MISMATCH REPAIR PROTEINS AND SPERMIOGENESIS

By

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Abstract

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In human males, one of the major causes of infertility is the abnormal development of immature sperm into fully motile, mature forms. Abnormalities such as round sperm heads and kinked or coiled sperm tails are all correlated with infertility [1-3]. These defects have been linked to disrupted formation of any of several essential structures, such as the acrosome, manchette, or cytoskeletal components within the immature sperm [4]. In previous immunostaining studies conducted in the Hassold/ Hunt Laboratory, certain DNA mismatch repair (MMR) proteins appeared to localize to different regions of testicular and epididymal sperm, e.g. to the acrosome or tail. These proteins are known to function in repair of DNA mismatches during mitosis and to play a role in meiotic recombination [1]. However there has been no reason to suspect any function in spermiogenesis, the complex process that involves the maturation of the immature spermatid to a fully motile, mature sperm [5, 6]. These new observations may shed some insight into the unknowns of spermiogenesis. Specifically, the localization pattern of MMR proteins MLH1, MLH3, MSH4, and MSH5, to testicular spermatids and epididymal sperm, and the differences in the patterns between the two, may signify a role for these proteins that is unrelated to typical MMR activity.

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I dedicate this thesis to my family for all of their support throughout my life, and their constant belief that I can accomplish any goal I set out to achieve. I also dedicate this thesis to my boyfriend, Andy Rowse, for his unerring emotional support over the last two years. I couldn't have done it without any of you.

CHAPTER ONE
INTRODUCTION

Spermatogenesis is a complex process that involves three major stages: mitotic proliferation, meiosis, and spermiogenesis [2]. Mitotic divisions occur during fetal development and begin again at the onset of puberty to maintain the stem cell pool that creates cells that undergo meiotic divisions. During meiosis diploid cells undergo two divisions to create four haploid gametes [7]. The last stage, spermiogenesis, occurs when the immature gametes undergo remarkable morphological changes, including formation of the tail and the loss of most of the cytoplasm [5, 6, 8].

The primary focus of the Hassold/Hunt laboratory is on the second of these stages, meiosis: how it proceeds and what happens if it is disturbed. As part of these studies, immunofluorescence techniques are used to examine localization patterns of specific proteins thought to be involved in the pairing, synapsis, and recombination of homologous chromosomes during meiosis I. The family of DNA MMR proteins is of particular interest due to the fact that these proteins are known to be involved in the meiotic recombination pathway [1, 9, 10]. While examining routine mouse testicular preparations, it became clear that some of the MMR proteins localized to sperm heads and tails. This was surprising because there is no obvious role for MMR proteins at this stage, since these cells are post-meiotic and presumably there should be minimal damage to DNA [2, 11]. This led us to hypothesize previously unidentified roles for MMR proteins, perhaps during spermiogenesis, a time when excess material is removed from the developing spermatozoa [8].

Sperm Development

Spermatogenesis

Spermatogenesis begins with the renewal of stem cells, called spermatogonia, through mitotic divisions. This creates a pool of cells with two different forms, type A spermatogonia which remain in the pool of cells to undergo further mitotic divisions, and type B spermatogonia which give rise to primary spermatocytes, from which meiosis proceeds [12] (Figure 1). Just prior to the first meiotic division, recombination occurs between homologous chromosomes [13, 14]. As discussed below, this is the point at which MMR proteins are known to function in meiosis [1, 9, 10]. The first meiotic division gives rise to the secondary spermatocytes which proceed through the second meiotic division. The daughter cells produced at the end of the second meiotic division are known as spermatids. These cells undergo remarkable morphological changes during spermiogenesis, the last stage of spermatogenesis, resulting in mature spermatozoa [6, 15]

Spermiogenesis

Spermiogenesis involves no cell divisions, but results in the transformation of a spherical, non-motile cell into a streamlined, fully motile agent of fertilization (Figure 2). Spermiogenesis can be broken down into four phases of morphological changes during transformation of the spermatid into the mature spermatozoa: formation of the acrosome, nuclear remodeling, development of the tail, and spermiation [5, 6].

Acrosome Formation

The head of the sperm consists mainly of the acrosome and the nucleus, with all extraneous material being shed during spermiogenesis. The acrosome is located on the anterior half of the sperm head and functions in oocyte fertilization. It is formed by the Golgi complex, which creates granules and a vacuole that are deposited at one

pole of the nucleus and spread out to form the acrosomal cap [16-18] (Figure 2A-E). The formation of the acrosome occurs throughout spermiogenesis with the acrosome expanding to cover the majority of the nucleus during nuclear remodeling [3] (Figure 2F-I). This is the general process of acrosome formation for all species, however due to developmental differences among species, the size of the acrosome varies greatly and may be modified as the spermatozoa pass through the epididymis [19] (Figure 3).

The acrosome plays an important role in sperm penetration of the outer membrane (zona pellucida) of the oocyte and fusion with the oocyte's inner plasma membrane [20]. This whole process is initiated by the acrosome reaction (AR), which consists of the exocytosis of the acrosome in response to an increase of intracellular calcium levels [21]. During exocytosis, many perforations are created in the acrosome. This allows the release of lysosomal enzymes that are essential in allowing the sperm to penetrate the zona pellucida of the oocyte [20]. The AR is crucial for fertility because only sperm that have completed the acrosome reaction can fuse with the oocyte inner membrane and commence fertilization [22].

Tail Development

The first stage of tail development occurs when the centriolar complex and the axial filament join together and become lodged at the pole of the nucleus opposite the acrosomal cap (Figure 2C-E). The development of the axial filament, the main component of the tail, begins during this early stage of spermiogenesis and projects outward from the surface of spherical spermatids [23] (Figure 2B). The mitochondria, which provide power for movement, move to surround the centriolar complex of the tail in a helical pattern [24] (Figure 2G-I). The cytoplasmic canal, which surrounds the middle piece of tail and houses the mitochondria, is formed by an invagination of the cell membrane that ends at the attachment to the annulus (Figure 2F). This combined structure allows for the articulation of the head and tail of the spermatozoon [23].

Motility is the main function of the tail and is an important component of the vitality of the sperm and is essential for its reproductive function [25]. Motility is responsible for the transportation of the sperm from the site of insemination, through the female reproductive tract, and passage through the zona pellucida of the oocyte [20]. Genetically determined abnormalities of this structure, such as a coiled tail or abnormal microtubules within the tail, can result in immotility of the sperm and therefore infertility [1, 2].

Nuclear Remodeling

The sperm nucleus is also modified greatly during the process of spermiogenesis. For example, it changes position from the center of the cell to a posterior position just in front of the tail (Figure 2A-E). Further, there is remarkably progressive condensation of the chromatin within the nucleus resulting in the DNA being sixfold more condensed than the DNA of a somatic cell [26, 27]. During this time the histones are replaced by transitional proteins, TP_1 and TP_2, which are evident between the time of removal of the histones and their replacement by protamines [28, 29]. This condensation is part of changes occurring in the DNA that cause stabilization and resistance to digestion by DNase [30, 31]. These changes occur late in spermiogenesis during a time of complete repression of gene transcription and physical re-shaping of the sperm nucleus [11]. The purpose for this compaction of the sperm DNA is to protect the genetic integrity of the paternal genome during its transport from the testes to the site of fertilization in the female reproductive tract [32].

Spermiation

The final phase of spermiogenesis, spermiation, is the process by which the residual cytoplasm and unneeded organelles are shed. This excess material is referred to as the residual body and generally contains the Golgi complex, manchette, ribosomes, mitochondria, remnants of the chromatid body, and the endoplasmic reticulum. During late spermiogenesis, the caudal spermatid cytoplasm is invaginated

with processes of Sertoli cell cytoplasm, and it has been postulated that this causes the excess cytoplasm to be pulled away from the spermatid. It is the loss of the residual body and contact with the Sertoli cell that signifies the completion of spermiogenesis and the spermatid is now referred to as a spermatozoon [8, 33, 34].

MMR Proteins: The Basics

DNA MMR is a crucial type of DNA repair that is conserved among species, from bacteria to humans. The MMR pathway targets base substitution mismatches and insertion/deletion loops (IDL). These mismatches occur during replication and homologous recombination, two processes fundamental to both mitosis and meiosis [35, 36].

The MMR proteins were first discovered in bacteria where their loss causes an accumulation of DNA replication errors over subsequent cell divisions. This accumulation of errors or mutations results in a phenotype referred to as a mutator (Mut) phenotype [9]. In *E. coli,* there are three major players: MutS, MutL, and MutH. MutS recognizes DNA mismatches and binds to them as a homodimer. The hydrolysis of ATP by MutS signals the recruitment of MutL, and together MutS and MutL activate MutH. MutH is an endonuclease that binds a methyl group on either side of the mismatch and preferentially nicks the unmethylated daughter strand to mark it for repair by DNA repair machinery [1, 36, 37].

Mammalian MMR

In mammals there are many MutS homologs (MSH) and MutL homologs (MLH), with active forms composed of heterodimers of two different MSH proteins or two different MLH proteins. Some are expressed specifically in mitotic cells, some specifically in meiotic cells, and some in both (Table 1). There is no MutH homolog in mammals. It has been hypothesized that its function is accomplished by nicks at the

5’ end of Okazaki fragments, which together with PCNA (a processing factor for DNA polymerases that is involved in the DNA re-synthesis step of MMR) are used for strand discrimination by the MSH/MLH proteins [9, 35, 36, 38].

The MMR proteins associated with mitosis include MutS homologs MSH2, MSH3, and MSH6 and MutL homologs MLH1, MLH3, PMS1, and PMS2. MSH2 forms heterodimers with both MSH6 (forming the MutSα complex) and MSH3 (forming the MutSβ complex). MutSα recognizes base substitution and small IDLs, and MutSβ recognizes only small IDLs.

MLH1 pairs with all other MutL homologs, with MLH1-PMS2 (MutLα) being the major participant in MMR in somatic cells [9, 36, 37]. Defects in MSH2, MSH6, MLH1, and PMS2 are each responsible for a proportion of cases of hereditary non-polyposis colorectal cancer (Lynch syndrome). These defects also predispose individuals to endometrial cancers and various other sporadic cancers [9, 38-40].

