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DNA Mismatch Repair, Genome Stability and Tumorigenesis

by

Marcia R. Campbell 

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ABSTRACT

A germline heterozygous alteration of any one of the human DNA mismatch repair (MMR) genes, *MSH2, MLH1, MSH6, PMS2* and *PMS1* has been associated with hereditary non-polyposis colorectal cancer (HNPCC).

Mice homozygous deficient for one of the MMR genes develop T-cell thymic lymphomas. I have evaluated the role of MMR in several cellular processes leading to the development of tissue-specific tumorigenesis. This thesis characterizes the genetic instability in the murine MMR deficient tumours and furthers our understanding of how tumorigenesis arises by i) molecular characterization of Msh2 deficient murine lymphomas ii) analysis of chromosomal instability in Msh2 deficient murine lymphomas as well as in a human cell-line lacking MSH2 iii) investigation of the involvement of MMR in telomere function iv) investigation of a role for MMR in centrosome stability and v) the generation of a novel mouse model to study HNPCC tumorigenesis.

I performed spectral karyotype analysis (SKY) on mouse tumours and SKY analysis of a cell line derived from a rare human patient deficient for MSH2, showed translocations and rearrangements. These results represent the first successful characterization of chromosomal instability in MMR deficient tumorigenesis. My results show that microsatellite instability (MSI+) as well as chromosomal instability (CIN) can co-exist and contribute to tumorigenesis.

Using immunofluorescence techniques I assayed the stability of centrosomal proteins in the absence of MMR in MEFs. For the first time I've shown that hyperamplification of

centrosome proteins occurs in the absence of MMR and may account for the increase in aneuploidy that I've observed in MMR deficient MEFs.

I created a novel animal model. I bred *Msh2*$^{-/-}$ and *Msh6*$^{-/-}$ mice to athymic nude mice. This animal model will allow us to further study the role of MMR proteins in the development of T and B-cells.

In conclusion, this thesis helps expand our understanding of the multi-functional roles that the MMR proteins have within a cell by identifying new areas, such as telomere regulation, T and B-cell development and centrosome regulation where mammalian MMR is required. Thus, in the absence of MMR, tumorigenesis arises from a combination of effects, underscoring the importance of MMR in neoplasia.

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ALT	Alternative lengthening of telomeres pathway
APC	Adenomatous polyposis coli gene
AT	ataxia telangiectasia
ATM	ataxia telangiectasia mutated gene/protein
ATR	ataxia telangiectasia related gene/protein
BASC	BRCA1-associated genome surveillance complex
bp	Base pairs
CGH	comparative genomic hybridization
DM1	Myotonic dystrophy gene
DNA	Deoxyribonucleic Acid
DNA pol δ	DNA polymerase delta
Dnmt1	DNA methyltransferase 1
ES	Embryonic stem
FAP	familial adenomatous polyposis
FISH	Fluorescent in situ hybridization
GI	Gastrointestinal
HD	Huntington disease
Hdh^{Q111}	Mouse with knocked-in human repeats
HNPCC	Hereditary non-polyposis colorectal cancer
HRR	Homologous recombination repair
IL	Interleukin
kDa	Kilo Dalton(s)
LBL	lymphoblastic lymphoma
LOH	Loss of heterozygosity
MBD4	methyl-CpG-binding domain protein 4 gene
MEFs	Mouse embryonic fibroblasts
MGMT	Methylguanine DNA methyltransferase gene
Min	Multiple intestinal neoplasia
MMR	DNA mismatch repair
MNNG	*N*-methyl-*N*'-nitroso-*N*-nitrosoguanidine
MSI/MSI+	Microsatellite instability
MSS	Microsatellite stable
NCBI	National Center for Biotechnology Information
NF1	Neurofibromatosis type 1
OMIM	On-line mendelian inheritance in man
PCR	Polymerase chain reaction
pTEN	phosphatase and tensin homolog deleted on chromosome ten gene
Rb	Retinoblastoma
RNA	Ribonucleic acid
SKY	Spectral Karyotyping
TdT	Terminal deoxynucleotidyl transferase
TRAP	Telomeric repeat amplification protocol
T-SCE	Telomere sister chromatid exchange
UV	Ultraviolet radiation

Chapter 1 ♦ Literature Review

Introduction

Neoplasia is widely accepted to result from the acquisition of numerous genetic lesions disrupting genes that regulate growth, the cell cycle or cell death. Hanahan and Weinberg suggest that there are six essential alterations in cell physiology regulatory pathways that when mutated lead to malignant growth. These pathways are: 1) self-sufficiency in growth signals 2) insensitivity to growth-inhibitory (antigrowth) signals 3) evasion of apoptosis 4) limitless replicative potential 5) sustained angiogenesis and 6) tissue invasion and metastasis (Hanahan & Weinberg, 2000). When one of these pathways becomes breached, the cell will no longer respond to one of these defense mechanisms. The altered regulation of the cell can lead to the acquisition of mutations that can lead to tumorigenesis. The mechanisms by which these mutations are generated are the subject of continuing debate. Based on the presence of genomic alterations, cancer cells are believed to be genetically unstable, and acquisition of genomic instability may represent an early stage of neoplasia (Loeb, 1991). Some have argued that underlying genetic instability is necessary to allow for the generation of the multiple mutations required for the progression of tumorigenesis (Cheng & Loeb, 1993; Hartwell, 1992). In contrast to this, it has also been hypothesized that intrinsic rates of mutation, when coupled with clonal expansion, are sufficient to allow for tumour formation (Tomlinson *et al.*, 1996). One problem with this concept is that it does not account for the frequent occurrence of different cells within a tumour population often harboring different mutations. To account for the large number of mutations in cancer cells, Loeb has hypothesized that cancer is manifested by a mutator phenotype (Cheng & Loeb, 1993; Loeb, 1991).

Genomic Instability

Using experimental evidence from others, Loeb has estimated that the spontaneous mutation rate observed in normal human cells is about 1.4×10^{-10} mutations/nucleotide/cell generation (Loeb, 1991). This mutation rate would result in approximately two to three mutations per cell and Loeb suggests this is inadequate to account for the six to nine chromosomal alterations that have been observed in many cancers or the larger number of

mutations that are predicted to accumulate during tumorigenesis (Loeb, 1991; Loeb & Christians, 1996). Initially, Loeb et al. and Nowell et al. postulated that during the growth of a tumour the mutation rate in the cancer cells must exceed that in the normal cells (Loeb & Christians, 1996; Loeb *et al.*, 1974; Nowell, 1974). But how does the mutation rate become elevated? Where do the multiple mutations found in cancer cells come from? Loeb and Christians (Loeb & Christians, 1996) propose several schemes that may account for the increase in mutations in cancer cells. 1) Mutational hotspots in the genome might exist. This might explain characteristic non-random mutations such as specific chromosomal translocations in several human lymphomas and leukemias. 2) Cancer cells might be uniquely susceptible to endogenous sources of DNA damage. 3) The types of mutations observed may be different from the spontaneous mutations that are found in normal somatic cells. 4) It is possible that each mutation in a tumour cell confers a profound growth advantage and clonal expansion is the driving force. 5) Stationary or 'adaptive' mutagenesis might be occurring so that cells that are not actively dividing are still accumulating mutations. 6) A mutator phenotype might exist that contributes to the accumulation of mutations (Cheng & Loeb, 1993; Loeb, 1991). This last concept is favoured by Loeb and colleagues. Early in the pathway to tumorigenesis, a gene involved in maintaining the stability of the genome may become mutated, resulting in a genome-wide increase in mutation rate. This increase in mutation frequency might cause other genes that are involved in maintaining stability to then become mutated, with the end result of irreversible, increased instability in the cell.

There are several forms of instability that have been seen in human cancers and although these observations do not prove that genetic instability is necessary for a tumour to develop, there is evidence to strongly support a role for genetic instability in the genesis of various human tumours. Instability as it is understood today refers to a dynamic rate of change (Jackson & Loeb, 1998). Coleman *et al.* propose that at least two broad types of genomic instability may exist: 1) progressive genomic instability, and 2) episodic (transient) genomic instability (Coleman & Tsongalis, 1999). Progressive instability defines an ongoing mutagenic process that persists over time in which new mutations are

occurring in each cell generation. This form of instability is associated with tumour populations that are compromised and not able to protect the integrity of their genome. Episodic or transient instability describes sporadic genetic damage and is associated with tumours that contain specific mutations or chromosomal alterations in the absence of wide-spread damage to the genome (Coleman & Tsongalis, 1999). Progressive instability can be expected to continue through subsequent rounds of DNA replication resulting in new genetic lesions and further instability where as episodic instability may occur once, resulting in a change to the genome that is propagated through several rounds of replication but may not lead to further instability.

Types of genetic alterations in tumours

Several forms of genetic alterations have been seen in human cancers. These can be divided into 2 major categories 1) simple sequence alterations and 2) gross genomic alterations.

Simple Sequence alterations. Sequence changes alter one or a few base pairs. They include base substitutions/point mutations or deletions/insertions of a few nucleotides. The maintenance of base pair fidelity is maintained by a balance between two separate yet linked processes - DNA polymerization and DNA repair. The proofreading function of DNA polymerases and the post-replicative DNA mismatch repair (MMR) machinery act on alterations that result from polymerase errors or DNA mutagens, preventing frameshift and base substitution mutations. This balance of replication and repair must be maintained to prevent genomic instability. Mono and di-nucleotide repeats (otherwise known as microsatellites) are prone to slippage during DNA replication, in the absence of functional MMR, resulting in small insertion/deletion loops. Left unrepaired, these changes lead to what is known as microsatellite instability or MSI+ (also known as the MIN phenotype). MSI+ is indicative of a loss of MMR. MSI+ tumours have mutation rates two to three times higher than normal (Parsons *et al.*, 1995). If these mutations occur in coding regions of genes, they can be catastrophic mutations resulting in altered gene function.

Gross genomic alterations. These changes include alterations in chromosome number, chromosome translocations and rearrangements as well as gene amplifications. Also known as chromosomal instability or CIN, this type of instability is found in the majority of solid human tumours (reviewed in Coleman & Tsongalis, 1999; Yunis, 1983). Only those chromosomal alterations that likely confer a proliferative advantage to the cell are likely to be preserved in a tumour. For example, the Philadelphia chromosome [trans(9;22)(q34;q11)], associated with chronic myelogenous leukemia, was the first non-random recurrent chromosomal alteration shown to be involved in the pathogenesis of disease (Nowell, 1974). Two types of experiments have shown that CIN in most cancers is not a transient occurrence but rather a state of instability that persists over time. Fluorescence *in situ* hybridization comparing aneuploid cancer cells with diploid cancer cells and normal cells as well as experiments that study the rate of LOH have both demonstrated that CIN is a feature of many neoplasias that is present over a continuing time period (Lengauer *et al.*, 1997; Phear *et al.*, 1996). Although CIN is known to be a common genotype of many cancers, how it occurs is still a topic of debate. Numerical chromosomal aberrations can be caused either by the loss of chromosomes at metaphase/anaphase or by multipolar divisions associated with abnormal number or structure of centrosomes. The molecular basis of CIN is unknown and just beginning to be studied (Lengauer *et al.*, 1998). The first report of a specific gene involved in the loss of function of a mitotic checkpoint showed that BUB1, when mutated, resulted in abnormal chromosome segregation (Cahill *et al.*, 1998). As well, structural rearrangements have been associated with chromosomal breakage-fusion-bridge (BFB) cycles. Cycles can be initiated by telomeric associations, dicentric chromosomes as well as ring chromosomes (reviewed in Gisselsson, 2003).

Chromosomal instability (CIN) versus microsatellite instability (MSI)

Previous research has led to the dogma that cancers demonstrating the microsatellite (MIN) phenotype generally do not show the chromosomal instability (CIN) phenotype and the inverse is true. Since early evidence showed that these instabilities rarely co-exist, it

appears that only one form of instability is necessary to drive tumorigenesis (Lengauer *et al.*, 1998). When CIN and MIN cells are fused, the resulting hybrids exhibit the CIN phenotype but not the MIN phenotype (Lengauer *et al.*, 1998). This is expected as the CIN cells are MMR proficient and can therefore complement the MMR deficient MIN cells. The fact that the MIN cells cannot complement the CIN cells suggests that the CIN phenotype is dominant and may result from a gain of function mutation(s) of an expressed protein rather than gene inactivation. Lengauer *et al.* suggested that this in turn may indicate that only a single mutational hit may be necessary to produce the CIN phenotype (Lengauer *et al.*, 1998). Although the CIN phenotype appears dominant, this does not exclude the possibility that both phenotypes may exist and present themselves together in the same cells. The fact that MIN cells can not correct the CIN phenotype when they are fused shows that either MMR does not play a role in the development of the CIN phenotype or it is involved and its absence further drives and maintains the CIN phenotype. Although thought to be exclusive, it is certainly possible that both MIN and CIN phenotypes coexist and may help drive the other phenotype. Recent experiments showing the physical interaction of several different repair proteins involved in initiating both CIN and MIN suggests that there may be overlap between these two phenomenon (Wang *et al.*, 2000). This concept will be explored further in Chapter 4.

DNA mismatch repair: Suppression of mutagenesis through mispair correction

MMR plays a critical role in maintaining the integrity of the genome in many organisms, from *Escherichia coli* to humans. DNA replication produces an error rate of one error in approximately 1 X 10^7 cells (Boland, 1997). In *E. coli*, MMR contributes almost 1000-fold to the fidelity of DNA replication (Modrich & Lahue, 1996). MMR is a post-replicative repair system that binds and initiates repair of small insertion/deletion loops and incorrectly paired nucleotides that are misincorporated during DNA synthesis. Cells lacking MMR display a mutator phenotype in which the frequency of spontaneous mutations is greatly elevated. In addition, cells without functional MMR present with microsatellite instability at mono and di – nucleotide repeats. Isolation of four mutator strains of *E. coli* led to the

identification of four genes that play key roles in MMR in *E. coli*: MutS, MutL, MutH and MutU (also known as UvrD) (Table 1-2) (Modrich & Lahue, 1996). Several orthologs of MutS, MutL and MutU have been identified in eukaryotic systems (Table 1-2). To date, no eukaryotic orthologs of MutH have been identified. Germline mutations in the human MMR genes are associated with the hereditary cancer syndrome hereditary non-polyposis colorectal cancer (HNPCC).

Hereditary Non-polyposis Colorectal Cancer (HNPCC)

Hereditary non-polyposis colorectal cancer: Knudson's modified two-hit hypothesis

Individuals with Hereditary non-polyposis colorectal cancer (HNPCC) inherit a heterozygous mutation in one of the DNA mismatch repair (MMR) genes and a second 'hit' silences the remaining allele, as in Knudson's two-hit hypothesis(Knudson, 1971). Knudson's two-hit model hypothesizes that cancer develops through the loss of function of both copies of a tumour suppressor gene. The first step occurs when one 'hit' happens in one allele, functionally disabling the gene. The second step occurs when the homologous gene becomes functionally mutated on the remaining allele in the same cell, i.e. two 'hits' have occurred. In HNPCC, the first 'hit' is the inheritance of the mutated MMR gene and the subsequent heterozygous inactivation of that particular MMR gene in all cells of the patient. The second 'hit' occurs when that MMR gene becomes inactivated in a tissue specific manner (colon, endometrium, ovary) in HNPCC patients. Inactivating mutations can include point mutations (base pair alterations, insertions/deletions), large deletions and hypermethylation of the promoter of MLH1, or one of the other MMR genes (Herman *et al.*, 1998). Inactivation of MMR leads to an increase in mutation frequency and subsequent mutations in various tumour suppressor genes lead to tumour formation.

Clinical features of Hereditary non-polyposis colorectal cancer

Hereditary non-polyposis colorectal cancer (HNPCC) is an autosomal dominant cancer syndrome that accounts for approximately 5-10% of sporadic colon cancers. It presents with an early age of onset (~40-45 years) and a high degree of penetrance. HNPCC patients have a greatly increased life-time risk of developing cancer of the colon (~70-85% of

patients), endometrium (~50% patients), ovary and GI tract (Aarnio *et al.*, 1999; Lynch & de la Chapelle, 1999; Vasen *et al.*, 1996). Genetic criteria for the diagnosis of HNPCC were outlined by the International Collaborative Group (ICG) in Amsterdam in 1990. Revised in 1999 to include cancers other than those of the colon, these criteria are now known as the ICG or the Amsterdam II criteria. The diagnosis of HNPCC requires that the patient fulfills the following criteria: 1) three or more relatives with an HNPCC-associated cancer (colorectal, cancer of the endometrium, small bowel, ureter, or renal pelvis) 2) cancer affecting at least two successive generations 3) of the family members affected with cancer, one person with cancer must be a first-degree relative of the other two 4) at least one case of colorectal cancer is under the age of 50 years 4) a diagnosis of familial adenomatous polyposis (FAP) has been excluded 5) tumours are verified by examination in a pathology laboratory (Vasen *et al.*, 1999). The criteria determine which patients should be considered for genetic testing. Other types of testing, in addition to genetic testing, analyze tumour samples from affected family members and aid in confirmation of samples as MMR deficient or not. Such tests include microsatellite analysis as well as immunohistochemistry analysis examining the expression levels of MMR genes. Microsatellite analysis measures genomic instability at the base pair level. Understanding how this genomic instability leads to tumorigenesis is fundamental to understanding the molecular pathways by which HNPCC tumours develop [and therefore further discussion will follow].

HNPCC is also known as Lynch syndrome I or Lynch syndrome II. Lynch syndrome I refers to HNPCC families that present only with colon cancers, whereas Lynch syndrome II refers to families that present with other cancers such as endometrial and ovarian cancers in addition to colon cancers. Muir-Torre syndrome is a rare variant of HNPCC in which patients present with symptoms similar to HNPCC patients, but in addition develop tumours of the sweat glands called sebaceous adenomas (reviewed in Akhtar *et al.*, 1999). As well, Muir-Torre patients develop specific skin tumours called keratoacanthomas. Like HNPCC patients, Muir-Torre patients have mutations in the MMR genes, MLH1 and MSH2. Another variant of HNPCC is Turcot syndrome. Patients develop primary brain

tumours as well as colon cancers and/ or multiple colorectal adenomas. In cases of Turcot syndrome with mutations in MMR genes, *MLH1* or *PMS2* are the genes involved (Hamilton *et al.*, 1995).

The prominent sites of tumour development in HNPCC patients are the colon and endometrium. This is intriguing as patients inherit a germline MMR mutation that results in heterozygous loss of function of that gene throughout the body. Why are the colon and endometrium the prominent sites of the 'second hit' to the affected MMR gene? Why are the above mentioned other tissues affected? Similar to HNPCC patients, mouse models of HNPCC display tissue specific tumorigenesis. However, mouse models do not display high rates of intestinal tumorigenesis but rather develop lymphomas, a different spectrum of tumours that will be discussed later in the chapter.

The colon tumours that arise in HNPCC patients are distinct from sporadic colorectal tumours and other inherited colon cancers in several ways. Two thirds of colorectal HNPCC tumours occur primarily in the proximal colon as opposed to the appearance of sporadic colorectal cancer that occurs primarily in the distal colon. HNPCC colon cancers have villous components, are more dysplastic and are poorly differentiated compared with adenomas in sporadic colon cancer (Vasen *et al.*, 1999). Multiple adenomas may be present but HNPCC patients do not generally present with abundant polypose distinguishing them from patients with another inherited colon cancer syndrome, familial adenomatous polyposis (FAP) (Vasen *et al.*, 1999). Despite these features as well as a generally younger age of onset, HNPCC patients have an improved survival compared with sporadic colon cancer patients (Vasen *et al.*, 1999). Evidence suggests that HNPCC tumours may invoke a lymphoid host response. Smyrk *et al.* (Lynch & de la Chapelle, 1999) describe a host lymphoid response with lymphocytic infiltration around HNPCC tumours. This lymphoid involvement in HNPCC tumour development may be of importance, as work presented in this thesis will show that Msh2 and Msh6 are important for normal T and B-cell development in the mouse (Chapter 6).

Microsatellite instability and cancer

Microsatellites are tandemly repeated mono, di, tri, tetra or pentanucleotide sequence motifs widely distributed throughout eukaryotic genomes (Umar & Kunkel, 1996). Microsatellites differ in length, sequence composition, and the number of repeating units. In the absence of MMR, the number of microsatellite repeat units is unstable and prone to strand slippage during DNA replication. Without MMR to repair the insertional/deletion loops, new microsatellite lengths are generated and detected as microsatellite instability (MSI+). Therefore, MSI+ is considered indicative of a loss of functional MMR and cancers that are found to be MSI+ are considered to be lacking functional MMR.

There is an ever growing list of sporadic cancers that demonstrate varying degrees of MSI+. The proportion of cancers with MSI+ (and therefore likely defects in MMR) is variable due to the tumour samples tested, methods of testing and criteria for establishing MSI+. However, when I started my Ph.D. in 1998, a proportion of sporadic cancers arising in many different tissues were MSI+: colon cancer, endometrial cancer, non-small cell lung cancer, stomach cancer, ovarian cancer, prostate cancer, esophageal cancer, squamous cell carcinoma, bladder cancer, gliomas and glioblastomas, cervical carcinoma and pancreatic adenocarcinoma, to name a few (Boyer *et al.*, 1995; Brentnall *et al.*, 1996; Chen *et al.*, 1996; dos Santos *et al.*, 1996; Egawa *et al.*, 1995; Ionov *et al.*, 1993; Leahy *et al.*, 2002; Mao *et al.*, 1996; Mironov *et al.*, 1995; Risinger *et al.*, 1993) etc. Today, this list has grown to include extensive studies of a wide variety of cancers, therefore broadening the likely role of MMR in human sporadic tumour development. For example, about 15% of sporadic colon cancer cases present with microsatellite instability, appearing to be the result of two somatic events inactivating MMR (Lynch & de la Chapelle, 1999; Umar *et al.*, 1994).

Dams *et al.* suggested that the lack of microsatellite alterations in most other cancers reflects near normal rates of mutation, but that these cancers evolve through clonal expansion during multiple rounds of cellular proliferation (Dams *et al.*, 1995). Although it is difficult to measure, cancers that do not have MMR deficiencies generally display subtle

sequence mutation rates equal to those of normal cells. If MSI+ is unlikely to be a dominating phenotype of many cancers, how do cancers acquire the mutations that are necessary to allow them to escape the normal growth constraints present within the cell? Unlike most tumours, MSI+ tumours most often have normal chromosome karyotypes (Abdel-Rahman *et al.*, 2001; Melcher *et al.*, 2002). In contrast, microsatellite stable (MMS) tumours often have a wide variation in chromosome number and compromised karyotype integrity (Melcher *et al.*, 2002).

Genetic aberrations causing hereditary non-polyposis colorectal cancer

Germline alterations of any one of the cloned human MMR genes, *MSH2, MLH1, MSH6, PMS2* and *PMS1* have been associated with HNPCC (Fishel *et al.*, 1993). However, only approximately 50% of HNPCC patients as defined by the Amsterdam II criteria reveal any genetic lesion in the known MMR genes suggesting mutations in other genes remain to be identified. Mutations in *MSH2* or *MLH1* account for approximately 90% of identifiable genetic aberrations (reviewed in Millar *et al.*, 1999). The remaining 10% of mutations have been identified in primarily MSH6 (Plaschke *et al.*, 2004), followed by mutations in *PMS2* and, one family, with a mutation in *PMS1* (Akiyama *et al.*, 1997b; Kolodner *et al.*, 1999; Miyaki *et al.*, 1997; Nicolaides *et al.*, 1998; Nicolaides *et al.*, 1994). Liu *et al.* assessed 84 HNPCC families with no identifiable mutations in either *MSH2, MLH1* or *MSH6* and found no mutations in *PMS1* or *PMS2* (Liu *et al.*, 2001). They suggest that mutations in these genes may not segregate with an HNPCC syndrome or that mutations in either *PMS1* or *PMS2* are a rare occurrence in traditional HNPCC families. The lack of mutations identified in HNPCC families suggests that other MMR genes remain to be identified or other means of inactivating known MMR genes have not been fully assessed.

HNPCC families may present with differences in phenotypes depending on which MMR gene is mutated. Recently Plaschke *et al.* investigated HNPCC families carrying a mutation in the *MSH6* gene and found that the median age of colorectal cancer onset in these families was 10 years higher (~54 years) compared with families carrying mutations in either *MSH2* or *MLH1* (~45 years). As well, the frequency of other, non-colorectal tumours

was higher in the *MSH6* families. The later age of onset and the increased frequency of non-colorectal tumours in families carrying a mutation in the *MSH6* gene suggests that these families, although similar to *MSH2* and *MLH1* families, may present with an altered HNPCC like phenotype. This altered phenotype (ie increase in time to tumorigenesis) may explain why *MSH6* mutations have been previously found at a lower frequency and why these patients may not have been classified clinically as HNPCC patients in the past.

Constitutive inactivation of DNA mismatch repair in homozygous hereditary non-polyposis colorectal cancer patients

Generally, HNPCC patients acquire a second inactivating mutation in the mutated MMR gene leading to tissue specific tumorigenesis. However, in a few rare cases, homozygous inheritance of a mutation in a MMR gene has been reported (Bougeard *et al.*, 2003; De Vos *et al.*, 2004; Gallinger *et al.*, 2004; Menko *et al.*, 2004; Ricciardone *et al.*, 1999; Vilkki *et al.*, 2001; Wang *et al.*, 1999a; Whiteside *et al.*, 2002). Specifically, in the earliest reports, consanguineous matings in two separate families, each carrying a mutation in the *MLH1* gene, were found to produce offspring who constitutionally lacked both wild-type alleles. All four children developed lymphoma or leukemia before the age of six years (average age two years). Three of the four children developed café au lait spots with two of those four concurrently developing neurofibromas all consistent with Neurofibromatosis type 1 (NF1) despite an absence of a family history of NF1 (Ricciardone *et al.*, 1999; Wang *et al.*, 1999a). Our lab described a novel homozygous mutation in the *MSH2* gene in a 24 month old proband. This individual presented with acute lymphocytic leukemia (T-cell ALL) and numerous café au lait spots consistent with NF1 (Whiteside *et al.*, 2002). Since these reports, Gallinger *et al.* have reported similar findings in other *MLH1* homozygous deficient children, although they report a later age of onset with the presence of gastrointestinal tumours: normally very rare in children (Gallinger *et al.*, 2004). As well, Bougeard *et al.* identified a second proband with a homozygous MSH2 deficiency presenting with a glioblastoma at age 15 months (Bougeard *et al.*, 2003). Most recently, a patient with a homozygous mutation in *MSH6* has been identified presenting with brain and rectal cancer as well as multiple café au lait spots (Menko *et al.*, 2004). Collectively,

homozygous mutations in *MLH1, MSH2, PMS2* and now *MSH6* have been described in this childhood cancer syndrome presenting with primarily leukemias/lymphomas as well as café au lait spots consistent with NF1. Thus constitutive loss of MMR results in genome wide instability but focuses tumorigenesis to specific tissues, primarily hematologic malignancy of B or T-cell origin. In contrast, HNPCC patients inheriting a heterozygous mutation in one of the MMR genes rarely develop hematological cancers, although they do occur (reviewed (Hirano *et al.*, 2002) (Rosty *et al.*, 2000). Finally, HNPCC patients do not develop café au lait spots as do the homozygous deficient patients. In conclusion, it is the different mode of inheritance (ie homozygous versus heterozygous) of mutations in MMR genes that leads to the different phenotypes.

