MicroRNA Expression Following Lipopolysaccharide-Induced Inflammation of Rat Testis

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Honors Thesis

Monmouth University

Biology

Spring 2016

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Note: This Honors Thesis was written in accordance with School of Science guidelines for the Honors Thesis.

This thesis is dedicated to my parents, Glenn and Elaine,

for helping and inspiring me to reach for the stars everyday.

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ABSTRACT

MicroRNAs (miRNAs) are small, double-stranded, non protein-coding RNAs that negatively regulate gene expression at the transcriptional and post-transcriptional levels. Reproductive biologists are interested in miRNAs because proper expression of these transcripts has been linked to normal testis development and spermatogenesis, while atypical expression of certain miRNAs has been implicated in testicular cancer formation and male infertility, among other roles. Our laboratory is interested in how miRNAs may be involved in defending the male reproductive tract from infection and the response to inflammation. We hypothesize that control of gene expression by miRNAs plays a significant role in the antimicrobial protection of rat testis and the response to inflammation. The overall goal of our research is to determine which miRNAs are involved in regulating inflammation of the testis in response to infection, and to discover the functional significance of such alterations. The objective of this study was to identify inflammatory-related miRNAs (miRNAs that regulate inflammatory genes) that are up-regulated and/or down-regulated following lipopolysaccharide (LPS)-induced inflammation of rat testis. LPS is a common component of bacterial cell walls that provokes a strong inflammatory response, in the testis and other tissues of the body, when introduced into the bloodstream. In this study, a Qiagen Rat Inflammatory Response & Autoimmunity miRNA PCR Array (MIRN-105Z) was used to evaluate the expression of 84 inflammatory-related miRNAs. Testis total RNA was purified from retired Sprague-Dawley breeder rats that were sacrificed 3 or 6 hours after receiving a 5 mg/kg injection of LPS (n=4) or saline (n=2), and examined by quantitative real-time polymerase chain reaction (qPCR). Results showed 5 inflammatory-related miRNAs with a greater than 2 fold down-regulation (p<0.05) in rats from the 3h group (let-7f-5p, miR-200c-3p, miR-23a-3p, miR-23b-3p, and miR-98-5p), and 5 inflammatory-related miRNAs with a greater than 2 fold down-regulation (p<0.05) in rats from the 6h group (miR-17-5p, miR-19a-3p, miR-34a-5p, miR-34c-5p, and miR-449a-5p). Review of the literature has revealed that these miRNAs also play major roles in the maintenance of fertility (let-7f, miR-200c, miR-17, miR-34a/c, and miR-449a), formation and elimination of cancer (all significant miRNAs), and development of the male reproductive tract (let-7f, miR-17, miR-19a, miR-34a/c, and miR-449a). Further study of these miRNAs, and their roles in male reproductive tissues, might lead to advanced therapeutics for treatment, novel biomarkers for detection, and a greater understanding of male reproductive biology and related health issues.

BACKGROUND

Introduction

Andrology is the study of male reproductive health and related issues of the male reproductive tract. Research into the defense mechanisms that the male reproductive organs use to safeguard against pathogens is of great interest to andrologists because bacterial and viral infections are known to cause infertility, cancers of the male reproductive tract, and erectile dysfunction, among other conditions. Understanding the ways in which the reproductive tissues fight off and defend against infection is central to the treatment and prevention of male reproductive diseases.

MicroRNAs (miRNAs) are a group of small RNA molecules that do not encode for proteins. Instead, they regulate gene expression at the transcriptional and post-transcriptional levels. Reproductive biologists are interested in miRNAs because proper expression of these transcripts has been linked to normal testis development and spermatogenesis. Atypical expression of certain miRNAs, however, has been implicated in testicular cancer formation and male infertility. Aberrantly expressed miRNAs are, therefore, being looked to as potential biomarkers for such pathologies. Additionally, miRNAs are being studied to discover novel therapeutics to treat cancers of the male reproductive tract and male infertility.

Research that examines the functional significance of miRNAs in defending the male reproductive organs from infection is yet to be published. In this study, lipopolysaccharide (LPS)-induced inflammation was used as a model to examine the effect of changes in testis miRNA expression following infectious insult of the rat male reproductive tract. We hypothesize that control of gene expression by miRNAs plays a significant role in the antimicrobial protection of rat testis. The overall goal of our proposed research was to determine which

miRNAs are involved in regulating inflammation of the testis in response to infection, and to discover the functional significance of such alterations. To achieve this goal, a number of objectives were performed to examine, at the molecular level, which miRNAs may be involved in manipulating the cellular responses of the testis to invading microbes. These studies, and others, will enable us to gain a greater understanding of male reproductive biology and related health issues.

The following topics will be covered in this thesis to help explain the full breadth of this research project: The Male Reproductive System, Immunophysiology of the Male Reproductive Tract, Control of Gene Expression by MicroRNAs, MicroRNAs in the Male Reproductive System, MicroRNAs in the Immune System, and Lipopolysaccharide-Induced Inflammation as a Model. We will begin with the male reproductive system in general, then make our way towards the immunophysiology of male reproduction, take a pause to discuss the basic biology of miRNAs and their known roles in the male reproductive and immune systems, and finish with a discussion of the model that will be used in this study to simulate immune activation of the male reproductive tract.

The Male Reproductive System

The main purpose of the male reproductive system is to support sexual reproduction. As a whole, the male reproductive organs work together to accomplish the two key steps of sexual reproduction: 1) producing gametes and 2) facilitating fertilization—the joining of male (a spermatozoan) and female (secondary oocyte) gametes. The process of sexual reproduction creates offspring that contain genetic information from both gamete-contributing parents. Sexual reproduction is an essential part of the human life cycle and continuation of our species existence (Jones and Lopez, 2014; Tortora and Derrickson, 2011).

The four main parts of the male reproductive system are 1) the testes (male gonads), 2) a system of ducts, 3) accessory sex glands, and 4) several supporting structures (Figure 1). The

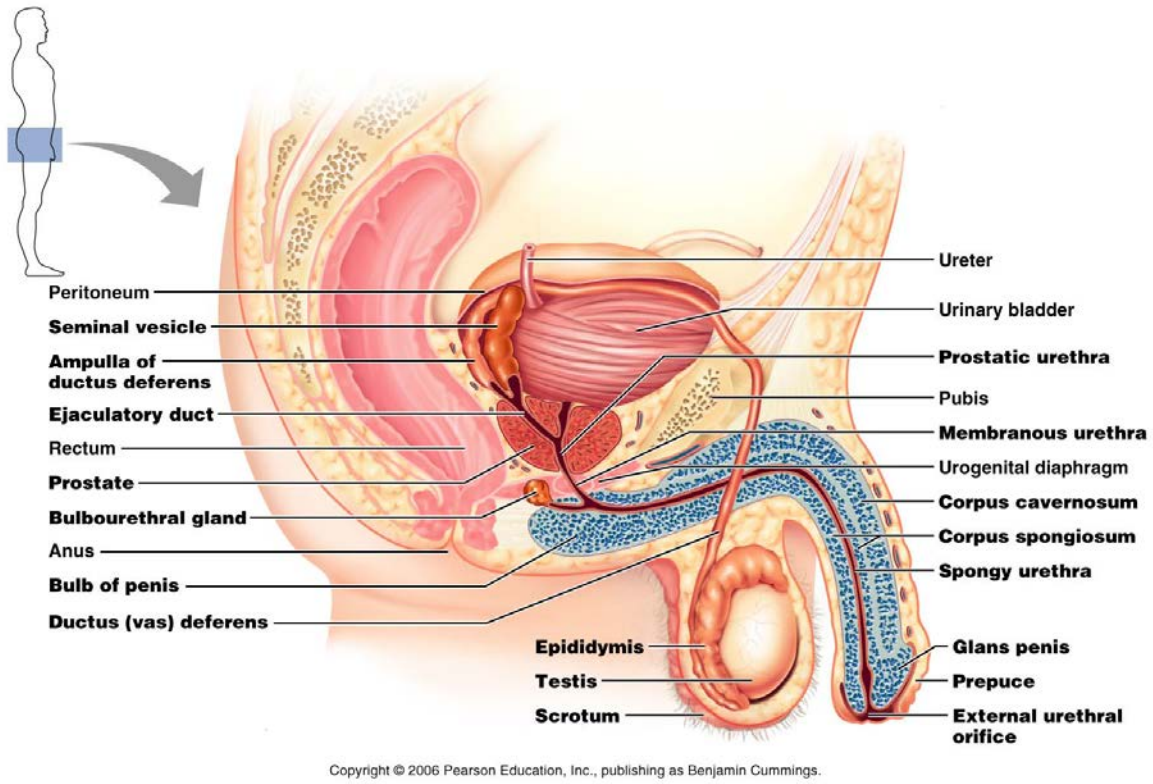

Figure 1. The Male Reproductive System. Marieb, E. N., & Hoehn, K. 2007. *Human Anatomy and Physiology*, 7th ed. Pearson Benjamin Cummings, San Francisco, CA.

testis (pl., testes) produces gametes and secretes sex hormones. The divisions of the duct system—the epididymis, ductus (vas) deferens, ejaculatory ducts, and urethra—store, assist in maturation of, and transport sperm to the exterior. Sperm is delivered in a secretion provided by the accessory sex glands (the seminal vesicles, prostate, and bulbourethral glands); the resulting combination is called semen. Semen is transferred to the female reproductive tract by the penis, a supporting structure. Another supporting structure, the scrotum, holds and protects the testes, while helping to create a thermostatic environment (2-3˚C below core body temperature) within these gonadic tissues—a requirement for normal sperm production (Jones and Lopez, 2014; Tortora and Derrickson, 2011).