Some of the mitotic mammalian homologs have also been recruited for the purpose of meiosis, such as MutL homologs MLH1, MLH3, and PMS2. In addition, the activities of two MutS homologs, MSH4 and MSH5, appear to be largely restricted to meiosis. These MMR proteins are of particular interest to our laboratory due to their roles in homologous recombination. MSH4 and MSH5 bind as a heterodimer and are thought to be involved in the processing of double strand breaks (DSB), specifically in the formation or stabilization of double Holliday junctions that form at DNA exchanges between homologous chromosomes. These exchanges can then either result in a crossover or non-crossover event. The binding of the MSH4-MSH5 complex with Holliday junction components causes ATP hydrolysis that stimulates a MSH4-MSH5 sliding clamp formation. MLH1-MLH3 heterodimers, with MLH3 recruited first followed by MLH1, are thought to stabilize these clamp structures and promote the DNA exchanges to result in a crossover event [1, 9, 10, 36, 37]. Defects in these MSH and MLH proteins cause meiotic failure and sterility in male and female mice [1, 9, 37].

Research Aims

The long term goals of this study were to determine if MMR proteins have a previously unknown function in the morphological change of the immature spermatid during spermiogenesis, and if the absence of the MMR proteins causes defects in sperm function. As summarized in Chapter 3, I conducted three types of studies to achieve these goals.

First, I conducted studies of the localization and expression of MMR proteins in mouse sperm and testis using immunofluorescence and immunohistochemistry. Localization patterns were determined for several MMR proteins, e.g. MLH1, MLH3, MSH2, MSH3, MSH4, MSH5 and MSH6, using immunofluorescence methodology to examine differentiating spermatids, testicular and epididymal sperm. Testicular cross sections were examined using immunohistochemistry to determine the stages of the developing spermatid at which MMR proteins were evident. These experiments have allowed me to determine which of the MMR proteins specifically and uniquely localize to sperm and to what stages of sperm development, thus implicating functional significance during spermiogenesis.

Second, I conducted experiments to determine whether MMR localization patterns were conserved among species. Specifically, I analyzed MMR protein localization patterns in sperm of three different mammalian species, mouse, rhesus macacque, and humans. My results indicated evolutionary conservation, consistent with our assumption that these proteins are important during spermiogenesis.

Finally, I examined the functional significance of MMR protein localization to sperm. In vitro experiments were used to determine if MMR proteins were involved in sperm capacitation or in the acrosome reaction. These studies helped shed insight into the potential function of MMR proteins in sperm processes beyond ejaculation.

In Chapter 3, I summarize the conclusions garnered from these experiments and examine future directions to explore the functional significance of MMR protein localization to sperm.

Figure 1: Spermatogenesis in the Testes

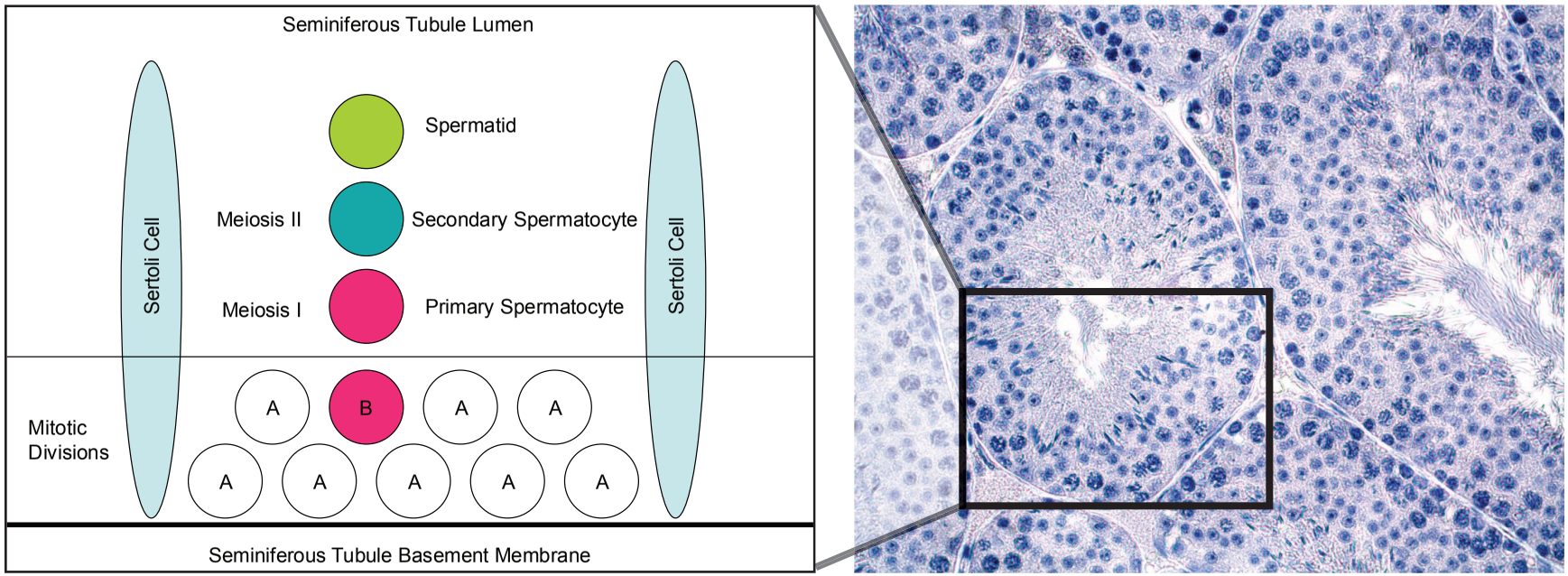


Figure 1 The process of spermatogenesis in the male testis as depicted by an image of a seminiferous tubule cross section and a graphical representation of the tubule. As the cells progress through mitotic and meiotic divisions, they move towards the lumen of the seminiferous tubule.

A = Spermatogonia A; B = Spermatogonia B

Figure 2: Spermiogenesis: The Process of Sperm Cell Development

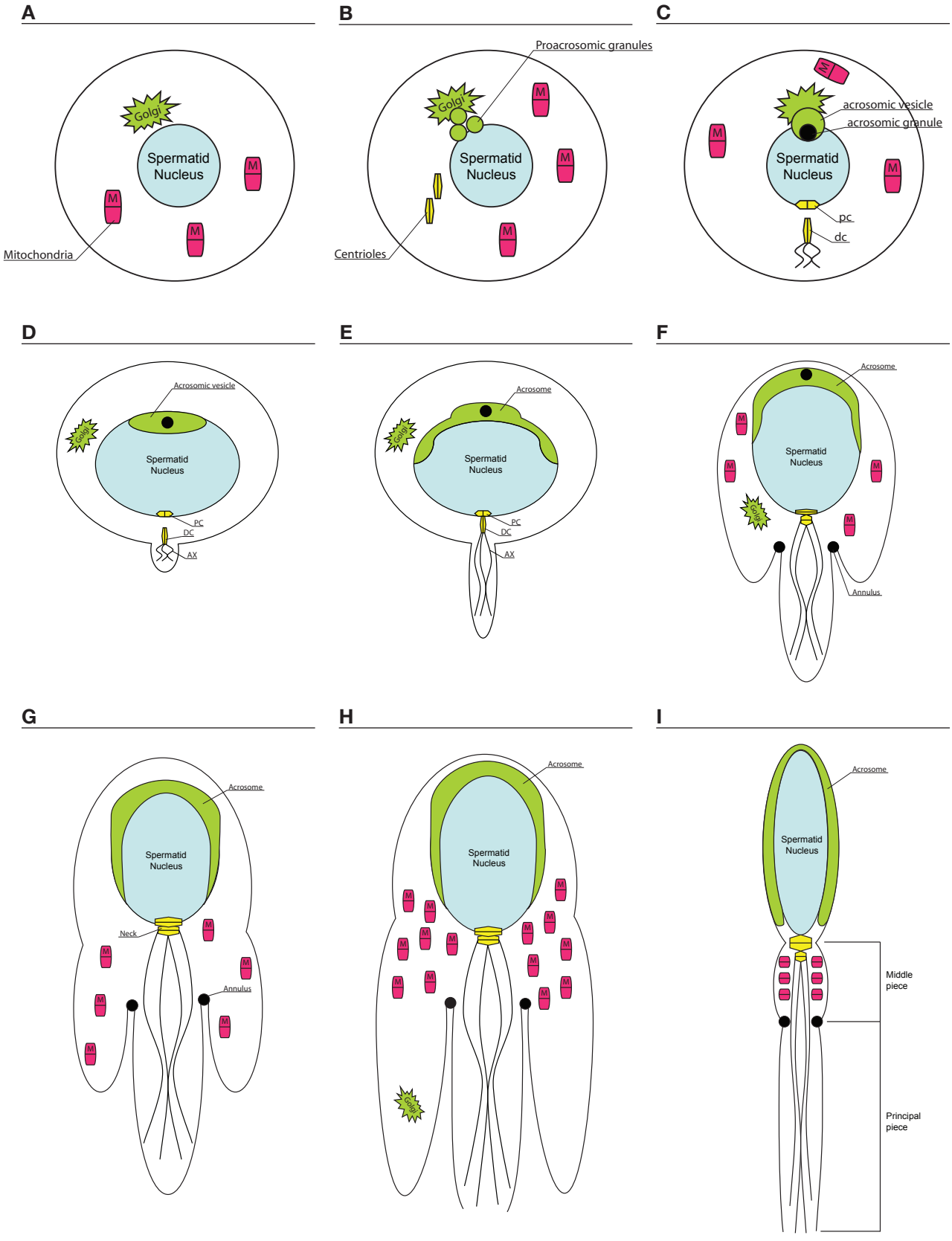


Figure 2

A) Newly formed spermatids have a well developed Golgi structure, mitochondria (M), and various other organelles.

B) Small vesicles of the Golgi bind together to form the proacrosomic granules.

C) This binding continues until a large acrosomic vesicle is formed containing a dense acrosomic granule. This is also the point at which the proximal centrioles (PC) and the distal centriole (DC) migrate to the opposite pole of the nucleus.

D) The remaining Golgi structure migrates toward the posterior end of the cell, and the acrosomic vesicle begins to flatten out.

E) The acrosomic vesicle now forms a distinctive cap structure over the nucleus, and the distal centriole (DC) forms the axial filament (AX).

F) The spermatid nucleus begins to elongate, and the axial filament begins to lengthen.

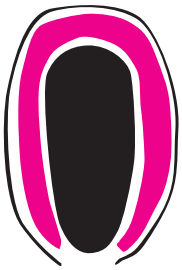
G) The acrosome now covers the majority of the anterior nucleus.

H&I) The middle piece is distinguished by the mitochondria that have assembled around the axial filament. The principle piece consists of the rest of the axial filament.

Adapted from (Senger 1997)

Figure 3: Sperm Acrosomal Variation Among Species

African Green Monkey



Bull

Human

Rat

Russian Hamster

Mouse

Figure 3 Variations in sperm acrosome structures and head shape in different species. Sperm heads are represented as a cross section with pink regions indicating the acrosome and black regions indicating the nucleus.