Molecular characteristics of hereditary non-polyposis colorectal cancer tumours

Over 90% of HNPCC tumours display MSI+ (Lynch & de la Chapelle, 1999). Instability at non-coding microsatellite sequences is a hallmark of HNPCC but does not contribute directly to tumorigenesis. Rather, MSI+ reflects a genome wide increase in mutation frequency that results in the subsequent increase in mutations in tumour suppressor genes (Peltomaki, 2001a). HNPCC tumours have been shown to demonstrate instability within coding repeat sequences, leading to pathogenic mutations. In recent years a number of genes containing coding repeats, targeted in the absence of MMR and selected for in subsequent tumour development, have been identified (Table 1-1). For example, the *TGFβRII* gene has been shown to be mutated in over 90% of HNPCC tumours, within a coding sequence of nine adenines. A frameshift within this coding repeat region results in a loss of functional protein (Parsons *et al.*, 1995). *TGFβRII* mutations were seen in a tissue specific manner; mutations were seen in MSI+ colon cancers but not in MSI+ gastric or endometrial cancers or in microsatellite stable (MSS) (ie microsatellite negative) tumours (Myeroff *et al.*, 1995). This tissue specificity difference suggests that TGFβRII mutations, when mutated in the colon, may confer a growth advantage and are selected for in HNPCC cancers of the colon but not in other MSI+ cancers (Myeroff *et al.*, 1995). This may help to explain the tissue specificity of the tumour spectrum in HNPCC. Grady *et al.* investigated the timing of *TGFβRII* mutations in MSI+ colon cancer onset (Grady *et al.*, 1998). No

TGFβRII mutations were detected in early MSI+ adenomas but mutations were detected in advanced adenomas and they concluded that *TGFβRII* mutations correlated with progression of colonic adenomas to cancer (Grady *et al.*, 1998). Although sporadic mutations in the *TGFβRII* gene appear to be associated with development of colorectal cancer, germline mutations in *TGFβRII* rarely lead to familial colorectal cancer (Lu *et al.*, 1998). More recently, mutational analysis of *TGFβRII* in 87 HNPCC patients who do not present with mutations in MSH2 or MLH1 identified no germline mutations in *TGFβRII*, demonstrating that inherited *TGFβRII* mutations are rare (Verma *et al.*, 2001). In conclusion, *TGFβRII* is a key gene involved in the progression of MMR deficient cancer but is rarely the source of a causative germline mutation in HNPCC.

Similar to the *TGRβRII* gene, frameshift mutations in coding mononucleotide runs of other genes with known roles in regulation of growth /apoptosis have now been identified, specifically in MMR deficient HNPCC colorectal tumours (Table 1-1). Thus, it appears that in the absence of MMR, accumulation of mutations in key tumour suppressor genes underlies tumour development.

The majority of target genes identified in HNPCC tumours have been those with mononucleotide repeat sequences with a small number of genes, such as *E2F4*, containing other repeats (Table 1-1). To test for microsatellite instability in human patients, non-coding mononucleotide repeat sequences are analyzed. For the most part, dinucleotide repeat sequences are used in the mouse when testing for microsatellite instability (Baker *et al.*, 1996; de Wind *et al.*, 1995; Reitmair *et al.*, 1995). The differential coding microsatellite sequence in human genes versus mouse genes might contribute to the different tissue specificity of tumorigenesis in human versus mouse. A comparison of human and mouse sequence in target genes shown to be mutated in human HNPCC tumours is shown (Table 1-1). In several genes the corresponding mouse sequence does not contain the same repeat as the human sequence but rather it is interrupted or truncated. Therefore, the downstream target genes that are commonly mutated in the absence of MMR may be different in the mouse. Different molecular pathways may be engaged in the murine system as opposed to

those in human patients explaining the tissue specific tumorigenesis. I undertook in depth microsatellite analysis as well as target gene analysis in MMR mouse models and this will be discussed in Chapter 2.

Table 1-1: Target Genes for frameshift mutations in the absence of MMR

Gene	Function	Human: Repeat Length	Mouse: Repeat Length	Proportion of human tumours with mutations	References
APAF1	Apoptotic protease activating factor	$(A)_9$	$(A)_8$	N/A	N/A
AXIN2	Wnt signaling	$(A)_6$, $(G)_7$, $(C)_6$,	$(A)_3G(A)_3$, $(G)_5$, $(G)_3(C)_2$	11/45 (24%)	(Liu, *et al.* 2000)
BAX	Promotes apoptosis	$(G)_8$	$(G)_2$ CT $(G)_4$	21/41 (51%)	Rampino *et al.*1997)
Caspase 5	Inflammation/apoptosis	$(A)_{10}$	no mouse ortholog	62%	(Schwartz *et al.* 1999)
CHK1	Role in G2 cell cycle checkpoint	$(A)_9$, $(A)_7$	$(A)_9$, $(A)_7$	3/17 (17.6%)	(Bertoni *et al* 1999)
E2F4	Cell cycle gene, family of transcription factors, involved in progression from G1 - S	$(AGC)_{13}$	$(AGC)_7(AGT)(AGC)_4$	35% 11/17 (65%)	(Johannsdottir *et al.* 2000) (Ikeda *et al* 1998)
IGFIIR	Tumour suppressor	$(G)_8$	$(G)_7$	3/35 (9%)	(Souza *et al* 1996)
MBD4	Methyl CpG binding protein, ability to remove Thymine or Uracil from mismatched CpG sites	$(A)_{10}$	$(A)_{10}$	10/23 (43%)	(Bader *et al* 1999)
MSH3	Mismatch repair protein	$(A)_8$	$(A)_5$ G $(A)_2$	16/41 (39%)	(Malkhosyan *et al.* 1996)
MSH6	Mismatch repair protein	$(C)_8$, $(T)_7$	$(C)_6$, $(T)_7$	12/40 (30%)	(Malkhosyan *et al.* 1996)
pTEN; Exons 7 & 8	Tumour suppressor	$(A)_6$, $(A)_6$	$(A)_5$, $(A)_6$	6/32 (19%)	(Guanti *et al.* 2000)
Riz; Exon 8	Tumour suppressor	$(A)_8$, $(A)_9$	$(A)_8$ $(A)_4$	9/24 (37.5%) 14/51 (26%)	(Chadwick *et al.* 2000)
TCF4	Transcription factor (Wnt signaling)	$(A)_9$	$(A)_9$	19/49 (39%)	(Duval *et al.*1999)
TGFβRII	Tumour suppressor	$(A)_9$	$(A)_4G(A)_5$, $(GT)_3$	7/7 (100%) 100/111 (90%)	(Markowitz *et al.*1995) (Parsons *et al.*1995)

N/A: not applicable
Adapted from (Peltomaki, 2001a)

The mechanism of DNA mismatch repair

Much of our understanding of the mechanism of mammalian MMR arose from work done in *E. coli* on the MutHLS mismatch repair pathway (see Table 1-2) (Modrich, 1991). MutS is the protein that recognizes and binds to the DNA mismatch and initiates repair (Parker & Marinus, 1992; Su & Modrich, 1986). In the presence of ATP, MutS recruits MutL and together they activate MutH (Figure 1-1). Initially, MutS undergoes a conformational change after ATP hydrolysis. Electron microscope data demonstrates that when MutS is bound to mismatched DNA it undergoes an ATP-driven translocation along the DNA, forming a loop structure. (Allen *et al.*, 1997) This process appears to be accelerated by MutL. MutL functions as a monomer in an open clamp formation (Bellacosa, 2001) and it is thought to be regulated through conformational changes that can be translated to MutH (Bellacosa, 2001). MutL forms a complex with MutS and activates MutH (Figure 1-1) (Umar *et al.*, 1996). MutH is the endonuclease that nicks the DNA at hemi-methylated d(GATC) sites. Thus in *E. coli*, the DNA mismatch repair system distinguishes between the newly synthesized daughter strand and the template strand using adenine methylation in d(GATC) sequences of the template strand. This differential methylation pattern directs MutH to nick the unmethylated strand and therefore repair is targeted to the newly synthesized daughter strand. Excision of the bases from the nick towards the mismatch requires the helicase UvrD (MutU) and a single-stranded DNA exonuclease. One of three exonucleases participates, depending on the position of the nick relative to the mismatch: 1) exonuclease I (3′-5′ excision) or 2) exonuclease VII (5′-3′ excision) or 3) *RecJ* exonuclease (5′-3′ excision) (Umar *et al.*, 1996). Re-synthesis of the gap is carried out by DNA polymerase III holoenzyme in the presence of single-strand DNA binding proteins (Lahue *et al.*, 1989). This process is reviewed in Grilley *et al.*, 1993).

Figure 1-1: The MMR system in *E. coli.*

Mismatches are recognized by dimeric MutS protein and hemimethylated sites are bound by MutH. The binding of ATP mediates formation of the MutS-MutL complex and ATP hydrolysis activation of MutH. MutH nicks the newly synthesized strand and DNA unwinding by helicase II occurs. One of the exonucleases degrades the single strand DNA and the template is protected by Ssb. Resynthesis is performed by polymerase III and the remaining nick is sealed by ligase. (adapted from Marti et al. 2002)

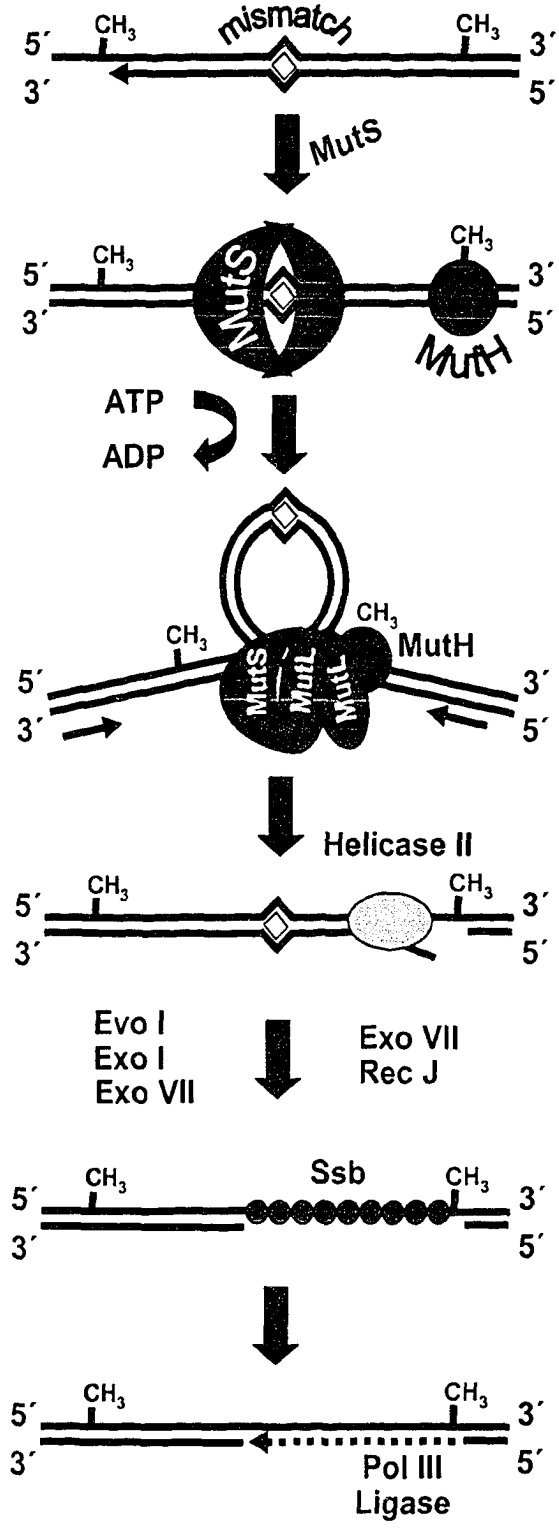

Table 1-2: MMR proteins in *E. coli* and their orthologs in eukaryotic systems

E. coli	*S. cerevisiae*	Mammalian	Function in Mammalian Systems
MutS	MSH1	?	suspected mitochondrial DNA MMR
MutS	MSH2	MSH2[a]	loop mismatch repair (with MSH3) single base mismatch repair (with MSH6)
MutS	MSH3	MSH3	loop mismatch repair (with MSH2)
MutS	MSH4	MSH4	meiosis (with MLH1)
MutS	MSH5	MSH5	meiosis (with MLH1)
MutS	MSH6	MSH6[a]	single base mismatch repair (with MSH2)
MutL	MHL1	MHL1[a]	mismatch repair
MutL	PMS1	PMS2[a]	mismatch repair (with MLH1)
MutL	MLH2	?	unknown function
MutL	MLH3	PMS1[a]	mismatch repair (with MLH1)
MutH	?	?	unknown
MutU (UvrD)	Exonuclease 1	Exonuclease 1	exonuclease 1

[a] Mutations found in cancer families

DNA mismatch repair in eukaryotes

Mammalian MMR is mediated by at least six proteins: Msh2, Msh3, Msh6, Mlh1, Pms1, Pms2, and Mlh3 (Table 1-2). The MutS homolog, MSH2, bound to either MSH6 (termed MutSα) or MSH3 (termed MutSβ), initiates the recognition of a mispair and the subsequent recruitment of additional MMR proteins to complete the repair process (reviewed in Buermeyer *et al.*, 1999). Although MutSα (MSH2/MSH6) has a preference for single base mismatches and MutSβ (MSH2/MSH3) preferentially binds to small loops, each heterodimer has some functional overlap (Buermeyer *et al.*, 1999). Insertion/deletion loops with one unpaired nucleotide are recognized by either MutSα or MutSβ whereas insertion/deletion loops of up to eight unpaired nucleotides are preferentially bound by MutSβ (Marti *et al.*, 2002). After the binding of either MutSα or MutSβ, a functional MutL heterodimer is recruited. MutLα is a functional heterodimer of MLH1 bound to PMS2, and biochemical studies in yeast (Flores-Rozas & Kolodner, 1998; Kramer *et al.*, 1989; Prolla *et al.*, 1994) and mammalian systems (Li *et al.*, 1998) have shown that MutLα is the MutL heterodimer predominately involved in mismatch correction. MutLβ (MLH1/MLH3 or MLH1/MLH2) plays a minor role in mismatch correction. The MutLβ heterodimer, MLH1/MLH3, may function in the repair of insertion/deletion loops recognized by MutSβ (Lipkin *et al.*, 2000) as well as play a role in meiotic recombination (Wang *et al.*, 1999b). Interestingly, in *S. cerevisiae* MutLα enhances affinity of MutSα for DNA mismatches and MutLβ enhances the affinity of MutSβ for loops. As well, the formation of the MutSα/MutLα complex but not the MutSβ/MutLβ complex is ATP dependent (Marti *et al.*, 2002). The binding of the MutL heterodimers are thought to facilitate the repair process through the recruitment of additional proteins necessary for excision, removal of the mispair/loop/damaged DNA and resynthesis of the DNA. These final steps however are not fully elucidated, but known steps of the repair mechanism will be discussed below.

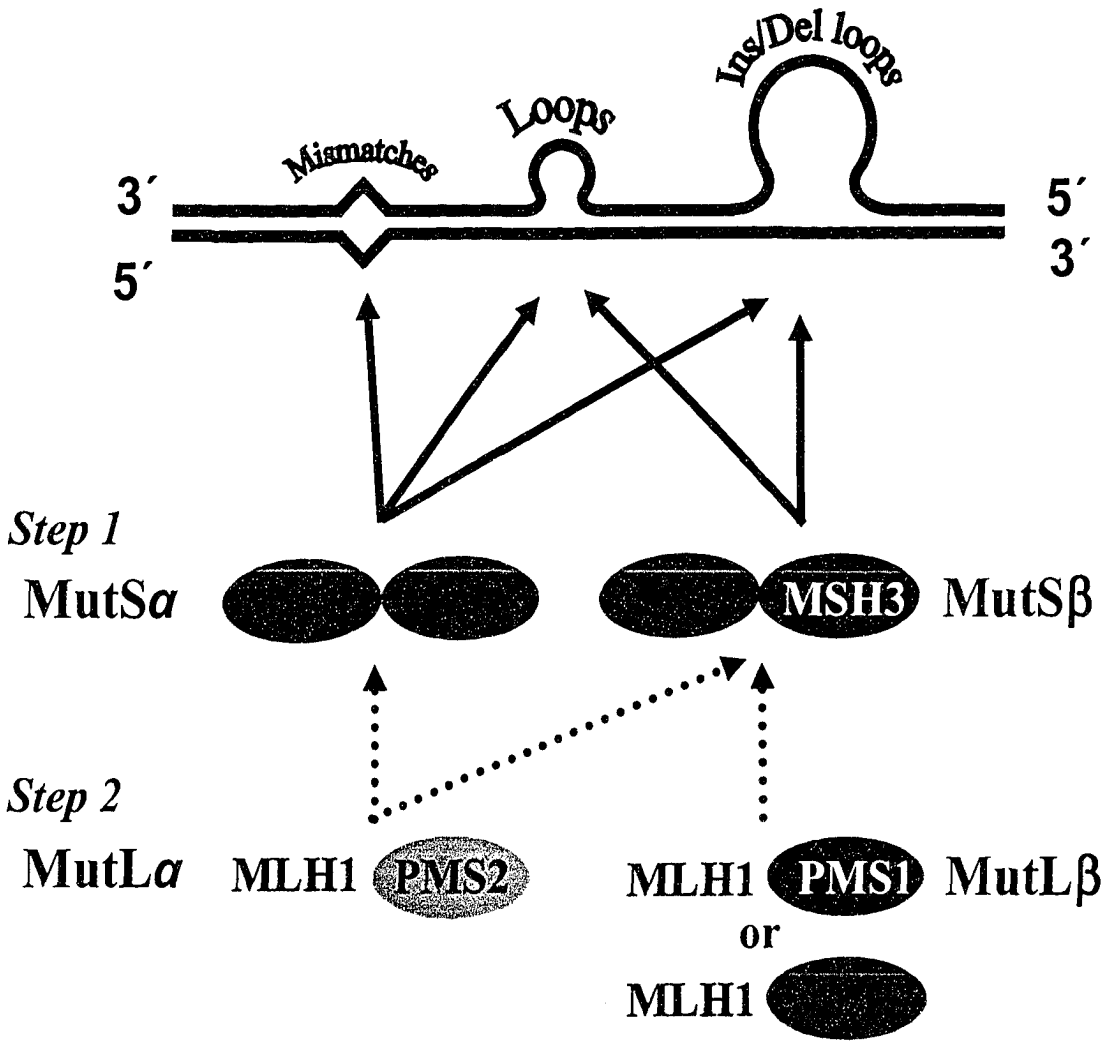

Figure 1-2: Mammalian DNA mismatch repair

Different types of mismatches (single base:base mismatches or one base pair insertion/ deletion loops or larger insertion/deletion loops) are preferentially recognized by either MutSα (MSH2:MSH6) or MutSβ (MSH2:MSH6) as indicated by the arrows. The MutS heterodimers recruit either MutLα (MLH1:PMS2) or MutLβ (MLH1:PMS1 or MLH1:MLH3) heterodimers as indicated by the dotted arrows.

Models of how MutSα transduces downstream signals in eukaryotes

The binding of MutSα to mismatched DNA simulates the intrinsic ATPase activity of the heteroduplex. *et al.* How eukaryotic MMR recognizes the small percentage of incorrectly paired or damaged DNA from the entire genome of normal DNA and triggers further downstream repair events is unknown. Several models of how MutSα transduces downstream signals include 1) the sliding clamp model 2) the ATP-dependent model and 3) the induced fit model (Bellacosa, 2001). In the sliding clamp model, MutSα (MSH2/MSH6) is hypothesized to exist in its ADP-bound state and readily recognizes mismatched DNA causing an ADP-ATP exchange and a subsequent conformational change of MutSα. The conformational changes result in reduced affinity for the mismatch, and MutSα moves freely along the DNA in an ATP independent manner to the next mismatch (Bellacosa, 2001). The second model, the ATP-dependent model, was initially based on electron microscopy data using MutS bound to heteroduplex DNA (Bellacosa, 2001). In an ATP hydrolysis-dependent manner, MutSα generates a DNA loop structure that contains the mismatch. In this model, the recognition of the mismatch by MutSα does not require ADP binding although the presence of ADP does increase mismatch binding specificity (Bellacosa, 2001). In both models 1 and 2, MutSα leaves the mismatch and travels along the DNA, allowing it to signal other downstream events. The third model is based on the crystal structures of MutS bound to DNA. The induced fit model argues that MutSα does not leave the mismatch but rather that the distortion of the DNA, as well as conformational changes of MutS on binding of DNA, allows MutSα to interact with downstream repair proteins such as MutLα without disassociating from the mismatched DNA. In this model, ATP is required for proofreading, to verify a mismatch and allow subsequent signaling events (Bellacosa, 2001).

Subsequent repair steps are less clear in eukaryotes. Currently, no homologs of MutU (UvrD) have been identified in eukaryotes. However, several candidate genes involved in excision and resynthesis of DNA have been identified. Three exonucleases that may serve

in mismatch correction have been identified; Exo1 and the proofreading exonucleases of DNA polymerase δ and DNA polymerase ε. EXO1 is a 5' – 3' exonuclease that has been shown in *Saccharomyces cerevisiae* to interact with MSH2, MLH1 and PMS1 (Schofield & Hsieh, 2003). Resynthesis of the excised strand appears to be undertaken by DNA polymerase δ (Modrich, 1997).

Strand discrimination in eukaryotes

As discussed previously, MutH in *E.coli* uses differential methylation of the daughter and template stands to target MMR to the newly synthesized strand. The lack of MutH orthologs in any eukaryotic organisms studied to date suggests that other proteins or other means substitute for MutH in eukaryotic MMR. How MMR distinguishes between the template and daughter strands in eukaryotes is not known. Orientation with respect to the replication machinery may be one possible mechanism by which MMR might distinguish template strand from daughter strand (Hsieh, 2001). MMR has been shown to be tethered to the replication machinery through its interaction with PCNA (Kleczkowska *et al.*, 2001; Umar *et al.*, 1996). Co-immunoprecipitation experiments with an anti-hMLH1 antibody co-immunoprecipitate PCNA together with hPMS2 and hMSH2, supporting the hypothesis that PCNA and MMR form a functional interaction (Gu *et al.*, 1998). As well, the localization of MMR to replication foci further links MMR to the replication machinery (Kleczkowska *et al.*, 2001).

Another mechanism by which MMR might distinguish between template and daughter strands is through the use of breaks/or nicks in DNA such as Okazaki fragments (Umar & Kunkel, 1996). *In vitro* experiments have shown that mammalian MMR can be directed to the DNA strand containing nicks (Holmes *et al.*, 1990; Thomas *et al.*, 1991). Indeed, nicked DNA can substitute for MutH in *E.coli* and serves to direct repair to the correct site in the DNA (Au *et al.*, 1992). Such experiments have led researchers to suggest that the MutS homologs, MutSα and MutSβ, recognize the mispaired DNA, and structural irregularities in the DNA such as Okazaki fragments direct repair to the daughter strand(Pavlov *et al.*, 2003; Wang & Hays, 2004). The difficulty with this hypothesis lies in

fact that DNA synthesis at the leading strand is continuous, with no irregularities and the hypothesis fails to suggest how repair is accurately directed to the correct base pairs on the leading strand. However, simple breaks in the DNA, such as would be present on the lagging strand, might be all that is needed to direct repair to the correct strand.

Multifunctionality of DNA mismatch repair

The main function of MMR appears to be the maintenance of DNA fidelity through the correction of base pairs and insertion and deletion loops that have been misincorporated during DNA replication. Without MMR, cells acquire a mutator phenotype in which the rate of spontaneous mutation is greatly elevated (Parsons *et al.*, 1995). This can be observed readily by microsatellite instability at mono and di-nucleotide repeats in MMR deficient cells. Mutations of such sequences found within coding repeats of key genes are associated with tumorigenesis as previously discussed. However, in addition to this, MMR is involved in several other cellular processes, that also contribute to tumorigenesis.

DNA mismatch repair and bulky adduct removal

In addition to the correction of misincorporated base pairs, MMR proteins are now known to play a role in the recognition and removal of endogenous or exogenously induced DNA lesions. Damage such as bulky adducts, oxidative damage, UV irradiation and alkylation damage have all been shown to result in lesions that are recognized by MMR (reviewed in Harfe & Jinks-Robertson, 2000). Some of the lesions induced by these damaging agents may not be recognized themselves but rather result in replicative mismatches that are recognized by MutSα (Hsieh, 2001).

SN1 alkylating agents such as N-methyl-N'-nitroso-N-nitrosoguanidine (MNNG) are known to result in O^6 methylguanine lesions in DNA. An absence of MMR confers high levels of resistance to such alkylating agents (up to 100 fold) in eukaryotes (Aquilina & Bignami, 2001). The absence of MMR allows for the accumulation of a larger number of DNA mutations prior to the signaling of cell death, and restoration of MMR function restores sensitivity to the alkylating agent (Aquilina & Bignami, 2001). Although the

absence of MMR is known to result in alkylation resistance, how this occurs is still in question. One model is the 'futile repair' model in which the O^6 methylguanine adduct on the template strand results in a mispair (most commonly to a T) and the induction of post-replicative MMR. The removal of the mispaired T on the daughter strand, rather than the removal of the O^6 methylguanine on the template strand allows for the re-insertion of the mispaired T by the DNA polymerase that reinitiates mispair recognition and removal of the mispaired T by MMR, leading to 'futile' cycles of repair with excision and resynthesis that result in the persistence of the lesion.

There is experimental evidence supporting the hypothesis that MMR is involved in the signaling of apoptosis in response to damaging agents such as alkylating agents (Hickman & Samson, 1999). Chromosomal aberrations and sister chromatid exchanges induced by alkylating agents have also been shown to be dependent on functional MMR (Galloway *et al.*, 1995). As well, MSH6 defective human cells perform less N-methyl-N'-nitroso-N-nitrosoguanidine (MNNG) induced homologous recombination (Zhang *et al.*, 2000). Thus, MMR appears to play many roles in response to DNA damage by SN1 type methylating agents.

DNA mismatch repair and apoptosis

Studies indicate that in addition to the correction of mispaired bases generated during DNA replication, MMR functions to mediate DNA damage-induced apoptosis as part of a cellular response to endogenous and exogenous agents (reviewed in Buermeyer *et al.*, 1999; Kolodner, 1996; Modrich, 1997). These studies have shown that cell lines deficient in MMR (human and mouse) are resistant to killing using genotoxic agents such as cisplatin, MNNG and oxidizing agents such as ionizing radiation (Aebi *et al.*, 1996; Aquilina & Bignami, 2001; Fritzell *et al.*, 1997). This decrease in apoptotic response was accompanied by an increase in the mutation rate in their genomes, showing that an absence of MMR allowed for the accumulation of more DNA lesions (Toft *et al.*, 1999). Using the *Dlb-1* locus as an *in vivo* mutation marker, Toft *et al.* showed that in *Msh2*$^{-/-}$ mice, normal intestinal epithelium had elevated levels of mutation rates endogenously as well as in

response to the genotoxin temozolomide (Toft *et al.*, 1999). A deficiency in MMR reduced the levels of apoptosis allowing for the accumulation of genetic mutations. Lin *et al.* generated $Msh2^{G674A}$ mice that carry a mutation in the *Msh2* gene causing a mutated protein to be produced; resulting in a strong cancer predisposition but allowing cells to respond normally to DNA damage, therefore maintaining a normal apoptotic response to DNA damaging agents (Lin *et al.*, 2004). Similarly, Yang *et al.* describe a dominant negative *Msh6* mutation in yeast and mouse cells that abolishes functional MMR while having no effect on the apoptotic response to DNA damaging agents (Yang *et al.*, 2004). These recent results show that in MMR, the functions of mispair correction and signaling of apoptosis can be disassociated from one another, demonstrating that the apoptotic response is a separate and distinct function.