The Testis

The testes (sing., testis) are paired oval glands that are located within the scrotum (Figure 2). Each testis is suspended from the body by a spermatic cord, a structure comprised of the

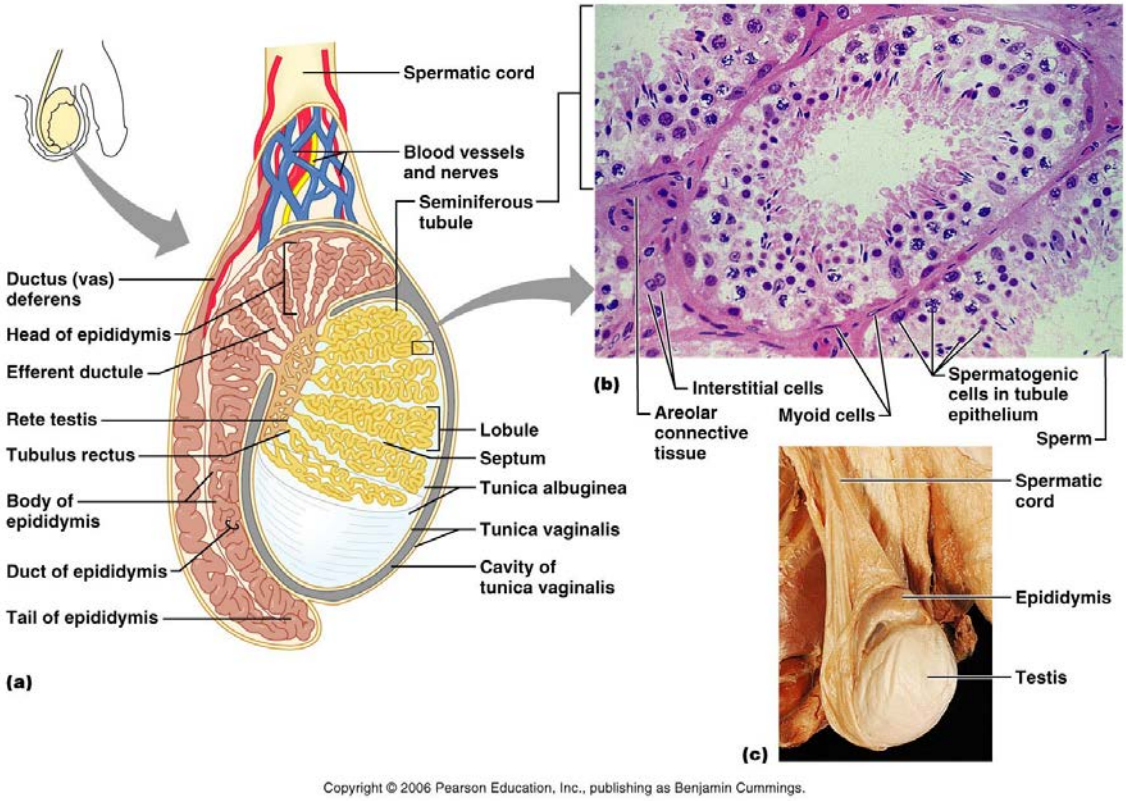

Figure 2. The Testis. Marieb, E. N., & Hoehn, K. 2007. *Human Anatomy and Physiology*, 7th ed. Pearson Benjamin Cummings, San Francisco, CA.

ductus deferens, a testicular nerve, and three coiled blood vessels. In humans, each testis contains 200-300 internal compartments (lobules), which are separated from each other by septa (tissue barriers). Within each lobule are one to three highly coiled seminiferous tubules—the site of sperm production (spermatogenesis). Two types of seminiferous tubule cells include: 1) spermatogenic cells—sperm-forming cells—and 2) Sertoli cells—spermatogenesis-supporting cells. Another cell type of the testis is the Leydig (interstitial) cell, which is located outside of the seminiferous tubules within the interstitial space. Leydig cells secrete testosterone, an androgen or sex hormone that promotes the development of male characteristics (Jones and Lopez, 2014;

Tortora and Derrickson, 2011). The testis is the male reproductive organ that will be the focus of this study.

Hormonal Control of the Testis

Hormones that are released by the hypothalamus and anterior pituitary (parts of the brain) control testis function. Release of gonadotropin-releasing hormone (GnRH) by the hypothalamus stimulates the anterior pituitary to secrete the gonadotropins 1) luteinizing hormone (LH) and 2) follicle-stimulating hormone (FSH). LH stimulates Leydig cells to secrete testosterone, while FSH indirectly incites spermatogenesis through Sertoli cells. Testosterone is required for spermatogenesis, in addition to stimulating the growth of male tissues, supporting secondary male sex characteristics, and regulating the physiology of other male reproductive organs (i.e epididymis, prostate, vas deferens, and seminal vesicles). The active metabolite of testosterone in peripheral tissues is dihydrotestosterone (DHT). All hormones involved in control of testis functions are regulated by negative feedback loops, either through nervous system control—as discussed above—or through the influence of local (paracrine) factors (Jones and Lopez, 2014; Tortora and Derrickson, 2011).

Spermatogenesis and Spermiogenesis

Spermatogenesis is the process in which sperm cells or spermatozoa (sing. spermatozoon) are made from male primordial germ cells by way of mitosis and meiosis. It begins with stem cells called spermatogonia (sing., spermatogonium) that arise from differentiated primordial germ cells during embryonic development of the testis. Spermatogonia remain dormant until puberty, when they are activated to begin producing sperm. The transition of spermatogonia to sperm occurs in a series of stages: spermatogonia develop into spermatocytes, primary

spermatocytes, secondary spermatocytes, and then into spermatids (Jones and Lopez, 2014; Tortora and Derrickson, 2011).

Spermatids mature into sperm cells or spermatozoa through a process called spermiogenesis. During spermiogenesis, an acrosome (cap-like vesicle filled with enzymes that help a sperm penetrate a secondary oocyte during fertilization) forms, nuclei condense and elongate, flagella develop, and mitochondria multiply, transforming spherical spermatids into elongated, slender sperm. After spermatogenesis, and subsequent spermiogenesis, spermatozoa are released into the lumen (inside space) of the seminiferous tubules and make their way through the male reproductive tract's system of ducts (Jones and Lopez, 2014; Tortora and Derrickson, 2011).

The Epididymis

The epididymis (pl., epididymides) is a comma-shaped organ consisting mostly of tightly coiled ducts. It is divided into four sections based on anatomical and functional differences: the 1) initial segment—which receives spermatozoa from the testis, 2) caput or head, 3) corpus or body, and 4) cauda or tail. The epididymis is the site of sperm maturation, the process by which sperm becomes motile and gains the ability to fertilize a secondary oocyte. Sperm is stored within the epididymis, where it remains viable for several months. During sexual arousal, the epididymis helps propel sperm into the ductus deferens, a major step in semen ejaculation (Jones and Lopez, 2014; Tortora and Derrickson, 2011).

The epididymis, like other male reproductive organs, contains many immune protective mechanisms. For example, during storage and maturation of spermatozoa in the epididymis, a number of immune system defenses help safeguard spermatozoa from a variety of insults (e.g. free radicals and bacterial infection). In the section below—Immunophysiology of the Male

Reproductive Tract—the unique relationship between the male reproductive and immune systems will be discussed. The hope is to set a framework for this research project, which will focus on the possible role of miRNAs in manipulating the cellular response of the testis to infection.

Immunophysiology of the Male Reproductive Tract

Part of maintaining homeostasis involves protecting tissues of the body from invading pathogens—disease-causing microbes and/or viruses. This task is accomplished by the immune system, which identifies and eliminates pathogens through the use of cells and/or molecules. Major cells of the immune system involved in fighting off disease-forming agents include: lymphocytes (white blood cells), macrophages, mast cells, natural killer (NK) cells, neutrophils, eosinophils, basophils, dendritic cells, T cells, and B cells. These cells circulate through the blood, lymph, and tissues to detect and remove disease-causing entities (Tortora and Derrickson, 2011).

The immune system can be divided into two subsystems: 1) the innate immune system and 2) the adaptive immune system. These two subsystems do not function in isolation, but rather have overlapping cellular and molecular pathways. The innate immune system provides non-specific responses to infection by recognizing conserved molecular markers across pathogens. Alternatively, the adaptive immune system initiates targeted attacks against particular pathogens by detecting specific molecular motifs (antigens) expressed by certain agents of disease. Infection is the term used to describe invasion and multiplication of a pathogen within a host's body tissues, while inflammation is the initial response to infection involving changes to homeostasis including: fever, activation of immune cells, and increased blood flow to the site of infection (Tortora and Derrickson, 2011).

Although the testis has properties of both innate and adaptive immunity, the immunophysiology of the male reproductive system is unique in comparison to other regions of the human body. Because sexual maturation begins long after the development of the immune system, and its establishment of immune tolerance (the ability of the immune system to recognize "self" from "non-self"), spermatogenic (sperm-forming) cells have the potential to be recognized as "foreign" and targeted for destruction by immune cells. Consequently, organs of the male reproductive tract must learn to strike a balance between *immunoregulatory* and *immunosuppressive* mechanisms to prevent loss of spermatogenic cells by autoimmune activities, while also protecting these tissues from pathological harm (Hedger, 2015).

Immune stability within the male reproductive system is maintained through a complex web of interactions involving somatic cells, resident immune cells, and circulating cellular elements of the immune system. Other means of protecting the organs of reproduction from infectious imbalance include physical barriers to pathogens, such as: 1) the long length of the male reproductive tract, 2) the high acidity of urine, and 3) the blood testis barrier (BTB)—occluding junctions between adjacent Sertoli cells. Although the male reproductive system has many methods of preventing infectious insult, invasion of reproductive tissues by pathogens can still occur by one or both of the following means: 1) retrograde movement through the tract and 2) arrival by systemic circulation (Hedger, 2015). The following two subsections provide a detailed discussion of the immunoregulatory and immunosuppressive mechanisms of the male reproductive tract, which are further reviewed by Mark P. Hedger in chapter 19 of *Physiology of Reproduction*, The Immunophysiology of Male Reproduction.

Immunoregulatory Mechanisms

One common theme of the male reproductive tract is a restraint of antigen-specific immunity that is counterbalanced by an enhancement in local innate immune mechanisms and conventional mucosal immunity. Innate immune cells, such as macrophages and granulocytes (eosinophils or basophils), are typical inhabitants of male reproductive tissues—particularly in the interstitial spaces of the testis—under normal (non-inflamed) conditions, and increase in number upon infection. Lymphocytes (adaptive immune cells), however, are only found in small amounts in the male reproductive tract. Additionally, many proteins are present within the male reproductive tract that assist in innate immune detection and extermination of invading pathogens; these include: toll-like receptors (TLRs), nitric oxide synthases (NOS), prostaglandins, caspases, mitogen-activating protein kinases (MAPK), and various cytokines, such as the interleukin (IL), tumor necrosis factor (TNF), and transforming growth factor-β (TNFβ) families of molecules. While Sertoli and Leydig cells are also involved in the fight against pathogens, they play more of an immunoregulatory role through the production and secretion of androgens and immunoregulatory molecules that help control inflammatory-related immune responses (Hedger, 2015).

Immunosuppressive Mechanisms

Another common theme of the male reproductive tract is reduced immunological activity. Compared with the female reproductive system, immunological activity within the male reproductive tissues is highly suppressed. Males have reduced cellular immunity and less effective responses to infection, which is the price paid for decreased incidence of autoimmune disease. In fact, immune cells that enter the male reproductive tract are modified to limit their pro-inflammatory activities—a safeguard to normal reproductive functions. Still, dysregulation

of the normal environment of the male reproductive tract can occur. Infection, inflammation (local or systemic), toxic insult, active immunization, or deletion of regulatory T cells, are just a number of factors that have been suggested to cause uncontrolled immunological activity within the male reproductive tract. If left unchecked, loss of immunosuppressive mechanisms within the tissues of male reproduction can lead to negative consequences such as: androgen insufficiency, infertility, or chronic inflammation. Scientists are, therefore, looking into the protective mechanisms of the male reproductive tract to unlock the secrets of how the male reproductive and immune systems work together to preserve a male's reproductive viability (Hedger, 2015).