Table 1: Mammalian MutS and MutL Homologs

E. coli	Mammals	Functions
MutS	MSH2	Forms heterodimers with MSH3 to remove nonhomologous tails. Forms heterodimers with MSH3 and MSH6 to: Repair replication errors Repair mismatches in recombination intermediates Inhibit recombination between nonidentical sequences Respond to DNA damage
	MSH3	Forms heterodimers with MSH2
	MSH4	Forms heterodimers with MSH5 to promote crossing-over in meiosis by stabilizing Holliday junctions
	MSH5	Forms heterodimers with MSH4
	MSH6	Forms heterodimers with MSH2
MutL	PMS1	Forms heterodimers with MLH1 to: Repair replication errors Repair mismatches in recombination specific intermediates
	PMS2	Forms heterodimers with MLH1 to: Repair replication errors Repair mismatches in recombination specific intermediates Inhibit recombination between nonidentical sequences Respond to DNA damage
	MLH1	Forms heterodimers with PMS1, PMS2, and MLH3
	MLH3	Forms heterodimers with MLH1 to: Repair replication errors Promote crossing-over in meiosis
MutH		Function replaced by nicks at the 5' end of Okazaki fragments and PCNA

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CHAPTER TWO
MISMATCH REPAIR PROTEINS AND SPERMIOGENESIS: A NEW ROLE FOR MISMATCH REPAIR PROTEINS?

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Note: This chapter is in preparation for submission to *Human Reproduction*.

Abstract

DNA mismatch repair (MMR) is a crucial type of DNA repair pathway that is highly conserved among species. MMR proteins appear to have at least two fundamentally different roles, one in repairing replication or recombination errors in somatic cells and a second in processing programmed double strand breaks (DSBs) in germ cells. However, immunostaining studies of testicular, epididymal, and ejaculated sperm suggest the possibility of another role for MMR proteins, one not associated with DNA repair. Specifically, MMR proteins MLH1, MLH3, MSH4, and MSH5 localized to different regions of sperm, e.g. to the acrosome or tail. Until now, there has been no reason to suspect any function for MMR proteins outside of typical MMR activity, but the findings in this study may signify a new role for these proteins in sperm development or sperm function.

Introduction

DNA mismatch repair (MMR) is a crucial type of DNA repair pathway that is conserved among species, from bacteria to mammals [1, 2]. In humans, mutations in loci encoding MMR proteins are best known for their association with cancer. Specifically, mutations in MLH1, MSH2, PMS2 or MSH6 are responsible for the vast majority of cases of hereditary non-polyposis colorectal cancer (HNPCC), a form of colon cancer inherited as an autosomal dominant disorder [3-6].

Beginning over a decade ago, HNPCC researchers initiated studies to model the condition in mice, analyzing knockouts for several of the MMR genes. Consistent with the human data, the knockouts exhibited an increase in tumors. However, unexpectedly, knockouts for the MMR-associated genes MLH1, MLH3, MSH4, MSH5 and PMS2 had an unrelated phenotypic abnormality – both males and females were infertile [7-11]. Subsequent analyses of murine spermatogenesis and oogenesis, coupled with analyses of meiosis in other model organisms, has led to an under-

standing of the basis of the infertility [1, 2, 7, 12]. That is, in addition to its repair role in somatic tissues, MMR proteins perform crucial functions in the meiotic recombination pathway. For example, MSH4 and MSH5 form a heterodimeric structure that is thought to be involved in the formation and/or stabilization of double Holliday junctions, intermediate structures in the genesis of cross-overs [1, 5, 12, 13]. Subsequently, MLH3 and then MLH1 are recruited to at least some of these sites, and these MLH3/MLH1 positive sites display localization patterns that conform to properties expected of meiotic cross-over associated proteins [1, 5, 7, 12, 14]. Thus, MMR proteins appear to have at least two fundamentally different roles, one in repairing replication or recombination errors in somatic cells and a second in processing programmed double strand breaks (DSBs) in meiocytes.

However, recent studies conducted in our laboratory suggest the possibility of another role for MMR proteins, one apparently not associated with DNA repair. Specifically, in immunostaining studies of MMR proteins in mouse testicular preparations, we observed localization of MLH3 to the acrosome and the tail of testicular sperm; i.e., to extra-nuclear regions of the cell. This surprising observation prompted us to conduct a systematic series of immunostaining studies of different MMR proteins at different stages in sperm development and in different species, as well as functional analyses to assess the role, if any, of MMR proteins in capacitation and in the acrosome reaction. As described in the present report, our analyses provide evidence of a role for MMR in spermiogenesis of multiple species, although its specific mode of action remains unclear.

Materials and Methods

Testicular Sperm Preparation

Surface spread preparations were made as described in Peters *et. al*. with minor modifications [15]. Briefly, the testes were removed from C57BL/6J mice, the tunica removed from each testis, and the seminiferous tubules placed in hypotonic buffer pH 8.2-8.4 [500 µl 600 mM Tris (pH8.2), 1 ml 500 mM sucrose, 1 ml 170 mM citric acid, 100 µl 500 mM EDTA, 50 µl 500 mM DTT, 50 µl 100 mM PMSF, 5 ml ddH_2O]. The tubules were then macerated and 10 µl of the cell suspension was added to a 1% paraformaldehyde (PFA) coated slide. Slides were placed in a humid chamber overnight to allow cell adherence, then air dried, soaked in 0.04% Kodak Photoflo solution for 2 minutes, and air dried again.

Epididymal Sperm Preparation

The cauda epididymi were removed from C57BL/6J mice and placed in 100 mM sucrose in separate watch glasses. Sperm were extracted from each cauda using forceps and a 1 ml pipette used to aspirate sperm and 500 µl of the sucrose mixture. This was then transferred to a 1.5 ml tube containing 500 µl of 100 mM sucrose. Slides were prepared as described for the testicular sperm preparations, with the exception that 20 µl instead of 10 µl of sperm suspension was pipetted onto each slide.

Ejaculated Sperm Preparation

Human semen was collected by masturbation and rhesus macacque (*Macaca mulatta*) semen was collected by electro ejaculation [16-18]. Semen was spun down at 400 x G, the supernatant poured off, and the sperm pellet re-suspended in a 100 mM sucrose solution. Slides were prepared as described for the testicular sperm preparations, with the exception that 20 µl instead of 10 µl of sperm suspension was pipetted onto each slide.

Immunofluorescence Staining

Testicular Sperm Immunostaining

Immunostaining was performed in a similar manner to Anderson *et. al.* [19]. Primary polyclonal antibodies against mouse MLH1 (Calbiochem), MLH3 [20], MSH2 (Santa Cruz Biotechnology, Inc.), MSH3 (Santa Cruz Biotechnology, Inc.), MSH4 [21], MSH5 [21], and MSH6 (Abcam) were generated in rabbit and polyclonal antibody against mouse SYCP3 (Novus Biologicals) was generated in goat. Secondary antibodies consisted of fluoroscein labeled donkey anti-rabbit and rhodamine labeled donkey anti-goat (Jackson ImmunoResearch). All primary antibodies were diluted to 1:75 in 1X ADB [10X stock contains 10 ml normal donkey serum (Jackson ImmunoResearch), 3 g BSA (Sigma), 50 µl Triton-X 100, and 90 ml of 1X phosphate buffered saline(PBS)], with the exception of MLH3 (diluted to 1:80) and SCP3 (diluted to 1:1000). Secondary antibodies to MLH1, MLH3, MSH4, and MSH5 were diluted 1:75 in 1X ADB and the secondary antibody to SYCP3 was diluted at 1:200.

For each experiment involving testicular sperm preparations, we examined one of the MMR proteins as well as SYCP3. Inclusion of SYCP3 provided an internal positive control, since MSH4 and MSH5 load on to synaptonemal complexes (SCs) in zygotene and MLH1 and MLH3 load on to SCs in pachytene. Antibodies were applied to microscope slides as follows: the primary antibody to either MLH1, MLH3, MSH4, or MSH5 was added, cover slips were applied, and the slides were incubated overnight; the primary antibody to SYCP3 was then added, cover slips were applied, and the slides were incubated for 2 hours; the secondary antibody to either MLH1, MLH3, MSH4, or MSH5 was added, cover slips were applied, and the slides were incubated overnight; and finally, the secondary antibody to SYCP3 was added, cover slips were applied, and the slides were incubated for 45 minutes. All incubations were done in a humid chamber at 37û C. Slides were then washed with 1X PBS, stained with DAPI

(Pierce Biotechnology), and individual drops of FluoroGuard Antifade Reagent (BioRad Laboratories) were applied to cover slips and placed on the slides.

Epididymal and Ejaculated Sperm Immunostaining

Immunostaining was performed similarly to the testicular sperm staining protocol with the exception that no SYCP3 primary or secondary antibodies were used. As internal positive controls for MSH2, MSH3, and MSH6, we applied 293T human embryonic kidney cells allowed to the slides before addition of PFA and sperm and monitored them for evidence of protein localization.

Analysis and Imaging of Immunofluorescence

Slides were examined on a Zeiss Axioimager M.1 epifluorescence microscope and imaged with a CCD camera and computer using Zeiss Axio Vision software. Two sets of slides, one for each testis and epididymis, from three animals were examined for all MMR proteins. A minimum of twenty-five sperm from each slide were scored for localization with each slide scanned completely to determine consistency of staining.

Immunohistochemistry

Immunohistochemistry was performed as described in Caires *et. al.* (in press). Briefly, C57BL/6J mouse testes were fixed in Bouin's solution and embedded in paraffin according to standard procedures. The tissue's were sectioned (5 µm thickness), deparaffinized and rehydrated. Antigen retrieval was done using 0.01M sodium citrate, and endogenous peroxidase activity was quenched using 3.0% H_2O_2 diluted with methanol. Sections were incubated for 20 minutes in 100 µl of 10% normal goat serum to block non-specific binding of primary antibodies. 100 µl of primary antibody was pipetted onto tissue sections (MLH1, MSH4, MSH5 1:75; MLH3 1:80 in 10% goat serum). As a negative control, the next serial section was processed without any primary antibody. Slides were incubated overnight in a humid chamber at 4û C.

Slides were washed and 100 µl of horseradish peroxidase conjugated donkey anti-rabbit secondary antibody (1:300; Santa Cruz Biotechnology) was pipetted onto both sets of tissue sections and incubated for 60 minutes at room temperature. Slides were washed and 100 µl of Immpact DAB substrate (1 drop concentrate in 1 ml diluent; Vector Laboratories) was pipetted onto both sets of tissue sections and the slides were incubated for 2-10 minutes. Slides were washed and counterstained using haematoxylin stain.