Mismatch repair and cell cycle arrest

In response to DNA damage such as alkylating agents, MMR proficient cells trigger cell cycle arrest at the G2 to M transition where as MMR deficient cells fail to induce such arrest (Adamson *et al.*, 2005; Hawn *et al.*, 1995). Several recent studies have shown that this MMR-dependent G2 arrest involves interaction of MSH2 with ataxia telangiectasia related protein (ATR) kinase after alkylation damage (Stojic *et al.*, 2004; Wang & Qin, 2003). MMR mediated G2 to M arrest induced by 6-thioguanine appears to require the proteins CHK1, CHK2 and ATR (Yamane *et al.*, 2004).

MMR is also implicated in triggering cell cycle arrest in response to other DNA damaging agents such as UV light (Lutzen *et al.*, 2004; van Oosten m, 2005). In response to ionizing irradiation, Davis *et al.* reported decreased survival as well as decreased G2-M cell cycle checks in MLH1 deficient human colon carcinoma (HCT116) cells (Davis *et al.*, 1998).

Fluorinated thymidines (eg. 5-fluorouracil and 5-fluoro-2'deoxyuridine), are widely used in the treatment of colon cancer (Meyers *et al.*, 2001). They are deoxyribonucleoside derivatives whose metabolites cause DNA cytotoxicity. In survival assays using 5-fluorouracil and 5-fluoro-2'deoxyuridine, MMR deficient cells are more resistant to killing

than MMR proficient cells (Meyers *et al.*, 2001). It is hypothesized that MMR deficient cells are 'tolerant' to more DNA damage due to the lack of MMR and its ability to 'signal' downstream events such as apoptosis and cell cycle check points. After treatment, MMR deficient cells had a 2 fold decrease in G2-M cell cycle arrest suggesting that MMR is involved in G2-M cell cycle checking in response to fluorinated thymidine (Meyers *et al.*, 2001).

Mismatch repair and meiosis

Yeast mutants defective in Mlh1 and Mlh3 and mice deficient in the MutL homologues, Mlh1, Mlh3 or Pms2, develop normally but exhibit a meiotic defect (Baker *et al.*, 1995; Edelmann *et al.*, 1996; Lipkin *et al.*, 2002; Wang *et al.*, 1999b). *Mlh1*$^{-/-}$and *Mlh3*$^{-/-}$ mice are sterile, with male *Mlh1*$^{-/-}$ sterility resulting from the failure of spermatocytes to progress beyond prophase I. Mlh1 requires Mlh3 for binding to meiotic chromosomes and therefore the cause of sterility in *Mlh3*$^{-/-}$ mice may be similar to that of the *Mlh1*$^{-/-}$ mice (Lipkin *et al.*, 2002). *Pms2*$^{-/-}$ male mice show abnormal chromosome pairing during meiosis and are sterile, where as females are fertile (Baker *et al.*, 1995).Yeast strains defective in the MutS homologues Msh4 and Msh5 show reduced rates of crossing over, increased chromosomal nondisjunction and reduced spore viability (Kneitz *et al.*, 2000). Both *Msh4*$^{-/-}$ and *Msh5*$^{-/-}$ mice are sterile and chromosome pairing during prophase I is thought to be the cause in both cases (de Vries *et al.*, 1999; Edelmann *et al.*, 1999a; Kneitz *et al.*, 2000). MMR proteins are essential for the proper functioning of meiosis and therefore maintaining the integrity of the DNA.

Double strand break repair

Double strand breaks in DNA can be formed by oxygen free radicals, DNA replication, ionizing radiation and other assaults on the DNA. In mammalian cells there are two mechanisms by which the cell attempts to repair a double strand break: 1) Nonhomologous end-joining and 2) Homologous recombination repair (reviewed in Pierce *et al.*, 2001). During nonhomologous end-joining, proteins recognize and bind to the exposed ends of the DNA at the site of the double strand breaks and function to bring the ends together so that a DNA ligase can fuse them together. To fill in the breaks in the DNA, the addition and

deletion of nucleotides is often required causing nonhomologous end-joining to be error prone. In contrast, homologous recombination repair is a precise repair mechanism as it uses the homologous DNA strand as a template to repair the double strand breaks. Homologous recombination repair can use sister chromatids, a homologous chromosome or the same chromosome as a template to repair the break. During repair, a gene conversion tract around the double strand break is formed where there is unidirectional transfer of sequence information from the template DNA strand to the DNA molecule containing the double strand break (Elliott & Jasin, 2001). In using these templates, varying degrees of sequence heterology may arise during the repair process. MMR is known to have an antirecombination role in bacteria and yeast (Evans & Alani, 2000). For example, recombination is decreased by approximately sixfold between sequences that are 1.2 % divergent (Elliott & Jasin, 2001). Other evidence has shown that in addition to the repair of base mispairs, MMR may play a role in the repair of double strand breaks through its role in homologous recombination repair. In *S.cerevisiae* MMR proteins interact with recombination proteins to regulate heteroduplex length (Alani *et al.*, 1994) and to suppress homologous recombination (Selva *et al.*, 1995). More recently, Elliot and Jasin used mouse embryonic stem (ES) cells to show that, similar to yeast, the frequency of recombination between divergent sequences in a gene targeting assay was reduced as the sequence divergence increased (Elliott & Jasin, 2001). However, in *Msh2* deficient ES cells the barrier to recombination was significantly reduced (a 15.3 fold difference) compared with wild type cells. *Msh2* deficient ES cells showed a tendency toward longer gene conversion tracts than wild type cells. Further evidence showing mismatch repair protein involvement in homologous recombination repair comes from work in *S. cerevisiae*. Marsischky *et al.* have shown that the MutSα heterodimer binds to holliday junctions with an affinity that is as high, if not higher, than its affinity for mispaired bases (Marsischky *et al.*, 1999). The involvement of MMR in homologous recombination repair means that a loss of functional MMR will have consequences beyond the conventional presentation of microsatellite instability. Incorrectly repaired double strand breaks can lead to chromosomal aberrations such as deletions, translocations and rearrangements as well as downstream events such as chromosomal aneuploidy. MMR functions as an inhibitor of homologous recombination

repair ensuring its accuracy and without MMR, homologous recombination repair will generate more errors leading to more mutations and possibly chromosomal abnormalities.

The above examples show the broad roles for the MMR proteins. Such multifunctionality suggests tumorigenesis can arise in the absence of MMR due to cumulative effects. For example, in the absence of accurate cell cycle check points, cells may not have time to completely assess cell viability factors such as DNA damage and signal the correct cellular response such as apoptosis or DNA repair. A reduction in cellular apoptosis would allow for cells to survive longer while accumulating more DNA damage. Damage that is left unrepaired may result in mutations in key genes necessary for the maintenance of cellular viability and may result in compromised cellular integrity leading to tumorigenesis.

DNA mismatch repair and interacting proteins

Although the mechanism of mammalian MMR is becoming clearer from evidence gathered in *E. coli*, *S. cerevisiae*, and mammalian studies, our understanding of the interactions of MMR with other nuclear proteins is just beginning. Data suggesting functional overlap between MMR and other DNA repair pathways, as well as cell cycle, apoptosis etc. is supported by evidence of physical interaction as well. For example, experiments show that MSH2 binds to the cell cycle protein CHK2 and that MLH1 associates with the DNA repair protein ataxia telangiectasia (AT) (Brown *et al.*, 2003). Also, human EXO1 interacts with MSH2, MSH3 and MLH1 suggesting that EXO1 may function as the endonuclease in mammalian MMR required for repair (Tishkoff *et al.*, 1998). As mentioned previously, human PCNA can be immunoprecipitated with MSH2, MLH1 and PMS2 suggesting that MMR is linked to the replication machinery through this interaction. In addition, the MSH2/MSH6 MutSα complex as well as MLH1 have been shown to be part of the larger BASC complex (BRCA1-associated genome surveillance complex) (Wang *et al.*, 2000). The BASC complex is a large 2 GDa protein complex of tumour suppressors, DNA damage repair proteins and signal transducing proteins. Given the roles that these proteins play in DNA repair and DNA damage surveillance, they may work in concert to monitor

the DNA and signal the appropriate responses i.e. cell cycle checks, apoptosis or DNA repair.

Mouse models of DNA mismatch repair

To study mammalian MMR, mice homozygous deficient for each of the MMR genes have been generated using gene targeting technology (Table 1-3) (Baker *et al.*, 1995; de Wind *et al.*, 1999; Edelmann *et al.*, 1996; Edelmann *et al.*, 1999a; Edelmann *et al.*, 2000; Edelmann *et al.*, 1997; Kneitz *et al.*, 2000; Narayanan *et al.*, 1997; Prolla *et al.*, 1998; Reitmair *et al.*, 1995). In contrast to the human HNPCC patients, mice heterozygous for a mutation in any one of the MMR genes develop normally and are not prone to tumorigenesis (reviewed in Wei *et al.*, 2002). However, mice homozygous deficient for any one of the MMR genes most commonly develop early onset thymic lymphomas, although other tumours such as ovarian and small intestinal tumours occur with a lower frequency (Table 1-3).

Msh2$^{-/-}$ and Msh6$^{-/-}$ mouse models

Msh2 is the protein responsible for the initial recognition of a mispair and is an essential component of the MutSα and MutSβ heterodimers that are required for subsequent recruitment of additional proteins necessary for functional MMR. Reitmair *et al.* and de Wind *et al.* generated mice lacking functional Msh2, hence generating mice nonfunctional for MMR (de Wind *et al.*, 1995; Reitmair *et al.*, 1995). *Msh2*$^{+/-}$ mice were normal with no increase in cancer and a normal life span comparable to *Msh2*$^{+/+}$ mice. *Msh2*$^{-/-}$ mice were viable, but rapidly developed thymic lymphoid tumours of T-cell origin starting at two months of age with a median time to tumorigenesis of 5.5 months. *Msh2*$^{-/-}$ mice rarely develop intestinal tumours, and no colorectal cancers have been observed (de Wind *et al.*, 1995; Lowsky *et al.*, 1997; Reitmair *et al.*, 1995).

Table 1-3: MutS homologs: Characteristics of mouse models of MMR

Genotype	*Msh2*$^{-/-}$	*Msh3*$^{-/-}$	*Msh4*$^{-/-}$	*Msh5*$^{-/-}$	*Msh6*$^{-/-}$
Protein Function	mismatch/loop recognition	loop repair as heterodimer with MSH2	meiosis	meiosis	base pair repair as heterodimer with MSH2
Authors	Reitmar *et al.* 1995 Reitmar *et al.* 1996 de Wind *et al.* 1995	de Wind *et al.* 1999 Edelmann *et al.* 2000	Kneitz *et al.* 2000	Endelman *et al.* 1999	Edelmann *et al.* 1997
Tumour Spectrum of Homozygotes	T-cell lymphomas colon cancers skin cancers uterine cancers 5.5 month median survival	no increase in tumour incidence	no increase in tumour incidence	no increase in tumour incidence	B-cell lymphomas T-cell lymphomas GI tract cancers 11 month median survival
MSI	high levels of MSI+ in tumours non-tumour tissue was stable	moderate MSI in old Msh3$^{-/-}$ tumours	not investigated	not investigated	no MSI+ in tumour tissue
Fertility of Males	fertile	fertile	sterile b/c of meiotic failure	sterile b/c of defect in chromosome pairing	fertile
Fertility of Females	fertile	fertile	sterile b/c of meiotic failure	sterile b/c of defect in chromosome pairing	fertile

Table 1-4: MutL homologs: Characteristics of mouse models of MMR

Genotype	*Mlh1*$^{-/-}$	*Pms1*$^{-/-}$	*Pms2*$^{-/-}$	*Mlh3*$^{-/-}$
Protein Function	forms heterodimer with PMS2, function unclear	forms heterodimer with MLH1, function unclear	forms heterodimer with MLH1, function unclear	forms heterodimer with MLH1, function unclear
Authors	Baker *et al.* 1995 Edelmann *et a*l. 1996 Prolla *et al.* 1998	Prolla *et al.* 1998	Baker *et al.* 1995 Prolla *et al.* 1998	(Lipkin *et al.*, 2002)
Tumour Spectrum of Homozygotes	T and B-cell lymphomas small intestine adenomas and colon cancers	no increase in tumour incidence	lymphomas cervical sarcomas not prone to intestinal tumorigenesis	no increase in tumour incidence
MSI	high levels of MSI+ in tumours	MEFs showed no MSI sperm showed some mononucleotide instability	thymic lymphomas showed MSI+ compared with normal tissue male germline shows MSI+	not investigated
Fertility of Males	sterile	fertile	sterile b/c defect in chromosome pairing	sterile, Mlh3 is required for Mlh1 binding to chromosomes
Fertility of Females	sterile	fertile	fertile	sterile

Msh6 is an essential component of the MutSα heterodimer. In the absence of Msh6, MutSα can not be formed and therefore the processing of mispaired nucleotides is absent (Edelmann *et al.*, 1997). However, due to the functional redundancy of MutSα and MutSβ. MutSβ can compensate to a degree for the loss of functional MutSα. Edelmann *et al.* used gene targeting technology to generate mice lacking functional Msh6, hence lacking functional MutSα but retaining functional MutSβ (Edelmann *et al.*, 1997). Similar to *Msh2*$^{+/-}$ mice, *Msh6*$^{+/-}$ mice were normal with no increase in cancer compared with *Msh6*$^{+/+}$ mice. Similar to *Msh2*$^{-/-}$ mice, *Msh6*$^{-/-}$ mice develop B and T-cell thymic lymphomas. However, *Msh6*$^{-/-}$ mice have an increased latency to tumour development with a median survival of 10 months compared with the *Msh2*$^{-/-}$ mice whose median survival is only 5.5 months. The increased latency to tumour development in the *Msh6*$^{-/-}$ mice suggests that, in contrast to the *Msh2*$^{-/-}$ mice, they do retain some functional MMR and are able to post-pone tumour development, although not indefinitely. This suggests that MutSβ is able to functionally compensate for the lack of MutSα. Edelmann *et al.* show that *Msh6-/-* cells are defective for the repair of single nucleotide mismatches while retaining the ability to repair insertion/deletion loops of 1, 2 and 4 base pairs (Edelmann *et al.*, 1997).

Cells lacking functional Msh3 cannot form functional MutSβ but retain the ability to form functional MutSα (de Wind *et al.*, 1999). Mice deficient in *Msh3-/-* are not predisposed to tumorigenesis (de Wind *et al.*, 1999). This suggests that MutSα is able to fully compensate for the loss of MutSβ but that MutSβ is not able to fully compensate for the loss of MutSα suggesting that MutSα does have some capacity to repair small insertion/deletion loops. In fact, MutSα has been shown to repair 1 base pair insertion deletion loops and most likely can repair larger loops in the absence of MutSβ (reviewed in Aquilina & Bignami, 2001). When *Msh3*$^{-/-}$ mice were bred to *Msh6*$^{-/-}$ mice the lack of Msh3 did not accelerate the time to tumorigenesis in *Msh6*$^{-/-}$;*Msh3*$^{-/-}$ animals but did change the spectrum of tumours. A larger percentage of mice developed intestinal tumours in *Msh6*$^{-/-}$;*Msh3*$^{-/-}$ than did in *Msh6*$^{-/-}$ mice (de Wind *et al.*, 1999). de Wind *et al.* contrast this to the intestinal tumour incidence of the *Msh2*$^{-/-}$ mice (de Wind *et al.*, 1998) and suggest that they have moved the spectrum of disease towards that of the *Msh2*$^{-/-}$ mice. The absence of both MutSα and MutSβ may be

necessary to foster tumour development in the gastrointestinal tract of the mouse. However, one must be careful when interpreting the intestinal tumour incidence in the MMR deficient mice, as some colonies see little to no intestinal tumour incidence (Lowsky *et al.*, 1997; Reitmair *et al.*, 1995).

Histologically, *Msh6*$^{-/-}$ lymphomas are very similar to the *Msh2*$^{-/-}$ lymphomas, presenting with a 'starry sky' appearance and numerous mitotic figures. Immunophenotyping of eight *Msh6*$^{-/-}$ thymic lymphomas revealed that 5/8 where B-cell in origin and only 3/8 where T-cell in origin (Edelmann *et al.*, 1997). This is in contrast to the 23 thymic lymphomas that have been immunophenotyped in *Msh2*$^{-/-}$ mice. Of the *Msh2*$^{-/-}$ thymic lymphomas that have been immunophenotyped, 22/23 were T-cell in origin and only 1/23 was B-cell in origin (Lowsky *et al.*, 1997; Reitmair *et al.*, 1995). This may be indicative of a bias for *Msh2*$^{-/-}$ mice to develop T-cell thymic lymphomas and for *Msh6*$^{-/-}$ mice to develop B-cell thymic lymphomas. More immunophenotyping of *Msh6*$^{-/-}$ lymphomas would be needed to confirm this.

Edelmann *et al.* hypothesized that because extracts from *Msh6*$^{-/-}$ cells repaired insertion/deletion mispairs, Msh6 deficient tumour cells would not show high levels of MSI+. They tested 12 different *Msh6*$^{-/-}$ tumours from six animals for microsatellite status using eight sets of microsatellite markers and concluded that *Msh6*$^{-/-}$ tumours showed little to no MSI+ (Edelmann *et al.*, 1997). This is in contrast to the *Msh2*$^{-/-}$ mice who show high levels of MSI+ in both tumour and non-tumour tissues.

DNA mismatch repair and inter-mouse modeling

To further study the role of MMR in the maintenance of genomic stability and tumorigenesis, many of the MMR mice have been bred to other mouse models (Table 1-4 and 1-5). In particular, *Msh2*$^{-/-}$ and *Msh6*$^{-/-}$ mice have been bred to other colon cancer mouse models as well as tumour suppressor models. Human patients heterozygous for a mutation in the *Apc* gene (adenomatous polyposis of the colon) develop familial adenomatous polyposis (FAP), an autosomal dominant disorder which typically presents

with extensive adenomatous polyps of the colon leading to colorectal cancer early in adult life. Mouse models heterozygous for a mutation in the *Apc* gene, also known as *Min* (multiple intestinal neoplasia) gene, develop intestinal tumours in a manner similar to human patients. In *Msh2*$^{+/-}$; *Apc*$^{+/Min}$ mice, loss of an Msh2 allele increased tumour multiplicity per animal and reduced survival (Table 1-5) (Reitmair *et al.*, 1996a). In *Msh6*$^{-/-}$; *Apc*$^{1638N/+}$ mice, Msh6 deficiency caused a 6-7 fold increase in tumour multiplicity per animal (Table 1-4) (Kuraguchi *et al.*, 2001). GI tumours that developed in *Msh6*$^{-/-}$ mice were found to express little to no Apc protein (Edelmann *et al.*, 1997). This is suggestive that mutations in the Apc gene may be causal in the development of the GI tumours seen in the *Msh6*$^{-/-}$ mice. However, only 2/30 (6.67%) of the intestinal tumours in *Msh2*$^{-/-}$;*Apc*$^{+/Min}$ mice showed LOH at the *Apc* locus (de Wind *et al.*, 1998), where as intestinal tumours arising in *Msh2*$^{+/+}$;*Apc*$^{+/Min}$ mice always demonstrated loss of heterozygosity (LOH) at the *Apc* locus. With such conflicting reports, further investigation is needed to determine the contribution of the *Apc* locus to MMR deficient tumorigenesis.

Msh2$^{-/-}$ mice and *Msh6*$^{-/-}$ mice have been bred to other tumour suppressor mouse models. Double knockout *Msh2*$^{-/-}$;*p53*$^{-/-}$ mice have been generated by two different groups (Table 1-5) (Cranston *et al.*, 1997; Toft *et al.*, 1998). Cranston *et al.* found that female mice deficient for both Msh2 and p53 were embryonic lethal. Males were viable but succumbed to tumours earlier than either single knockout model. In contrast, Toft *et al.* produced female double knockout *Msh2*$^{-/-}$;*p53*$^{-/-}$ mice with a similar male:female ratio as those seen in *p53*$^{-/-}$ mice demonstrating that Msh2 deficiency did not further alter the male:female ratio. Both studies reported that the double knockout mice developed predominately lymphomas with a decreased latency to tumour development compared with either gene knockout alone.

The above studies demonstrate that tumour spectrum and survival can be altered by breeding MMR deficient mice to other murine models. I hypothesized that MMR deficient mice succumb to thymic lymphomagenesis at too young an age to allow for the development of gastrointestinal tumours similar to human HNPCC patients. If mice are prevented from or delayed in developing lymphomas, they may develop GI cancer and

therefore present as a model with which to study MMR deficient GI tumour development. To test this hypothesis, I bred Msh2 and Msh6 deficient mice, to athymic nude mice to observe if a lack of a thymus and reduction of the number of T-cells per animal would prevent thymic lymphomagenesis and may therefore result in an altered spontaneous tumour spectrum. This is further investigated in Chapter 6.

Table 1-5: *Msh2*$^{-/-}$ mice bred to other mouse models

Msh2	Resultant Phenotype
Msh2$^{-/-}$; *DM1*$^{knock\text{-}in}$ (Savouret *et al.* 2004) (Savouret *et al.* 2003)	Msh2 deficiency shifts the CTG instability from expansions to contractions
Msh2$^{-/-}$; *Hdh*$^{Q111Knock\text{-}in}$ (Wheeler *et al.* 2003)	Shows Msh2 as a factor that strongly modifies the timing of onset of HD
Msh2$^{-/-}$; *Mbd4*$^{-/-}$ (Sansom *et al* 2004)	Mbd4 loss does not alter tumour spectrum or MSI status of *Msh2*$^{-/-}$ mice
Msh2$^{-/-}$; *p53*$^{-/-}$ (Toft *et al* 1998) (Cranston *et al* 1997)	{Cranston *et al.*1997): embryonic lethal in females males succumb to tumours earlier than either gene alone (Toft *et al.* 1998) both males and females susceptible to lymphomagenesis
Msh2$^{-/-}$; *Tap1*$^{-/-}$	No lymphoma development; Tap deficiency caused *Msh2*$^{-/-}$ mice to develop HNPCC-like tumours
Msh2$^{-/-}$; *Xpa*$^{-/-}$ (Yoshino *et al.*, 2002)	Xpa deficiency had no additive effect on spontaneous skin tumorigenesis in *Msh2*$^{-/-}$ mice
Msh2$^{-/-}$; *Xpc*$^{-/-}$ (Meira *et al.*, 2001)	Addition of Msh2 deficiency decreased time to skin tumour incidence
Msh2$^{-/-}$;*Dnmt1* (Trinh *et al.*, 2002)	Increase in lymphomagenesis and a decrease in intestinal tumours compared with *Msh2*$^{-/-}$ mice
Msh2$^{-/-}$ *Mth1*$^{-/-}$ (Tsuzuki *et al.*, 2001)	↑ in G:C → T:A transversions over *Msh2*$^{-/-}$ mice alone
Msh2$^{-/-}$;*Rb*$^{+/-}$ (Nikitin *et al.*, 2002)	Deceleration in the development of lymphomas compared with *Msh2*$^{-/-}$ mice and a longer life span no change in neuroendocrine neoplasia compared with *Rb*$^{+/-}$ mice
Msh$^{+/-}$; *Apc*$^{+/Min}$ (Reitmair *et al.*, 1996a) (de Wind *et al.*, 1998)	Msh2 deficiency increased tumour multiplicity and reduced survival

Table 1-6: *Msh3*$^{-/-}$ and *Msh6*$^{-/-}$ mouse models bred to other mouse models

Msh3	Resultant Phenotype
Msh3$^{-/-}$; *Msh6*$^{-/-}$ (Edelmann *et al.*, 2000) (de Wind *et al.*, 1999)	Tumour phenotype is the same as *Msh2*$^{-/-}$ or *Mlh1*$^{-/-}$ mice
Msh3$^{-/-}$;*Apc*$^{1638N/}$ (Kuraguchi *et al.*, 2001)	No difference in tumour multiplicity with Msh3 deficiency
Msh3$^{-/-}$; *DM1* $^{knock\text{-}in}$ (van den Broek *et al.*, 2002)	Msh3 deficiency blocked the DM1 knock-in gene repeat instability in somatic tissues

Msh6	Resultant Phenotype
Msh3$^{-/-}$;*Msh6*$^{-/-}$ *Apc*$^{1638N/}$ (Kuraguchi *et al.*, 2001)	Survival is further reduced compared with *Msh6*$^{-/-}$; *Apc*$^{1638N/+}$ mice
Msh6$^{-/-}$; *Apc*$^{1638N/+}$ (Kuraguchi *et al.*, 2001)	6-7 fold increase in tumour multiplicity with Msh6 deficiency
Msh6$^{-/-}$; *DM1* $^{knock\text{-}in}$ (van den Broek *et al.*, 2002)	Msh6 deficiency resulted in an increase in instability in the DM1 knock-in gene in somatic tissues

Table 1-7: MMR mouse models bred to other mouse models

Mlh1	Resultant Phenotype
Mlh1$^{-/-}$;*Pms2*$^{-/-}$ (Yao *et al.*, 1999)	Similar mutator phenotype to *Mlh1*$^{-/-}$ mice
Mlh1$^{-/-}$; *Apc*$^{1638N/+}$ (Kuraguchi *et al.*, 2000) {Edelmann, 1999 #908	GI tumour incidence increased 40-100 fold compared with *Mlh1*$^{-/-}$ mice
Mlh1$^{-/-}$; *Mgmt*$^{-/-}$ (Kawate *et al.*, 1998)	Deleting Mlh1 causes mice to become resistant to MNU
Mlh1$^{-/-}$; *Mbd4*$^{-/-}$ (Sansom *et al.*, 2004)	Mbd4 loss does not alter tumour spectrum or MSI status
Mlh1$^{-/-}$;*Nf1*^{s} (Gutmann *et al.*, 2003)	Mice die significantly earlier than either alone due to myeloid leukemia

Pms2	Resultant Phenotype
Apc$^{+/Min}$;*Pms2*$^{-/-}$ (Baker *et al.*, 1998)	Develop 3-4 times the number of GI tumours than Apc/Min mice alone

Hypothesis and Summary of Studies

Such wide prevalence of instability in all types of cancers supports the idea that an early mutational event causing genetic instability may be necessary for the progression of tumorigenesis. Genetic instability drives the progression as well as accounts for the heterogeneity of tumours. MMR is one system whereby its inactivation leads to genome instability. Individuals with germline mutations in the MMR genes are predisposed to HNPCC and develop a broad range of tumours including colorectal and endometrial cancer in the absence of MMR. To further understand the relationship between MMR, genomic instability and tumorigenesis I have used mouse models deficient in the MMR proteins Msh2 and Msh6. MMR null mice are predisposed to hematological malignancy – primarily B and T-cell lymphomas.

To help explain the tissue specificity of the murine thymic lymphomas arising in the absence of MMR, I used several techniques to characterize the genomic instability arising in the tumours. I screened genes known to be downstream targets in human HNPCC tumours, for mutations in *Msh2*$^{-/-}$ thymic lymphomas in an attempt to identify downstream target genes that may have become mutated in mouse model and contributed towards lymphoma development. I analyzed the degree of microsatellite instability (MSI+) in the *Msh2*$^{-/-}$ thymic lymphomas, comparing MSI+ seen in tumours with MSI+ seen in normal embryonic stem cells . As well, using spectral karyotyped analysis, I assayed for the presence of chromosomal instability in *Msh2*$^{-/-}$ thymic lymphomas. Using MMR proficient and deficient mouse cells, I investigated the effect a loss of MMR has on telomere fucntion. I studied centrosomal protein alterations in the absence of MMR and analyzed cellular aneuploidy in mouse embryonic fibroblasts. I bred *Msh2*$^{-/-}$ and *Msh6*$^{-/-}$ mice to athymic nude mice to determine if tumours would arise in other tissues in the absence of a thymus and greatly reduced T-cell component. In conclusion, I have furthered our understanding of how tumorigenesis arises in a background lacking MMR and have developed a new animal model that can be used for future studies to elucidate possible role for MMR in T and B cell development.