Control of inflammatory gene expression by miRNAs may be one method that the male reproductive system employs to maintain immune stability within its tissues. In the sections following—Control of Gene Expression by MicroRNAs, MicroRNAs in the Male Reproductive System, and MicroRNAs in the Immune System—the basic biology of miRNAs, in addition to the functional role of these molecules in the male reproductive and immune systems, will be discussed. This will set a concrete background for the research project, which focused on discovering which miRNAs may be involved in regulating the cellular response of the testis to infection.

Control of Gene Expression by MicroRNAs

MicroRNAs (miRNAs) are a group of small (~17-25 ribonucleotides long), double-stranded, non-protein coding RNAs that negatively regulate gene expression at the transcriptional and post-transcriptional levels. RNA interference (RNAi) is the term used to describe post-transcriptional repression of gene expression, while RNA-induced gene silencing is the phrase used to depict transcriptional suppression of gene expression. In RNAi, antisense miRNAs bind to endogenous sense mRNAs blocking translation or causing their degradation. In

RNA-induced gene silencing, miRNAs alter chromatin structure in the nucleus preventing transcriptional access to specific parts of DNA (Klug et al., 2014; Thieman and Palladino, 2012).

Formation of microRNA

The creation of miRNAs begins in the nucleus as pri-miRNA—a single-stranded RNA with a double-stranded stem-loop (hairpin loop) structure—is transcribed from genomic DNA (Figure 3). Then Drosha, a nuclease enzyme, processes pri-miRNA separating its single-stranded and double-stranded, stem-loop portions; the resulting product is called pre-miRNA. Afterwards, the formed pre-miRNA molecule is exported from the nucleus where it is broken up by cytoplasmic Dicer (another nuclease enzyme) into a short, linear, double-stranded miRNA molecule. Over 1500 eukaryotic miRNAs have already been discovered. These miRNAs are estimated to regulate the expression of more than half of all protein-coding genes (Klug et al., 2014; Thieman and Palladino, 2012).

RNA Interference (RNAi)

The process of RNAi begins as a miRNA molecule associates with a complex of proteins called the RNA-induced silencing complex (RISC) (Figure 3). RISC denatures the double-stranded miRNA and degrades its sense stand. Once formed, the miRNA-RISC complex can target mRNA molecules complementary to the antisense miRNA strand contained within the RISC. If exact complementarity is achieved, the targeted mRNA will be cleaved by the RISC and the resulting mRNA fragments will be degraded by ribonucleases. If the antisense miRNA strand contained within the RISC does not exactly match the target mRNA sequence, the RISC complex will stay bound to the mRNA molecule and prevent translation by interfering with ribosomal binding (Klug et al., 2014; Thieman and Palladino, 2012).

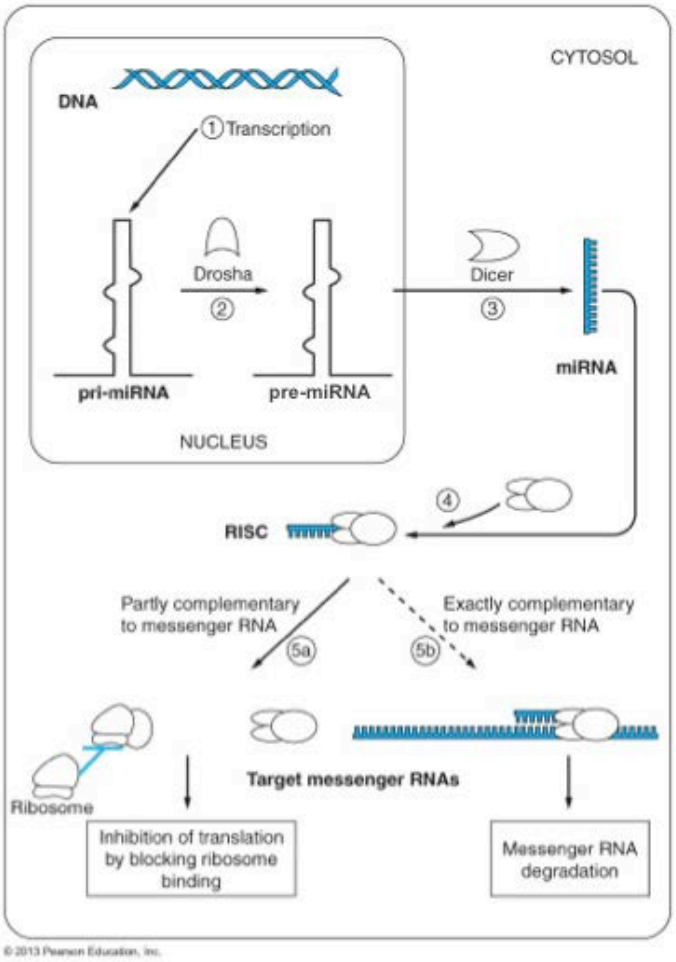

Figure 3. Formation of miRNA and RNA Interference. Thieman, W. J., Palladino, M.A. 2012. *Introduction to Biotechnology*, 3rd ed. Benjamin Cummings, Boston, MA.

RNA-Induced Gene Silencing

RNA-induced gene silencing can occur by two means. The first approach involves a complex of proteins called the RNA-induced transcription silencing complex (RITS). Molecules of miRNA can associate with the RITS, and the antisense strand of the miRNA molecule that binds to the RITS can target the complex to specific gene promoters or larger regions of chromatin. Enzymes that modify chromatin structure—by methylating histones and/or DNA—will then be recruited by the RITS to create heterochromatin, thereby silencing transcription. The second way that RNA-induced gene silencing can be achieved is by RNAi-mediated silencing of transcription factors. If RNAi methods prevent the formation of transcription factors,

transcription of genes whose expression are dependent on such proteins may be prevented (Klug et al., 2014).

MicroRNAs in the Male Reproductive System

MiRNAs that are predominately derived from the testis and epididymis are involved in regulating normal functions of the male reproductive system (Nixon et al., 2015a; Yang et al., 2013). Through studies over the past few years, miRNAs have been implicated in control of androgen signaling (Hu et al., 2013a) and the repression of cell proliferation and oncogenic pathways within the male reproductive tract (Nixon et al., 2015b). Highly regionalized patterns of miRNA expression within the male reproductive system demonstrate that each tissue within the tract plays a specific role in preserving normal reproductive functions. Especially in the epididymis, segmental patterns of miRNA expression have been seen to parallel the maturation process of sperm (Nixon et al., 2015a). Moreover, miRNAs have been found in seminal plasma, signifying their importance in fertilization and embryo development (Hu et al., 2013a). These miRNAs found within semen were shown to come from the epididymis, marking this organ as an important site for the formation of the sperm epigenome—a major regulator of sperm functionality and viability (Nixon et al., 2015b). Because of their importance in regulating normal reproductive function, miRNAs are being studied to understand their role in male fertility, testicular cancer formation, and development of the male reproductive tract. Brief summaries of the roles of miRNAs in these processes are provided in the next few subsections.

Roles of microRNAs in Male Fertility

Growing research has demonstrated that miRNAs play a major role in regulating normal spermatogenesis and male fertility. Expression of certain miRNAs has been shown to be associated with the processes of spermatogonial stem cell (SSC) renewal, spermatogonial mitosis,

spermatocyte meiosis, and spermiogenesis (McIver et al., 2012; Wang and Xu, 2015). MiRNAs control these processes by epigenetically regulating target genes involved with the mitotic, meiotic, and post-meiotic (spermiogenesis) stages of spermatogenesis (Wang and Xu, 2015; Yao et al., 2015). Since spermatogenesis is characterized by high transcriptional activity and suppressed translation activity (Wang and Xu, 2015), it is no surprise that miRNAs are involved in regulating fertility. In fact, Dicer—which is needed to form mature miRNAs—has been shown to be essential for Sertoli cells to mature, survive, and sustain germ cell development. Alternatively, ablation of Dicer has been implicated in male infertility (McIver et al., 2012; Wang and Xu, 2015). Given the fact that miRNAs have increasingly emerged as major players in the processes of normal spermatogenesis and male fertility, miRNAs are being looked to for potential treatments of male infertility and development of male contraceptives (McIver et al., 2012; Papaioannou and Nef, 2010). Additionally, research has confirmed that certain miRNA signatures can be linked to obstructive and non-obstructive azoospermia (having no measurable sperm in semen), making miRNAs possible biomarkers and therapeutics for male infertility (Abu-Halima et al., 2014; Lian et al., 2009).

microRNAs and Cancer of the Male Reproductive System

Abnormal expression of miRNAs in the male reproductive system has been shown to play a role in tumorigenesis. Studies have demonstrated cancer-specific expression of miRNAs in tumors of the prostate and testis (Schaefer et al., 2010), and have shown a unique connection between miRNAs, infertility, and testicular germ cell tumor (TGCT) formation (Bezan et al., 2014; Wang and Xu, 2015). Deregulated miRNAs (recently coined “oncomirs”) in the male reproductive tract have been thought to result in cancer by interfering with the normal cellular processes of growth, apoptosis, differentiation, and proliferation (i.e. giving them oncogenic or

removing tumor suppressive capabilities). Various mechanisms of miRNA dysregulation have been proposed including: genomic alterations, gene mutations, and epigenetic modifications. Although data is relatively sparse in regards to miRNAs and urological cancers, scientists are hopeful that further studies in this area of research will lead to the discovery of novel diagnostic, prognostic, and predictive biomarkers for urological tumors, in addition to potential therapeutics for such cancers (McIver et al., 2012; Schaefer et al., 2010).

microRNAs and Development of Male Reproductive Organs

Various miRNAs have been shown to be involved in the development of male reproductive organs (Buchold et al., 2010; Yan et al., 2007). Specifically, studies have revealed differential expression of miRNAs between prepubertal (immature) and adult (mature) testis (Yan et al., 2007); adult testis have a more complex miRNA profile (Buchold et al., 2010). Still, after birth, miRNAs have been shown to be abundantly expressed in the male testis, demonstrating their potential importance in the development of testis tissue (Buchold et al., 2010). One notable finding regarding sexual development and miRNAs has been a robust, male-biased expression of miRNAs encoded on the X chromosome, which could be one method of male resistance to meiotic sex chromosome inactivation. Research has shown that approximately 79% of miRNA genes expressed in testis tissue are derived from the X-chromosome, while, surprisingly, no miRNA expression have been shown to come from the Y chromosome (Mishima et al., 2008). Interestingly, these X-linked miRNAs have been shown to have higher substitution rates than autosomal miRNAs, implying an important role in male reproductive function and a possible mechanism for speciation in mammals (Guo et al., 2009). Given that there is a distinguishable difference in miRNA signatures between adult testis and ovaries, miRNAs may play an essential part in the development of male sexual characteristics (Mishima et al., 2008).