Seven sets of slides from 5 animals were examined for each of the four MMR proteins. Representative seminiferous tubules from each stage of spermatogenesis were examined for each MMR protein. Digital images for immunohistochemistry were captured using a Leica DFC 280 camera and a Leica DME compound microscope (Leica Microsystems Imaging Solutions Ltd.) at 400x magnification. Tubule staging was determined using the method of Kotaja *et. al.* [22].

In Vitro Capacitation and Acrosome Reaction

The in vitro capacitation and acrosome reaction protocol was adapted from Larson *et. al.* [23]. C57BL/6J mouse cauda epididymi were removed and placed in 100 mM sucrose. The sperm were squeezed from each cauda using forceps and aspirated along with 500 µl of the sucrose mixture using a 1 ml pipette. This was transferred to a 2 ml tube containing 1 ml of Krebb's Ringer Bicarbonate Buffered solution [KRBT; 1 package of KRBT media mix (Sigma-Aldrich), 1.26 g sodium bicarbonate, 0.25 g calcium chloride, 90 ml ddH_2O]. Control slides were made as described for epididymal sperm with the exception that some slides were allowed to air dry for Coomassie staining.

The sperm were washed twice in KRBT media by centrifuging at 400 x G for 8 minutes. Sperm were resuspended using KRBT media and incubated for 1 hour in a 37û C water bath to allow for capacitation. After incubation, slides were made as

described above. Ca^{2+} ionophore (5mg/ml) was added to the capacitated sperm and incubated for an additional 1 hour to allow for the acrosome reaction. Additional slides were made after this incubation as described above.

Control, capacitated, and acrosome reacted sperm slides were stained for MLH1, MLH3, MSH4, and MSH5 as described in the epididymal and ejaculated sperm staining protocols. Slides were examined using a Zeiss Axioimager M.1 epifluorescence microscope and imaged with a CCD camera and computer using Zeiss Axio Vision software.

Additional air dried slides for control, capacitated, and acrosome reacted sperm were stained using Coomassie blue. The air dried slides were placed in freshly made Coomassie stain [0.2 g Coomassie Blue G-250 (MP Biomedicals), 50 ml methanol, 10 ml glacial acetic acid, 40 ml ddH_2O] for 2 minutes. The slides were washed in running ddH_2O and allowed to air dry before examination using a Leica DFC 280 camera and a Leica DME compound microscope (Leica Microsystems Imaging Solutions Ltd.) at 1000x magnification.

Results

Localization of MMR Proteins to Testicular and Epididymal Mouse Sperm

Immunofluorescence was used to determine initial MMR protein localization patterns in both testicular and caudal epididymal sperm in the C57BL/6J mouse. Three categories of MMR proteins were analyzed for localization to sperm: somatic cell specific, meiocyte specific, and proteins that have roles in both somatic cells and meiocytes (Table 1A and 1B).

Somatic cell MMR proteins MSH2, MSH3, and MSH6 were tested and had no localization to either testicular or caudal epididymal sperm (Figure 1). In these analyses,

293T human embryonic kidney cells represented a somatic control and as expected, displayed protein localization throughout the cytoplasm.

The meiocyte specific MMR proteins, MSH4 and MSH5, typically heterodimerize during homologous recombination. Unexpectedly, these proteins displayed localization to different regions of testicular and cauda epididymal sperm. In testicular sperm, MSH4 localized to the posterior head and principle piece of the tail, while MSH5 localized only to the acrosome (Figure 2). In cauda epididymal sperm, the localization patterns for these two proteins changed from the patterns seen in testicular sperm. MSH4 localized to the acrosome, posterior head, and middle piece of the tail. MSH5 had similar localization to MSH4 in the tail, but localized to the acrosome and in a line in the middle of the head (Figure 3).

The MMR proteins MLH1 and MLH3 also heterodimerize with each and have functions in both somatic cells and in meiocytes. Like MSH4 and MSH5, the MLH proteins had different localization patterns in testicular and cauda epididymal sperm. In testicular sperm, MLH1 was absent, whereas MLH3 showed localization to the acrosome and to the principle piece of the tail (Figure 2). In cauda epididymal sperm, MLH1 exhibited localization to the middle piece of the tail, and MLH3 localized to the acrosome and middle piece of the tail (Figure 3).

Antibody reactivity analysis for MMR proteins MLH1, MLH3, MSH4, and MSH5 is summarized in Table 2 A and B.

Localization of MMR Proteins in the Absence of Pms2

PMS2 is one of the MMR proteins involved in meiosis and is thought to function in the synapsis of chromosomes and the correct pairing of homologues. However, due to the absence of useful antibodies for this protein, its exact function in meiosis is still unknown [9, 14]. Our observation in our immunofluorescence results that MMR proteins do not appear to heterodimerize with their normal partners suggests that they may pair

with different MMR proteins such as PMS2. To examine this hypothesis we chose to characterize localization patterns of MMR proteins in the Pms2$^{-/-}$ mouse sperm.

Pms2 $^{-/-}$ mice are sterile but do manage to produce a small amount of abnormally shaped sperm. The sperm heads are severely malformed and the tails are extremely truncated or missing entirely. As a result of the truncated/missing tails in Pms2 $^{-/-}$ mouse sperm, we focused on the localization of the MMR proteins that had distinct localization to the head of the sperm. Localization of MLH3 and MSH5 were similar in abnormally formed Pms2$^{-/-}$ testicular sperm when compared to the Pms2$^{+/-}$ and the C57BL/6J wild type mouse testicular sperm. MLH3 localized to the acrosomal region of the sperm head in the Pms2 $^{-/-}$ mouse as seen in the wild type mouse. The localization of MLH3 appeared to be unaffected by the absence of the PMS2 protein or the abnormal shape of the sperm head in the Pms2$^{-/-}$ mouse. MSH5 localization to the sperm head was also similar to the wild type mouse, with localization to the acrosomal region unaffected by the abnormal sperm head shape or absence of the PMS2 protein in the Pms2 $^{-/-}$ mouse (Figure 4).

Determination of Stage Specific Localization of MMR Proteins During Spermiogenesis

To verify our immunofluorescence results we conducted Immunohistochemical (IHC) studies of MLH1, MLH3, MSH4, and MSH5 on testes sections of C57BL/6J mice. This approach also allowed us to view several sections of an intact seminiferous tubule and all of the stages of spermiogenesis within that tubule. Therefore, IHC was additionally used to determine if MMR proteins were present in specific stages of sperm development. All four proteins are known to localize to meiotic stage cells in the testes, but have not previously been associated with any other stages of sperm development [1, 5].

MLH1 localized only in primary spermatocytes (Figure 5A), whereas MLH3 had distinct localization in primary spermatocytes, the developing acrosome of round spermatids, and the tails and heads of elongated spermatids and spermatozoa (Figure 5B). MSH4 had dispersed cytoplasmic localization in primary spermatocytes and round spermatids and localization to the tails of spermatozoa (Figure 5C). MSH5 localization was dispersed in the cytoplasm of primary spermatocytes and round spermatids, and evident in small quantities in the head of spermatozoa (Figure 5D). The results for all four MMR proteins in seminiferous tubule sections confirm the immunofluorescence data previously presented for testicular spermatozoa. In addition, the localization of MMR proteins in cells other than spermatocytes implies a potential functional role for MMR proteins in spermiogenesis.

Evolutionary Conservation of MMR Protein Localization

To determine if MMR protein localization was similar in sperm of different species, we examined their immunofluorescence localization patterns in ejaculated sperm samples of Rhesus macaque and human males. Localization patterns of MLH1, MLH3, MSH4, and MSH5 were examined and compared to each other and mouse cauda epididymal sperm. Results of these localization patterns are summarized in Table 3 A-D. Although specific localization patterns varied among the three species, general patterns were similar. Localization of MLH1 on the human sperm heads was unique, while all three species analyzed had localization of MLH1 to the sperm tails (Figure 6A). Little difference among species was detected with respect to MLH3 localization, which was abundant in the sperm heads and tails (Figure 6B). Mouse and monkey had localization of MSH4 on the sperm heads, and mouse and human had MSH4 localization to the sperm tails (Figure 6C). All species had MSH5 localization to the sperm tails; while both mouse and human displayed additional localization of MSH5 on the sperm heads (Figure 6D).

MMR Protein Localization after Capacitation and Acrosome Reaction of Sperm

Capacitation of sperm is necessary for the acrosome reaction to occur [24-26]. The acrosome reaction, in turn, is necessary for the sperm to penetrate the oocyte for fertilization [27-29]. Due to the importance of these sperm functions, the capacitation and acrosome reaction of C57BL/6J mouse sperm was examined to determine if MMR proteins might have a functional role in preparation of the sperm for fertilization of the oocyte.

The results for individual protein localization on capacitated and acrosome reacted sperm is summarized in Table 4. MLH1 was not examined for these experiments due to the fact that it has no MMR protein localization to the head of the sperm and both reactions occur in this region. Capacitation and acrosome reaction of the sperm was determined using Coomassie staining. Three hundred sperm were examined for control, capacitated, and acrosome reacted sperm for Coomassie and MMR protein staining (Figure 7). An analysis of the data was performed using 3 data pairs (i.e., control vs each of the MMR proteins) with the Coomassie staining acting as the control. Of the three comparisons, only the MSH5 vs control reached significance (chi square = 4.45; p= 0.03). However, after using the Bonferroni correction to account for multiple tests, none of the individual comparisons were statistically significant. This suggests that there is not a significant difference between the Coomassie results and the MMR protein localization, meaning that the results for MMR protein loss from the head of sperm correlate with the capacitation of the sperm.

Discussion

The present study had three main objectives: first, to characterize the localization patterns of MMR proteins in mouse testicular and epididymal sperm, second, to determine if these localization patterns were conserved among different species and third, to assess potential functional roles for these MMR proteins during spermiogenesis and after ejaculation.

MMR Proteins Show Localization to Both Testicular and Epididymal Sperm

The goal of this part of the study was to use immunofluorescence to determine localization patterns of MMR proteins to both testicular and epididymal sperm in a standard inbred mouse strain (C57BL/6J). The localization of MMR proteins in these cells is unusual since they are post meiotic and should have no DNA damage due to transcriptional silencing of their DNA [24, 25]. Surprisingly, MMR proteins appeared not to be associated with the DNA; instead they showed localization to the acrosomal region of the head, the neck region, and the tail of mouse sperm. Importantly, temporal variation of MMR protein localization patterns was observed between testicular sperm and epididymal sperm. The fact that MMR were visualized in testicular, epididymal, and ejaculated sperm suggests a functional role during capacitation, the acrosome reaction, or fertilization of the oocyte.