Chapter 2 ♦ Molecular characterization of *Msh2*$^{-/-}$ deficient murine thymic lymphomas *

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* *Parts of this chapter have been published or are being prepared for publication*
Marcia R. Campbell, Patrick N. Nation, Susan E. Andrew. (2005)

Leah C. Young, Kyle Thulien, Marcia R. Campbell, Victor A. Tron and Susan E. Andrew. (2004) DNA mismatch repair proteins promote apoptosis and suppress tumorigenesis in response to UVB irradiation: an in vivo study. Carcinogenesis vol.25 no.10 pp.1821--1827

Fernando Benavides, Monica Zamisch, Monica Flores, Marcia R. Campbell, Susan E. Andrew, Joe M. Angel, Julien Licchesi, Gabriel Sternik, Ellen R. Richie, and Claudio J. Conti (2002) Application of Inter–Simple Sequence Repeat PCR to Mouse Models: Assessment of Genetic Alterations in Carcinogenesis. Genes, Chromosomes & Cancer 35:299–310

All of the experiments in this chapter were performed by Marcia Campbell with the exception of the histological identification which was performed by Dr. Nation and the inter-simple sequence-PCR gels which were run by Dr. Benavides.

Introduction

Several strains of mice homozygous deficient for the MMR protein Msh2 have been developed (de Wind *et al.*, 1995; Edelmann *et al.*, 1997; Reitmair *et al.*, 1995; Smits *et al.*, 2000). When I started my Ph.D. in 1998 two Msh2 knockout mouse models had been generated (de Wind *et al.*, 1995; Reitmair *et al.*, 1995). Msh2 deficient mice develop predominately thymic lymphomas with an average age of onset of 5.5 months. However, characterization of the *Msh2*$^{-/-}$ mice and the thymic lymphomas they developed was limited, as outlined below when this thesis began. I undertook molecular characterization of the thymic lymphomas arising in the *Msh2*$^{-/-}$ mice in an attempt to better understand the molecular pathways by which tumorigenesis is occurring in the absence of MMR.

Previous analysis with T and B-cell markers demonstrated that murine *Msh2*$^{-/-}$ deficient thymic lymphomas were very homogeneous. All *Msh2*$^{-/-}$ thymic lymphomas were found to be uniform in appearance, displaying a primitive lymphoblastic morphology (Lowsky *et al.*, 1997). Thymic lymphoma cells had reduced cytoplasm, enlarged nuclei and numerous mitotic figures (Reitmair *et al.*, 1995). Histological examination revealed that the thymic lymphomas in *Msh2*$^{-/-}$ mice were widely disseminated, with metastases to the liver, spleen, kidney and lung (Reitmair *et al.*, 1995). Atypical cells could be identified in the bone marrow; however definite involvement could not be established by microscopic examination (Reitmair *et al.*, 1995). de Wind *et al.* analyzed B and T-cell populations in normal two month old *Msh2*$^{-/-}$ mice (de Wind *et al.*, 1995). Histologically cells were normal as well as having normal proportions of B and T-cell populations (de Wind *et al.*, 1995). As well, tumours from *Msh2*$^{-/-}$ mice had the full spectrum of immunophenotypes characteristic of lymphoblastic lymphoma (Lowsky *et al.*, 1997; Reitmair *et al.*, 1995). From this it is believed that thymic lymphomas in *Msh2*$^{-/-}$ deficient mice evolve from a background of normal hematopoietic progenitors to become tumours (Reitmair *et al.*, 1995). Lowsky *et al.* evaluated the expression of TdT to confirm that *Msh2*$^{-/-}$ thymic lymphomas arose from precursor T-cells and not post-thymic T-cell lymphocytes (Lowsky *et al.*, 1997). Given that *Msh2*$^{-/-}$ thymic lymphomas appear as sheets of monotonous cells

and uniformly express TdT, Lowsky *et al.* concluded that they closely represent human precursor T-cell lymphoblastic lymphomas (LBL).

The first analysis of genomic instability arising in the Msh2 null mice, tested the microsatellite instability status of normal and tumour tissue in both strains of Msh2 mice (de Wind *et al.*, 1995; Reitmair *et al.*, 1995). Reitmar *et al.* analyzed various tissues for microsatellite status from six *Msh2*$^{-/-}$ mice that developed thymic lymphomas. All six thymic lymphomas displayed MSI+ when tested using nine dinucleotide markers (*D1Mit4, D2Mit16, D3Mit11, D5Mit10, D6Mit8, D7Mit12, D8Mit4, D9Mit17, D10Mit2*). Tissues that were histologically found to be infiltrated with tumour also displayed MSI+. Normal tissues were MSS (Reitmair *et al.*, 1995). However, the frequency and/or degree of instability were not discussed. de Wind *et al.* used two dinucleotide markers to analyze microsatellite stability, *D14Mit15* and *D7Mit17*. Microsatellite status was analyzed in 24 *Msh2*$^{-/-}$ embryonic stem (ES) cell lines. Of these lines, 8/24 (33%) were MSI+ at the *D7Mit17* locus and 6/24 (25%) were MSI+ at the *D14Mit15* locus. All *Msh2*$^{+/+}$ cell lines were MSS (de Wind *et al.*, 1995). It is difficult to compare the results from the two groups, however, as each group used different markers and more importantly, different tissue types; tumour versus non-tumour. Non-tumour ES cells showed a relatively high degree of instability with 25%-33% of cell lines demonstrating instability. In all cases, only di-nucleotide repeat markers were analyzed (de Wind *et al.*, 1995; Reitmair *et al.*, 1995). Therefore, many questions arise from these experiments. If a broader spectrum of markers were tested would *Msh2*$^{-/-}$ tumour and non-tumour tissues show a different degree of MSI+? Are mice more susceptible to repeat slippage at di-nucleotide repeats compared to mononucleotide repeats? I hypothesized that Msh2 null tumour tissue would display higher levels of instability than normal Msh2 null ES cells. To test this hypothesis I have used mono, di, tri and tetra-nucleotide microsatellite markers and tested *Msh2*$^{-/-}$ thymic lymphomas for the presence of MSI+. In the absence of MMR, I hypothesized that both spontaneous and induced tumours would display high levels of MSI+. Using UVB induced *Msh2*$^{-/-}$ deficient skin tumours I assayed for the presence of microsatellite instability and compared these results to those from spontaneously arising *Msh2*$^{-/-}$ thymic lymphomas.

Inter-simple sequence (inter-SSR) PCR was developed by Zietkiewicz and colleagues as a new fingerprinting approach utilizing $(CA)_n$ repeats as primer sites for intermicrosatellite amplification (Zietkiewicz *et al.*, 1994). This technique has several advantages: (1) no sequencing is required to design the primers; (2) primers are anchored at the termini of the $(CA)_n$ repeats and extend into the flanking sequence by two nucleotide residues; (3) complex, species-specific patterns are obtained from a variety of eukaryotic taxa; and (4) intraspecies polymorphism is observed, and it segregates as Mendelian markers. Inter-SSR PCR has been proved to be a fast and reproducible technique for quantitation of amplifications, deletions, translocations, and insertions in human sporadic colorectal cancer (Anderson *et al.*, 2001; Basik *et al.*, 1997; Stoler *et al.*, 1999). The genomic instability detected by this technique is independent of microsatellite instability because only inter-microsatellite DNA segments are analyzed. Prior to this work, the use of inter-SSR PCR in mouse models had never been described. The aims of this study were (1) to apply inter-SSR PCR to the mouse and (2) to evaluate the use of inter-SSR fingerprints for the analysis of genomic instability mouse tumours. In collaboration with the Conti laboratory we were successful in adapting inter-SSR PCR for the analysis of spontaneous and chemically induced mouse tumours by use of several single microsatellite-anchored primers. We established the best PCR conditions for each primer and critically evaluated the reproducibility of the band patterns and the absence of spurious bands.

As discussed previously, it is hypothesized that MMR deficient cells have acquired additional genetic changes in the pathway from normal tissue to tumour that may include mutations in various known tumour suppressor or growth control genes. In human MMR deficient colorectal and endometrial cancers, mutations in homopolymeric sequences have been identified in *BAX, caspase-5, CHK1, IGFRII, MBD4, TGFβRII, MSH6* and *MSH3* (Akiyama *et al.*, 1997a; Bader *et al.*, 1999; Bertoni *et al.*, 1999; Markowitz *et al.*, 1995; Percesepe *et al.*, 1998; Rampino *et al.*, 1997; Souza *et al.*, 1996; Takenoshita *et al.*, 1997) These downstream genes have been shown to be non-random targets for mutation in the absence of MMR. To determine if MMR deficient murine lymphomagenesis develops

along similar molecular pathways to human MMR deficient malignancies, I investigated the involvement of *Bax, Chk1, IgfrII, TgfβrII, pTEN*, and *Riz* in murine *Msh2*$^{-/-}$ thymic lymphomas. I compared human sequence to murine sequence to determine if the repetitive coding repeats are conserved between human and mouse (Table 1-1). As seen in Table 1-1, several of the genes in the mouse do not contain the corresponding repeat sequence as in the human gene. Notably, *TGFβRII* has a coding repeat of nine adenines in the human sequence and that same repeat is interrupted in the mouse sequence by a guanine. As length of the repeat is a critical factor in the likelihood of DNA polymerase slippage, I hypothesized that different genes are susceptible to mutagenesis in the absence of MMR in the mouse compared with humans and this may contribute to the tissue specificity difference of tumours between the two species.

Mouse models provide a valuable model to examine the deregulation of the genome that occurs when MMR is lost. Using *Msh2*$^{-/-}$ tumours and various means to test instability such as microsatellite instability analysis, inter-SSR PCR and candidate gene screening, *Msh2*$^{-/-}$ thymic lymphomas appear more stable than predicted. In this study I demonstrate that although *Msh2*$^{-/-}$ thymic lymphomas do demonstrate MSI+, levels of instability are not higher than those found in normal *Msh2*$^{-/-}$ ES cells. As well, analysis of candidate genes hypothesized to be mutated in the absence of MMR revealed low levels of mutation. These genes, identified to be mutated in human HNPCC tumours, are not found to have high mutation rates in the murine *Msh2*$^{-/-}$ thymic lymphomas. Thus, either other target genes are mutated in the mouse that gives rise to tumorigenesis, or alternative pathways exist for tumorigenesis arising in the MMR null mouse.

Materials and Methods

Examination of Mice

Mice were monitored daily for signs of poor health/grooming or tumorigenesis. Mice were sacrificed by isoflurane overdose upon signs of lethargy, weight loss or physical demonstration of a tumour. Tumour tissue specimens were confirmed to be lymphomas

after histological examination or by gross thymus enlargement. Histological analysis was performed on 5μm sections stained with hematoxylin and eosin using standard methods (P.N.). Genomic DNA was extracted from thymic lymphoma tissues using Qiaquick DNA extraction columns (Qiagen Inc.). DNA was eluted in water and stored at -20°C. DNA was quantified using a spectrophotometer.

UVB treatment of Mice

The UVB irradiation of mice experimental protocol was approved by the Health Science Animal Policy and Welfare Committee of the University of Alberta, and all animals cared for according to the guidelines of the Canadian Council on Animal Care. *Msh2*$^{-/-}$ and *Msh2*$^{+/+}$ control mice were UVB treated over a course of 8-10 months: The posterior two-thirds of the back of each mouse was shaved with an electric razor as required. Mice were irradiated with 4000 J/m2 of UVB three times per week (Monday, Wednesday and Friday) until the first presentation of a skin lesion or until 37 weeks of UVB irradiation. At the first presentation of a skin lesion, the cumulative UVB exposure of the mouse was noted and the mouse continued to receive chronic UVB radiation until the lesion reached ~0.5 cm in diameter. After death, the tumour was excised, a sample prepared for histological examination and UVB induced skin tumours were harvested upon presentation As well, tissues harvested from controls included surrounding UVB treated normal skin and non-UVB treated normal abdominal skin. DNA was extracted from harvested tissues using Qiaquick DNA extraction columns (Qiagen Inc.). DNA was eluted in water and stored at -20°C. DNA was quantified using a spectrophotometer.

Microsatellite Instability Analysis

Matched normal brain samples and tumour biopsies were harvested from nine *Msh2*$^{-/-}$ mice and were analyzed for MSI+. DNA was extracted using Qiaquick DNA extraction columns (Qiagen Inc.). Microsatellite loci were amplified using fluorescently labeled Licor PCR primers and products were analyzed on a Licor electrophoresis gel system (Licor-Biosciences). Ten loci were investigated for microsatellite status using primers as follows: 1) Five primer pairs used amplified mononucleotide repeats: JH101, JH102, JH103, JH104 (Edelmann *et al.*, 1997) and U12235 (5'GCTCATCTTCGTTCCCTGTC-3' and 5'-

CATTCGGTGGAAAGCTCTGA-3') (Edelmann *et al.*, 2000). 2) Three primer pairs amplified dinucleotide repeats: D1mit83, D7mit17, D7mit91 (Whitehead Institute: www-genome.wi.mit.edu/cgi-bin/mouse/sts_info). 3) One primer pair, Tcrb, amplified a trinucleotide repeat $(GCT)_{12}$, [Tcrb Forward 5'-AGTTTTAGGCTATAGGTT-3' and Tcrb Reverse 5'- TGATCTAGAGAAAGGGTAGGTCTA-3'] (Hearne *et al.*, 1991) and 4) one primer pair, Cyp1a2, amplified a tetranucleotide repeat $(CAAG)_{10}$, [Cyp1a2 Forward 5'-TGGCAGGACTGCACCTAAGCT-3' and Cyp1a2 Reverse 5'-ACTGGAACCTTAGAGCATGAG-3'] (Hearne *et al.*, 1991).

Inter-Simple Sequence Repeat (SSR) PCR

Genomic DNA from 12 *Msh2*$^{-/-}$ thymic lymphomas was analyzed by inter-SSR PCR. Four degenerate primers were used that amplify simple repeat sequences such as $(CA)_n$. [primer 1: $(CA)_8$RY, primer 2: $(CA)_8$RG, primer 3: $(AAC)_6$Y, and primer 4: $(AGC)_4$Y] [R = purine; Y = pyrimidine]. The 3'-anchored primers amplify adjacent repeat sequences that are less than 2 kb apart and arranged tail to tail. This PCR amplification yields a large number of products. Amplifications were carried out in 25-µl reactions containing 50–100 ng of genomic DNA, 2.5 µl 10 X PCR buffer (15 or 20 mM MgCl2), 2% formamide, 0.2 mM dNTP, 1 µM primer, and 1 U *Taq* polymerase. Testing PCR conditions were as follows: initial denaturation 3 min at 94°C, 30 cycles of 30 sec at 94°C/45 sec at 52–60°C/2 min at 72°C, and final extension 7 min at 72°C. I tested two thermal cyclers: a Perkin–Elmer Model 9600 DNA thermal cycler (Perkin Elmer Cetus Instruments, Norwalk, CT) and a RoboCycler 96 Temperature Cycler (Stratagene, La Jolla, CA), and two DNA *Taq* polymerases: AmpliTaq (PE Applied Biosystems, Foster City, CA) and FastStart Taq (Roche, Indianapolis, IN). Both machines and enzymes yield similar but not identical band patterns under the same conditions. Because of a better reproducibility, I decided to continue the assessment of genetic alterations with the RoboCycler 96 Temperature Cycler (at 54°C of annealing temperature) and FastStart *Taq* polymerase (hot-start PCR). PCR products were analyzed by electrophoresis through non-denaturing 8–10% polyacrylamide gels using Sequi-Gen Sequencing Cell (BioRad) and Criterion precast 10% polyacrylamide gels (BioRad) and visualized by silver staining (Amersham-Pharmacia Biotech,

Piscataway, NJ) and with Vistra Green staining (Amersham international, Buckinghamshire, UK). Gels were scanned or captured with a digital camera. Intensity changes were scored by means of fluor imaging with Vistra Green with a FluorImager apparatus (Molecular Dynamics, Sunnyvale, CA) and IQ Mac 1.2 software (Bio-Image, Ann Arbor, MI). A change was considered significant when at least a twofold increase or decrease in band intensity was observed in the tumour as compared to normal tissue DNA. For the analysis of variability within inbred strains, the difference value (*D*) of the fingerprints was calculated as the number of fragments that differed between two strains divided by the total number of fragments present in the two strains. The average percentage difference (APD) is simply the average of all *D* values for the group of strains (Gilbert *et al.*, 1990). Assays were repeated six times for each tumour/normal pair to ensure reproducibility. Tumour-specific alterations were detected as gains, losses, or intensity changes in bands, and only those alterations that recurred in at least four of the six assays were counted in the estimation of the instability index. The amount of alterations were quantitated by dividing the number of altered bands in the tumour by the total number of bands in matched normal DNA, generating a genomic instability index (Basik *et al.*, 1997; Kahlenberg *et al.*, 1996; Stoler *et al.*, 1999).

Amplification and Sequence Analysis of Candidate Genes from Genomic DNA

DNA was extracted as described previously. PCR amplification of candidate genes (*Bax, Chk1,IgfrII, pTEN Exon7/8, Riz*) was performed using approximately 30 ng of genomic DNA per 25 µl reaction. The amplification conditions were 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds for a total of 30 cycles. To analyze the murine *TgfβrII* gene the following primers were used tgfβrII-F-5'-GAAGATGCCGCTTCTCCCAA-3' and TgfβrII-R 5' GCTGGTGGTGTATTCTTCCG-3' to amplify a region of the murine *TgfβrII* gene (GeneBank Accession #69 114) containing the mononucleotide (A) repeat corresponding to the $(A)_{10}$ repeat of the human *TGFβRII* gene (Lowsky *et al.*, 2000). To amplify the $(GT)_3$ repeat in the murine *TgfβrII* gene the following primers were used TgfβrII-GT-F-5'GAGACTTTGACCGAGTGCTG-3' and TgfβrII-GT-R-5'GCAGAGCGCTTCAGTGAGCT-3' (Lowsky *et al.*, 2000). All PCR products were

separated on a 1.5% agarose gel and amplification products were excised. Products were extracted from the gel using Qiagen Gel extraction columns (Qiagen Inc.). DNA was eluted from the columns using 30 µl of H_2O. Sequencing of extracted PCR products was performed using 5 µl of DNA. PCR products were sequenced using the ABI Prism Dye Terminator Cycle Sequencing kit with AmpliTaq Fs (Perkin Almer ABI, Foster City, CA) and analyzed on a 310 Genetic Analyzer (Perkin Almer ABI).

PCR Assay for DHJH Rearrangement Status

D_HJ_H rearrangement analysis was performed as previously described (Chang *et al.*, 1992). Briefly, two forward primers were used that are immediately 5' of the D_H elements (DSF: 5'-AGGGATCCTTGTGAGGGATCTACTACTGTG-3' and Dq52: 5'-GCGGAGCACCACAGTGCAACTGGGAC-3'). These primers are based on a consensus sequence and together recognize all of the D_H elements. The reverse primer (J_H4: 5'-AAAGACCTGCAGAGGCCATTCTTACC-3') is immediately 3' of the J_H4 element. The DSF/J_H4 primer pair amplifies recombination products between DSP and DFL D_H gene elements and all of the J_H elements. The Dq52 and J_H4 primer pair amplifies recombination products between Dq52 and all J_H4 elements. If rearrangement of the D_HJ_H locus has occurred, one of four PCR product sizes (0.12kb to 1.3 kb) will be observed depending on which J_H element was used. DNA from NIH-3T3 cells was used as a germline control and mouse spleen DNA was used as a positive control.

Western Blot Analysis

Tumour tissue and control tissue (as described previously) was solubilized by sonication in the presence of lysis buffer and protease inhibitor. Proteins were quantified using the BioRad Detergent Compatible protein assay. Protein samples were stored at -80°C. Equal amounts of protein from tumour samples were separated on a 12% SDS-polyacrylamide gel and transferred onto a PVDF membrane. Prior to immunoblotting, membranes were stained with amido black and photographed to measure for equal loading. The membrane was blocked in Tris-buffered saline (TBS), 0.1% Tween-20, 4% nonfat dry milk, and incubated with primary antibody in TBS: Msh2, 1:2500 (PharMingen), Msh6, 1:2000 (BD

BioScience), Pms2 1:2000 (BD BioScience). Bound protein was detected by chemiluminescence using an anti-mouse IgG peroxidase conjugate (BD Bioscience).

Results

Microsatellite Instability Analysis of Lymphoblastic Lymphoma

I hypothesized that *Msh2*$^{-/-}$ thymic lymphomas would display high levels of microsatellite instability and wanted to assess the effect of loss of MMR on different types of repetitive DNA tracts (mono-, di-, tri- and tetranucleotide markers). Using 10 *Msh2*$^{-/-}$ spontaneously arising thymic lymphomas from 10 different mice, I investigated the stability of microsatellite sequences at 10 loci. I analyzed five mononucleotide repeats, three dinucleotide repeats, one trinucleotide repeat and one tetranucleotide repeat. I used non-tumour matched brain as a control to compare to the size of the PCR product from the tumour. Tumours were considered microsatellite unstable low (MSI-L) if one or two markers out of 10 tested showed instability. Tumours were considered microsatellite unstable high (MSI-H) if three or more markers out of ten tested showed instability. One tumour tested showed no instability at any marker tested and was classified as microsatellite stable. Six out of ten tumours tested showed instability at one or two markers and were classified as MSI-L where as three of ten tumours tested showed instability at greater than three markers tested and were classified as MSH-H (Table 2-2), (Figure 2-1). Overall, there were 19/100 unstable reactions corresponding to 19% instability (Table 2-3).

As well, I assayed for the presence of microsatellite instability (using the previously mentioned markers) in five UVB induced *Msh2*$^{-/-}$ murine skin tumours compared with non-tumour tissue. No instability was seen at any marker tested in the UVB induced skin tumours (Figure 2-1).

Inter-SSR PCR

Using inter-SSR PCR I analyzed 12 *Msh2*$^{-/-}$ thymic lymphomas compared with normal non-tumour tissue. Three of the 12 thymic lymphomas were analyzed with primers

$(CA)_8RY$, $(CA)_8RG$, and $(AGC)_4Y$ (as tumour/normal DNA pairs) and showed evidence of genomic instability as measured by inter-SSR PCR, with instability indices of 4, 5 and 8.3% (Figure 2-2).

Table 2-1: Proportion of *Msh2*$^{-/-}$ thymic lymphomas classified as MSI-Low or MSI-Hi

	MSS (no markers tested showed instability)	MSI-L (1 or 2 out of 10 markers tested unstable)	MSI-H (3 or more out of 10 markers tested unstable)
Thymic Lymphoma	1/10 (10%)	6/10 (60%)	3/10 (30%)

Table 2-2: The degree of microsatellite instability seen in *Msh2*$^{-/-}$ thymic lymphomas

Type of Microsatellite Marker	Primer Name	MSI Frequency in *Msh2*$^{-/-}$ Thymic Lymphomas n=10
Mononucleotide	JH101	0/10
	JH103	0/10
	JH103	6/10
	JH104	0/10
	U12235	4/10
Dinucleotide	D7mit17	5/10
	D7mit91	0/10
	D1mit83	3/10
Trinucleotide	Tcrb (GCT)	1/10
Tetranucleotide	Cyp1a2 (CAAG)	0/10

Figure 2-1: Microsatellite instability analysis of spontaneous *Msh2*$^{-/-}$ thymic lymphomas as well as UVB induced *Msh2*$^{+/+}$ skin tumours

A) Murine *Msh2*$^{-/-}$ thymic lymphomas showing microsatellite instability using a mononucleotide marker (U12235) and a dinucleotide marker (D7mit17). Dotted lines indicate normal allele size in each mouse (DNA from non-tumour brain tissue) with arrows indicating unstable alleles.
B) Microsatellite stable (MSS) murine *Msh2*$^{-/-}$ thymic lymphomas using the same markers. Dotted lines indicating the normal allele size in the control tissue.
C) Microsatellite instability assayed in UVB induced *Msh2*$^{-/-}$ tumours as well as control tissues; non-UVB exposed abdomen and UVB exposed non-tumour tissue. All tissues including tumour tissue showed stable microsatellites.