MicroRNAs in the Immune System

Although research into the impact of miRNAs on cellular immunity is still in its early stages, we have already learned so much about the role of miRNAs in the development of the immune system and regulation of immune functions (Pedersen and David, 2008; Sonkoly et al., 2008). Many miRNAs have already been discovered to be involved in a number of immune system processes: proliferation of monocytes and neutrophils, maintaining the cell fate of immune cells, controlling TLR signaling and cytokine responses associated with innate immunity, regulating central elements of the adaptive immune response (e.g. antigen presentation), controlling B and T cell differentiation and development, facilitating T cell receptor signaling, enabling antibody switching, and promoting the release of inflammatory mediators (Lindsay, 2008; Pedersen and David, 2008; Sonkoly et al., 2008).

Additionally, miRNAs have been shown to assist in anti-viral defense by directly impinging on the viral life cycle, viral tropism, and the pathogenesis of viral diseases (Taganov et al., 2007). Alternatively, bacteria and viruses have been seen to significantly modify the expression profile of host miRNAs, changing the host's normal proliferation, differentiation, and apoptotic pathways—an implicated technique for viral immune escape (Staedel and Darfeuille, 2013). Furthermore, expression patterns of certain miRNAs have been seen to change following chronic infection and inflammation. Given the fact that specific miRNAs can be regulated by inflammatory stimuli, and certain miRNAs can act as mediators of inflammation, it is no surprise that alterations in inflammatory-related miRNA expression can generate negative immune-associated effects such as: uncontrolled cell proliferation and apoptosis, inflammation, oxidative stress, and inflammation-associated carcinogenesis (Schetter et al., 2010).

Because miRNAs are know to be involved in immune system functions throughout the human body, it is reasonable to think that the same holds true in the testis. In this research project, rats were used as a model to examine the role of miRNAs during innate immune activation of the testis. Rats are ideal animals to perform genetic studies on because they share 80% of their genes with humans and contain a similar physiological makeup (Thieman and Palladino, 2012). In the next section, the method that was used in this study to induce inflammation of rat testis will be discussed.

Lipopolysaccharide-Induced Inflammation as a Model

A component of Gram-negative bacterial cell walls, lipopolysaccharide (LPS) is a common antigen that—when introduced into the bloodstream—provokes a strong inflammatory response in the testis and other tissues of the body. LPS additionally has the effect of reducing testosterone production by inhibiting steroidogenesis (the creation of androgens) within Leydig cells (Bachir and Jarvi, 2014). Low testosterone levels, therefore, can be used as an indicator that an inflammatory response has occurred in the testis. Moreover, systemic administration of LPS has been shown to affect spermatogenesis and reduce sperm concentration, motility, and viability (Hedger, 2015). *Pseudomonas aeruginosa* is a bacterium that is known to contain LPS within its outer-membrane (Mittal et al., 2009).

Molecules of LPS are detected by the receptor protein TLR4, causing a biochemical cascade that triggers innate immune responses. Past studies in our laboratory, determined that TLRs—one being TLR4—are abundantly expressed throughout the male reproductive tract, implicating their importance in innate immune activation of the testis (Palladino et al., 2007, 2008). Additionally, further research in our laboratory discovered up- and down- regulation of a number of inflammatory genes following LPS-induced inflammation of rat testis (Fasano, 2014).

Since TLR4 is abundantly expressed in the male reproductive tract, and administration of LPS into the bloodstream has proven to cause a robust inflammatory response within these tissues, LPS-induced inflammation was used as a model in this research to examine changes in inflammatory-related miRNA expression following innate immune activation of the testis.

Summary

Proper immune system balance within the male reproductive tract is essential to the maintenance of normal reproductive functions. Since, miRNAs have been shown to be important regulators of gene expression in the immune and male reproductive systems, it should follow that miRNAs manipulate inflammation of the testis in response to infection. In this research, LPS-induced inflammation was used as a model to study changes in miRNA patterns of expression following innate immune activation of the male reproductive tract, and the resulting effect of this regulatory adaptation. We hypothesize that control of gene expression by miRNAs plays a significant role in the antimicrobial protection of rat testis. To test this hypothesis, various techniques were used to study changes in inflammatory-related miRNA expression following LPS-induced inflammation of rat testis. The hope was to identify miRNAs that are essential to the preservation of normal immune function within the male reproductive tract, and use the data acquired to discover novel detection and treatment methods for male reproductive diseases and related health issues.

OBJECTIVES

Hypothesis

We hypothesize that control of gene expression by miRNAs plays a significant role in the antimicrobial protection of rat testis.

Goals

1. Discover which inflammatory-related miRNAs are significantly up-regulated and/or down-regulated following LPS-induced inflammation of rat testis.
2. Construct a pathway map connecting significantly up-regulated and/or down-regulated inflammatory-related miRNAs to their known target inflammatory genes.
3. Determine if any of the known target inflammatory genes for significantly up-regulated and/or down-regulated inflammatory-related miRNAs also showed significant changes in expression.
4. Uncover the combined effect of changes in expression of (i) inflammatory-related miRNAs, and (ii) their known target inflammatory genes, on the levels of their down-stream inflammatory proteins.

Objectives

1. Determine changes in expression of inflammatory-related miRNAs following LPS-induced inflammation of rat testis.
2. Use computer-based (*in silico*) methods to discover known target inflammatory genes of significantly up-regulated and/or down-regulated inflammatory-related miRNAs.
3. Compare results to data collected in previous studies that examined changes in expression of inflammatory genes following LPS-induced inflammation of rat testis.

4. Determine changes in levels of notable, down-stream inflammatory proteins (i.e. inflammatory proteins coded for by significantly up-regulated and/or down-regulated inflammatory genes whose known target miRNA also showed significant up-regulation and/or down-regulation) following LPS-induced inflammation of rat testis.

MATERIALS AND METHODS

Based on time constraints, only objectives 1-3 of this research were completed. The materials and methods for objectives 1-3 are provided below.

Animals

In this study, LPS-induced inflammation was used as a model to examine changes in testis miRNA expression following innate immune activation of the male reproductive tract. The model organisms that were used in this study were adult, male, retired breeder Sprague-Dawley rats (approximately 60 days old). These rats were chosen because—as retired breeders—they proved to have healthy, well-functioning reproductive systems. Past work in our laboratory received approval from the Monmouth University Institutional Animal Care and Use Committee (IACUC) for the use of 12 rats in LPS-induced inflammation model experiments. No new animals were used in this study.

During a previous study in our laboratory, 8 of 12 rats were injected intraperitoneally with LPS from *P. aeruginosa* at a dosage of 5 mg/kg body weight (experimental group), while 4 of 12 rats were injected with saline (control group). Half of the rats in each group (i.e. 4 of 8 rats from the experimental group and 2 of 4 rats from the control group) were sacrificed 3 hours post-injection, while the other half (i.e. other 4 rats from the experimental group and other 2 rats from the control group) were sacrificed 6 hours post-injection (Figure 4, 5). These time points were selected based on results from past experiments in the laboratory and studies from the literature (O'Bryan et al., 2000); tissues from rats sacrificed at 3 hours post LPS-induced inflammation is known to represent a snapshot of an "immediate response" to infection, while tissues from rats sacrificed at 6 hours post LPS-induced inflammation is known to represent a snapshot of a "long-term or acute-phase response" to infection. After rats were sacrificed, testis tissue from each rat

was surgically removed and frozen at -80°C for RNA isolation purposes. In this study, a number of molecular and computer-based techniques were used to accomplish certain objectives that allowed us to determine, at the molecular level, how miRNAs manipulate the cellular responses of the testis to invading microbes.

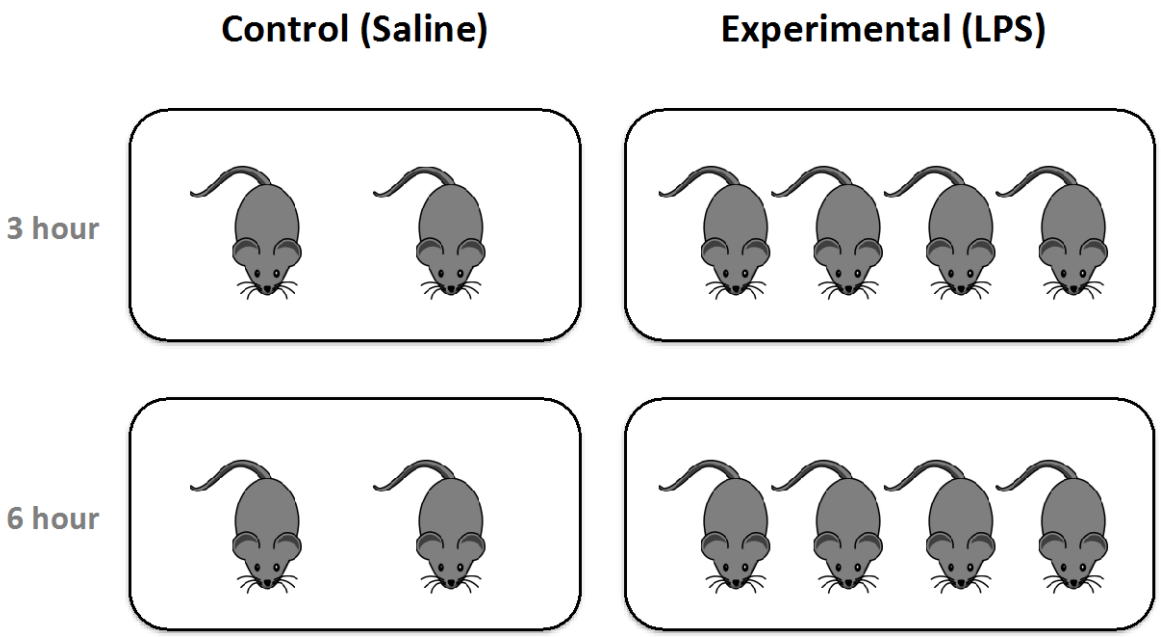

Figure 4. Experimental setup. Rats were divided into groups in a way that maximized the number of experimental samples, while maintaining a sufficient number of control samples.

Objective 1

The first objective of this research was to determine changes in expression of inflammatory-related miRNAs following LPS-induced inflammation of rat testis. The goal of this objective was to discover which inflammatory-related miRNAs are significantly up-regulated and/or down-regulated following LPS-induced inflammation of rat testis. To complete this objective the following procedures were performed: RNA Isolation, Synthesis of

Complementary cDNA, Real-Time Quantitative Polymerase Chain Reaction (qPCR), and Statistical Analysis of Data (Figure 5).

RNA Isolation

RNA was extracted from testis tissue as per a protocol already established in our laboratory and according to manufacturer's (Invitrogen) instructions. The procedure utilized a chemical solution called TRIzol, which protects RNA integrity during the homogenization process while destroying other cellular components. The total isolated RNA contained testis miRNA in it.