To confirm the immunofluorescence results, MMR protein localization was examined in seminiferous tubule cross-sections. In addition to their localization in the spermatozoa, MMR proteins were present throughout various stages of the developing sperm. This suggests that there may be some functional role for them in spermiogenesis. Of the MMR proteins examined, MSH4 is a likely candidate for a functional role in spermiogenesis due to its interaction with VBP1, a highly conserved protein that is thought to promote the formation of α- and γ-tubulins [32, 33]. During spermiogenesis a major restructuring of the sperm cell cytoskeleton takes place, transforming a round cell to a streamlined, flagellated cell [34, 35]. This association with VBP1 could indicate a role for MSH4 in the restructuring of the sperm.

MMR Protein Localization is Conserved Among Species

Evolutionary conservation of MMR protein localization to sperm was compared in three species, mouse, rhesus macaque, and human. The specific localization patterns for the three species varied, but the patterns were similar across all three. For example, MLH3 localized to the sperm head and middle piece of the tail in mouse, rhesus

macaque, and human. This shows that the localization of these MMR proteins to sperm has been conserved throughout evolution. However, due to differences in specific localization, their function in association with sperm may vary.

MMR Proteins may be Involved in Capacitation or Acrosome Reaction

The goal of the final experiment was to examine functional roles of MMR proteins in capacitation, the acrosome reaction, or fertilization of the oocyte using mouse sperm. When sperm were capacitated and acrosome reacted in vitro, it was evident that MMR proteins were lost from the sperm head during the capacitation event. These findings seem to rule out a function for these MMR proteins during fertilization of the oocyte. However, there could still be localization of MMR proteins to the sperm head that escape our level of detection using immunofluorescence. Although the MMR proteins may not be involved in oocyte fertilization, there could be a functional role for these proteins in the capacitation or acrosome reaction of the sperm. It has been determined that another protein associated with the sperm head, Crisp-1, acts to inhibit premature capacitation and acrosome reaction of the sperm [26, 30, 31, 36]. The role for MMR proteins could be similar to that of Crisp-1, or alternatively, they may act to facilitate the capacitation or acrosome reaction of sperm.

Conclusions

As a result of this study, new roles for MMR proteins beyond the previously known somatic and meiotic functions have been proposed. The localization patterns of MMR proteins in mouse, rhesus macaque, and human sperm have been determined, indicating that this localization is evolutionarily conserved among mammals. Evidence of MMR proteins in various stages of spermiogenesis also indicates a potential functional role for them during sperm development. Additionally, in vitro studies seem to rule out the possibility of the MMR proteins having a function in fertilization of the oocyte, but may imply a role for them in the capacitation or acrosome reaction of the sperm.

Figure 1: Somatic Cell MMR Protein Localization in Testicular and Epididymal Sperm

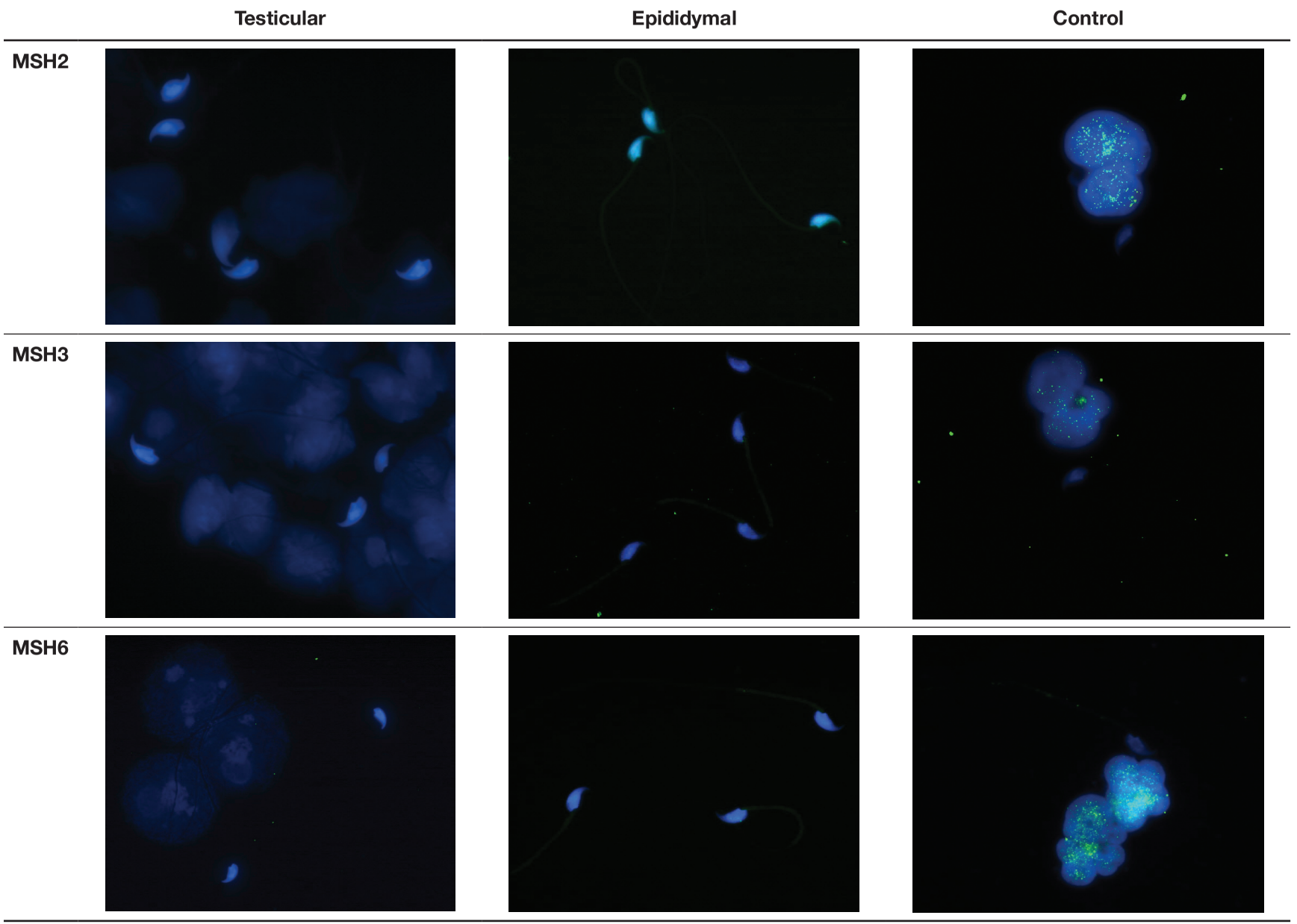


Figure 1 Somatic Cell Specific MMR protein localization in C57BL/6J mouse testicular and cauda epididymal sperm. DNA (detected by DAPI) is in blue, and MMR proteins (detected by FITC labeled anti-MSH2, MSH3, and MSH6 antibodies) are in green. As a control, human embryonic kidney cells were fixed to the slide with sperm. Neither testicular or epididymal sperm show localization of MSH2, MSH3, or MSH6. Localization of all three MMR proteins can be seen in the control human embryonic kidney cells as punctuate staining throughout the cytoplasm of the cell.

Figure 2: Meiotic MMR Protein Localization in Testicular Sperm

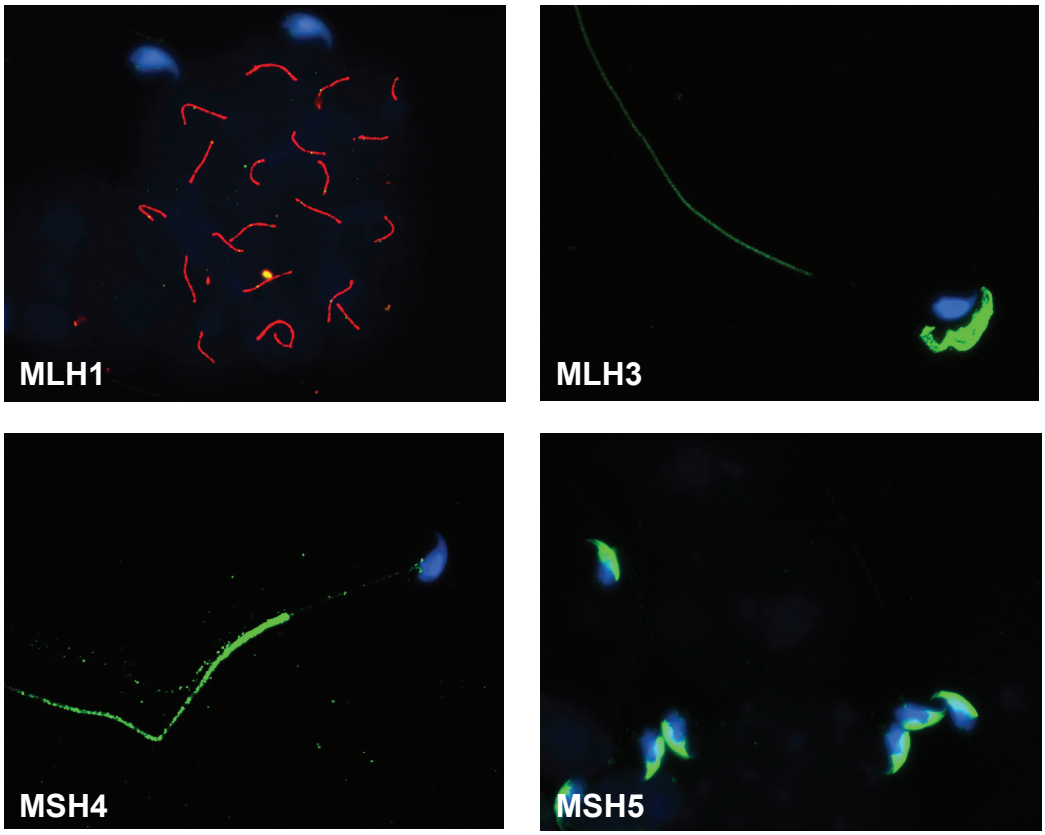


Figure 2 Localization patterns of MMR proteins MLH1, MLH3, MSH4, and MSH5 in C57BL/J6 mouse testicular sperm. DNA (detected by DAPI) is in blue, and MMR proteins (detected by FITC labeled anti-MLH1, MLH3, MSH4 and MSH5 antibodies) are in green. As a control, SYCP3 (which detects axial elements of the synaptonemal complex) is shown in red. All MMR proteins but MLH1 are present on sperm. Localization appears to be in regions not associated with DNA.