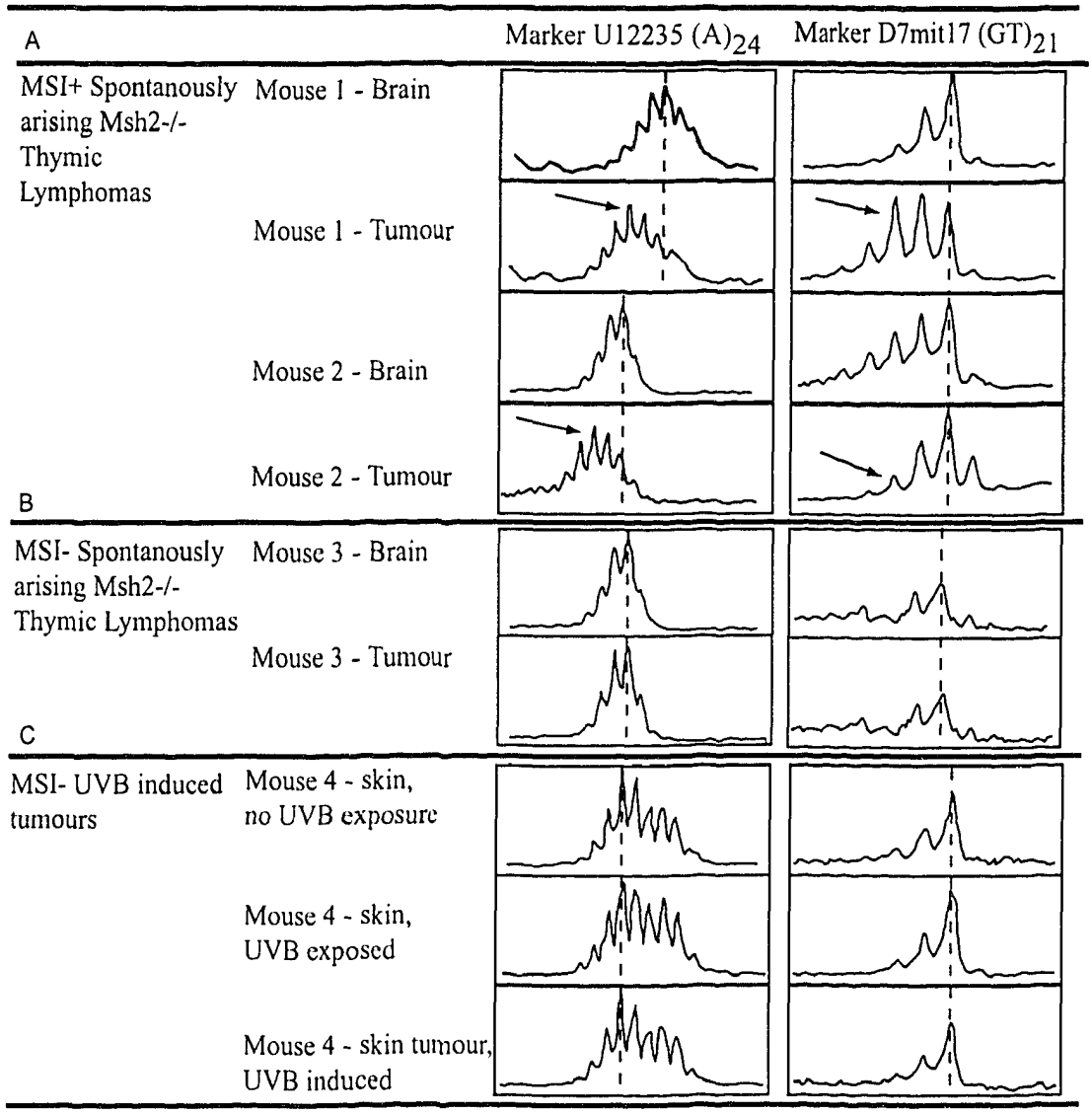
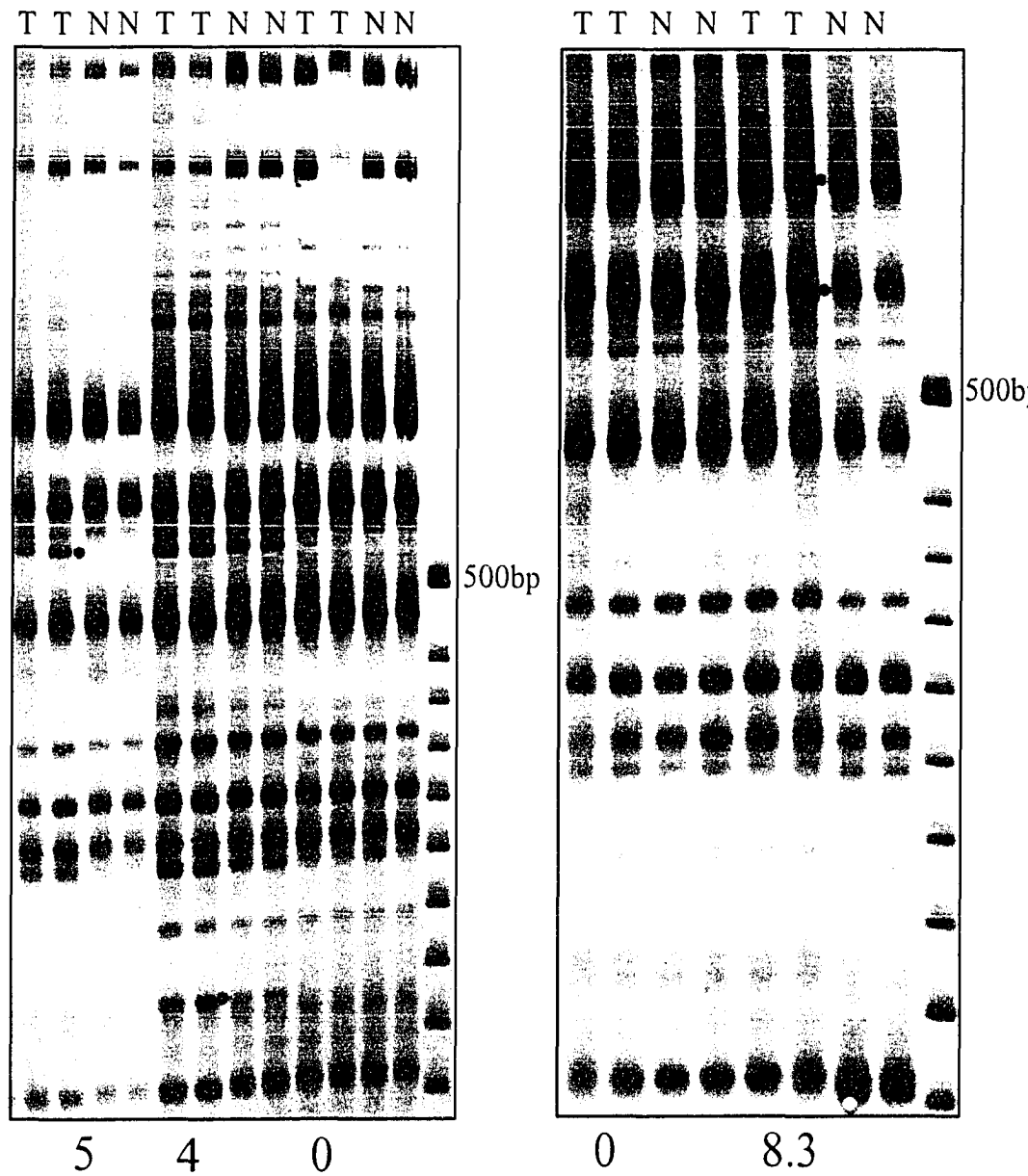


Figure 2-2: Inter-SSR PCR analysis of spontaneous *Msh2*$^{-/-}$ thymic lymphomas.

Duplicate normal (N) and tumour (T) pairs examined with primers $(CA)_8RG$ are shown. Genomic instability (%) is shown under duplicate tumour samples. Dots (•) placed alongside lanes indicate bands with twofold increases or decreases in intensity. [Tumour cell and DNA preparation by Marcia Campbell, inter-SSR PCR and gel was run by Dr. Benavides]



Sequence Analysis of Candidate Genes from Genomic DNA.

A comparison between the repetitive tracts of human genes known to be mutated in HNPCC tumours and the corresponding murine sequence of the orthologus gene is shown is Table 1-1. The sequence of the (A) repeat of the murine *TgfßrII* gene 5'AAAAGAAAAG-3' differs from the human $(A)_{10}$ repeat in the *TGFßRII* gene because it contains guanine nucleotides at the positions corresponding to the 5th and 10th adenine nucleotides of the human repeat. I have analyzed 20 murine *Msh2*$^{-/-}$ thymic lymphomas for insertion/deletion loop mutations within a 250 bp fragment of the *TgfßrII* gene containing the (A) repeat (Table 2-4 and data not shown). Comparison of tumour DNA with non-malignant DNA showed tumour DNA to have no shifts and therefore this repeat $[(A)_4G(A)_4G]$ is not mutated in *Msh2*$^{-/-}$ thymic lymphomas. I sequenced the $(GT)_3$ repeat in the *TgfßrII* murine gene as well as in the 20 *Msh2*$^{-/-}$ thymic lymphomas. No insertion or deletion mutations were identified in this repeat (data not shown).

In seven *Msh2*$^{-/-}$ thymic lymphomas, I analyzed five other genes known to contain mononucleotide repeats and be targeted for mutation in human HNPCC colon tumours: *Bax, Chk1, IgfrII, pTEN* Exons 7 and 8, and *Riz*. Analyzing the coding repeat region in each gene I found one tumour was unstable. It contained deletion mutations at the coding repeat regions in *IgfrII* and *pTEN* Exon 8 (Table 2-3) (Figure 2-3).

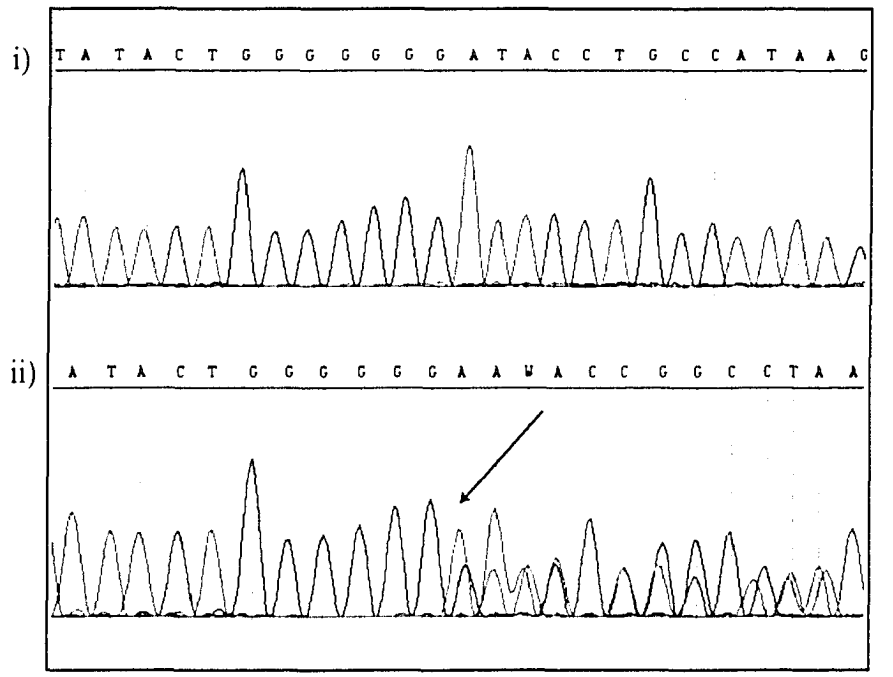
Table 2-3: Results of candidate gene analysis.

	Bax	*Chk1*	*IgfrII*	*pTEN Ex 7*	*pTEN Ex 8*	*Riz*	*TgfβrII*
Tumour							
Msh2$^{-/-}$ #1	√	√	√	√	√	√	√
Msh2$^{-/-}$ #2	√	√	√	√	√	√	√
Msh2$^{-/-}$ #3	√	√	√	√	√	√	√
Msh2$^{-/-}$ #4	√	√	Mutation	√	Mutation	√	√
Msh2$^{-/-}$ #5	√	√	√	√	√	√	√
Msh2$^{-/-}$ #6	√	√	√	√	√	√	√
Msh2$^{-/-}$ #7	√	√	√	√	√	√	√

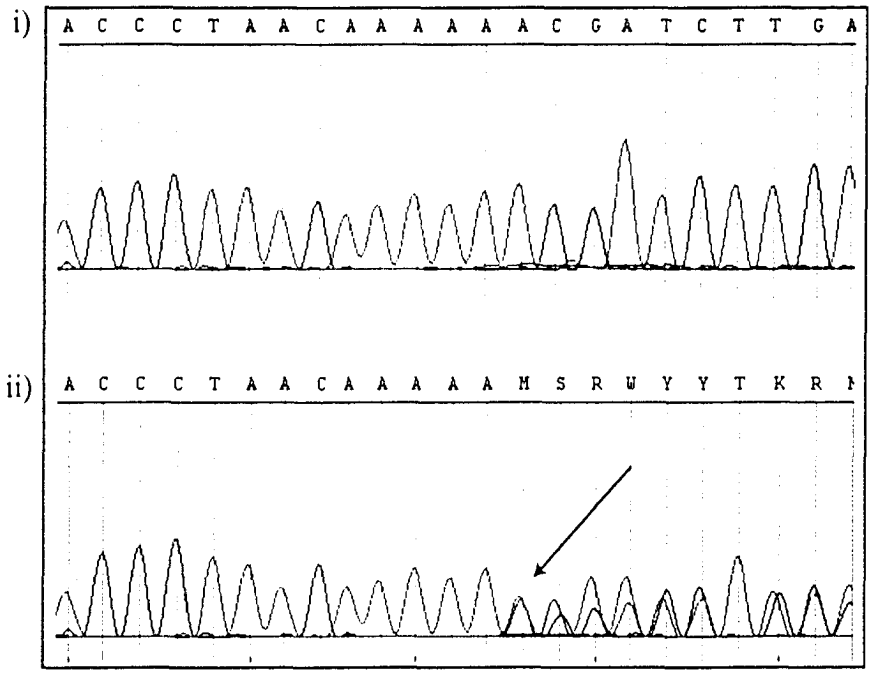
Figure 2-3: Sequence analysis of the *IgfRII* and *pTEN* genes in *Msh2*$^{-/-}$ thymic lymphomas.

Examples of genomic sequence profile obtained from tumour DNA specimens demonstrating coding repeat tract mutations indicating that a contraction of the microsatellite sequence has occurred. A) Analysis of the murine *IgfrII* gene $(G)_7$ repeat tract. A i) wildtype *IgfrII* $(G)_7$ coding repeat sequence from normal *Msh2*$^{-/-}$ brain tissue. A ii) corresponding *Msh2*$^{-/-}$ thymic lymphoma tissue with mutant *IgfrII* sequence showing a contraction at the coding $(G)_7$ repeat tract as indicated by the arrow. B) Analysis of the murine *pTEN* gene (A)6 repeat tract. B i) wildtype *pTEN* $(A)_6$ coding repeat sequence from normal *Msh2*$^{-/-}$ brain tissue. B ii) corresponding *Msh2*$^{-/-}$ thymic lymphoma tissue with mutant *pTEN* sequence showing a contraction at the coding $(A)_6$ repeat tact as indicated by the arrow.

A



B



D_HJ_H Gene Rearrangement Analysis of Lymphoblastic Lymphomas.

The presence of D_H to J_H gene rearrangements was assayed in 10 *Msh2*$^{-/-}$ thymic lymphomas as well as in six normal *Msh2*$^{-/-}$ thymocytes samples (age 8 weeks) and 3 *Msh2*$^{+/+}$ normal thymocyte (age 8 weeks) samples. All of the 10 tumours analyzed showed D_H to J_H gene rearrangements at at least one locus (Table 2-3). Four of the 10 tumours investigated had matched normal brain control DNA. One of the four matched normal brain samples showed the same D_H to J_H gene rearrangement products found in the corresponding tumour tissue leading us to suggest that tumour metastasis to the brain has occurred as the brain is not expected to have undergone DH to JH gene rearrangement. In the normal thymocyte samples, all possible D_H to J_H gene rearrangements were found regardless of genotype. Therefore there was no difference in D_H to J_H gene rearrangements in the absence of MMR in 8 week old normal thymocytes.

Table 2-4: D_HJ_H rearrangements in tumours from *Msh2*$^{-/-}$ thymic lymphomas

		D_HJ_H rearrangements per tumour									
		DSF/JH4 primer pair					DQ52/JH4 primer pair				
		DJH1	DJH2	DJH3	DJH4	germline	DJH1	DJH2	DJH3	DJH4	germline
Tumour ID											
tumour	588		■	■	■		■	■	■	■	■
tumour	125					■		■			■
tumour	3						■				
tumour	4		■	■				■	■		■
tumour	7		■	■	■		■	■	■	■	
tumour	11									■	
tumour	12				■			■			
tumour	17	■	■	■	■		■	■	■		■
tumour	21		■	■						■	
tumour	83					■	■	■	■	■	■

■ represents rearrangement products that were present

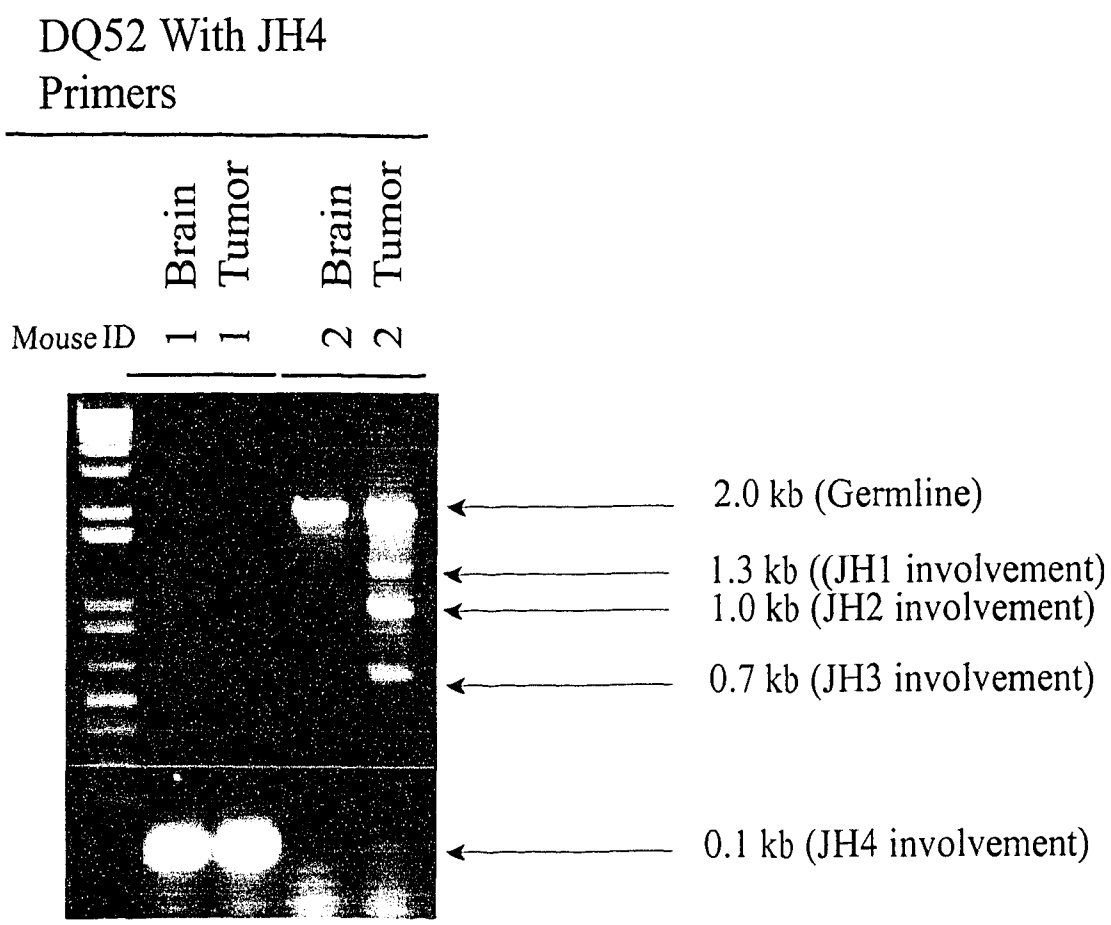


Figure 2-4: DJ recombination in *Msh2*$^{-/-}$ thymic lymphomas.

PCR amplification of DNA from *Msh2*$^{-/-}$ thymic lymphomas using the DQ52/JH4 primer pair. Normal brain was compared with tumour samples. Mouse 1: both the brain and tumour display rearrangement between Dq52 elements and the JH4 element suggesting that there has been metastasis to the brain. Mouse 2: the brain displays germline configuration (no rearrangements) with the corresponding tumour sample showing all possible rearrangements, including the germline configuration.

Protein expression of the MMR proteins Msh2, Msh6 and Pms2.

MMR protein expression levels were analyzed in normal murine tissues. 70 μg of protein for each tissue was loaded into each lane and tissue type marked as indicated in Figure 2-4. Proteins were equally loaded in each lane as indicated by amido black staining (data not shown). Thymus showed high levels of Msh2, Msh6 and Pms2 protein expression. Four week old thymus (pre-sexual maturity) as well as 8 week old thymus (post-sexual maturity) both showed high levels of MMR protein expression. 1 year old thymus showed lower levels of expression compared with young 4 and 8 week old thymus. In addition, spleen showed high levels of MMR proteins levels (Figure 2-4).

Cellular Changes in Colonic Tissues.

To investigate the possibility of early stages of tumour formation in the *Msh2*$^{-/-}$ colon, I undertook a hyperproliferation study of the colon hypothesizing that early cellular changes may be occurring that are not seen macroscopically. *Msh2*$^{-/-}$ mice were sacrificed at 24 days, 8 weeks, and 3 months old and each colon was divided into three sections. Three mice for each age group were used to account for individual variation between mice. In all colon sections examined there were no unusual cellular changes or thickening of the epithelial cell wall. I found no evidence of cellular hyperproliferation or pre-cancerous lesions in the colon.

Figure 2-5: Detection of MMR proteins in normal mouse tissues by western blot analysis.

Protein extracts are from normal MMR proficient mice. Thymus and spleen show high levels of Msh2, Msh6 and Pms2 protein expression as indicated by (*).

Discussion

To further characterize the role that MMR plays in the development of tumours, mice deficient in the MMR gene *Msh2* have been generated (de Wind *et al.*, 1995; Reitmair *et al.*, 1995; Smits *et al.*, 2000). In contrast to the GI tumours that develop in human HNPCC patients, MMR deficient mice develop predominately thymic lymphomas. Since MMR is thought to have a ubiquitous role in DNA repair and mutation avoidance, the tissue specificity of tumorigenesis in human HNPCC patients as well as in MMR deficient mice is unexpected.

The thymic lymphomas arising in the Msh2 null mice can be characterized with respect to the degree of genomic instability to gain insights into why these particular tissues are targeted in the absence of MMR for malignancy. I hypothesized that *Msh2*$^{-/-}$ thymic lymphomas would display a high degree of MSI+, an obvious indication of high levels of overall genomic instability. I choose to screen 10 *Msh2*$^{-/-}$ thymic lymphomas for MSI using a panel of 10 markers. Hypothesizing that different types of repeats may be susceptible to slippage in the mouse genome compared with the human genome, I used mono, di, tri and tetra nucleotide repeat markers to assay for MSI in MMR deficient mouse tumours. I used five mononucleotide markers that had been used previously JH101, JH102, JH103, JH104 and U12235 (Edelmann *et al.*, 2000; Edelmann *et al.*, 1997). I chose a dinucleotide marker that had shown instability in several of my earlier experiments (D1mit83), as well as two markers that had been shown informative in published work (D7mit91, D7mit17) (de Wind *et al.*, 1995; Edelmann *et al.*, 1996; Edelmann *et al.*, 1997). The tri and tetranucloetide markers were chosen from published work describing microsatellites in the mouse genome and to my knowledge they have not been used for MSI analysis (Hearne *et al.*, 1992).

The majority of tumours tested demonstrated some degree of microsatellite instability, however less instability was observed than expected based on earlier work with *Msh2*$^{-/-}$ ES cells. Of the 100 reactions carried out, 19 were shown to be unstable i.e. 19% of reactions

were unstable. However, 6/10 tumours did show MSI-L to MSI-H. de Wind *et al.* saw between 25-33% instability in normal ES cells. However, these were cultured ES cell lines and were considered MSI+ when they showed instability at one marker. In this study, 3/5 mononucleotide markers did not show instability in any reactions suggesting that they are not informative for MSI in the mouse. Also, D7mit91 did not show instability and is likely not informative. Only one tumour tested was stable (MSS).

Particular mononucleotide markers (JH101, JH103 and U12235) and dinucleotide markers (D1mit83 and D7mit17) showed greater levels of instability than comparable markers, suggesting that some and not all microsatellites are informative when assaying for MSI. Instability was not detected in the trinucleotide or tetranucleotide marker, in any tumour tested suggesting that these tri and tetra nucleotide markers are not susceptible to MMR dependent MSI+ or that these markers are not informative. In addition, the length of a microsatellite may play a role in its susceptibility to instability. In *Msh2*$^{-/-}$ thymic lymphomas, instability was not seen in the shorter mononucleotide repeats (JH102 and JH104, 8 and 10 base pairs respectively) but was seen in the longer mononucleotide repeats (JH103 and U12335, 13 and 24 base pairs respectively).

Due to the low levels of instability seen, I cannot draw any conclusions as to whether mono, di, tri or tetra nucleotide markers are differentially susceptible to instability in the absence of MMR. From the microsatellite instability patterns seen in human HNPCC tumours, it was expected that the majority of microsatellites would be unstable in the absence of MMR in the mouse. Thus, the surprising overall stability led me to hypothesize that murine MMR deficient tumorigenesis may develop along different molecular pathways than in humans. Other types of genomic instability may be occurring concurrently with MSI+ to lead to tumorigenesis This will be further discussed in Chapter 4.

The latency of UVB-induced MMR deficient tumorigenesis is significantly longer than the time to develop spontaneously arising thymic lymphomas in MSH2 null mice (Young *et al.*, 2004). The longer length of time to tumorigenesis would suggest that UVB induced

tumours have had more time to acquire microsatellite mutations. I tested five UVB-induced skin tumours with two microsatellite markers and showed that all samples were stable. My results show that even with the increased latency to tumorigenesis, UVB induced skin tumours do not acquire microsatellite mutations, suggesting that the molecular pathways leading to tumorigenesis in spontaneous versus induced MMR deficient tumorigenesis are different. Although UVB induced DNA damage such as thymine dimers and 6-4 photoproducts is not primarily repaired by MMR, the resulting apoptosis and cell cycle arrest may well be mediated by MMR. The absence of MMR may lead to the accumulation of UVB induced DNA damage that is not detected by our microsatellite analysis and results in skin tumorigenesis.

In previous studies with human cancer specimens, more than 80% of the sporadic colorectal carcinomas evaluated by inter-SSR PCR showed at least one altered band. Based on these results, Stoler and colleagues reported that typical sporadic colorectal cancers contain at least 11,000 genomic alterations per cell and that this is an early event in tumour development (Stoler *et al.*, 1999). Through our collaboration with Benavides *et al.*, I conclude that inter-SSR PCR is a suitable technique for the assessment of the degree of genomic damage in mouse models by unbiased DNA fingerprinting (Benavides *et al.*, 2002). In comparison with classic DNA fingerprinting techniques, inter-SSR PCR is much simpler and requires less DNA as template (two orders of magnitude). It also has an advantage over AP-PCR (random amplified polymorphic DNA-PCR) because of the high stringency conditions used in the PCR amplification. These stringent PCR conditions avoid the problems of primer competition that are probably responsible for the appearance of spurious bands observed in the AP-PCR procedure. However, because reproducibility of the DNA patterns is also a concern for inter-SSR PCR, repeated (parallel) amplifications and the use of tumour and normal DNA from the same animal are highly recommended. I have shown, using inter-SSR PCR, that 25% of *Msh2*$^{-/-}$ thymic lymphomas are genetically unstable. This supports my hypothesis that in the absence of MMR various forms of genomic instability are driving tumorigenesis.

There are a number of human target genes for which mutations appear to be selected in the absence of MMR (Peltomaki, 2001a). Such genes contain coding microsatellite repeat sequences that undergo frameshift mutation. An example of one such gene is *TGFβRII.* There is strong selection for this gene, greater than 90% of HNPCC colon cancers have mutations in this gene (Parsons *et al.*, 1995). Five genes, known to contain mononucleotide repeats and be targeted for mutation in human HNPCC colon tumours include *BAX, CHK1, IGFRII, pTEN* Exons 7 and 8, *TGFβRII* and *RIZ.* Comparative genomic analysis allowed me to assay for the corresponding sequences in the murine genome (Table 1-1). Mutational analysis of the corresponding repeats in *Msh2*$^{-/-}$ thymic lymphomas showed one tumour to be unstable. We investigated *Tgfβr1I* as a candidate gene that may be involved in MMR deficient murine thymic lymphomagenesis. Sequencing of both microsatellites revealed no mutations in 20 thymic lymphomas. We conclude that instability at microsatellite sequence in the *Tgfβr1I* gene does not contribute to MMR deficient thymic lymphomagenesis. This is surprising as *Tgfβr1I* is mutated in over 90% of human HNPCC tumours. In contrast to my findings, Lowsky *et al.* analyzed 10 *Msh2*$^{-/-}$ thymic lymphomas at the *Tgfβr1I* gene and found 6/10 to contained insertion/deletion mutations (Lowsky *et al.*, 2000). However, the lack of mutations in my analysis may help explain the differential tumour spectrum seen in murine vs. human MMR deficient tumours. Secondary downstream genes such as *Tgfβr1I* may predominate in the human MMR deficient tumorigenesis pathway but may not play a role in murine tumorigenesis. In addition, the lack of mutations may indicate a different degree of susceptibility to instability with different types of microsatellites (ie mononucleotide vs. dinucleotide) and/or different degrees of susceptibility in human vs. murine microsatellites. Overall, the lack of mutations in the corresponding mouse genes leads me to suggest that different genetic pathways may be involved in MMR deficient tumorigenesis in the mouse compared with man.

As discussed previously, MMR may play a role in double strand break repair. In the overlap with double strand break repair, the possibility exists for MMR to play a role in the repair of DNA lesions generated during such processes as D_HJ_H rearrangement. Typical B-cell development is characterized by the rearrangement and expression of immunoglobulin

heavy and light chain genes. The first Ig gene rearrangement is the joining of D_H (diversity) gene elements to J_H (joining) gene elements. It is possible that in the absence of MMR, immunoglobulin gene rearrangement is incorrectly regulated, resulting in recurring mistakes at this stage of development leading to tumorigenesis. Approximately 40% of normal T-cells have undergone D-J rearrangements (Corcoran *et al.*, 2003). I found no difference in the levels of DHJH rearrangement in normal 8 week old *Msh2*$^{+/+}$ thymocytes compared with normal *Msh2*$^{-/-}$ 8 week old thymocytes. In 10 *Msh2*$^{-/-}$ thymic lymphomas all possible D_HJ_H rearrangements were present. I conclude that D_HJ_H rearrangement analysis does not appear to be a reliable indicator of MMR dependent instability in thymic lymphomagenesis.