The concentrations of testis RNA samples were determined using a ThermoScientific, NanoDrop 2000/2000c Spectrophotometer. The benefit of using this device is that it only requires a one-µl sample to determine the RNA concentration of an isolate. Additionally, since nucleic acids absorb light at 260 nm, and proteins absorb light at 280 nm, a 260/280 ratio of 1.8 or above was used as indictor that the isolation procedures resulted in purified RNA, and not protein.

Synthesis of Complementary DNA (cDNA)

To prepare RNA to be analyzed for miRNA expression, total RNA was first converted to complementary DNA (cDNA) through a reverse transcription reaction. To do this, a miScript II RT Kit was used. The procedure followed was that for "Pathway Profiling of Mature miRNA." Volumes containing equivalent amounts of RNA (125-250 ng), were diluted with RNase-free water to make 50 µl RNA samples with concentrations of 50 ng/µl. Five µl of each diluted RNA sample was then mixed with 4 µl of 5x miScript HiSpec Buffer, 2 µl of 10x Nucleics Mix, 7 µl of RNase-free water, and 2 µl of miScript Reverse Transcriptase Mix. The resulting reverse-transcription reaction solutions (20 µl for each sample) were then incubated at 37˚C for 60

minutes (activation stage), and at 95˚C for 5 minutes (inactivation stage), in a Bio-Rad T100 Thermal Cycler, to form cDNA for each sample. The cDNA samples were then diluted with RNase-free water to total volumes of 200 µl, and stored at -20˚C prior to expression profiling.

Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Real-time quantitative polymerase chain reaction (qPCR) is a technique used to determine comparative expression of specific RNA or DNA molecules. In this study, inflammatory-related miRNA patterns of expression for rats treated with either LPS (experimental) or saline (control) were determined using qPCR. A 96-well "Rat Inflammatory Response & Autoimmunity miRNA PCR Array" (MIRN-105Z) was purchased from Qiagen, which was used to determine the expression of 84 inflammatory-related miRNAs. The array also contained 2 Positive PCR Controls (PPC), 2 Reverse Transcription Controls (RTC), and 8 other controls, which were used to normalize and conclude the validity of the qPCR results. Samples for qPCR analysis were prepared using a miScript SYBR Green PCR Kit; one sample (corresponding to one of the 12 cDNA samples) was created for each array (12 in total). The protocol involved mixing 100 µl of template cDNA with 1375 µl of 2xQuantiTect SYBR Green PCR Master Mix, 275 µl of 10x miScript Universal Primer, and 1000 µl of RNase-free water; 25 µl of this mixture was then loaded into each well of a plate. A Stratagene Mx3005P qPCR instrument with laptop and software was used to perform the qPCR reaction. The cycling conditions were set as follows: 15 minutes at 95˚C (initial activation) and 40 cycles of 3-step cycling involving 15 seconds at 94˚C (denaturation), 30 seconds at 55˚C (annealing), and 30 seconds at 70˚C (extension). The raw data from the qPCR reactions were represented in critical threshold (C_t) values—the amount of cycles needed to detect variations in expression. These values were converted to fold-change differences by comparing C_t values of LPS and saline

treated samples and analyzed for statistical significance using a software program provided by Qiagen.

Statistical Analysis of Data

Once inflammatory-related miRNA patterns of expression for rats treated with either LPS or saline were determined, expression profiles of rats sacrificed within the same amount of time after treatment (3 or 6 hours) were compared to each other through a software program provided by Qiagen. miRNAs with a 2 fold change in expression (up- or down-regulation) and $p<0.05$ (t-test) were considered as having significant alterations in expression.

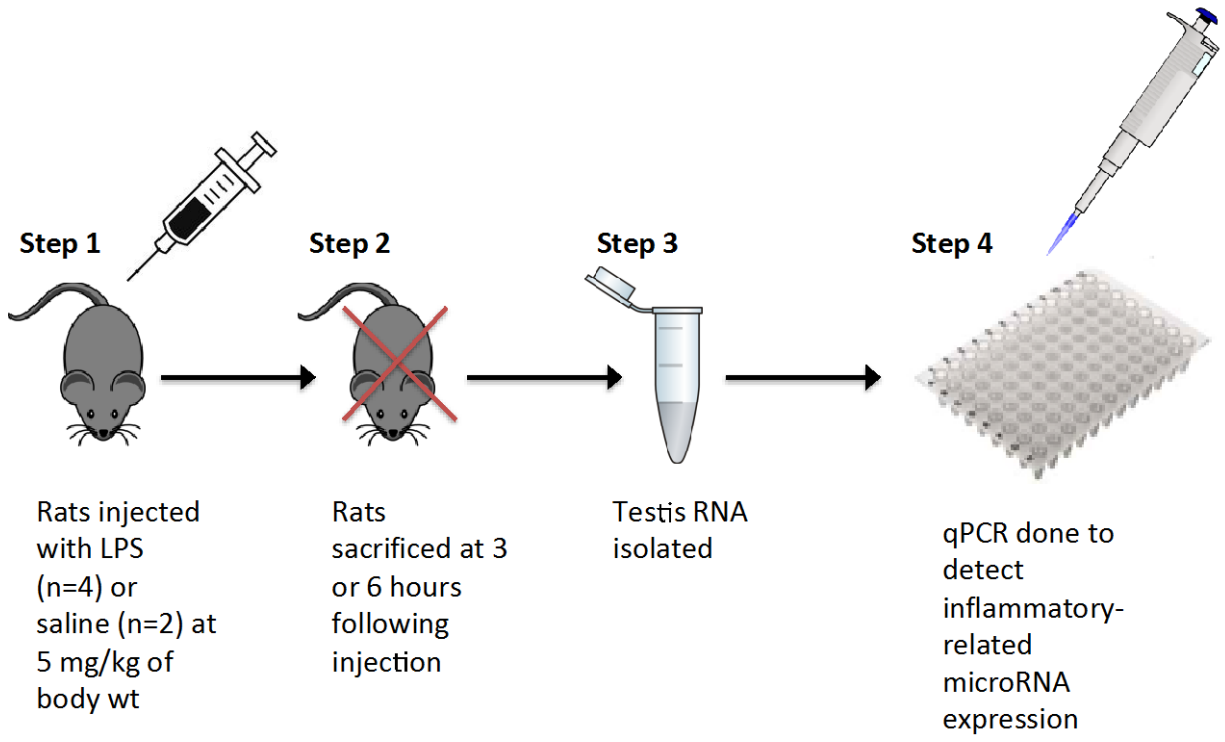

Figure 5. Methods. The following steps were performed to detect inflammatory-related miRNA expression following LP-induced inflammation of rat testis.

Objective 2

The second objective of this research was to use computer-based (*in silico*) methods to discover known target inflammatory genes of significantly up-regulated and/or down-regulated

inflammatory-related miRNAs. The goal of this objective was to construct a pathway map connecting significantly up-regulated and/or down-regulated inflammatory-related miRNAs to their known target inflammatory genes. To complete this objective the following procedure was be performed: Computer-Based (*In Silico*) Methods.

Computer-Based (In Silico) Methods

Qiagen provides a list of known target inflammatory genes for the inflammatory-related miRNAs analyzed in their array. Using this information, a pathway map was constructed connecting known target inflammatory genes to significantly up-regulated and/or down-regulated inflammatory-related miRNAs.

An online program called UniProt was used to determine the coding protein and function of the known target inflammatory genes. An attempt was made to connect the common pathways that these target inflammatory genes regulate (using an online program called Reactome), however, the results were too scattered to include in this paper.

Objective 3

The third objective of this research was to compare the results of this research to data collected in previous studies that examined changes in expression of inflammatory genes following LPS-induced inflammation of rat testis. The goal of this objective was to determine if any of the known target inflammatory genes for significantly up-regulated and/or down-regulated inflammatory-related miRNAs also showed significant changes in expression. To complete this objective the following procedure was performed: Identifying Interactions Between miRNAs and Target Inflammatory Genes from Previous Studies.

Identifying Interactions Between miRNAs and Target Inflammatory Genes from Previous Studies

Results from this study were compared to data from previous studies in our laboratory, which identified a number of up- and down-regulated inflammatory genes following LPS-induced inflammation of rat testis. Known inflammatory target genes of significantly up-regulated and/or down-regulated inflammatory-related miRNAs that also showed significant expression changes were noted, and the data was included in the results of this study.

RESULTS

Down-Regulation of Inflammatory-Related microRNAs at 3 and 6 Hours Following LPS-Induced Inflammation

Using qPCR analysis, the expression of inflammatory-related miRNAs in rats sacrificed at 3 and 6 hours following LPS-induced inflammation was determined. As shown in Table 1, various miRNAs were down-regulated at 3 and 6 hours post LPS-induced inflammation. The miRNAs that were down-regulated in the 3h group were let-7f-5p, miR-98-5p, miR-23a-3p, miR-23b-3p, and miR-200c-3p, while the miRNAs that were down-regulated in the 6h group were miR-17-5p, miR-19a-3p, miR-34a-5p, miR-34c-5p, and miR-449a-5p. Significant miRNAs had fold changes between -2.13 and -3.77 in the 3h group and between -2.02 and -2.12 in the 6h group. All significant miRNAs had a $p<0.05$ (Table 1). No miRNAs were shown to have increased expression based on our selection criteria.

Significant microRNAs Had Overlapping Clusters of Target Inflammatory Genes

Based on a list provided by Qiagen, the target inflammatory genes of the significant miRNAs were determined (Figure 6). Most of the significant miRNAs had overlapping clusters of target inflammatory genes. For example, let-7f-5p and miR-98-5p (3h group) were found to target the same 7 genes (*Ccr7, Faslg, Fgf5, Gdf6, Il13, Olr1,* and *Osmr*); miR-23a-3p and miR-23b-3p (3h group) were found to target the same 12 genes (*Ccl7, Cxcl12, Fas, Grem1, Il11, Il3, Il6ra, Kitlg, Mstn, Prkca, Prok2,* and *Stat5b*); and miR-34a-5p, miR-34c-5p, and miR-449a-5p (6h group) were found to target the same 7 genes (*Areg, Bmp3, Il6ra, Kitlg, Nampt, Nfe2l1,* and *Serpinf2*). Other significant miRNAs, however, targeted their own cluster of inflammatory genes. For instance, miR-200c-3p (3h group) was found to target a certain group of 6 genes (*Il13, Lepr, Ntf3, Prkca, Ripk2,* and *Vegfa*); miR-17-5p (6h group) was found to target a certain group of 4

genes (*F3, Il25, Mgll,* and *Osm*); and miR-19a-3p was found to target a certain group of 4 genes (*Bmp3, Cast, Cntfr, and F3*). Some target inflammatory genes were found in multiple clusters (*Il13, Il6ra, Prkca, Kitlg, Bmp3, and F3*). For other inflammatory target genes, the gene coding for the receptor and related ligand were found in separate clusters; these included the genes *Faslg* (ligand) and *Fas* (receptor), and *Osm* (ligand) and *Osmr* (receptor).

