the impact of TCDD on *Cyp1A1* in conjunction with glucose transporter expression. While we had qualitative evidence of *Glut1* in spermatogonia, and decreased *Glut1* presence in spermatocytes and spermatids, other facilitative glucose transporters SLC2A expression patterns require further exploration. SLCA8 (*Glut8*) and SLCA9b (*Glut9*) displayed decreased expression in the testes of diabetic mice when compared to controls (Kim & Moley, 2008). While assays to discover the impact of *Ahr* activation on *Glut1* localization in the blastocyst stage embryo and the uterine environment were incorporated into the HSRD funded protocol *Paternal Environmental Exposures and Reproductive Outcomes: A Comparison of in Vivo and in Vitro Fertilization*, the assessment of TCDD on *Slc2A* expression and localization during spermatogenesis can also be incorporated into the VISN 15 funded protocol *Paternal Environmental Exposures and Gene Expression during Spermatogenesis*. When combined with IHC to localize SLC2A expression, it is expected that these findings will significantly increase our understanding of the effects of altered glucose metabolism on spermatogenesis, and inform our understanding of the mechanisms whereby TCDD induces diabetes and generates oxidative stress. It is believed that the qRT-PCR two hit toxicology plate will be an important part of the proposed comprehensive research framework that will evaluate the effects of paternal exposures on spermatogenesis and spermiogenesis and suggest avenues for treatment and prevention.

## *Translational Clinical Research*

Establishing a paradigm whereby paternal preconception exposures impact gene expression during spermatogenesis is problematic. First, ethical considerations prevent the intentional exposure of humans to environmental hazards, and preclude access to sufficient quantities of human testicular tissue for the study of gene expression during spermatogenesis. This is further complicated by genetic susceptibilities or personal health habits such as cigarette smoke, which is known to be an AHR ligand and that the exposure to multiple substances may occur simultaneously (Kitamura & Kasai 2007). In addition, the PBPK model related to TCDD toxicokinetics found that peak human exposures were closely correlated to the doses used in animal toxicity studies (IOM 2007). Thus basic laboratory science in non human mammals is essential to establishing a comprehensive research framework upon which male environmental exposures can be quickly evaluated. The current approach is to base risk assessment upon the response of the most sensitive animal species in order to protect vulnerable human populations (Aylward et al., 2005).

*Paternal Environmental Exposures and Reproductive Outcomes: A Comparison of in Vitro and in Vivo Fertilization* was presented at a poster session at the American College of Medical Genetics annual clinical genetics meeting in Tampa Florida March 25$^{th}$ to 29$^{th}$, 2009. During the discussion of this project with clinicians during the poster session, it was apparent that there was limited data to explain the mechanisms of paternal risk. While there was significant evidence from animal models to support the role of male mediated developmental toxicity, the mechanisms of action required further elucidation.

To our knowledge there were no other studies which utilized LMD to examine the role of paternal preconception exposures and gene expression during spermatogenesis, nor were there any published studies of reproductive outcomes which segregate sperm related factors from seminal fluid factors through the process of IVF. Consequently our studies hold the potential to a) establish LMD as an essential tool in the assessment of male gene expression toxicology assays, b) elucidate the biologic mechanisms whereby paternal exposures elicit adverse male mediated reproductive effects, c) inform preconception counseling in the setting of paternal risk, d) impact reproductive decision making, and e) build upon existing male reproductive toxicology assays to establish a framework which can be used to target prevention. However, toxicology assays demand stringent transparent protocols for animal care and housing, sample collection and processing, consumables, extraction methods, and downstream applications, in order to demonstrate due diligence in all laboratory methods and to facilitate replication. Therefore *Spermatogenesis: Laser Microdissection Optimization* provides a model of transparent integrity which sets the standard for laboratory bench science within the framework of toxicology assays. It is a versatile model that can be translated to a variety of future laboratory applications.

### *Conclusion*

Finally, translational research conveys a sense of immediacy and research that is cutting edge with the timely translation of information obtained from animal models to human systems (Schuster & Powers, 2005). The exposure of Vietnam veterans to Agent Orange occurred between the years of 1962 to 1970 (Schecter, et al., 1996). In 1997, Public law 104-204 provided presumptive service connected status to children with Spina

Bifida if their parent was an Agent Orange exposed Vietnam Veteran (Benefits for Children of Vietnam Veterans 2003). Thus legislation was enacted 35 years after the first veterans were exposed, and 27 years after the known of exposure of military personnel to Agent Orange ended. Consequently, the concerns about the primary reproductive outcomes and the reproductive fitness of Agent Orange exposed Vietnam Veterans were ending. Gulf War Veterans and their families are facing similar delays. Gulf War environmental exposures occurred between January and February 1991, yet there continued to be inadequate or insufficient evidence to permit a conclusion regarding the existence of an association between paternal preconception exposures and male mediated reproductive effects (IOM, 2005). These delays, in part, can be attributed to conflicting data as evidenced by the 1996 Agent Orange report in which the committee found that the Ranch Hand Study, the CDC Birth Defect Study, and the CDC Vietnam Experience Study provided suggestive evidence that paternal TCDD exposure led to NTD (IOM, 2001); while another study concluded that there was little or no evidence to support the hypothesis that exposure to Agent Orange resulted in birth defects (Wolfe et al., 1995). This was further complicated by the failure to recognize the contribution of paternal environmental exposures to chemicals and ionizing radiation to developmental end points such as miscarriage, birth defects, growth retardation, and cancer (IOM 1995). Because science informs policy, and policy informs funding decisions and resource allocation, *Paternal Environmental Exposures and Reproductive Outcomes: A Comparison of In Vivo and In Vitro Fertilization*, the companion VISN 15 funded project entitled *Paternal Environmental Exposures and Gene Expression during Spermatogenesis*, and this dissertation entitled *Spermatogenesis: Laser Microdissection Optimization* are essential