Figure 3: Meiotic MMR Protein Localization in Epididymal Sperm

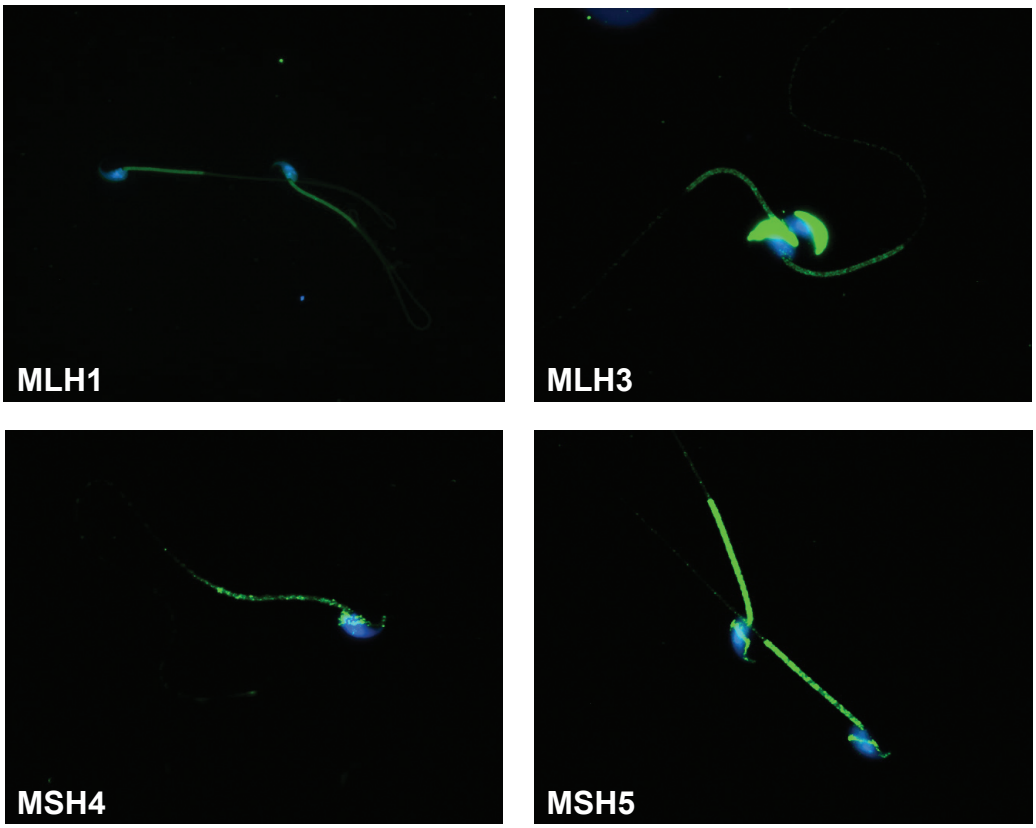


Figure 3 Localization of MMR proteins MLH1, MLH3, MSH4, and MSH5 in C57BL/6J mouse caudal epididymal sperm. DNA (detected by DAPI) is in blue, and MMR proteins (detected by FITC labeled anti-MLH1, MLH3, MSH4 and MSH5 antibodies) are in green. All MMR proteins are present on sperm in regions not associated with DNA.

Figure 4: MMR Protein Localization in the Pms2 Mouse

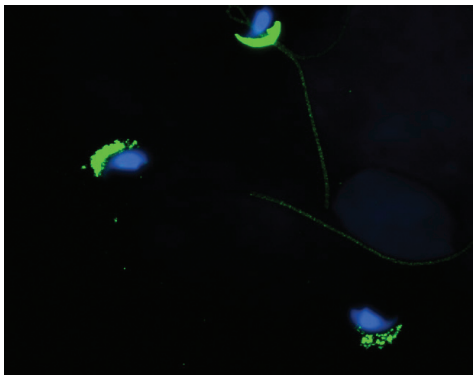
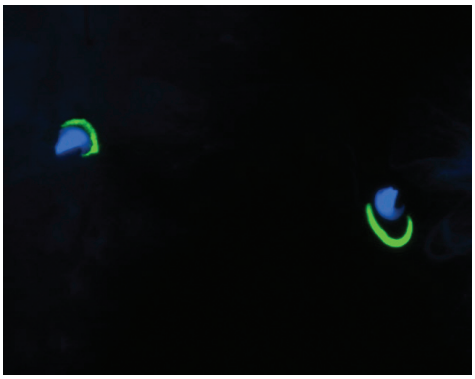
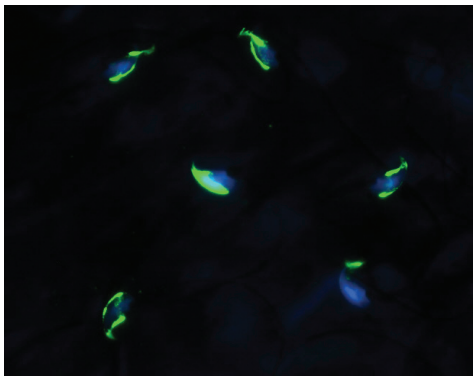
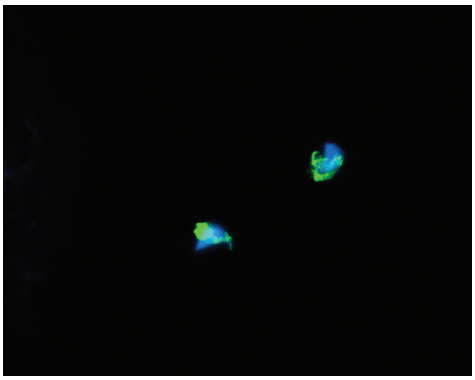
	(+/-)	(-/-)
MLH3		
MSH5		

Figure 4 Localization of MMR proteins MLH3 and MSH5 in Pms2 heterozygous (+/-) and homozygous null (-/-) mouse testicular sperm. DNA (detected by DAPI) is in blue, and MMR proteins (detected by FITC labeled anti-MLH3 and MSH5 antibodies) are in green. Pms2 (+/-) mouse testicular sperm have MMR protein staining that matches that of C57BL/6J mouse testicular sperm. Regardless of abnormal sperm formation, Pms2 (-/-) mouse sperm has similar MMR protein staining to C57BL/6J and Pms2 (+/-) testicular sperm.

Figure 5: Stage Specific Localization of MMR Proteins During Spermiogenesis

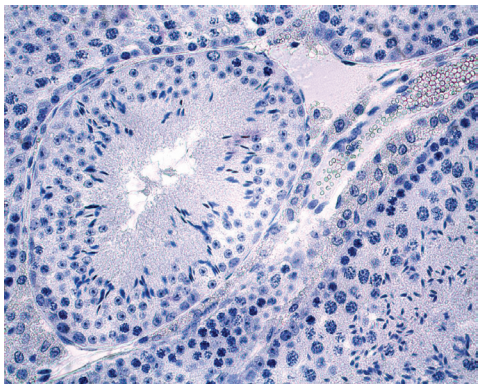
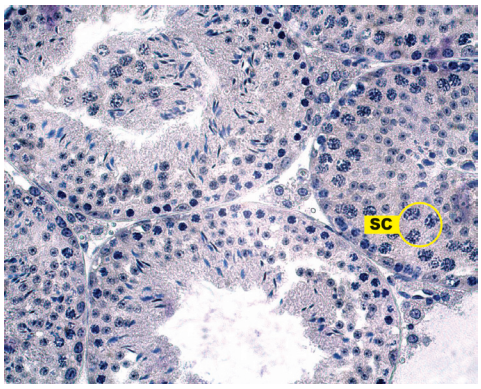
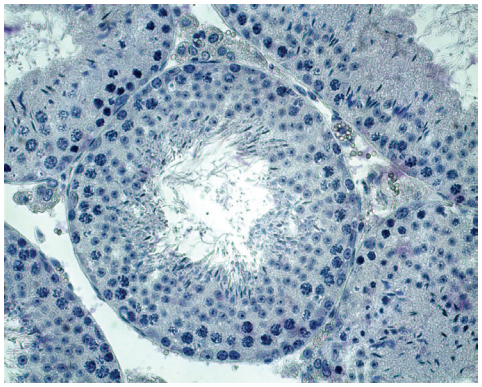
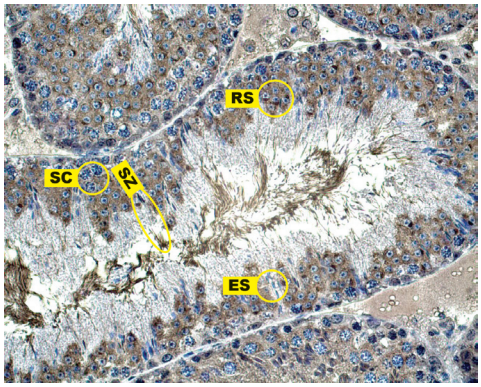
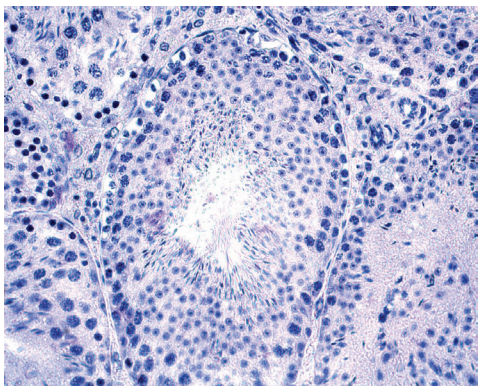
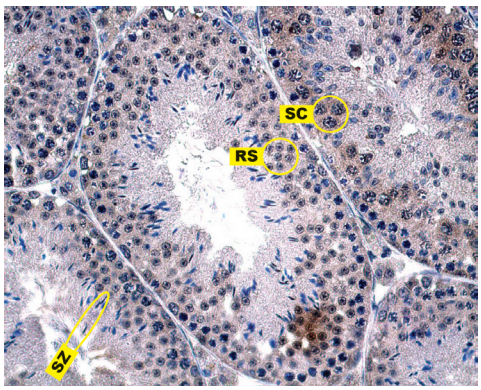
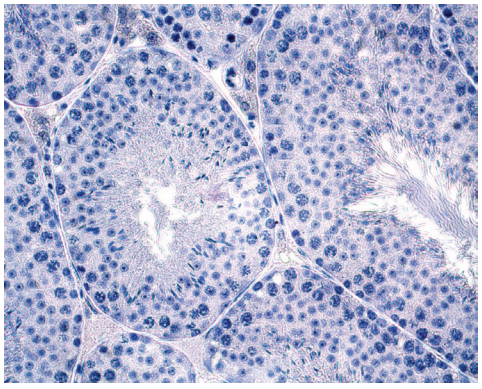
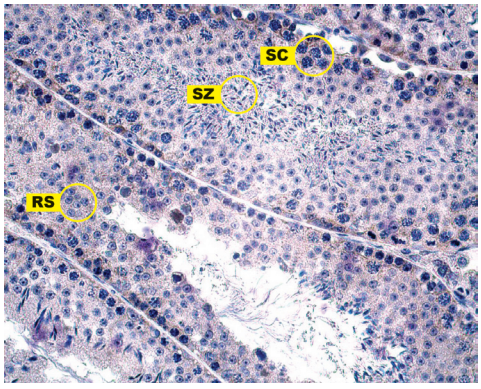
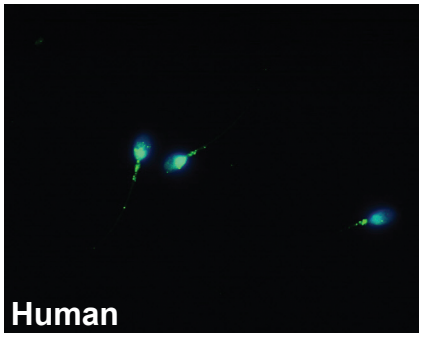
	Control	Experimental
MLH1		
MLH3		
MSH4		
MSH5		