The target inflammatory genes of significant miRNAs and their coded proteins and functions are listed in Table 2.

No Interactions Seen Between Significant microRNAs and Inflammatory Genes From Previous Study

Results from this study were compared to data from previous studies in our laboratory, which identified a number of up- and down-regulated inflammatory genes following LPS-induced inflammation of rat testis. No known target inflammatory genes of significant miRNAs also showed significant expression changes in past studies. However, only 2/29 target inflammatory genes were included in the PCR array used in the past study (Qiagen Rat Innate & Adaptive Immune Responses PCR Array).

Table 1. Down-regulation of inflammatory-related microRNAs following 3 and 6 hours of LPS-induced inflammation. Fold changes were determined by qPCR analysis using a Qiagen Rat Inflammatory Response & Autoimmunity Array (MIRN-15Z). miRNAs with a fold up-regulation or down-regulation greater than 2 and $p<0.05$ (t-test) were accepted as statistically significant. The significant miRNAs at 3 (left) and 6 (right) hours were all different, however, all significant miRNAs showed down-regulation of expression. Down-regulation of the significant miRNAs ranged from -2.02 to -3.77.

3h Group (*n=4*)

miRNA ID	Fold Change
let-7f-5p MIMAT0000778	-3.00 (*p*=0.024)
miR-98-5p MIMAT0000819	-3.77 (*p*=0.031)
miR-23a-3p MIMAT0000792	-2.41 (*p*=0.038)
miR-23b-3p MIMAT0000793	-2.13 (*p*=0.014)
miR-200c-3p MIMAT0000873	-2.16 (*p*=0.016)

6h Group (*n=4*)

miRNA ID	Fold Change
miR-17-5p MIMAT0000786	-2.11 (*p*=0.021)
miR-19a-3p MIMAT0000778	-2.12 (*p*=0.043)
miR-34a-5p MIMAT0000815	-2.02 (*p*=0.035)
miR-34c-5p MIMAT0000814	-2.09 (*p*=0.021)
miR-449a-5p MIMAT0001543	-2.09 (*p*=0.021)

Figure 6. Significant microRNAs and their known target inflammatory genes. Target inflammatory genes were provided in a list compiled by Qiagen. A number of the significant miRNAs had overlapping target inflammatory genes, while others did not. The number of miRNAs that target the same inflammatory genes ranged from 2-3, while the number of inflammatory genes that each miRNA targets ranged from 4-12. Target genes that were found in multiple clusters were starred (*). A carrot (^) was put next to target inflammatory genes that code for a receptor that had the gene coding for its ligand in a separate cluster.

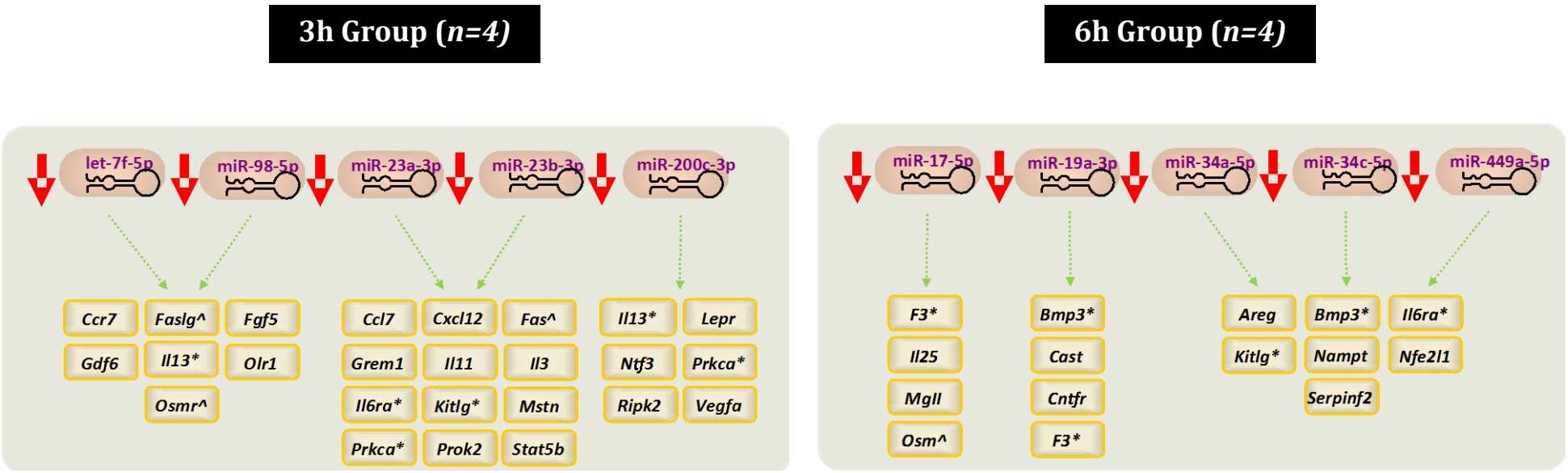

Table 2. Target inflammatory genes of significant microRNAs and their coded proteins and functions. Coded proteins and gene functions were based on *in silico* analysis using the online database UniProt. The only gene that was not included was *Cast*, since it was not found in the UniProt database.

Target Gene	Protein	Function
Areg	Amphiregulin	Ligand of the EGF receptor/EGFR.
Bmp3	Bone morphogenetic protein 3	Negatively regulates bone density.
Ccl7	C-C motif chemokine 7	Chemotactic factor that attracts monocytes and eosinophils, but not neutrophils.
Ccr7	C-C chemokine receptor type 7	Receptor for the MIP-3-beta chemokine.
Cntfr	Ciliary neurotrophic factor receptor subunit alpha	Binds to CNTF.
Cxcl12	Stromal cell-derived factor 1	Chemoattractant active on T-lymphocytes, monocytes, but not neutrophils.
F3	Tissue factor	Initiates blood coagulation by forming a complex with circulating factor VII or VIIa.
Fas	Tumor necrosis factor receptor superfamily member 6	Receptor for TNFSF6/FASLG.
Faslg	Tumor necrosis factor ligand superfamily member 6	Cytokine that binds to TNFRSF6/FAS, a receptor that transduces the apoptotic signal into cells.
Fgf5	Fibroblast growth factor 5	Plays an important role in the regulation of cell proliferation and cell differentiation.
Gdf6	Growth/differentiation factor 6	Growth factor that controls proliferation and cellular differentiation in the retina and bone formation.
Grem1	Gremlin-1	Acts as inhibitor of monocyte chemotaxis
Il11	Interleukin-11	Cytokine that stimulates the proliferation of hematopoietic stem cells and megakaryocyte progenitor cells and induces megakaryocyte maturation resulting in increased platelet production
Il13	Interleukin-13	Cytokine. Inhibits inflammatory cytokine production.
Il25	Interleukin-25	Induces activation of NF-kappa-B and stimulates production of the proinflammatory chemokine IL-8.
Il6ra	Interleukin-6 receptor subunit alpha	Part of the receptor for interleukin 6.

Kitlg	Kit ligand	Ligand for the receptor-type protein-tyrosine kinase KIT. Plays an essential role in the regulation of cell survival and proliferation, hematopoiesis, stem cell maintenance, gametogenesis, mast cell development, migration and function, and in melanogenesis.
Lepr	Leptin receptor	Receptor for hormone LEP/leptin.
Mgll	Monoglyceride lipase	Converts monoacylglycerides to free fatty acids and glycerol.
Mstn	Growth/differentiation factor 8	Acts specifically as a negative regulator of skeletal muscle growth.
Nampt	Nicotinamide phosphoribosyltransferase	Catalyzes the condensation of nicotinamide with 5-phosphoribosyl-1-pyrophosphate to yield nicotinamide mononucleotide, an intermediate in the biosynthesis of NAD.
Nfe2l1	Nuclear factor erythroid 2-related factor 1	Activates erythroid-specific, globin gene expression.
Ntf3	Neurotrophin-3	Seems to promote the survival of visceral and proprioceptive sensory neurons.
Olr1	Oxidized low-density lipoprotein receptor 1	Receptor that mediates the recognition, internalization and degradation of oxidatively modified low density lipoprotein (oxLDL) by vascular endothelial cells.
Osm	Oncostatin-M	Growth regulator. Inhibits the proliferation of a number of tumor cell lines. Stimulates proliferation of AIDS-KS cells. It regulates cytokine production, including IL-6, G-CSF and GM-CSF from endothelial cells.
Osmr	Oncostatin-M-specific receptor subunit beta	Associates with IL31RA to form the IL31 receptor.
Prkca	Protein kinase C alpha type	Calcium-activated, phospholipid- and diacylglycerol (DAG)-dependent serine/threonine-protein kinase that is involved in positive and negative regulation of cell proliferation, apoptosis, differentiation, migration and adhesion, tumorigenesis, cardiac hypertrophy, angiogenesis, platelet function and inflammation.
Prok2	Prokineticin-2	May function as an output molecule from the suprachiasmatic nucleus (SCN) that transmits behavioral circadian rhythm.

Ripk2	Receptor-interacting serine/threonine-protein kinase 2	Serine/threonine/tyrosine kinase that plays an essential role in modulation of innate and adaptive immune responses.
Serpinf2	Alpha-2-antiplasmin	Serine protease inhibitor.
Stat5b	Signal transducer and activator of transcription 5B	Carries out a dual function: signal transduction and activation of transcription.
Vegfa	Vascular endothelial growth factor A	Growth factor active in angiogenesis, vasculogenesis and endothelial cell growth.

DISCUSSION

Results showed down-regulation of a number of inflammatory-related miRNAs at 3 and 6 hours following LPS-induced inflammation (Table 1). Previous studies in our laboratory on gene expression following LPS-induced inflammation of rat testis demonstrated up-regulation of a number of inflammatory genes. Since miRNAs negatively control gene expression, we propose that the levels of all of the significant, inflammatory-related miRNAs might be decreasing in the testis following LPS-induced inflammation to increase the net number of target inflammatory genes available for translation. This would ultimately lead to the creation of more proteins that help protect these tissues from infection and preserve their normal reproductive functions (Figure 7).