Previous RNA expression analysis showed Msh2 expression to be ubiquitous (Varlet *et al.*, 1994). However, thymus was not included in this experiment and the experimental results for the spleen were unclear. Using normal mouse protein extracts, I showed high levels of Msh2, Msh6 and Pms2 MMR protein in murine thymus and spleen (Figure 2-4). I hypothesize that these high levels of expression are most likely due to the high expression of MMR proteins in T-cells and suggest a particular requirement for MMR in B and T-cells. Further work in Chapter 6 highlights the importance of the role of MMR in developing B and T-cells.

A lower level, but still significant expression, of the three MMR proteins was demonstrated in colon compared with thymus (Figure 2-4). However, in contrast to HNPCC individuals, *Msh2*$^{-/-}$ mice develop lymphoid tumours with no indication of primary intestinal tumour formation (de Wind *et al.*, 1995; Reitmair *et al.*, 1995). Reitmair *et al.* examined older *Msh2*$^{-/-}$ mice (6-9 months) that succumbed to thymic lymphomas for presence of intestinal tumours and found that 15/22 (68%) showed evidence of intestinal involvement (Reitmair *et al.*, 1996b).

I hypothesized that *Msh2*$^{-/-}$ mice may develop primary tumours of the colonic epithelium similar to human HNPCC patients. Thymic lymphomas in *Msh2*$^{-/-}$ mice may be developing

so quickly so to obscure the investigation of colon tumour formation in these mice. Of note, on histological examination, colons from several *Msh2*$^{-/-}$ mice presenting with thymic lymphomas did show lymphoid infiltration of the lamina propria of intestinal villi and the presence of lymphoid follicles in the mucosa with increased prominence of submucosal lymphoid follicles. However, no mice showed primary colonic tumours. In all colon sections examined there were no unusual cellular changes or thickening of the epithelial cell wall. We found no evidence of cellular hyperproliferation or pre-cancerous lesions in the colon and conclude that the colon is not susceptible to tumour formation in these mice.

Chapter 3 ♦ Candidate mutator genes in the development of mismatch repair deficient murine thymic lymphomas: no evidence of mutations in the DNA polymerase δ gene*

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* *A version of this chapter has been published.*

Marcia R. Campbell, Thy Y Thang, Frank R. Jirik, Susan E. Andrew (2000) *Carcinogenesis* vol 21 no 12 pp : 2281-2284

All of the experiments presented in this chapter were done by Marcia Campbell. I supervised a summer student Thy Thang in assisting me with PCR of DNA polymerase delta exons.

Introduction

Tumour development is hypothesized to require the accumulation of multiple genetic alterations within a particular cell. A mutator phenotype, characterized by an increased mutation rate, increases the tempo of mutation accumulation, allowing for the acquisition of sufficient mutations for tumorigenesis. Thus, a mutator phenotype is hypothesized to be a common feature in the development of many neoplasias (Loeb, 1991). It is thought that the subsequent genetic alterations within a particular cell confer a proliferative advantage to that cell through the accumulation of mutations within key tumour suppressors or oncogenes.

Using internal genes to measure endogenous mutation rates, Msh2 deficient mice have been shown to have mutation frequencies 5-15 fold higher than repair proficient mice (Andrew *et al.*, 1997). In addition to the already elevated mutation frequency in *Msh2*$^{-/-}$ mice, DNA isolated from *Msh2*$^{-/-}$ thymic lymphomas revealed an even greater increase (18-32 fold) in mutation frequency than that observed in other *Msh2*$^{-/-}$ tumours (Baross-Francis *et al.*, 1998). The dramatically increased mutational frequencies compared to other *Msh2*$^{-/-}$ tumours and *Msh2*$^{-/-}$ normal thymic tissue suggested that an additional mutator phenotype may have been acquired in a tissue specific manner (Baross-Francis *et al.*, 1998). The greatly increased mutation frequency seen in the thymic lymphomas is hypothesized to result from a mutation in a gene that contributes to further elevation of the mutation rate in the thymus leading to an additional mutator phenotype and subsequent tumorigenesis (Baross-Francis *et al.*, 1998). The thymic lymphoma tumours showed exclusive mutation patterns that consisted of multiple mutations within short regions of DNA. The fact that this mutation pattern was observed exclusively in the thymic tumours and not in other *Msh2*$^{-/-}$ tumours or normal tissue suggested that a gene(s) associated with the replication machinery might have become altered during tumorigenesis (Liu *et al.*, 1999). Thus, I hypothesized that mutations in DNA polymerase genes that alter the enzyme fidelity could be responsible for the above characteristics of the thymic lymphomas. An example of reduced enzyme fidelity resulting in a mutator phenotype, DNA polymerase β mutations have been shown

to result in a mutator phenotype associated with several forms of cancer (Dobashi *et al.*, 1994; Matsuzaki *et al.*, 1996; Wang *et al.*, 1992). The most conserved eukaryotic replicative DNA polymerase, DNA polymerase δ (DNA pol δ), is another potential polymerase that has an intrinsic 3' to 5' exonuclease activity and has been implicated in DNA repair (Chang *et al.*, 1995; Cullmann *et al.*, 1993). The catalytic subunit of *Mus musculus* DNA pol δ is a 1105 amino acid protein. When mutated, the catalytic properties of DNA pol δ have been shown to be error prone suggesting a potential mutator activity possibly resulting in a mutator phenotype (Popanda *et al.*, 1999). These results are consistent with the findings that *Saccharomyces cerevisiae* strains with mutations in the 3'-5' exonuclease domain of DNA pol δ are hypermutable (Simon *et al.*, 1991). Supporting this result, Tran *et al.* have shown that an error prone DNA pol δ results in a mutator phenotype in *S. cerevisiae* and double *Msh2*$^{-/-}$ */Polδ* mutations have hyper-mutation frequencies above either mutation alone suggesting Msh2 and DNA pol δ act synergistically (Tran *et al.*, 1999). Da Costa *et al.* examined the 3'-5' exonuclease domain of DNA pol δ and found that 3 out of 7 colon cancer cell lines contained a DNA alteration causing an amino acid change suggesting that a mutated DNA pol δ leads to tumorigenesis (da Costa *et al.*, 1995). Further implicating DNA pol δ in tumorigenesis, Flohr *et al.* identified point mutations in the catalytic subunit of DNA pol δ in human colon cancer cell lines as well as primary human colon cancers (Flohr *et al.*, 1999).

It has previously been shown that in the absence of MMR, some genes are non-random targets for mutations due to the presence of coding mononucleotide repeats of 5 base pairs (bp) or greater (Table 1-1). As well, mutations in homopolymeric sequences have been identified in BAX, caspase-5, *TGFβRII, IGFRII, MSH6* and *MSH3* in microsatellite unstable (MSI+) colorectal and endometrial cancers (Akiyama *et al.*, 1997a; Markowitz *et al.*, 1995; Percesepe *et al.*, 1998; Rampino *et al.*, 1997; Souza *et al.*, 1996; Takenoshita *et al.*, 1997). In the *Mus Musculus* catalytic genomic DNA pol δ sequence there are six exons with mononucleotide runs of five bp or greater. This makes DNA pol δ a likely target gene in the absence of MMR. From the above data, and the knowledge that the catalytic subunit

of DNA pol δ contains six exons with mononucleotide runs of five bp or more, I hypothesized that a mutated DNA pol δ is a likely candidate mutator gene in *Msh2*$^{-/-}$ murine thymic lymphomas. It is likely that a mutation in the catalytic subunit of DNA pol δ would cause decreased DNA copying fidelity and as such, cause additional mutator activity leading to increased genomic instability and progression to malignancy. I have therefore sequenced the 26 exons of DNA pol δ catalytic subunit from nine *Msh2*$^{-/-}$ thymic lymphomas. No mutations were found in the nine thymic lymphomas screened indicating that DNA pol δ likely does not contribute to the increased mutator phenotype seen in *Msh2*$^{-/-}$ tumours.

Materials and methods

Msh2$^{-/-}$ Transgenic Mice

Msh2$^{-/-}$ mice were crossed with the BC-1 *lacI* transgenic mouse line as previously described (Andrew *et al.*, 1998; Reitmair *et al.*, 1995). *Msh2* genotyping was carried out using a PCR assay with earclip DNA and the following three primers: U771 Forward (5'-GCTCACTTAGACGCCATTGT-3') and L926 Reverse (5'-AAAGTGCACGTCATTTGGA-3') amplifying the wild type allele and U771 Forward and Neo Reverse (5'-TGG AAG GAT TGG AGC TAC GG-3') amplifying the targeted allele. *Msh2$^{-/-}$ /lacI* mice were sacrificed when moribund, thymic lymphomas were confirmed by histology and thymic lymphoma genomic DNA extracted from nine animals as previously described (Kohler *et al.*, 1990). Similarly, control DNA from normal *Msh2$^{+/+}$* liver and brain tissue was obtained from two mice; littermates of the mice in which the tumours arose.

PCR Amplification and Purification of Exons

I analyzed the catalytic subunit of the DNA pol δ gene in nine thymic lymphomas and two *Msh2$^{+/+}$* normal tissues by PCR amplification and manual DNA sequencing. I designed twenty-six sets of primers (Table 3-3 and Table 3-4) to amplify each exon of the DNA pol δ gene by PCR based on the *Mus musculus* DNA pol δ catalytic subunit genomic DNA sequence (Table 3-4)(Goldsby *et al.*, 1998) (Genbank accession number AF024570). A standard 25*ul* PCR was performed using approximately 5*ng* of genomic thymic lymphoma DNA or control DNA for each reaction. Conditions included a denaturation time of 30 seconds at 95^0 C, primer annealing time of 15 seconds at various temperatures, and an extension time of 20 seconds at 72^0 C (Table 3-4). Thirty cycles were run in an Eppendorf Scientific Mastercycle gradient thermal cycler. PCR modifications for individual exon annealing temperatures, reaction components and concentrations are shown in Table. PCR products were subsequently analyzed on a 2% agarose gel containing ethidium bromide. Each band was excised from the agarose gels after electrophoresis and purified using the QIAquick Gel Extraction Kit (Qiagen, Santa Clarita, CA). Purified DNA was released

from the extraction column by elution in 30*ul* of water and stored at -20^0 C to prevent degradation of product.

DNA Sequencing

Each of the 26 exons of DNA pol δ were sequenced from nine *Msh2*$^{-/-}$ thymic lymphomas as well as non-tumour liver and brain controls. Potential DNA alterations were re-sequenced to confirm results. Sequencing was performed using a USB Thermo Sequenase Radiolabeled Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech Limited, Cleveland, Ohio). Reactions were performed according to manufacturer instructions. Sequencing reactions were separated in a 6% denaturing polyacrylamide gel at approximately 1300 V. Gels were subsequently dried and exposed to x-ray film for 24-36 hours.

Results

To identify the possible role of DNA pol δ in the development of *Msh2*$^{-/-}$ thymic lymphomas, the 26 exons of the gene (genomic organization of DNA pol δ displayed in Table 3-1) were sequenced from genomic DNA in nine *Msh2*$^{-/-}$ thymic lymphomas as well as two control (non-tumour) liver and brain samples from *Msh2*$^{+/+}$ litter mates. Unfortunately matched non-tumour *Msh2*$^{-/-}$ DNA was not available for analysis. 26 sets of intronic primers were used to amplify gDNA by PCR (Table 3-3) and products were examined for mutations by sequencing.

When I started this project in 1998 an exhaustive search of the NCBI (National Center for Biotechnology Information) nucleotide database revealed that the catalytic subunit of DNA pol δ had been sequenced in ten organisms; *Mus musculus, Rattus norvegicus, Mesocricetus auratus, Homo sapien, Bos taurus, Saccharomyces cerevisiae, Saccharomyces pombe, Droshophila. Melanogaster, Plasmodium falciparum*, and *Glycine max*. With the advancement of sequencing techniques, today there are many organisms for which the catalytic subunit of DNA pol δ has been sequenced. However, the previously identified functional domains and conserved residues remain the same (Table 3-1) (Figure 3-1). In 1998, analysis comparing the two published sequences of DNA pol δ in *M. musculus* (one sequence was from cDNA and one sequence was from gDNA) revealed seven locations that contained differing sequence, likely polymorphisms (Table 3-2).

Exons 3, 8, 16, 18 and 22 contained mononucleotide repeats of five or greater bp, which were hypothesized to be non-random locations for polymerase slippage. However, no additions or deletions of bases were seen at these locations, in normal or tumour DNA. Sequence differences from either of the two published DNA pol δ catalytic subunits sequences were found at 10 locations (Table 3-5). In five of these instances, DNA alterations resulted in an amino acid difference from one of the published sequences (Table 3-5). Sequencing was repeated to confirm differences. Four changes were silent and one was intronic (Table 3-5). Only one alteration (exon 13) differed from both the genomic

and cDNA sequences published. However, in exon 13, both control tissues as well as tumours B, C, D and H demonstrated the same nucleotide sequence and thus the alteration is not considered disease associated (Table 3-5). However, this alteration is located adjacent to a conserved putative DNA polymerization domain as seen in Figure 3-1 and it has not been ruled out that this point mutation could affect enzyme activity.

Contrary to my hypothesis, no disease associated mutations were found in the coding sequence of DNA pol δ in the 9 *Msh2*$^{-/-}$ thymic lymphomas that were screened (Table 3-5).

Table 3-1: Genomic structure of the *Mus musculus* DNA Pol δ gDNA

Exon Number	Position of Coding Nucleotides[2]	Exon Size	Intron Number	Intron Size
1	316-511	195 bp		
2	874-987	113 bp	1	363 bp
3	1502-1648	146 bp	2	515 bp
4	1728-1853	125 bp	3	80 bp
5	1930-2098	168 bp	4	77 bp
6	2181-2262	81 bp	5	83 bp
7	2348-2477	129 bp	6	86 bp
8	2741-2907	166 bp	7	264 bp
9	3122-3226	104 bp	8	215 bp
10	4240-4380	140 bp	9	1014 bp
11	4578-4688	110 bp	10	198 bp
12	4778-4969	191 bp	11	90 bp
13	5050-5138	88 bp	12	81 bp
14	5299-5415	116 bp	13	161 bp
15	5639-5752	113 bp	14	224 bp
16	5989-6136	147 bp	15	257 bp
17	7718-7813	95 bp	16	1582 bp
18	7908-8045	137 bp	17	95 bp
19	8552-8727	175 bp	18	507 bp
20	9022-9174	152 bp	19	295 bp
21	9251-9353	102 bp	20	77 bp
22	9721-9853	132 bp	21	368 bp
23	9940-10053	113 bp	22	87 bp
24	10312-10364	52 bp	23	259 bp
25	10436-10533	97 bp	24	72 bp
26	10843-10948	105 bp	25	310 bp

[1] The first nucleotide if the ATG start codon is designated as +316 from the genomic published sequence

[2] The published sequence by Goldsby, R. E. *et al.* 1997

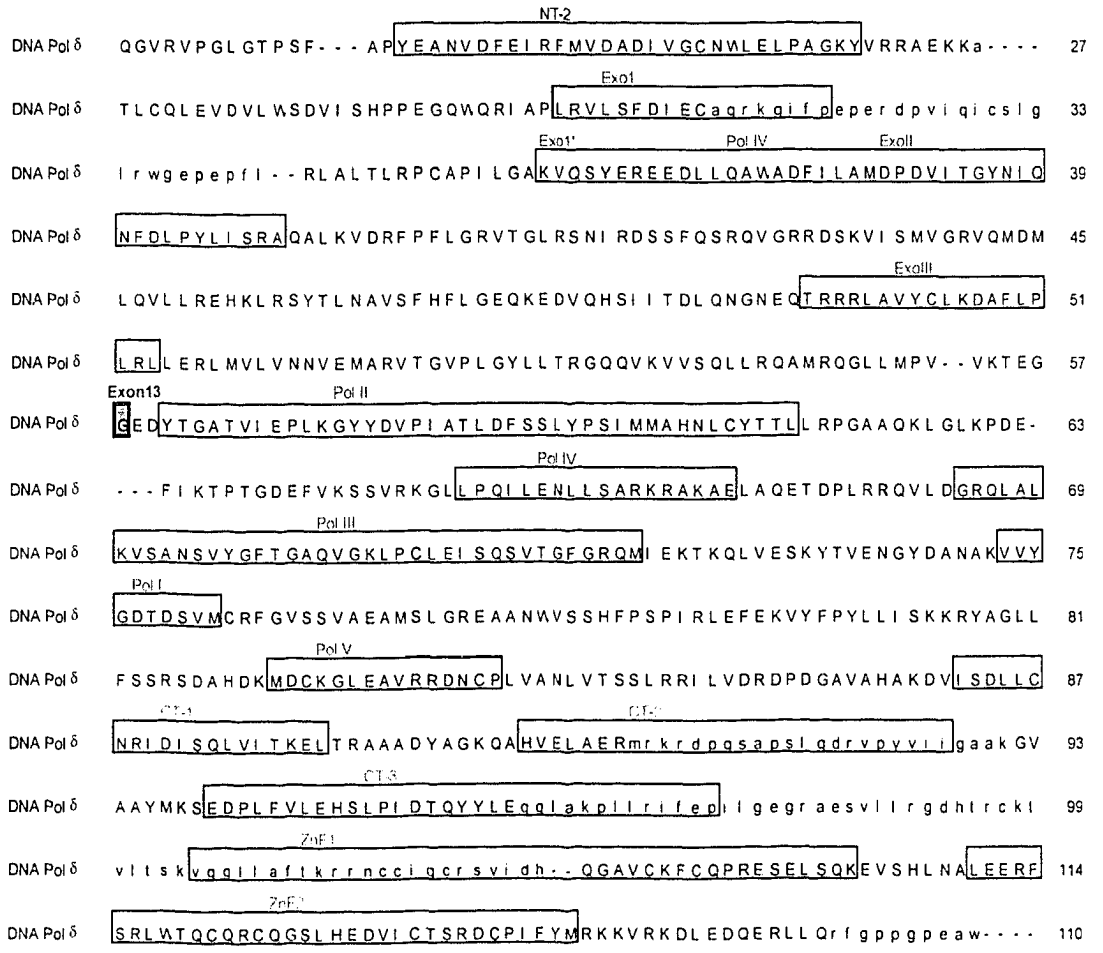

Figure 3-1: Amino acid sequence comparison of DNA Pol δ.

The regions of similarity have been boxed to ease recognition of homologous regions. Regions of high homology are shown in capital letters. This alignment shows the high degree of sequence similarity between eukaryotic DNA polymerases and the conserved functional regions. Abbreviations used stand for: **Exo:** Exonuclease domain, **Pol:** polymerization domain, **CT:** highly conserved δ-like regions named by Yang *et al.* as CT-1, CT-2, CT-3 and ZnF2, **ZnF**: potential zinc finger domains.
Sources for the DNA pol δ sequences were: Mouse (*Mus musculus*, Genbank accession number Z21848), Rat (*Rattus norvegicus*, Genbank assession number O54747), Hamster (*Mesocricetus auratus*, Genbank assession number P97283), Human (*Homo sapiens*, Genbank assession number AAA58439; Chung *et al.* 1991), Calf Thymus (*Bos taurus*, Genbank assession number M80395; Zhang *et al.* 1991), *S. cerevisiae* (Genbank assession number X15477; Morrison and Sugino, 1992), *S. pombe*, (Genbank assession number AL121815; Pignede *et al.* 1991), Drosophila (*Drosophila melanogaster* Genbank assession number P54358; Chiang *et al.* 1996)

* Location of the exon 13 DNA alteration is shown next to the Pol II domain.

Table 3-2: Comparison of the two published *Mus musculus* DNA Polymerase δ cDNA and gDNA sequences

Exon Location	Nucleotide Position	Genomic DNA		cDNA	
		Nucleotide	Amino Acid	Nucleotide	Amino Acid
3	1525	**G**GG	Glycine	**C**GG	Arginine
16	6127	GA**G**	Glutamic Acid	GA**A**	Glutamic Acid
18	8040	A**A**G	Lysine	**G**AG	Glutamic Acid
20	9124	CGA	Arginine	CG**C**	Arginine
23	9990	**T**TC	Phenylalanine	**C**TC	Leucine
24	10354	TA**T**	Tyrosine	**T**CT	Serine
25			AWKNGSLRF		ALEERFSRL

[1] The first nucleotide if the ATG start codon is designated as +316 from the genomic published sequence
[2] The published sequence by Goldsby *et al.* 1997 Genbank Accession number AF024570
[3] The published sequence by Cullmann et al. 1993
Only the nucleotides and corresponding amino acids that are different among the two sequences are shown
Contradictory nucleotides are indicated in blue

Table 3-3: Intronic Primer sets for amplification of exons 1-26 of the DNA pol δ gene

Exon	Position	Sequence of Primer[1]	Nucleotide Number[2]
1	Upstream	5'- GAGCACCACCTTGCCCACAG-3'	296-315
	Downstream	5'- GGATCTCTGTATGGGTGGG-3'	535-553
2	Upstream	5'- GCCTCTCATTTCAATCCAGGG- 3'	855-875
	Downstream	5'- TGCCTCCCCTGCCAACTCACC-3'	987-1007
3	Upstream	5'- GTGTGGCCTCCGGTCCTGAGGG- 3'	1457-1478
	Downstream	5'- CAGGTCAGGGAACACTCACC-3'	1648-1667
4	Upstream	5'- GACTGGCCTCCTACACCAGG- 3'	1709-1728
	Downstream	5'-CCACACTCACTCTCACGG-3'	1846-1865
5	Upstream	5'- CAACCTCCTTTCTCCGCAGG-3'	1911-1930
	Downstream	5'- AGGGGAGACAGGACAGCACC-3'	2098-2117
6	Upstream	5'- CTGACACGCCACTCTTCCAG-3'	2161-2180
	Downstream	5'- CACCAGGTCCTGGCTCCC-3'	2287-2304
7	Upstream	5'- ACCCGCTACCTTTCCCCAGG-3'	2329-2348
	Downstream	5'- GAGAAGGAGCCCAGGCTCCC-3'	2487-2506
8	Upstream	5'- CCCTGTGTCTTCCTCTCTGC-3'	2719-2738
	Downstream	5'- GACACAGGGCAGGTGGCACC-3'	2926-2945
9	Upstream	5'- TGTGTCCGTCCCCTCCACAGG-3'	3102-3122
	Downstream	5'-AAGATCCATCTTCCCTCACC-3'	3226-3245
10	Upstream	5'- CTCTCCCCATGCTCCTCAGG-3'	4221-4240
	Downstream	5'- CGAGTGGGGTTCCTACATACC-3'	4380-4399
11	Upstream	5'- TTGCGTGGAGATACCCTTCC-3'	4519-4538
	Downstream	5'- CTTGGTGGAGTGGCGGCAC-3'	4689-4707
12	Upstream	5'- CTGACCCCACATGCCCATCC-3'	4754-4773
	Downstream	5'- ACCAGGTGCTGTCCCTCACC-3'	4969-4988
13	Upstream	5'- ACTCCGCCCTTTCTCCAGG-3'	5032-5050
	Downstream	5'- GTGCCCAGCACAGTCCTTACC-3'	5138-5158
14	Upstream	5'- TCCCCCATTCTCCCTCAGG-3'	5281-5299
	Downstream	5'- CTAGATAAAGGCAGCATACC-3'	5415-5434
15	Upstream	5'- CCTGTGTCTCCTTCCCCAGC-3'	5620-5639
	Downstream	5'- GGCAGAGCCCACAGCCCACC-3'	5751-5771
16	Upstream	5'- TCATCCTGCCTCCACCTAGG-3'	5970-5989
	Downstream	5'- TCGGGGTCCTGAGGGCTTACC-3'	6135-6156
17	Upstream	5'- CTCCCCGACCCAGCGGAT-3'	7657-7676
	Downstream	5'- GCTGCCATGGTAGGAAGCC-3'	7844-7863
18	Upstream	5'- CCTCACGCATGTACCCTGGC-3'	7886-7905
	Downstream	5'- TGAGTACAGAGGATGCACGC-3'	8048-8067
19	Upstream	5'- GCCCATGGGTCCTTTGCAGG-3'	8533-8552
	Downstream	5'- ACTTCTTGGCCTAGGTGGG-3'	8768-8786
20	Upstream	5'- CTGTGGACATCCTCCCTCCC-3'	8987-9006
	Downstream	5'- AAGCTGTGTGGCTGCCCACC-3'	9174-9193
21	Upstream	5'- CCCCAAACCTCATCCAACCC-3'	9119-9139
	Downstream	5'- CAGCCCAGGTGGGCCTGACC-3'	9353-9372
22	Upstream	5'- CATTGGTGACCCTGGCCTGC-3'	9695-9714
	Downstream	5'- TCCTGGGCGGTCCACTTACG-3'	9853-9872
23	Upstream	5'- TTCAGCTTCCCTCCTCTAGG-3'	9921-9940
	Downstream	5'- GGCTGTAACTCGGTCTCACC-3'	10053-10072
24	Upstream	5'- ACCTCTGTGCTCACCCACCC-3'	10283-10302
	Downstream	5'- CCCTCCCTGCCTCACTCACC-3'	10364-10383
25	Upstream	5'- CAAGCCACTGTGTCCCCAGG-3'	10417-10436
	Downstream	5'- GGAGTCTGATGGCAGGAGGG-3'	10550-10565
26	Upstream	5'- ACCACCATCCTCCGCAGCC-3'	10825-10844
	Downstream	5'- ATTCCTTGTCCCGTGTCAGG-3'	10949-10969

[1]primer sequences correspond to the gDNA reported by Goldsby *et al.* 1998; Genbank Accession number AF024570.
[2] the first nucleotide of the ATG start codon is designated +316.