to the advancement of the knowledge of environmental exposures and male mediated reproductive events, and to provide timely and relevant scientific data which can then be quickly translated into policy and legislation. The importance of timely relevant decisions was emphasized in the statement of the Secretary of Veterans Affairs, Eric Shinseki in which he noted that that we are still trying to discover the health consequences of Agent Orange exposure more than 40 years after the exposure of veterans to Agent Orange ended (Vietnam Veterans of America, 2009).

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# APPENDIX A

## Animal Welfare Assurance and IACUC Approvals

**UNIVERSITY OF MISSOURI - ST. LOUIS**

**OFFICE OF RESEARCH ADMINISTRATION**
**INSTITUTIONAL ANIMAL CARE & USE COMMITTEE**

TO: Deborah Hansen, Nursing

FROM: Tim Farmer, IACUC Chairman

DATE: November 30, 2009

RE: Protocol Review

This is to certify that the University of Missouri - St. Louis Institutional Animal Care and Use Committee (IACUC) which is officially charged with the review of proposals involving animal experimentation has reviewed and approved the following protocol:

Protocol No.: **09-11-10**
Title: **Spermatogenesis: Laser Microdissection Optimization**
Start Date: **11/30/2009** Expiration Date: **11/30/2012**

This proposal was approved for a period of three years. Although the IACUC recognizes that the nature of creative research sometimes requires minor adjustments in experimental procedures, **substantial changes should be reported to the IACUC in the form of a revised protocol or an amendment to the original protocol, as appropriate to the extent of the changes.** Annual continuation forms are sent to all Principal Investigators around the anniversary of the protocol start date. Annual continuation forms must be filed with the IACUC for the duration of the protocol, and the IACUC should be notified when the work covered by the protocol has been completed.

This action is officially recorded in the minutes of the IACUC.

| **Signature – Chair** | **Date** |
| --- | --- |
| Timothy A. Farmer | 30 November 2009 |

APPENDIX A

Continued

Washington University in St. Louis
**Animal Studies Committee**

Animal Welfare Assurance # A-3381-01
April 27, 2009

To: Kelle H Moley, MD Campus Box 8064
From: Dana R. Abendschein, Ph.D., Chairman
Subject: Approval of Protocol for Experiments Utilizing Animals

Agency/Title: DEPARTMENT OF VETERAN AFFAIRS
2008 Department of Veterans Affairs VISN 15 Research Award
Paternal Environmental Exposures and Gene Expression during Spermatogenesis

The Animal Studies Committee of Washington University has reviewed this protocol for the use of animals in conjunction with the research project named above. Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of this protocol, you will be asked to submit an annual progress report describing any changes in this protocol.

**Approval Date:** **04/27/2009** **Approval No. 20080290**
**Expiration Date:** **04/27/2012**
Approved with addendum
SPECIES:
Mouse

It is your responsibility to see that all persons who use animals under your direction understand and follow the approved protocol. Should it become necessary to make substantial changes in this protocol, you must submit a new protocol. **Failure to comply with these provisions can result in suspension of the research.**

Campus Box 8025, 660 South Euclid Avenue, St. Louis, Missouri 63110-1093,
(314)362-3229, *Fax* (314)454-6617, http://asc.wustl.edu

APPENDIX A

Continued

Washington University in St. Louis
**Animal Studies Committee**

Animal Welfare Assurance # A-3381-01
April 27, 2009

To: Kelle H Moley, MD Campus Box 8064
From: Dana R. Abendschein, Ph.D., Chairman
Subject: Approval of Protocol for Experiments Utilizing Animals

Agency/Title: DEPARTMENT OF VETERAN AFFAIRS
Department of veterans' Affairs Office of Research and Development (VA-ORD).
Paternal Environmental Exposures and Reproductive Outcomes: A Comparison of in vitro and in vivo Fertilization

The Animal Studies Committee of Washington University has reviewed this protocol for the use of animals in conjunction with the research project named above. Federal laws and guidelines require that institutional animal care and use committees review ongoing projects annually. For the first two years after initial approval of this protocol, you will be asked to submit an annual progress report describing any changes in this protocol.