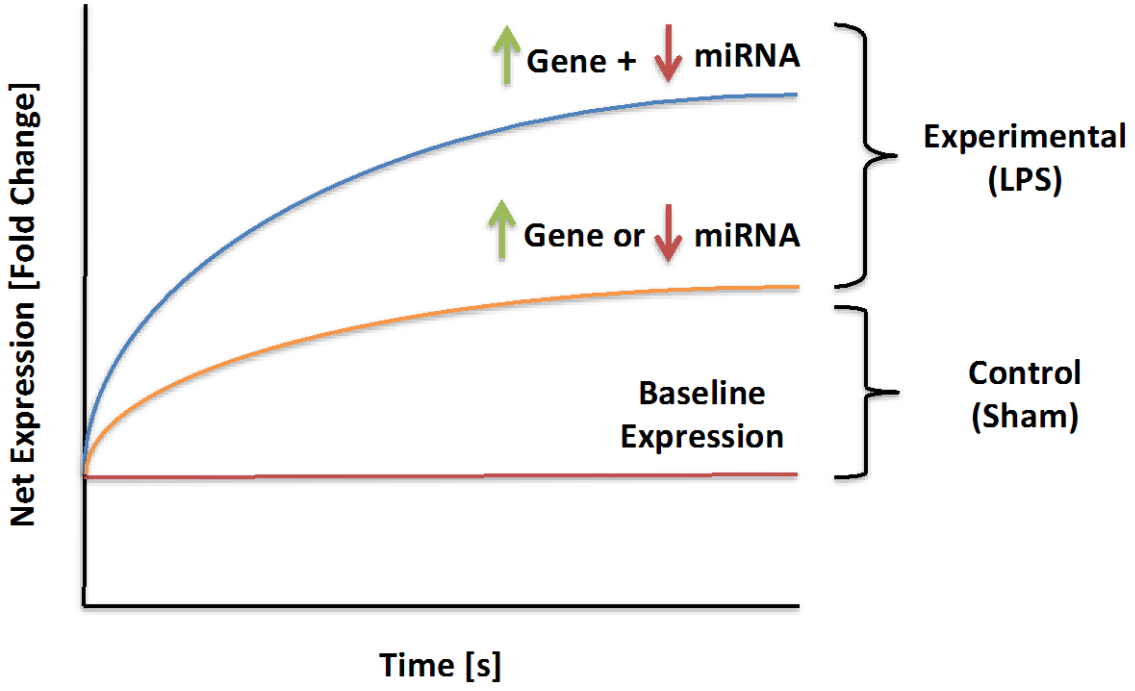

Figure 7. Hypothetical model of gene expression following LPS-induced inflammation. In a hypothetical model, control treatment (sham) results in no change in net expression (fold change), and baseline expression (red) is kept. However, in a hypothetical model, treatment with LPS (experimental) results in a net change in expression due to either gene up-regulation and miRNA down-regulation (yellow), or the combined effect of the two (blue).

It is known that miRNAs act pleiotropically, meaning that each miRNA targets multiple genes. What is interesting about our results of this study is that a number of the significant, inflammatory-related miRNAs target the same clusters of inflammatory genes (Figure 6). For example, miR-34a/c and miR-449a target the same 7 inflammatory genes, while miR23a/b target the same 12 inflammatory genes (Figure 6). Additionally, some target inflammatory genes were found in multiple clusters, while the gene coding for a ligand and respective receptor were found in separate clusters. This is exciting because it could indicate that the overlapping pathways that these miRNAs regulate could be essential to controlling normal immune system functions within the male reproductive tract.

Below the roles of the significant miRNAs in regulating immune system functions will be discussed. Furthermore, since some of the significant miRNAs also play major parts in the maintenance of fertility, formation and elimination of cancer, and development of the male reproductive tract, these additional roles will be discussed (Table 3). The target inflammatory genes of significant miRNAs, however, will not be a focus of the discussion, unless their interactions are cited in the literature and seem to be of importance.

Significant microRNAs Regulate Immune System Function

Given that the miRNAs detected in this research were inflammatory-related, it is no surprise that they play major roles in the regulation of immune system function. However, what is interesting is that majority of the significant miRNAs are involved in supporting the innate immune response, while only one of the significant miRNAs (miR-23a) has been shown to be involved in the adaptive immune response—by targeting CD8+ T lymphocytes (Lin et al., 2014). Below a number of the significant miRNAs will be highlighted because of their importance in

proper immune system balance. Additionally, a few important miRNAs that were not detected in our research will be discussed.

A number of the significant, inflammatory-related miRNAs have been shown to sustain the innate immune response through LPS-dependent (let-7, miR-200c, and miR-34a; Guennewig et al., 2014; Staedel and Darfeuille, 2013; Wendlandt et al., 2012) and LPS-independent (miR-17 and miR-19a; Collins et al., 2013; Xu et al., 2013) mechanisms. Most notably, TLR signaling by bacterial LPS has been demonstrated to repress let-7 expression, which leads to a greater net expression of IL-6 (pro-inflammatory) and IL-10 (ant-inflammatory; Staedel and Darfeuille, 2013). This is interesting because: 1) our study showed concurrently that let-7 is down-regulated upon LPS administration, and past research in our laboratory demonstrated that *Il-6* and *Il-10* are up-regulated under the same conditions (Fasano, 2014); and 2) it supports the idea that let-7 is involved in tightly balancing immunoregulatory and immunosuppressive mechanisms within the male reproductive tract.

Other significant miRNAs that are involved in LPS-dependent regulation of the immune system include miR-200c and miR-34a (Guennewig et al., 2014; Wendlandt et al., 2012). miR-200c has been shown to modify the efficiency of the TLR4 pathway—which is activated by LPS detection—and decline of miR-200c has been demonstrated to alter target genes that control nuclear factor-κB (NF-κB; a transcription factor) signaling pathway, inflammation, cell cycle, and migration (Wendlandt et al., 2012). This is interesting because: 1) NF-κB levels have been shown to increase upon LPS administration (Frede et al., 2006); and 2) past research in our laboratory studied the crosstalk between hypoxic and inflammatory pathways through the proteins NF-κB and HIF-1α (hypoxia inducible factor-1α; Patel, 2012). Another significant miRNA that has been implicated in LPS-dependent regulation of the immune system is miR-34a.

miR-34a has been shown to promote endothelial inflammation (Rippo et al., 2014) and LPS-activation of primary monocyte-derived macrophages (Guennewig et al., 2014). This shows that miR-34a is important for protection of the male reproductive tract from infection because macrophages have been shown to play a major role in innate immune activation of the testis (Hedger, 2015).

Two significant miRNAs that have been shown to be involved in LPS-independent regulation of the immune system include miR-17 and miR-19a (Collins et al., 2013; Xu et al., 2013). The first significant miRNA, miR-17 has been demonstrated to control monocyte-to-macrophage differentiation—through targeting of the Hif-system (Poitz et al., 2013)—and the proangiogenic function of macrophages—via targeting of HIF-2α (Xu et al., 2013). This is fascinating because: 1) miR-17 usually works in conjunction with miR-20a, which showed no significant change upon LPS administration; 2) it further proves that there is a crosstalk between hypoxic and inflammatory pathways involving the Hif-pathways, which was studied in the past in our laboratory (Patel, 2012); and 3) it demonstrates an important role for miR-17 in regulating macrophage activity within the testis, which is an essential part of the innate immune response within these tissues (Hedger, 2015). The second significant miRNA, miR-19a has been shown to inhibit TLR2-triggered cytokines and kinases (He et al., 2014) and cause augmentation of inflammatory-related pathways, such as JAK-STAT signal transduction (Collins et al., 2013). This is important because it demonstrates that protection of the testis from LPS is instigated in part by the down-regulation of miR-19a and subsequent immune activation of these tissues.

Some miRNAs that and are involved in controlling immune responses did not show significant fold changes following LPS-induced inflammation of rat testis. For example, miR-181a, which has been implicated in B and T cell development (Sonkoly et al., 2008); miR-142,

which has been associated with T cell development (Sonkoly et al., 2008); and miR-16, which provides constitutive modulation of inflammation (Lindsay, 2008); among other inflammatory-related miRNAs; did not show significant fold changes upon LPS administration. This could have been a result of our small tissue sample size, or the fact that these miRNAs, in fact, do not play significant roles in regulating immune system functions, specifically within the male reproductive tract, at least under conditions of our experimental system.

Other miRNAs that are involved in controlling immune responses were not detected by the PCR array used in this study. For example, miR-155 and miR-146a, which regulate innate immune system function and are both regulated by downstream targets of TLR4 (Lindsay, 2008); miR-150, which has been implicated in B cell differentiation (Sonkoly et al., 2008); and miR-223, which has been connected to granulocytic and T cell differentiation (Sonkoly et al., 2008); among other inflammatory-related miRNAs; were not detected by the PCR array used in this study. Thus, further research could be dedicated to delving into the expression of these miRNAs following LPS-induced inflammation.

Significant microRNAs are Important to the Maintenance of Male Fertility

As discussed beforehand, miRNAs have been shown to play major roles in regulating normal male reproductive functions. After review of the literature, it has become apparent that a number of the significant, inflammatory-related miRNAs (3h group: let-7, miR-200c; 6h group: miR-17, miR-34a, miR-34c, and miR-449a) also have overlapping regulatory roles in the area of male fertility. In general, these significant miRNAs have been shown to control male germ cell development (differentiation), maintenance, and self-renewal (let-7, miR-200c, miR-34a/c; Yao et al., 2015); prevent germ cell apoptosis (miR-34c; Yang et al., 2013); and target various stages of spermatogenesis and the cell cycle (miR-34a/c; Bouhallier et al., 2010). For instance, miR-34c

has been demonstrated to regulate NOTCH1 and Nanos2, which are key regulators of germ cell differentiation (Mishima et al., 2008); BCL2 and ATF1 (activating transcription factor 1), which are involved in regulating germ cell apoptosis (Yang et al., 2013); and cdk4, myc, tgif2, and *notch1/2*, which control cell cycle progression and spermatogenesis (Bouhallier et al., 2010). Although majority of these significant miRNAs are localized to the testis (let-7f, miR-17, miR-34c, and miR-449a; Buchold et al., 2010; Yang et al., 2013), a number of them have been observed to originate from the epididymis (miR-34c) and sperm itself (miR-34a/c and miR-449a; Nixon et al., 2015b). In terms of clinical significance, all of this is important because inactivation of certain miRNAs has been shown to trigger azoospermia (miR-34c and miR-449a; Lian et al., 2009), a major cause of male infertility.

Significant microRNAs Play Roles in the Formation and Elimination of Cancer

It is known that there is a difference in miRNA expression between normal and cancerous tissues within the male reproductive tract (Bezan et al., 2014; Gillis et al., 2011). What we found to be interesting, however, is that all of the significant, inflammatory related miRNAs studied in this research seem to play important roles in the formation of cancer and the process of tumor progression. For instance, miR-19a expression has been implicated in seminoma formation (Gillis et al., 2011). Below is a detailed discussion of how the significant miRNAs contribute to cancer creation and prevention.