Figure 5 Stage specific localization of MMR proteins during spermiogenesis in C57BL/6J mice. MMR proteins were labeled in serial C57BL/6J mouse testicular sections using an HRP/chromagen system (brown). Haematoxylin (blue) was used as a counter stain. Control tissue sections had no primary antibody added, but went through all other staining procedures. All MMR proteins localized to spermatocytes (SC). MLH3 localized to the developing acrosome in round spermatids (RS), and the tails and heads of elongated spermatids (ES), and spermatozoa (SZ). MSH4 localized diffusely in the cytoplasm of round spermatids (RS) and to the tails of spermatozoa (SZ). MSH5 localized diffusely throughout the cytoplasm of round spermatids (RS) and in small quantities to the head of the spermatozoa (SZ).

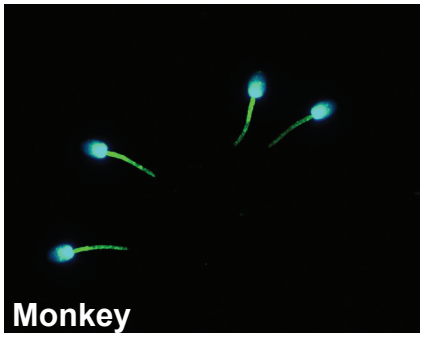
Figure 6: Comparison of MMR Protein Localization Among Species

Figure 6A MLH1

Human MLH1



Monkey MLH1



Mouse MLH1

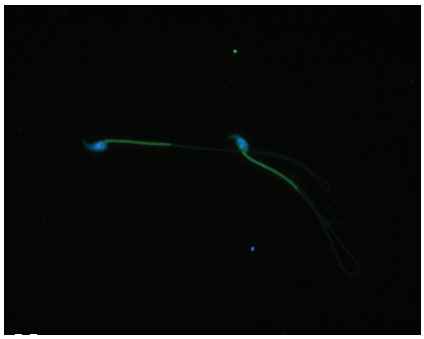
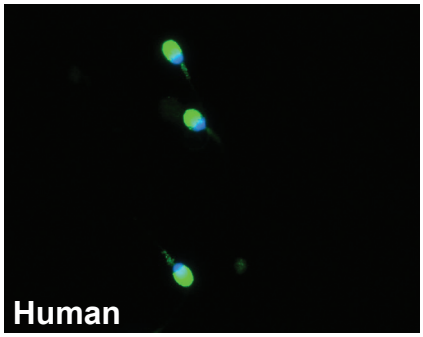
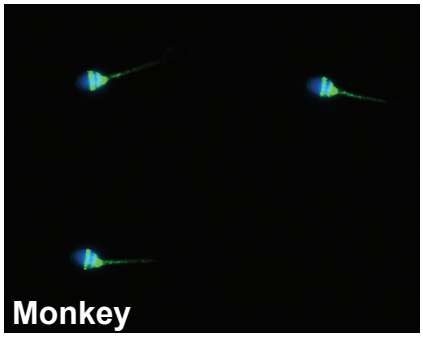


Figure 6B MLH3

Human MLH3



Monkey MLH3



Mouse MLH3

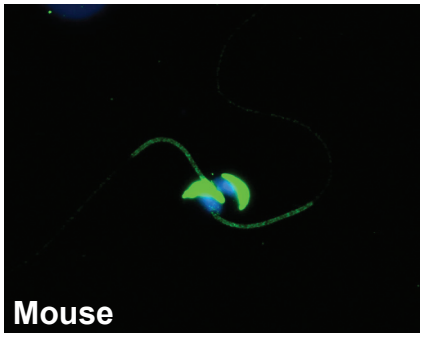
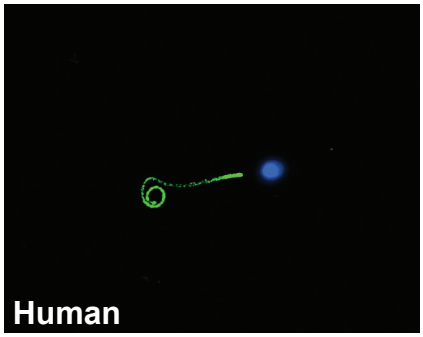
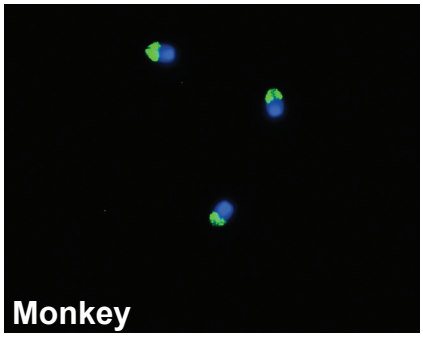


Figure 6C MSH4

Human MSH4



Monkey MSH4



Mouse MSH4

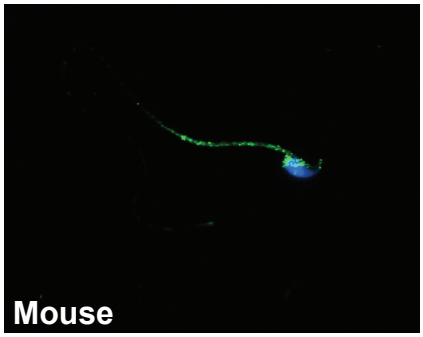
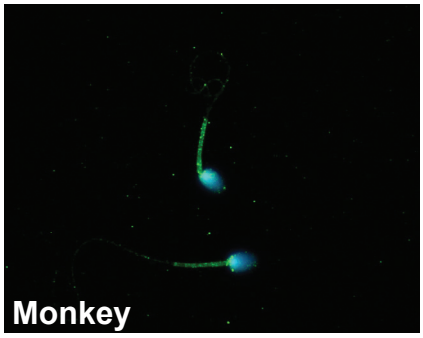


Figure 6D MSH5

Human MSH5

Monkey MSH5



Mouse MSH5

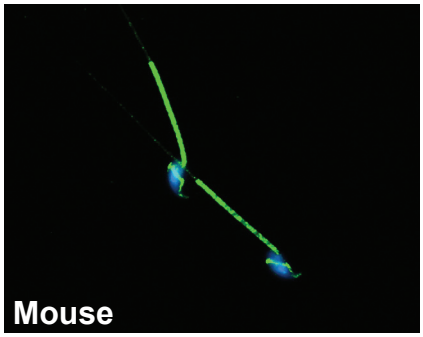


Figure 6 Conservation of MMR protein localization to sperm among three mammalian species. DNA (detected by DAPI) is in blue, and MMR proteins (detected by FITC labeled anti-MLH1, MLH3, MSH4 and MSH5 antibodies) are in green. Although specific patterns vary among mouse, monkey and human, general localization of MMR proteins to sperm is similar.