**Approval Date:** **04/27/2009** **Approval No. 20090071**
**Expiration Date:** **04/27/2012**
Approved with addendum and suggestions
SPECIES:
Mouse

It is your responsibility to see that all persons who use animals under your direction understand and follow the approved protocol. Should it become necessary to make substantial changes in this protocol, you must submit a new protocol. **Failure to comply with these provisions can result in suspension of the research.**

Campus Box 8025, 660 South Euclid Avenue, St. Louis, Missouri 63110-1093,
(314)362-3229, *Fax* (314)454-6617, http://asc.wustl.edu

APPENDIX A

Continued

**Department of Veterans Affairs** | **Memorandum**

Date: February 3, 2010

From: Acting Associate Chief of Staff, Research and Development Service (RDC) (151/JC)

Subj: Notification of Protocol Approvals and Permission to Initiate Research

To: Deborah Hansen, PhDc, RN (00QM/JC)

1. This is to notify you that your protocols entitled "Paternal Environmental Exposures and Gene Expression during Spermatogenesis" and "Paternal Environmental Exposures and Reproductive Outcome: A Comparison of in Vitro and in Vivo Fertilization" have been reviewed and approved by the appropriate research committees. As the VA Office of Research Oversight has now permitted our Medical Center to review and approve new research involving animals, as of February 3, 2010 you may initiate research on these protocols.

2. If you have additional questions or concerns, please contact me at laura.kroupa@va.gov.

Laura J Kroupa

LAURA J. KROUPA, MD
Acting ACOS, Research and Development Service

Cc: RDC Chairman/Administrator
SRS Chairman/Administrator
IACUC Chairman/Administrator

# APPENDIX B

## LMD Protocol Time Line

## Laser Micro Dissection using Leica LMD 6000

1. Tissue fixation: ~30min
2. UV Treatment to membrane slide: 30min
3. Cryosectioning: ~1-2.5h
4. Staining of the membrane slide:  (Staining with H & E) ~30min
5. Preparation for LMD and Laser capture: ~2h
6. RNA Isolation using PicoPure kit: ~1.5h
7. Quantitation by Nanodrop method: ~10min
8. integrity analysis by Agilent bioanalyzer using Nano kit: ~40min + 45min

Total time ~ 8 hours

# APPENDIX C

## Modified CAT Hematoxylin and Eosin (H&E) staining protocol

H & E Staining of mouse testis Frozen sections for LMD (Short Protocol ~25 min)

| | | |
|---|---|---|
| 1. | Testis blocks are stored at -80$^{O}$C freezer before use. | |
| 2. | Cold acetone (-20$^{O}$C) (Fixation) | 1min |
| 3. | CAT-Hematoxylin | 5min |
| 4. | RNase-free Dis. H2O | 1 slow dip |
| 5. | Eosin-Y (Sigma) | 5sec |
| 6. | 75% Et-OH | 30 sec |
| 7. | 95% Et-OH | 30 sec |
| 8. | 100% Et-OH | 30 sec |
| 9. | Xylene | 5min |
| 10. | Air-drying | 10min |

# APPENDIX D

## Modified Reverse Transcriptase Protocol

RT cDNA using superscript III with modifications for ABI random hexamers

1. Prepare on dry ice. Stored as 20 µl aliquots in – 80.
2. Pre-treat all plastic ware with RNase zap and rinse x 2 with RNase free H20.
3. First strand cDNA to prepare 20 µl reaction using random hexamers. Plan per 96 well plate. Color code tubes for RT master mix to avoid confusion. Number all PCR tubes which will store cDNA before start.
4. Dilute ABI random hexamers 1:10 in 10 mM Tris HCL buffer, pH 8.3.
5. Add 1.5 µl of 1:10 random hexamers to achieve 150 ng of random hexamers.
6. Add 10 pg – 5 µg of total RNA (1 µl per reaction). Use the following dilutions (expected from LCM), 5 10, 20, and 50 ng/µl. Confirm with starting RNA quantity with NanoDrop.
7. Add 1 µl 10 mM dNTP mix. Quick spin.
8. Aliquot needed amount (20 µl) and return the rest to – 20.
9. Add sterile RNAse/DNAse free water to total 13 µl (9.5 µl).
10. Heat to 65 C x 5 minutes.
11. Incubate on ice x 1 minute.
12. Collect the contents of the tube by brief centrifugation and add the following:

| | Amount for single reaction | Amount for 20 rx MM |
|---|---|---|
| a. | 4 µl 5x first strand buffer | 80 µl |
| b. | 1 µl 0.1 M DTT | 20 µl |
| c. | 1 µl RNaseOUT | 20 µl |

d. 1 µl super script III RT (200 units/µl) 20 µl

13. Mix by pipetting up and down. If using random hexamers, incubate at 25 c for 5 minutes.
14. Incubate 50c for 30-60 minutes. Increase the reaction temperature to 55 C for gene specific primer or for difficult templates with high secondary structure.
15. Inactivate by heating at 70 C x 15 min.
16. Time summary
    a. Step 1 – 65 x 5 min, ice x at least 1 minute
    b. Step 2
        i. 25 c x 5 min
        ii. 50 C x 30 to 60 min (45 minutes used)
        iii. 70 c x 15 minutes

.