Table 3-4: DNA polymerase δ PCR conditions

Exon Number	Annealing Temperature	$MgCl_2$ Concentration
Exon 1	*TD 51° C	1.0 mM
Exon 2	60° C	1.0 mM
Exon 3	*TD 51° C	1.0mM
Exon 4	57° C	1.0 mM
Exon 5	57° C	1.0 mM
Exon 6	59° C	1.0 mM
Exon 7	57° C	1.0 mM
Exon 8	57° C	0.75 mM
Exon 9	57° C	1.0 mM
Exon 10	59° C	1.0 mM
Exon 11	58° C	1.0 mM
Exon 12	60° C	1.5 mM
Exon 13	*TD 60° C	1.0 mM
Exon 14	60° C	1.0 mM
Exon 15	57° C	1.0 mM
Exon 16	58° C	1.5 mM
Exon 17	57° C	1.0 mM
Exon 18	57° C	0.75 mM
Exon 19	56° C	0.75 mM
Exon 20	52° C	0.75 mM
Exon 21	58° C	1.0 mM
Exon 22	*TD 51° C	1.0 mM
Exon 23	58° C	2.0 mM
Exon 24	58° C	1.0 mM
Exon 25	58° C	1.0 mM
Exon 26	54° C	1.5 mM

*TD - Touchdown PCR protocol - decreasing annealing temperatures starting at 64° C with replication cycles occurring at indicated temperature
$MgCl_2$-final $MgCl_2$ concentration in 25*ul* PCR reaction

Table 3-5: Nucleotide sequence variations found in *Msh2*$^{-/-}$ thymic lymphomas and *Msh2*$^{+/+}$ control tissues vs. DNA polymerase δ published sequences

	Msh2$^{-/-}$ Thymic lymphomas [1]			*Msh2*$^{+/+}$ Controls [2]		gDNA published sequence [3]		cDNA published sequence [4]		Effects of Sequence Alterations in tumours
Exon	Nucleotide Position *	Nucleotide Sequence	Amino Acid	Tumour ID	Nucleotide Sequence	Amino Acid	Nucleotide Sequence	Amino Acid	Nucleotide Sequence	Amino Acid	
3	1517	CCT	L	B, H, I	CTG	L	CTG	L	CTG	L	silent change
12	4876	AAT	N	B,C,D,G	AAT	N	AAC	N	AAC	N	silent change
13	5095	AGT	S	B,C,D,H	AGT	S	GGT	G	GGT	G	conservative amino acid change
17 intronic	7713	TAA	N/A	B,D,I	TAA	N/A	TGA	N/A	N/A	N/A	intronic polymorphism
3	1525	GGGs	G	ALL	GGG	G	GGG	G	CGG	R	
24	10354	TAT	Y	ALL	TAT	Y	TAT	Y	TCT	S	
18	8040	GAG	E	ALL	GAG	E	AAG	K	GAG	E	
23	9990	CTC	L	ALL	CTC	L	TTC	F	CTC	L	

[1] *Msh2*$^{-/-}$ thymic lymphoma DNA polymerase δ sequence
[2] *Msh2*$^{+/+}$ normal brain or liver DNA polymerase δ sequence
[3] DNA polymerase δ published sequence; Goldsby *et al.* 1998; GenBank accession number AF024570
[4] DNA polymerase δ published sequence; Cullmann *et al.* 1993; GenBank accession number Z21848
*Nucleotide position based on Goldsby *et al.* 1998 published sequence; the first nucleotide of the ATG start codon is designated +316
Contradictory nucleotides are indicated in blue

Discussion

Mice lacking the DNA MMR gene, *Msh2*, have been developed as a model for studying the relationship between hypermutability and hereditary cancer development. The *Msh2*$^{-/-}$ mice develop predominantly thymic lymphomas from 2-6 months of age. It is hypothesized that the hypermutability resulting from the absence of post-replicative DNA MMR leads to a greater overall genomic mutation rate, hastening the acquisition of mutations that result in loss of tumour suppressor genes and/or activation of oncogenes, and propensity towards uncontrolled proliferation and tumorigenesis. *Msh2*$^{-/-}$ thymic lymphomas demonstrate a hypermutator mutation frequency 3 to 17 fold higher than non-tumour *Msh2*$^{-/-}$ tissues (Baross-Francis *et al.*, 1998). The hypermutator effect seen in *Msh2*$^{-/-}$ thymic lymphomas is specific to MMR deficient thymic lymphomas (Baross-Francis *et al.*, 2000). Interestingly, thymic lymphomas in p53 deficient mice do not demonstrate an increased mutation frequency (Buettner *et al.*, 1996). In *Msh2*$^{-/-}$ thymic lymphomas I hypothesized that an additional mutator gene (in conjunction with MMR loss) was responsible for this increased hypermutator effect. DNA replicative slippages as a result of a mutated DNA polymerase may very well account for the observed hypermutator effect and therefore a likely hypothesis to explain the increase in mutation frequency. Previous studies of human MMR deficient tumours have made use of the likelihood of DNA polymerase slippage and frame shift mutations occurring at short (greater than five bp) mononucleotide repeats to successfully identify downstream genes involved in tumourigenesis (Akiyama *et al.*, 1997a; Markowitz *et al.*, 1995; Percesepe *et al.*, 1998; Rampino *et al.*, 1997; Souza *et al.*, 1996; Takenoshita *et al.*, 1997). This justified a similar candidate gene approach for a mutated DNA polymerase in murine MMR deficient thymic lymphomas.

In an effort to identify genes mutated in murine thymic lymphomas lacking MMR, I chose to investigate the catalytic subunit of DNA pol δ as a likely candidate gene based on a) the presence of six coding mononucleotide repeats greater than five bp in length b) the hypermutator phenotype of *S. cerevisiae* DNA pol δ mutants c) the synergistic effect of

DNA pol δ and *Msh2*$^{-/-}$ in *S. cerevisiae* d) likelihood a polymerase mutation could account for the clustering of DNA mutations seen in the thymic lymphomas and e) DNA pol δ is mutated in human sporadic colorectal cancers and colorectal cell lines, thus implicating it in tumorigenesis.

I sequenced all 26 exons of the DNA pol δ gene in genomic DNA from nine *Msh2*$^{-/-}$ thymic lymphomas and two wild type control tissues from *Msh2*$^{+/+}$ littermates. No mutations believed to be associated with lymphomagenesis were found in the coding region of DNA pol δ. Notably, the six coding mononucleotide repeat sequences were not mutated, suggesting that polymerase slippages in the absence of MMR do not result in mutations of the mononucleotide tracts of DNA pol δ.

Several polymorphic DNA alterations were seen in the tumours and controls (Table III). However, the identified changes seen in the lymphomas are unlikely to be disease-associated as they were observed in both tumour and non-tumour control tissue. Four of the alterations differ from either the genomic DNA or cDNA published sequence (exons 3, 18, 23, 24 and intron 17) and most likely demonstrate sequencing errors of the cDNA or gDNA or differences in genetic backgrounds of the mice. The *Msh2* mice are on a mixed genetic background of BalbC and 129 mouse strains. The published genomic sequence of DNA pol δ is from the 129SVJ mouse strain and the cDNA sequence from the BalbC/9 strain. It is possible however, that the DNA alterations resulting in amino acid changes may affect fidelity of the enzyme itself, however, further functional analyses are required to confirm this.

The intronic sequence alteration seen in intron 17 is believed to be non-pathogenic as it does not alter the splice site junction of intron 17-exon 17. A DNA alteration in exon 13 resulted in a polymorphic amino acid in 4/9 tumours sequenced. This sequence was not in either the genomic or cDNA published sequence but is not believed to be disease associated as it was also found in *two* separate non-tumour tissues. Furthermore, this DNA alteration results in a conservative substitution of a serine replacing a glycine. Both are

neutral, uncharged amino acids and the substitution is not predicted to result in altered protein function. Although this change is not likely associated with tumorigenesis, it is located adjacent to the hypothesized polymerization II (Pol II) domain of DNA pol δ (Figure 3-1) (Cullmann *et al.*, 1993). The close proximity of this polymorphism to the Pol II polymerization domain suggests that this change could subtly affect enzyme function or fidelity contributing to the development of neoplasia.

In the absence of MMR, mononucleotide repeats of five bp or greater are prone to mutation through expansion and contraction of these repeats. Evidence supporting the involvement of DNA pol δ in *Msh2*$^{-/-}$ thymic lymphoma development and the presence of six coding mononucleotide repeats led to the sequencing of the 26 exons of the DNA pol δ gene in nine *Msh2*$^{-/-}$ thymic lymphomas. No lymphoma associated mutations were found in the coding region of DNA pol δ. The absence of repeat tract mutation indicates that the DNA pol δ gene is not a non-random target for hypermutation-driven mutagenesis in *Msh2*$^{-/-}$ murine thymic lymphomas. Continued investigation of other possible candidate genes is required to further understand the molecular events underlying thymic lymphoma development in the Msh2 deficient mouse.

Chapter 4 ♦ Spectral Karyotype analysis of murine *Msh2*$^{-/-}$ thymic lymphomas and a rare human individual with constitutive loss of MSH2*

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* *A version of this chapter has been submitted for publication*

Marcia R. Campbell, Jane Bayani, Jeremy Squire, Susan E. Andrew (2005)

All of the experiments presented in this chapter were done by Marcia Campbell. while on exchange to Dr. Jeremy Squire's lab at the Ontario Cancer Institute, Toronto, Ontario Canada

Introduction

It has been proposed that tumours require genomic instability in order to accumulate sufficient mutations required for transformation (Cheng & Loeb, 1993; Hartwell, 1992). This can be at the level of microsatellite instability (MSI+) or at the chromosomal level.

Mice deficient in *Msh2*$^{-/-}$, *Mlh1*$^{-/-}$, *Pms2*$^{-/-}$ and *Msh6*$^{-/-}$;*Msh3*$^{-/-}$ demonstrate MSI+ (Baker *et al.*, 1995; Edelmann *et al.*, 1996; Edelmann *et al.*, 2000; Prolla *et al.*, 1998; Reitmair *et al.*, 1995). Mice that exhibited increased levels of mutation were all prone to tumorigenesis (Buermeyer *et al.*, 1999). Although *Msh6*$^{-/-}$ mice do not display a MSI+ phenotype they have been shown to be deficient in base mispair repair and are therefore predicated to have increased base substitution mutations (Edelmann *et al.*, 1997). In contrast, *Msh3*$^{-/-}$ and *Pms2*$^{-/-}$ mice do not display MSI+ and are in turn not prone to tumorigenesis (Edelmann *et al.*, 2000; Prolla *et al.*, 1998). Thus, the tumour and mutator phenotypes correlate in MMR-deficient mice, supporting the concept that genomic instability is necessary for tumorigenesis (Buermeyer *et al.*, 1999). Are the levels of MSI+ seen in normal and tumour tissues in MMR deficient mice high enough to allow for the cell to acquire numerous genetic lesions disrupting genes that regulate such functions as growth, cell cycle or cell death and therefore lead to the dramatic tissue specific tumorigenesis that is seen in the mouse models of MMR? I suggest that given the levels of instability necessary to drive tumorigenesis and that human lymphomagenesis and leukemiagenesis often coincides with the presence of chromosomal instability (CIN), MMR deficient lymphomas may present with CIN as well as MSI+. However, no cytogenetic analysis of murine MMR deficient tumours has been undertaken and is the focus of this chapter.

Little is known of cytogenetic changes underlying the development of T cell lymphomas in humans. Normal human T-cells do not express the genes *Rhombotin-2 (RBTN-2), HOX-11* or *TAL-1*. However, these proto-oncogenes have been identified as activating events in the development of human precursor T-cell neoplasias (Cline, 1994). Chromosomal rearrangements, deletions or point mutations are thought to cause aberrant expression of

RBTN-2, HOX-11 and *TAL-1* but the molecular mechanism underlying abnormal expression is not well understood. Lowsky *et al.* have shown that the expression profiles of *RBTN-2, HOX-11* and *TAL-1* in murine *Msh2*$^{-/-}$ thymic lymphomas were nearly identical to that of human lymphomblastic lymphomas (LBLs) (Lowsky *et al.*, 1997). The finding that 20% of human LBLs tested had mutations within the coding sequence of *MSH2*, predicted to result in loss of gene expression (Lowsky *et al.*, 1997) is also supportive of a role for MMR in lymphomagenesis. The absence of MSH2 expression may contribute to chromosomal aberrations and instability leading to tumorigenesis.

It is possible that loss of MMR results in mutations in other genes associated with the CIN phenotype. Cahill *et al.* have shown that the loss of function of a mitotic check point is consistently associated with CIN (Cahill *et al.*, 1998). In some human cancers with the CIN phenotype, mutation of the human BUB1 gene is hypothesized to be the cause (Cahill *et al.*, 1998) as BUB1 is known to control mitotic checkpoints and chromosome segregation in yeast. In MSI+ cells with normal mitotic check points, transfer of mutant hBUB1 alleles from CIN tumours results in abnormal check points possibly leading to aneuploidy (Cahill *et al.*, 1998).

Early experiments demonstrated that MMR deficient cell lines and/or tumors from HNPCC patients are generally characterized by diploid or near diploid karyotypes compared to microsatellite stable (MSS) cell lines that are often characterized by higher levels of chromosomal instability (Abdel-Rahman *et al.*, 2001). However, several groups have shown CIN in cell lines or tumours characterized by MSI+ (Abdel-Rahman *et al.*, 2001; Curtis *et al.*, 2000; Melcher *et al.*, 2002; Planck *et al.*, 2000; Tsushimi *et al.*, 2001). Several MSI+ cell lines have been karyotyped; LoVo (*MSH2*$^{-/-}$), HCT-15 (*MSH6*$^{-/-}$), HCT116 (*MLH1*$^{-/-}$) (Abdel-Rahman *et al.*, 2001; Melcher *et al.*, 2002; Tsushimi *et al.*, 2001). The LoVo cell line has been characterized as aneuploid with all three groups reporting structurally aberrant chromosomes; translocations, deletions, and breakpoints (Abdel-Rahman *et al.*, 2001; Melcher *et al.*, 2002; Tsushimi *et al.*, 2001). HCT116 was found to contain translocations as well partial chromosome gains and losses as resolved by

comparative genomic hybridization (CGH) analysis (Abdel-Rahman *et al.*, 2001). HCT-15 was generally diploid (45-47) in addition to containing translocations, inversions and deletions (Chen *et al.*, 1995; Melcher *et al.*, 2002). Planck *et al.* investigated CIN in a cell line cultured from a colon tumour from a HNPCC *MLH1*$^{+/-}$ patient. Flow cytometric analysis revealed stable numerical changes (Planck *et al.*, 2002). Recently, Abdel-Rahman *et al.* showed that restoring MMR in a CIN cell line (HCA7) deficient in MMR (*MLH1*$^{-/-}$) does not restore chromosomal stability but rather promotes numerical alterations after irradiation (Abdel-Rahman *et al.*, 2005). These results suggest that in the absence of MMR, chromosomal numerical changes as well as structural aberrations may be occurring leading to tumorigenesis. I hypothesize that in MMR deficient tumour development, sufficient genomic instability required for transformation is occurring through both MSI+ and CIN.

Microsatellite analysis of *Msh2*$^{-/-}$ thymic lymphomas (Chapter 2) demonstrates these tumours are microsatellite unstable (MSI+), although are more stable than predicted However, the lower degree of MSI+ may indicate an alternative genomic instability pathway that also contributes to tumorigenesis in the murine MMR deficient tumours. I hypothesized that in addition to MSI+, *Msh2*$^{-/-}$ thymic lymphomas are characterized by chromosomal instability (CIN) and together these two pathways of genomic instability are driving tumorigenesis in this murine model. In the experiments described here, spectral karyotyping (SKY) was used to karyotype thymic lymphomas arising in the MSH2 deficient mice and in a cell line derived from a rare human individual with constitutive loss of MSH2 to test this hypothesis.

Materials and Methods

Examination of Mice

Mice were monitored daily for signs of poor health/grooming or tumorigenesis. Mice were sacrificed by isoflurane overdose upon signs of lethargy, weight loss or physical demonstration of a tumour. Murine tissues were fixed in 10% buffered formalin and embedded in paraffin blocks. Histological analysis was performed on 5μm sections stained with hematoxylin and eosin using standard methods. Tumour tissue specimens were

confirmed to be lymphomas after histological examination or by gross thymus enlargement.

Human cell culture

Whole blood from an MSH2 null individual was used to generate an EBV transformed cell line (The Hospital for Sick Children in Toronto). This cell line was designated KM and was used for the SKY experiment performed here. An MMR competent EBV transformed cell line was used as a control.

Murine Cell Culture

Upon necropsy, sections of thymic lymphomas were removed, and placed in small petri dishes with ~1-1.5 ml RPMI 1640 cell culture media at 37°C with 100U/ml antibiotic in media. Sterile scissors were used to mince the tissue and release cells into the cell culture media. A rubber policemen was used to disaggregate the tumour. As well, the solution was pipetted up and down several times in a 5 or 10 ml pipette and filtered through a 70μm cell culture filter to collect single cells into a 50ml conical tube. Cells were counted using a hemocytometer cell counter. Cells were grown at a concentration of 5 million cells/ml in 6 well suspension plates with IL2 to a final concentration of 30U/ml and IL7 to a final concentration of 50U/ml. IL2 and IL7 was added every 3-4 days when the cells were split.

Metaphases preparation from patient KM lymphocytes

Cells were grown in RPMI 1640 until media started to turn colour, approximately 48hrs. Cell were split 1:2 and harvested for metaphases the following day as described below.

Metaphase preparation from murine thymic lymphoma cells

Cells were grown in culture for 48-72 hrs. until media started to change colour. Cells were then split 1:2 or 1:3 and treated with IL2 and IL7 (30U/ml and 50U/ml respectively). 24 hrs. after cells were split, cells were harvested for metaphases. To harvest cells, cells were pelleted and the supernatant was removed leaving 1ml of solution remaining in which to resuspend cells. Cells were hypotonically swollen with 75 mM KCL at 37°C for 20-25 minutes. Cells were pre-fixed by adding 5-10 drops of fixative (3:1 acetic acid:methanol at

-20°C) and then pelleted. Cells were resuspended in fixative and left overnight at -20°C. Metaphase spreads were obtained by dropping fixed cells onto wet slides and air drying the slides.

Spectral Karyotyping (SKY)

Fresh cytogenetic slides were made from thymic lymphoma and patient KM samples that produced metaphases. Care was taken to ensure slides had dense metaphase counts with low cytoplasm surrounding the metaphases.

Hybridization and detection of the SKY probe (Applied Spectral Imaging Inc., San Diego, CA) was done according to the manufactures instructions with modifications as described. *Pepsin Treatment:* To permeabilize the chromosomes so the SKY probes can bind the DNA, slides were treated with 0.003% pepsin dissolved in 10mM HCL solution at 37°C for 5 min. Slides were washed in PBS for 5 minutes. Slides were then washed in $MgCl_2$/PBS for 5 minutes. Slides were then treated with 1% formaldehyde for 10 minutes according to manufacturer's instructions. Slides were washed in PBS for 5 minutes. Slides were then dehydrated through a series of ethanol washes by placing each slide in 70%, 90% and finally 100% ethanol. Slides were air dried. *Chromosome Denaturation*: Denaturation solution (70% formamide/2X SSC) was heated to 75°C. Slides were placed in solution for 1.5 minutes and immediately placed in cold ethanol; 70%, 80%, 100% ethanol for 2 minutes each and allowed to air dry. *Probe Denaturation*: 10µl of mouse SKY probe (Applied Spectral Imaging) was placed in a 0.6ml tube , mixed well and placed at 80°C. After heating it was placed at 37°C until ready for use. The probe (10µl) was applied to a 24 X 30 cover slip and a slide with the denatured chromosomes was placed on top pressing out any bubbles. Edges were sealed with rubber cement to ensure that the probe did not dry out. Slides were placed at 37°C for 48 hrs. *Probe Detection*: Slides were washed with 50% formamide/2 X SSC for 15 minutes at 45°C then washed twice with 1XSSC at 45°C and finally washed with 4 XSSC/0.1% tween 20 at 45°C for 2 minutes. *Antibody application*: Excess solution was drained from the slides. 80µl of manufacturer's 'vial 3' was applied and incubated in a humidified chamber for 50 minutes at 37°C. Slides were washed in 4 X

SSC/0.1% tween 20 at 45°C for 3 minutes and repeated 3 times. Slides were then incubated in 'vial 4' for 1 hour at 37°C. Slides were washed in 4 X SSC/0.1% tween 20 at 45°C for 3 minutes and repeated 3 times. Slides were washed briefly in water and air dried. Metaphases were counter stained with DAPI/antifade (Vectashield mounting medium, Vector Laboratories). Images were acquired using the Skyvision spectral imaging system (Applied Spectral Imaging).

Results

SKY analysis of Msh2$^{-/-}$ murine thymic lymphomas

Msh2$^{-/-}$ thymic lymphomas were collected and cells cultured for SKY analyses. Over 30 thymic lymphomas were originally harvested for culture, however, many of the primary cultures did not survive long enough for analysis. Only ten cultures resulted in metaphases and seven *Msh2*$^{-/-}$ murine thymic lymphomas were successfully characterized by SKY analysis. Results are based on the analysis of ten metaphases per thymic lymphoma unless otherwise stated and cytogenetic aberrations are listed in Table 4-1.

Table 4-1: Summary of SKY data on *Msh2*$^{-/-}$ murine thymic lymphoma tumours

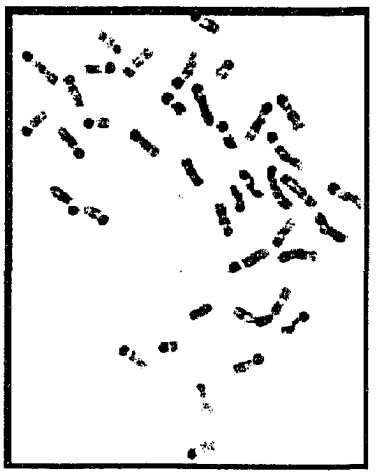
Tumour ID	Number of Metaphases Analyzed	SKY	
1072	16	Clonal Karyotype:	39, XX,der(3)t(3;11),-11 [5/16] (Figure 4-1)
		Variant Karyotype:	40,XX,der(3)t(3;11),+5,-11 [3/16]
		Other Karyotypes:	39,XX,balanced robertsonian translocation ch 2 [3/16]
		Normal:	40,XX [2/16]
		Other:	Remaining cells show non-random chromosomal changes including unbalanced translocations, whole chromosomal gains and losses
1211	10	Clonal Karyotype	40,XY,der(7)(t(7;16),-16,der(18)t(12;18),+del(18)(?) (Figure 4-2)
		Other:	Remaining cells show whole chromosomal gains and losses
1257	16	Other:	No clonal aberrations detected. Unbalanced translocations and whole chromosome gains and losses were detected. Ploidy ranged from 39-44.
1183	7	Other:	No clonal aberrations detected. Unbalanced translocations and whole chromosome gains and losses were detected. Ploidy ranged from 37-40.
2N539	12	Other:	No clonal aberrations detected. Whole chromosome gains and losses were detected.
1184	10	Normal:	Of the 10 cells karyotyped, all were normal: 40,XX
1259	10	Normal:	Of the 10 cells karyotyped, all were normal: 40,XX

In summary, 6/8 *Msh2*$^{-/-}$ thymic lymphomas were found to have aberrant karyotypes. In all five cases, ploidy changes were present with whole chromosome gains and losses present. 4/8 *Msh2*$^{-/-}$ thymic lymphomas analyzed had translocations and rearrangements (Figures 4-1 and 4-2). 2/8 had clonal rearrangements. No chromosomal alterations involving a specific chromosome were observed.

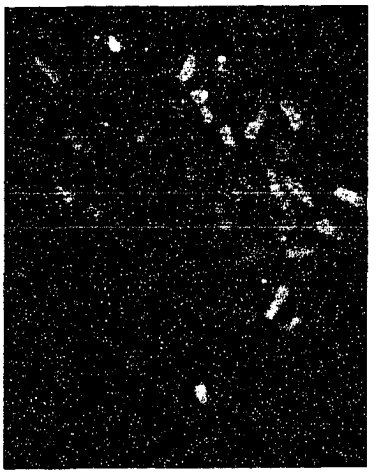
Figure 4-1: Spectral Karyotyping of an *Msh2*$^{-/-}$ thymic lymphoma (#1072).

A). One representative metaphase shown, counter stained with dapi
B) The same metaphase as shown in A), with the fluorescent chromosome paints shown.
C) Karyotype of the metaphase shown in A) and B). The karyotype is representative of the most common clonal karyotype seen in thymic lymphoma #1072 (5/16 metaphases analyzed): 39, XX,der(3)t(3;11),-11

A



B



C

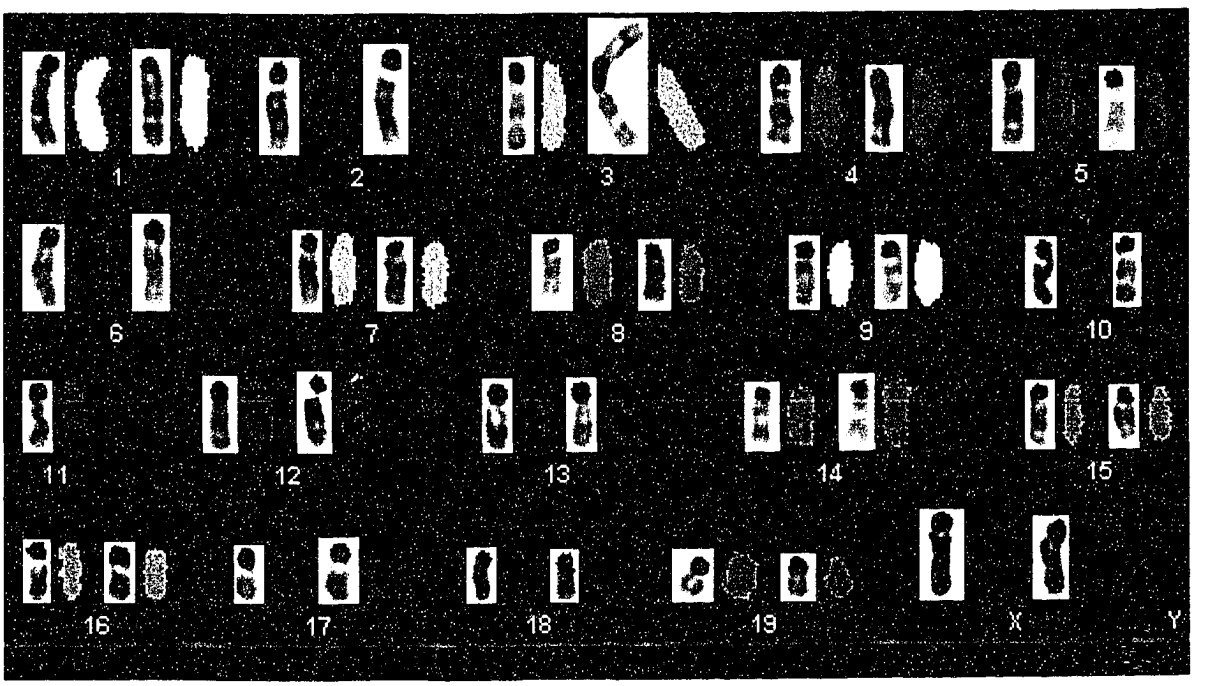
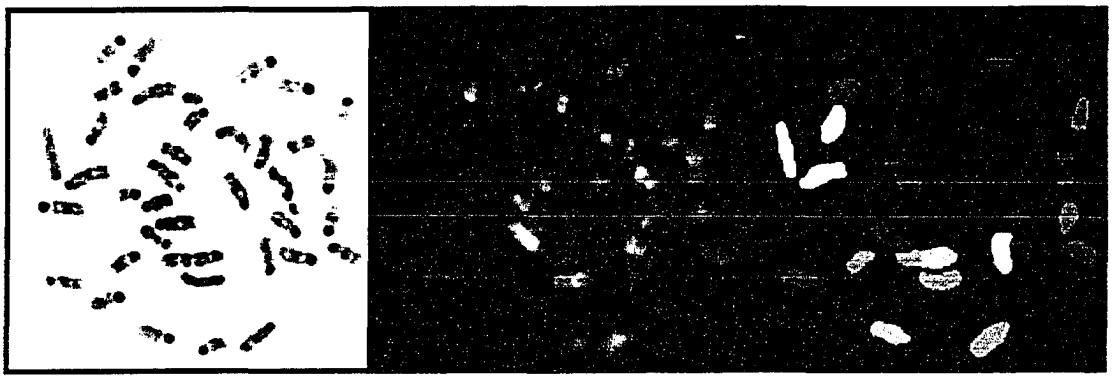


Figure 4-2: Spectral Karyotyping of an *Msh2*$^{-/-}$ thymic lymphoma (#1211).