Figure 7: MMR Protein Localization to Capacitated and Acrosome Reacted Sperm

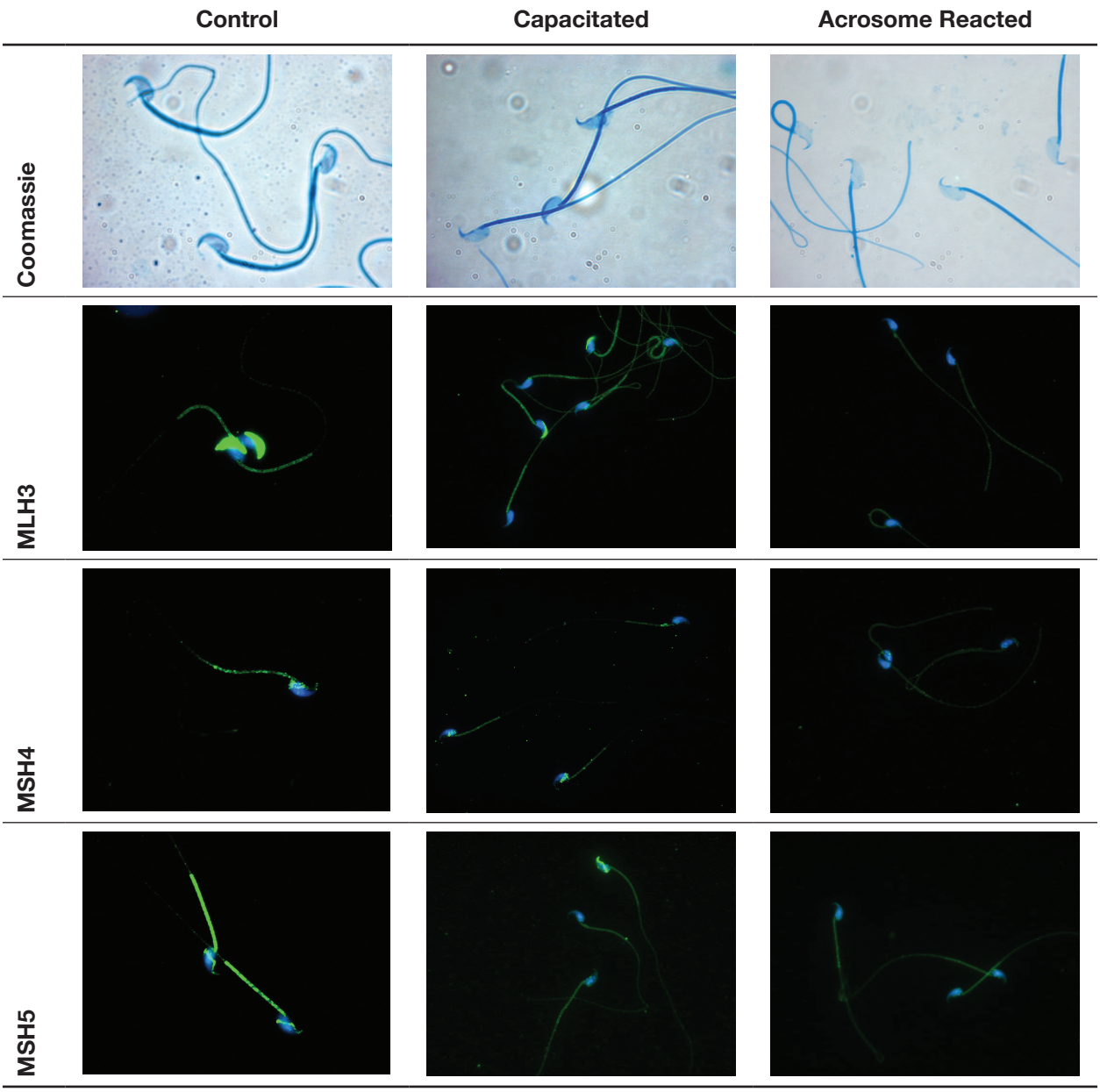


Figure 7 Effect of in vitro capacitation and acrosome reaction on MMR protein localization to C57BL/6J mouse caudal epididymal sperm. DNA (detected by DAPI) is in blue, and MMR proteins (detected by FITC labeled anti-MLH1, MLH3, MSH4 and MSH5 antibodies) are in green. Control sperm were fixed immediately after removal from the cauda epididymis. At capacitation stage (after 1 hr. incubation at 37^0 C in KRBT media), approximately 50% of sperm have lost MMR staining on the head. By acrosome reaction stage (after addition of Ca^{2+} ion to KRBT media and another 1 hr. incubation at 37^0 C), 100% of sperm have lost MMR staining on the head.

Table 1: MMR Protein Localization in Mouse Sperm

A. Bl/6 Mouse Testicular Sperm

	Acrosome	Posterior Head	Anterior Head	Middle Piece	Principle Piece
MLH1					
MLH3	X				X
MSH4		X			X
MSH5	X				

B. Bl/6 Mouse Epididymal Sperm

	Acrosome	Posterior Head	Anterior Head	Middle Piece	Principle Piece
MLH1				X	
MLH3	X			X	
MSH4	X	X		X	
MSH5	X	Line Between		X	

Table 2A: Antibodies for MMR Protein Analysis

MMR Proteins	Epitope	Ab reacts
MLH1	83 kDa protein	Mouse, Human
MLH3	20 aa at c-terminus of peptide	Mouse
MSH4	920-936 aa of peptide	Human
MSH5	109 aa at c-terminus and 103 aa at n-terminus of peptide	Human

Table 2B: Protein Homology Among Species

	Human	Rhesus macaque	Mouse
Human	100%	97%	88%
Rhesus macaque	97%	100%	85%
Mouse	88%	85%	100%

Table 3: Comparison of MMR Protein Localization Among Species

A. MLH1

	Acrosome	Posterior Head	Anterior Head	Middle Piece	Principle Piece
Mouse				X	
Rhesus				X	
Human	X			X	

B. MLH3

	Acrosome	Posterior Head	Anterior Head	Middle Piece	Principle Piece
Mouse	X			X	
Rhesus		Line Between		X	
Human	X			X	

C. MSH4

	Acrosome	Posterior Head	Anterior Head	Middle Piece	Principle Piece
Mouse		Line Between		X	
Rhesus	X				
Human					X

D. MSH5

	Acrosome	Posterior Head	Anterior Head	Middle Piece	Principle Piece
Mouse	X	Line		X	
Rhesus				X	
Human		Line		X	

Table 4: In Vitro Capacitation and Acrosome Reaction of Sperm

 # Sperm with No Staining Present out of 300

Label	Control	Capacitated	Acrosome Reacted
Comassie	0	175	300
MLH3	0	192	300
MSH4	0	169	300
MSH5	0	201	300

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CHAPTER 3
SYNOPSIS AND FUTURE DIRECTIONS

The studies presented in the previous chapter had three main objectives: first, to characterize the localization patterns of MMR proteins in mouse testicular and epididymal sperm, second, to determine if these patterns were conserved across species and third, to examine potential roles for these MMR proteins during spermiogenesis and fertilization. This chapter summarizes this work and discusses possible future studies.

Localization of MMR Proteins in Mouse Sperm

In initial studies described in Chapter 1, I examined localization patterns of MMR proteins in both testicular and epididymal sperm in a ÒstandardÓ inbred mouse strain (C57BL/6J). While MMR proteins are typically known to associate with DNA, these proteins were instead present in the acrosomal region of the head, the neck region, and the tail of mouse sperm. Importantly, these proteins displayed temporal variation between testicular sperm and epididymal sperm. The fact that MMR proteins were visualized in both testicular and epididymal sperm and, indeed, in ejaculated sperm raises the question of a functional role in capacitation, the acrosome reaction, or fertilization.

In addition, I examined MMR protein localization in seminiferous tubule cross-sections from C57BL/6J mice to determine if these proteins might be involved in specific stages of sperm development. As expected, all four MMR proteins were observed in primary spermatocytes undergoing meiosis I. Three of the four proteins examined MLH3, MSH4, and MSH5, were present in various stages of developing sperm including round spermatids, elongated spermatids, and mature testicular spermatozoa. The presence of these MMR proteins throughout the various stages

of the developing sperm suggests that there may be a functional role for them in spermiogenesis.

Future investigations using Western blots to analyze specific localization of MMR proteins to sperm components would be a logical next step in this project. As described in Nixon *et. al.* 2006, sperm membranes, heads, and tails can be separated using sonication and centrifugation techniques. The proteins contained on each of these components can then be resolved using SDS PAGE gel electrophoresis and Western blotting [1]. This would allow us to make two determinations: first, it would confirm our immunofluorescence results; and second, it would reveal any monomeric, homodimeric, or heterodimeric structures being formed by the MMR proteins in sperm.

One protein in particular, MSH4, displays attributes that would make it a good candidate for a specific role in spermiogenesis. MSH4 has been shown by Her *et. al.* 2003 and Neyton *et. al.* 2007 to interact with VBP1, a highly conserved protein known to be expressed in the testis that is thought to promote the formation of α- and γ-tubulins. MSH4 has also been shown to be present in both the cytoplasm and nucleus of testicular cells [2, 3]. During spermiogenesis, there is a major restructuring of the cytoskeletal structure of the sperm, with one major player in this process being the manchette structure. The manchette is thought to be involved in the final restriction of the cytoplasm near the middle piece of the tail resulting in the removal of excess cytoplasm from the sperm cell [4, 5]. The fact that MSH4 is found in the cytoplasm of testicular cells and is known to associate with VBP1 could mean that MSH4 may have a role in the physical reshaping of the sperm cell during spermiogenesis, perhaps in association with the manchette structure. It would be interesting to see if MSH4 and VBP1 are present during different stages of spermiogenesis, and through Western Blot analysis determine if they are indeed interacting. In addition, if the interaction between MSH4 and VBP1 exists at various stages in the developing sperm, it could support a role for MSH4 in the physical reshaping of the sperm during spermiogenesis.

Conservation of MMR Protein Localization to Sperm

In the second part of this study, I examined the evolutionary conservation of MMR proteins by examining their localization in sperm of three species, mouse, rhesus macaque, and human. Localization patterns of MMR proteins in rhesus macaque and human ejaculated sperm were determined and compared to the localization patterns in mouse caudal epididymal sperm. The specific localization patterns for the three species varied, but the patterns were similar across all three. For example, MLH3 localized to the sperm head and middle piece of the tail in all three species. These findings show that MMR protein localization to sperm is conserved among species and furthermore implies functional significance for this localization.

Investigations into non-mammalian species would be of interest to determine if MMR protein localization patterns observed in mammals extends to other classes of animals. Another avenue would be to examine a defined subset of mammals, specifically the hominids. Since there is a distinct break between old and new world monkeys, it would be interesting to see if there is a specific change in localization of MMR proteins between these two subsets.

Localization of MMR Proteins to Capacitated and Acrosome Reacted Sperm

The third part of the study in Chapter 2 was designed to examine the functional roles of MMR proteins in capacitation, the acrosome reaction, or fertilization using C57BL/6J mouse sperm. When sperm were capacitated in vitro, acrosomal staining of MMR proteins was diminished by approximately fifty percent compared to uncapacitated sperm. Once the acrosome reaction occurred, all of the acrosomal labeling was abolished. This rules out a function for MMR proteins during fertilization of the oocyte. However, this could imply a functional role for these proteins in causing the capacitation or acrosome reaction of sperm to occur.

It is has been shown that capacitation and the subsequent acrosome reaction of the sperm occur only under specific conditions. Cholesterol must be removed from

the membrane of the sperm causing the induction of a signaling pathway consisting of adenylyl cyclase (AC)/cAMP/protein kinase A (PKA). Downstream signaling results in the tyrosine phosphorylation of several proteins in the plasma membrane and intracellular structures [1, 6, 7]. Due to the importance of these events in the capacitation and acrosome reaction of the sperm, it would be interesting to determine if the MMR proteins might play a role in one of these processes.

Several experiments could further elucidate a functional role for MMR proteins during the capacitation and acrosome reaction of sperm. It would be interesting to use the method described in Roberts *et. al.* 2003 to see if continuous addition of MMR proteins during in vitro capacitation and acrosome reaction of sperm could block the tyrosine phosphorylation reaction [6]. Alternatively, MMR proteins may be involved in either facilitating the cholesterol removal or tyrosine phosphorylation of the sperm. Initially MMR proteins on sperm heads could be blocked using anti-MMR protein antibodies and then capacitated and acrosome reacted in vitro. This would determine if blocking of these proteins results in the sperm being unable to capacitate or acrosome react.

Conclusions

These studies identified the localization patterns of MMR proteins in mouse, monkey, and human sperm and indicate and evolutionarily conserved function among mammals. We have also determined that MMR proteins are evident in various stages of spermiogenesis, indicating potential functional roles for them in this process. Additionally, in vitro studies ruled out the possibility that MMR proteins have a function in fertilization of the oocyte, but may indicate a role in the capacitation and acrosome reaction of the sperm.

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