A number of the significant miRNAs have been demonstrated to act as tumor suppressors (let-7f, miR-34a/c, miR-17, and miR-449a; Cloonan et al., 2008; Hagman et al., 2013; Lizé et al., 2010; Roush and Slack, 2008)—through the targeting of p53 (miR-34a/c, miR-449a; Ji et al., 2009; Lizé et al., 2010; Zhang et al., 2014), p21 (miR-17; Wang et al., 2010), and pRb (miR-449a; Noonan et al., 2010)—and regulators of oncogenic pathways (miR-200c, miR-

17, and miR-34a/c; Cloonan et al., 2008; Kopp et al., 2013; Wang et al., 2010)—by manipulating the activities of CRKL (miR-200c; Tamura et al., 2015), RAS (miR-200c; Kopp et al., 2013), and c-myc (miR-17 and miR-34a/c; Cannell et al., 2010; O'Donnell et al., 2005; Wang et al., 2010), among other proteins. Additionally, a number of the significant miRNAs have been seen to regulate tumor angiogenesis (miR-98, miR23b, and miR-34a/c; Siragam et al., 2012; Wang et al., 2015; Zhang et al., 2014), which is essential for tumor growth and metastasis. For example, miR-98 has been shown to regulate tumor angiogenesis and invasion through repression of ALK4 (activin receptor-like kinase-4) and MMP11 (metalloproteinase-11; Siragam et al., 2012). Moreover, some of the significant miRNAs have been seen to directly mediate tumor growth (miR-23b, miR-17, and miR-34a/c) and metastasis (mi-23b and miR-34a/c), in addition to facilitating apoptosis of certain cancer cells (let-7, miR-98, miR-17, miR-34a/c, and miR-449a; Hermeking, 2010; Lizé et al., 2010; Novotny et al., 2007; Wang et al., 2015, 2011; Zhang et al., 2011). Most notably, miR-17 and miR-449a have been demonstrated to control the expression of ETF1, a transcription factor associated with cell proliferation and apoptosis in carcinomas *in situ* cells of the testis (Lizé et al., 2010; Novotny et al., 2007), while let-7 and miR-98 have been demonstrated to regulate the expression of Fas, an important mediator of apoptosis (Wang et al., 2011). Another critical contributor to tumor metastasis, epithelial to mesenchymal transition (ETM), is repressed by the negative regulation of the transcription factor ZEB1/2 (zinc finder E-box-binding homeobox 1 and 2) through the actions of miR-200c and miR-23b (Ceppi and Peter, 2014; Radisky, 2011; Shi et al., 2014). Furthermore, one of the significant miRNAs (miR-23a) has be shown to regulate telomere length and cellular senescence through the targeting of TR2F (telomeric repeat binding factor 2; Luo et al., 2015), an important contributor to cancer immortality.

The overarching theme that is seen with the significant miRNAs and cancer is that they regulate carcinogenesis in different ways based on context. For instance, miR34a/c has been widely implicated in the control of the Wnt/β-catenin signaling pathway, which has oncogenic hallmarks—such as cell proliferation, metastasis, angiogenesis, and telomerase activity—and tumor suppressive hallmarks—such as apoptosis (Wang et al., 2015). Further study of these significant miRNAs, and their role in tumorigenesis must continue to be studied in order to identify novel diagnostic and prognostic cancer signatures, in addition to anti-cancer therapies.

Significant microRNAs Control Development of the Male Reproductive Tract

There is not much direct evidence that connects miRNA regulation to control of development of the male reproductive tract. Still, some of the significant, inflammatory-related miRNAs show expression features that implicate them in development of the testis and other male reproductive organs (3h group: let-7f; 6h group: miR-17, miR-19a, miR-34a/c, and miR-449a). Some of the significant miRNAs were demonstrated to have different levels of expression between prepubertal and adult testis (let-7f, miR-34a/c, and miR-449a; Buchold et al., 2010; Mishima et al., 2008); let-7f was up-regulated, while miR-34a/c and miR-449a were down-regulated. Other significant miRNAs have been implicated in control of androgen signaling (miR-17, miR-19a, and miR-34a; Hu et al., 2013b; Nixon et al., 2015b), which is important for the development of normal male sexual characteristics. For example, miR-19a has been shown to regulate testosterone production by Leydig cells by targeting genes involved in lipid metabolism and steroidogenesis (Hu et al., 2013b). Most importantly, there is a difference in significant miRNA expression between the male testis and female ovary (miR-19a, miR-34a/c, and miR-449a; Mishima et al., 2008), which could connect these miRNAs to development of male specific features. More research needs to be done to understand what pathways miRNAs regulate

in controlling development of the male reproductive tract, however, it is easy to see that dysregulation of certain miRNAs could lead to developmental issues.

Table 3. Roles of significant microRNAs in the maintenance of fertility, formation and elimination of cancer, and development of the male reproductive tract. Roles were determined through *in silico* analysis by PubMed searches of the literature.

miRNA ID	Fertility	Cancer	Development
let-7	Controls male germ cell development	Acts as a tumor suppressor and facilitates apoptosis of cancer cells	Has a difference in expression between pre-pubertal and adult testis
miR-98	-	Regulates tumor angiogenesis and facilitates apoptosis of cancer cells	-
miR-23a	-	Regulates telomere length and cellular senescence	-
miR-23b	-	Regulates tumor angiogenesis, directly mediates tumor growth and metastasis, and contributes to epithelial-to-mesenchymal (EMT) transition	-
miR-200c	Controls male germ cell development	Regulates oncogenic pathways and contributes to epithelial-to-mesenchymal (EMT) transition	-
miR-17	-	Acts as a tumor suppressor, regulates oncogenic pathways, directly mediate tumor growth, and facilitates apoptosis of cancer cells	Has a difference in expression between pre-pubertal and adult testis and controls androgen signaling
miR-19a	-	Found in seminomas	Has a difference in expression between pre-pubertal and adult testis, controls androgen signaling, and has difference in expression between male testis and female ovary
miR-34a	Controls male germ cell development and targets various stages of spermatogenesis and the cell cycle	Acts as a tumor suppressor, regulates oncogenic pathways, regulates tumor angiogenesis, directly mediates tumor growth and metastasis, facilitates apoptosis of cancer cells, and regulates telomere length and cellular senescence	Has a difference in expression between pre-pubertal and adult testis, controls androgen signaling, and has difference in expression between male testis and female ovary
miR-34c	Controls male germ cell development, prevents germ cell apoptosis, targets various stages of spermatogenesis and the cell cycle, and triggers azoospermia	Acts as a tumor suppressor, regulates oncogenic pathways, regulates tumor angiogenesis, directly mediates tumor growth and metastasis, facilitates apoptosis of cancer cells, and regulates telomere length and cellular senescence	Has a difference in expression between pre-pubertal and adult testis and has difference in expression between male testis and female ovary
miR-449a	Triggers azoospermia	Acts as a tumor suppressor and facilitates apoptosis of cancer cells	Has a difference in expression between pre-pubertal and adult testis and has difference in expression between male testis and female ovary

CONCLUSIONS

Before conducting this study, it was known that proper expression of certain miRNAs within the male reproductive tract is linked to normal testis development and spermatogenesis, while atypical expression of certain miRNAs is connected to testicular cancer formation and male infertility. In addition, a distinct relationship was made between miRNAs and regulation of immune system functions in other tissues of the body. However, what was not known is the role that miRNAs might play in regulating the immunophysiology of the male reproductive tract.

We hypothesize that control of gene expression by miRNAs plays a significant role in the antimicrobial protection of rat testis, and the response to inflammation. Using an LPS model of inflammation, we demonstrated, in rats, that miRNAs of the testis might be involved in regulating the immunophysiology of these tissues. Down-regulation of a number of inflammatory-related miRNAs at 3 (let-7f, miR-98, miR-23a/b, and miR-200c) and 6 (miR-17, miR-19a, miR-34a/c, and miR-449a) hours post LPS injection proved that infectious insult of the testis causes a change in genetic expression within these tissues. Even though we know the target genes of these significantly, down-regulated miRNAs, the question still remains what this change in genetic expression actually means for organisms at the molecular, cellular, and physiological levels.

After review of the literature, it has become apparent that the significant miRNAs also play important roles in the maintenance of fertility (let-7f, miR-200c, miR-17, miR-34a/c, and miR-449a), formation and elimination of cancer (all significant miRNAs), and development of the male reproductive tract (let-7f, miR-17, miR-19a, miR-34a/c, and miR-449a). Following this connection, it is not unreasonable to speculate that infectious insult of the testis might lead to pathologies in these areas by deregulation of certain miRNAs essential to normal testis function.

Further research must be performed to better understand if this connection exists and how it might manifest.

Although we do not know what cell types these changes in miRNA expression are localized to, we now have a list of significant, inflammatory-related miRNAs that show promise as important regulators of immune function within the testis. This will save future researchers in this area a lot of time, money, and resources, because there is now a list of key miRNAs to focus on. Future research will be dedicated to better understanding how these miRNAs contribute to protecting the testis from infection and the response to inflammation. Eventually, the hope is that further study of these miRNAs, and their roles in male reproductive tissues, might lead to advanced therapeutics for treatment, novel biomarkers for detection, and a greater understanding of male fertility and related health issues.

ACKNOWLEDGMENTS

This research was supported by Monmouth University's School of Science, Bristol-Myers Squibb, and Merck. Special thanks to Genevieve Fasano for acting as a mentor to me and preparing all of the tissue samples used in this study, and Rekha Penmetcha for assisting me in research prior to this thesis project. Thank you ICFNJ and Novartis for funding me as an undergraduate research student through the Novartis Science Scholarship. To my parents, grandparents, siblings, cousins, and friends, your support and enthusiasm for my work is what pushed me to reach this point in my academic career. I could not have done it without you all. To my teachers, everything that I have written in this thesis includes something that you have taught me in my time at Monmouth. Thank you for giving me a love for lifelong learning and growth and a substantial base of knowledge to build off of for the future. To Koorleen Minton, Beth Brody, Cynthia Plantamura, and Margaret Cate, you have been my fans and advocates, cheering me on every step of the way. You are forever family in my heart. To Anne Marie Lavin, Patricia Hicks, and Karen Bentley, you have always been there for me to give me advice, in and out of the lab. Thank you for stopping your days to have a conversation with me and to help me troubleshoot any issues that I may have had. To Dean Dooley, Reenie Menditto, and Erin Hawk in the Honors School, you have been there for me from day one, making sure that my experience at Monmouth was the best that it could be. Just as you were always there for me, I will always be there for you with anything that you need. To my second reader, Dr. Jeffrey H. Weisburg, thank you for helping me throughout the entire process of organizing and writing my thesis. Your enthusiasm, guidance, and insight brought ease to the writing process for me. Most importantly, I would like to thank my teacher, coach, mentor, sponsor, and friend Dr. Michael A. Palladino. From the day that you opened the doors of your lab to me, you taught me more than I could have

ever have imagined, given me insights that most people do not see until much later in life, and provided me with experiences that students at the undergraduate level only dream of. Because of you, I want to pursue medical research in the future, and make a contribution to the field of science. Thank you so much for helping and guiding me with my thesis project and inspiring me to reach my greatest potential everyday. I will never forget my experience as part of the “repro gang.” It has changed my life and will always hold a special place in my heart.

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