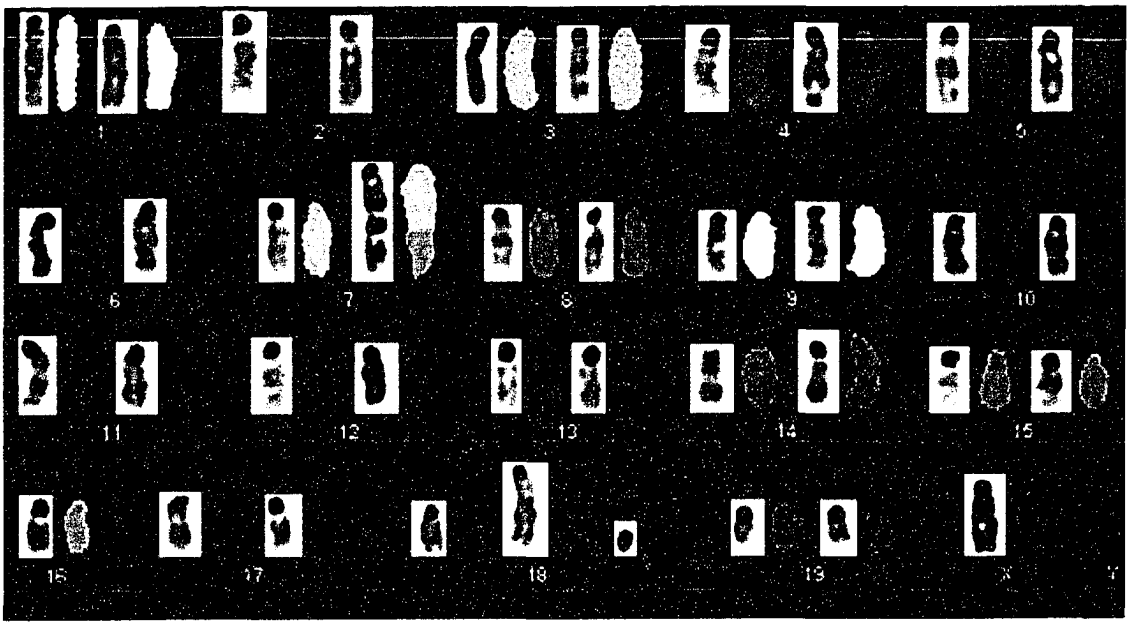
A). One representative metaphase shown, counter stained with dapi
B) The same metaphase as shown in A), with the fluorescent chromosome paints shown.
C) The same metaphase as shown in A) and B) with the chromosomes artificially coloured
D) Karyotyped metaphase from A) and B) representative of the most common clonal karyotype seen in the thymic lymphoma #11211: 40,XY,der(7)(t(7;16),-16,der(18)t(12;18),+del(18)(?)

Of the ten metaphases analyzed there were also whole chromosomal gains and losses

A B C



D



A cell line established from lymphoblasts from a rare individual with constitutive loss of MSH2 was also analyzed by SKY (Figure 4-3). Twenty one metaphases were karyotyped. 5/21 of the metaphases were abnormal, with the following karyotypes:

46,XY,dic(7;22)(q36.3;p13)
46,XY,dic(3;22)(p26;q13.3),
46,XY.dic(2;3)(q37.3;p26.3),
46,XY,dic(7;7)(q36.3;q36.3),
33, incomplete r(8).

All aberrations involved telomeres. Of the 4 dicentric chromosomal aberrations detected, 2 involved the same chromosome 22, but different arms. Chromosome 3 was also involved in 2/4 dicentric chromosomes involving q26.3. It was not possible to tell whether the same chromosome 3 was involved in the two aberrations.

Chromosome 7 was involved in 2/4 dicentric chromosomes, 1 involving itself at q36.3 and the other with chromosome 22 at q36.3 (Figure 4-3). It's not possible to tell whether the same chromosome 7 was involved in the aberrations (Figure 4-3). One ring chromosome of chromosome 8 was detected in a partial metaphase (Figure 4-3). One endoreduplicated metaphase was detected and was normal.

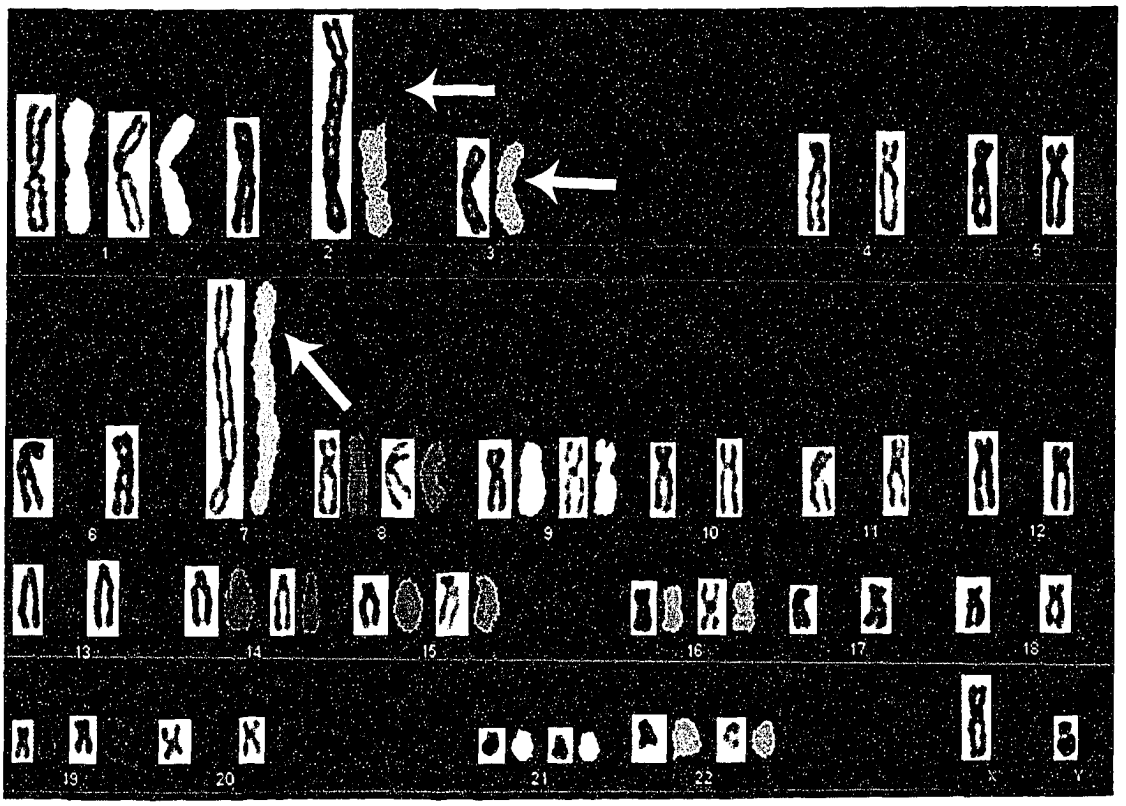
Figure 4-3: Spectral Karyotype Analysis of a cell line derived from a human patient deficient in MSH2.

A) A karyotyped metaphase seen in the human patient cell line deficient in MSH2. 46,XY.dic(2;3)(q37.3;p26.3),dic(7;7)(q36.3;q36.3) Arrows indicate the involved chromosomes.

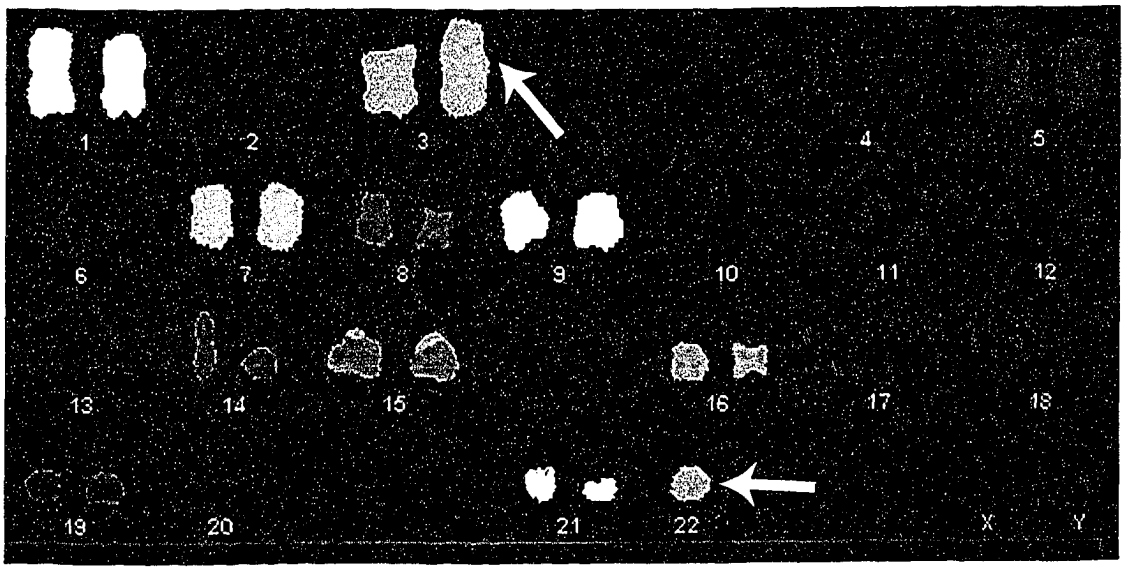
B) A karyotyped metaphase seen in the human patient cell line deficient in MSH2. 46,XY,dic(3;22)(p26;q13.3). Arrows indicate the involved chromosomes.

All aberrations in this cell line involved the telomeres with no whole chromosomal gains or losses occurring

A



B



Discussion

Earlier research has led to the dogma that cancers demonstrating the microsatellite unstable phenotype generally do not show the chromosomal instability (CIN) phenotype and the inverse is true (Lengauer *et al.*, 1998). Cells without functional MMR present with MSI+ at mono and dinucleotide repeats. However, results presented in chapters 2 and 3 show that in the absence of MMR murine thymic lymphomas demonstrate lower than expected levels of MSI+ as well as low mutation rates of coding microsatellites. Given that human lymphomagenesis and leukemiagenesis often coincides with the presence of CIN, MMR deficient lymphomas may present with CIN, as well as MSI+. I hypothesized that the CIN phenotype may occur in MMR deficient thymic lymphomas.

Chromosomal instability has never before been investigated in MMR deficient murine lymphomas. I propose that the chromosomal instability seen in the *Msh2*$^{-/-}$ thymic lymphomas is a 'dynamic' or 'continuous' form of instability that is constantly changing. I believe this contributed to the difficulty in the culturing of primary cells from the thymic lymphomas, making analysis extremely difficult. However, I was successful in karyotyping eight *Msh2*$^{-/-}$ thymic lymphomas. Six of the eight demonstrated aberrant karyotypes with various chromosome gains and losses, translocations and rearrangements. No chromosomal alterations involving a specific chromosome were observed suggesting no particular chromosomal alteration underlies lymphomagenesis. This novel finding shows that MSI+ and CIN may be functioning together to generate sufficient genetic instability that tumorigenesis is induced. My results suggest that CIN and MSI+ do not have to be mutually exclusive pathways, but that both types of genomic instability can arise and contribute to tumorigenesis.

Data in Chapter 5 shows that in the absence of MMR, hyperamplification of centrosome proteins occurs. This could result in abnormal numbers of functional spindle poles leading to inaccurate chromosomal segregation during cell division. I suggest that this increase in centrosome numbers may lead to chromosomal aneuploidy seen here as well as

translocations and rearrangements. Further analysis is necessary to confirm that centrosome abnormalities exist in the *Msh2*$^{-/-}$ thymic lymphoma tumours.

Previous investigation of a connection between MMR and CIN has used cultured cell lines that have been grown for successive generations and may have acquired alterations that are due to cell culture and are unrelated to MMR status. For example, the LoVo cell line (*MSH2*$^{-/-}$) has been characterized as aneuploid with structurally aberrant chromosomes; translocations, deletions, and breakpoints (Abdel-Rahman *et al.*, 2001; Melcher *et al.*, 2002; Tsushimi *et al.*, 2001). I have used primary culture to assay for instability and it is unlikely that the aberrant chromosomes arose as a result of cell culture effects, suggesting the aberrant karyotypes are associated with tumorigenesis arising in the absence of MMR.

In collaboration with Drs. Ross Macleod (U of Calgary) and Gail Graham (U of Ottawa), our laboratory identified a 24 month old proband with a novel homozygous mutation in the *MSH2* gene. This individual presented with acute lymphocytic leukemia (T-cell ALL) and was successfully treated by chemotherapy (Whiteside *et al.*, 2002). A lymphoblastoid cell line derived from this patient was analyzed by SKY. Although, this cell line was derived from normal lymphoblasts, I hypothesized that chromosomal instability may be occurring for several reasons i) constitutive loss of MMR in human patients has been shown to result in hematological malignancy (Bougeard *et al.*, 2003; De Vos *et al.*, 2004; Gallinger *et al.*, 2004; Ricciardone *et al.*, 1999; Vilkki *et al.*, 2001; Wang *et al.*, 1999a; Whiteside *et al.*, 2002). Thus, the lymphoblastoid cell line was created from a cell type that is prone to tumorigenesis in the absence of MMR (further discussed in Chapter 6) and an underlying level of ongoing instability may be occurring. ii) CIN is a common feature of hematological malignancy iii) our laboratory found no evidence of MSI+ using DNA from this patient suggesting that other forms of instability are functioning to drive tumorigenesis (Whiteside *et al.*, 2002) and iv) chromosomal instability analysis on murine MMR deficient lymphomas indicates that CIN may be a feature of MMR deficient hematological malignancies and may be an important mechanism generating genomic instability in the pathway to tumorigenesis. SKY analysis of the cell line derived from this patient revealed

that almost one quarter (5/21) of the metaphases analyzed demonstrated abnormal karyotypes with four of the five abnormal metaphases presenting with translocations and rearrangements. Of the 21 metaphases examined, dicentric chromosomes were observed with high frequency suggesting chromosomal breakage and improper re-annealing has occurred. As well, all of the aberrations involved the telomeres. This suggests that MMR may play a role in the maintenance of telomere function and in its absence increased telomere rearrangements may suggest a role for MMR in the maintenance of telomere function (described in more detail in Chapter 5).

The existence of chromosomal aberrations (chromosome gains, losses, rearrangements) in both murine Msh2 null tumours and a human lymphoblastoid cell line lacking MSH2 is a novel finding. This suggests that the coexistence of both a MS+ and CIN phenotype can occur, raising the instability index and suggesting that together these two instability processes contribute to malignancy.

These results suggest that in the absence of MMR, chromosomal numerical changes as well as structural aberrations may be occurring leading to tumorigenesis. I hypothesize that in MMR deficient tumour development, sufficient genomic instability required for transformation is occurring through both MSI+ and CIN.

Chapter 5 ♦ Loss of mismatch repair results in centrosome amplification and chromosomal abnormalities but does not effect telomeres or telomerase activity*

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* *A version of this chapter has been submitted for publication*

Marcia R. Campbell, Susan E. Andrew, Yie Liu (2005) *PNAS* (submitted).

In this chapter I worked with Dr. Yie Liu. I carried out immunofluorescent staining of centrosome proteins. as well, G-banding and karyotype analysis of MEFs. Dr. Yie Liu performed the telomere length and telomerase assays.

Introduction

Normally, human somatic cells have a limited proliferative capacity (Hayflick, 1965). To move beyond this limit and become immortalized, cells must overcome two barriers, termed senescence and crisis. Crisis is the result of telomere shortening which occurs with each successive round of DNA replication due to the inability of DNA polymerases to completely replicate the ends of chromosomes (reviewed in Stewart & Weinberg, 2000). Critically short telomeres will not protect the ends of chromosomes from rearrangement, and the subsequent loss of telomere function may result in chromosomal rearrangements and genomic instability. To overcome crisis, cells can maintain telomere length by 1) activating telomerase or 2) through recombination of chromosome ends using the alternative lengthening of telomeres (ALT) pathway.

Telomerase is a ribonucleoprotein reverse transcriptase that adds DNA telomere repeats to the ends of chromosomes to maintain and/or extend telomere sequences (Greider & Blackburn, 1987). With few exceptions, most human somatic cells do not express telomerase. Therefore in normal somatic tissues, telomeres become critically short signaling to cells that a state of crisis as been reached. In contrast, greater than 90% of human cancers express telomerase *in vivo* allowing cells to maintain telomere length and escape crisis. However, not all cancers express telomerase. Telomere maintenance can occur in the absence of telomerase through ALT pathway. Using the ALT pathway telomere repeat lengths can vary widely from one cell cycle to the next, up to several kilo bases. In contrast, tumour cells that have activated telomerase display shorter but more stable telomere lengths (Bryan *et al.*, 1997; Bryan *et al.*, 1995; Murnane *et al.*, 1994). The instability seen in ALT cells is suggestive of a recombination mechanism of maintaining telomeres as first suggested by Le *et al.* (Le *et al.*, 1999). Although the majority of human cancers maintain telomere length through the activation of telomerase, the ALT pathway is an important alternative mechanism of telomere maintenance. In the advent of telomerase directed therapy, the ALT mechanism may serve as selective resistant pathway that is refractory to telomerase directed treatment.

The homologous recombination used by the ALT pathway would involve many of the same mechanisms and proteins involved in mitotic and meiotic homologous recombination. MMR is known to be involved in homologous recombination (Elliott & Jasin, 2001; Mohindra *et al.*, 2002; Schofield & Hsieh, 2003) and MMR's anti-recombination activity may act to inhibit the ALT pathway. In the absence of telomerase activation, MMR may inhibit the ALT pathway therefore acting to protect cells from potential immortalization. Using telomerase defective *Saccharomyces cerevisiae*, a recent report has shown that, in the absence of MMR, proliferation is enhanced (Rizki & Lundblad, 2001). This suggests that removal of MMR's anti-recombination activity allowed for the activation of the ALT pathway and an escape from crisis. To test this hypothesis, Bechter *et al.* (Bechter *et al.*, 2004) used an MSH6 deficient human cell line and showed that the inhibition of telomerase resulted in the activation of telomere sister chromatid exchange (T-SCE), suggesting that the ALT pathway had been activated. In addition to MMR's anti-recombination activity, MMR is hypothesized to be involved in telomere function.

Several other studies have investigated the possibility of a connection between MMR and telomere function (Cheng *et al.*, 1998; Li *et al.*, 1996; Takagi *et al.*, 2000). Cheng *et al.* hypothesized that in the absence of MMR, human HNPCC tumours would not require telomerase activity because MMR defects favor malignant transformation by generating genomic instability. In contrast to this hypothesis Cheng *et al.* (Cheng *et al.*, 1998) found that human HNPCC tumours express telomerase at a rate similar to other tumours. This implies that telomerase inhibitors can be used on both groups of tumours but selective pressure on HNPCC tumours (ie MMR deficient tumours) may allow for the activation of the ALT pathway. However, Cheng *et al.* found that normal tissues from human HNPCC patients were more likely to express telomerase than normal tissues from non-HNPCC patients. This supports the hypothesis that a genetic defect affecting MMR function in HNPCC facilitates the activation of telomerase. Takagi *et al.* studied the relationship between microsatellite instability (MSI+), telomere length and telomerase activity in colorectal cancer. MSI+ correlated with short telomeres but there was no correlation

between strength of telomerase activity and the microsatellite status of the tumours. These data led Takagi *et al.* to suggest that there may be a relationship between MMR and the ALT pathway (Takagi *et al.*, 2000).

I hypothesized that the increase in cellular proliferation seen in MMR deficient *S. cerevisiae* by Rizki and Lundblad may be the result of an increase of telomere fusions and chromosomal instability. MMR proteins may function in mammalian cells to suppress genomic instability through the regulation of telomere length. The ability of MMR proteins to inhibit recombination may function to stabilize the genome. In order to maintain telomere length in the presence of MMR, mechanisms other than telomere recombination, such as the activation of telomerase, may be necessary.

I used MMR deficient mouse embryonic fibroblasts (MEFs), thymocytes and splenocytes to investigate the affect of a loss of MMR on chromosomal stability and telomere function. Using telomere specific probes it was found that in the absence of MMR there was a significant increase in cellular aneuploidy as well as an increase in the number of telomere fusion events that were confirmed by G-banding. This led to the hypothesis that there may be an innate difference in telomere length between MMR proficient versus MMR deficient cells leading to telomeric fusions and chromosomal instability. However, no difference in telomere length and no difference in telomerase activity were observed in MMR proficient versus MMR deficient cells. These results suggest that although a lack of MMR promotes telomere fusion events and the development of chromosomal aneuploidy, the absence of MMR does not appear to affect telomere length or telomerase activity in mammalian cells.

Centrosomes are critical in maintaining proper chromosome segregation during mitosis and centrosome aberrations have been implicated in the development of tumour aneuploidy. Centrosome alterations are often found in conjunction with alterations in chromosome numbers (Brinkley, 2001; Carroll *et al.*, 1999; Ghadimi *et al.*, 2000; Lingle *et al.*, 2002; Montagna *et al.*, 2002; Neben *et al.*, 2003; Xu *et al.*, 1999; Zhou *et al.*, 1998). I found that MMR deficient MEFs had increased numbers of centrosomes compared to wildtype MEFS

and I hypothesize that this increase in centrosome numbers may be driving the development of aneuploidy in MMR deficient MEFs.

From the experiments described here, I conclude that although mammalian telomerase activity does not appear to be affected by the loss of MMR, chromosomal stability is altered in mammalian cells lacking MMR leading to telomeric associations and cellular aneuploidy. Genomic instability plays an important role in multi-step tumorigenesis by allowing for and promoting dysregulation of key downstream oncogenes and tumour suppressor genes. The finding of chromosomal instability and microsatellite instability (MSI+) in MMR deficient tumours suggests these two pathways are not mutually exclusive and when occurring simultaneously can hasten the progression from normal to a tumorigenic state.

Materials and methods

Cell Preparation and Telomere Length Analysis

Using 8 week old *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ mice, single cell suspensions were prepared from thymus and spleen. Tissues were minced with scissors in cell culture medium (RPMI 1640 with 10% FBS and 0.1% 2-mercaptoethanol) and filtered using 40 μm cell strainers (Becton Dickinson Cat#352350). Single cell suspensions were centrifuged at 200g for 6 minutes and cells resuspended in freezing media (RPMI 1640 medium with 40% FBS and 10% DMSO) and frozen until use.

Telomere length measurements by fluorescence in situ hybridization

The average telomere fluorescence in isolated cell populations of splenocytes and thymocytes was measured by Flow-FISH (Rufer *et al.*, 1998) with minor modifications. A telomere specific FITC conjugated $(CCCTAA)_3$ peptide nucleic acid (PNA) probe (0.3 μg/ml) (Perseptive Biosystems) was employed.

Metaphase spreads, FISH and image analyses of MEFs were performed as described (Blasco *et al.*, 1997; Zijlmans *et al.*, 1997). The Cy-3-labeled $(CCCTAA)_3$ PNA (Applied

Biosystems) was used as a probe. Cells were examined with a Zeiss axiophot fluorescence microscope.

Chromosome Orientation FISH (Co-FISH) has been described in detail previously and was used here with some modification (Bechter *et al.*, 2004). Briefly, MEFs were subcultured into medium containing a 3:1 ratio of 5'-bromo-2'-deoxyuridine:5'-bromodeoxycytidine - (BrdU; Sigma) at a total final concentration of 1 x 10^{-5} M and collected at 24 hours. Colcemid was added for the final 4 hours. Metaphase spreads are prepared as mentioned above and then stained with the DNA-binding fluorescent dye Hoechst 33258, exposed to UV light, and digested with exonuclease III to remove newly synthesized DNA strands according to the previous protocols. An additional denaturation in 70% formamide, 2xSSC at 70°C for one minute was performed, followed by dehydration in a cold ethanol series (70%, 85%, 100%). The Cy-3-labeled $(CCCTAA)_3$ PNA (Applied Biosystems) was used as a probe. Probe hybridization and wash conditions were identical to that described above for FISH experiments (Blasco *et al.*, 1997; Zijlmans *et al.*, 1997).

Cell lysate preparation and telomerase assays

Cultured MEFs and freshly dissected mouse testes were lysed and prepared in a buffer containing 0.5% w/v CHAPS as described (Kim & Wu, 1997) (Intergen, Inc.). Cell extract was assayed for the presence of telomerase activity using the TRAP assay following manufacturer's instructions (Intergen, Inc.) (Kim & Wu, 1997). A titration of cell extract was used to demonstrate that the TRAP products were in the near-linear range.

Tissue culture and Metaphase preparation using MEFs

Msh2$^{+/+}$ and *Msh2*$^{-/-}$ MEFs were cultured in T-150 tissue culture coated flasks in DMEM media supplemented with 20% FBS. Cultures were split 1:3 when 100% confluent. The following day, MEFs were cultured for 4-6 hours with colcemid to a final concentration of 0.1ug/ml. To harvest cells, media was removed and cells were washed with PBS, treated with trypsin and then pelleted. The supernatant was removed leaving 1ml of solution remaining in which to resuspend cells. Cells were hypotonically swollen with 75 mM KCL at 37°C for 25-30 minutes. Cells were pre-fixed by adding 5-10 drops of fixative (3:1 acetic

acid:methanol at -20°C) and then pelleted. Cells were resuspended in fixative and left overnight at -20°C. Metaphase spreads were obtained by dropping fixed cells onto wet slides and air drying the slides. Slides were stained with Giemsa and photographed and analyzed using Cytovision™ software (Applied Imaging Corp.). Three individual embryos were analyzed for each genotype (*Msh2*$^{+/+}$ and *Msh2*$^{-/-}$). Approximately 10 metaphases were scored for each embryo. A two sided Fisher's exact test was used to compare normal and abnormal numbers of chromosomes per cell.

Centrosome Immunohistochemistry on MEFs

Msh2$^{+/+}$ and *Msh2*$^{-/-}$ MEFs were cultured on glass slides in DMEM media supplemented with 20% FBS. Cells were washed with PBS when 50% confluent and fixed with Methanol:Acetone 1:1 at -20°C for 10 minutes. Cells were washed in PBS and blocked in PBS containing 1% bovine serum albumin (BSA) and 0.1% IGEPAL CA-630 (Sigma # I-3021) for 30 minutes at 37°C. Centrosome complexes were detected by incubation with an anti-mouse monoclonal anti-γ-tubulin antibody overnight at 4°C (Sigma #T6557) (diluted 1:1000 in block solution). Slides were washed 3 X 5 minutes in PBS. The antibody complexes were detected using Rhodamine Red ™ – conjugated secondary goat anti-mouse (IgG) antibody (Molecular Probes R-6393) for 30 minutes at 37°C. Slides were washed 3 X 5 minutes in PBS and the cells were counter stained with DAPI (Vector Labs H-1200) and visualized using a fluorescence microscope (Axioskop; Zeiss). Grey level images were acquired and pseudo colored using Adobe™ Photoshop software. Three separate embryos were scored for each genotype (*Msh2*$^{+/+}$ and *Msh2*$^{-/-}$). Centrosome counts were scored using 200 cells for each embryo; approximately 600 cells per genotype. Differences in the number of cells with centrosome aberrations between *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ genotypes were analyzed by the application of the student's t-test for independent samples. All student's t-test analyses were performed using the following web site from the Department of Physics at the College of Saint Benedict Saint John's University at Collegeville, Minneapolis http://www.physics.csbsju.edu/stats/t-test_bulk_form.html.

Results

Chromosomal abnormalities –telomere association, chromosomal breakage

I hypothesized that the increase in cellular proliferation seen in *S. cerevisiae* by Rizki and Lundblad (Rizki & Lundblad, 2001) in the absence of MMR may be the result of increased telomere fusions and overall chromosomal aberrations. To investigate in mammalian cells, PNA-FISH was used on *Msh2*$^{-/-}$ MEFs and *Msh2*$^{+/+}$ MEFs (Figure 5-1). In *Msh2*$^{-/-}$ MEFs an increase in telomere fusion events was observed (Table 5-1) with no fusion events occurring in wild type cells. In addition, a higher level of aneuploidy was seen (55.7%) in *Msh2*$^{-/-}$ MEFs compared to *Msh2*$^{+/+}$ MEFs (16%) (Table 5-1).

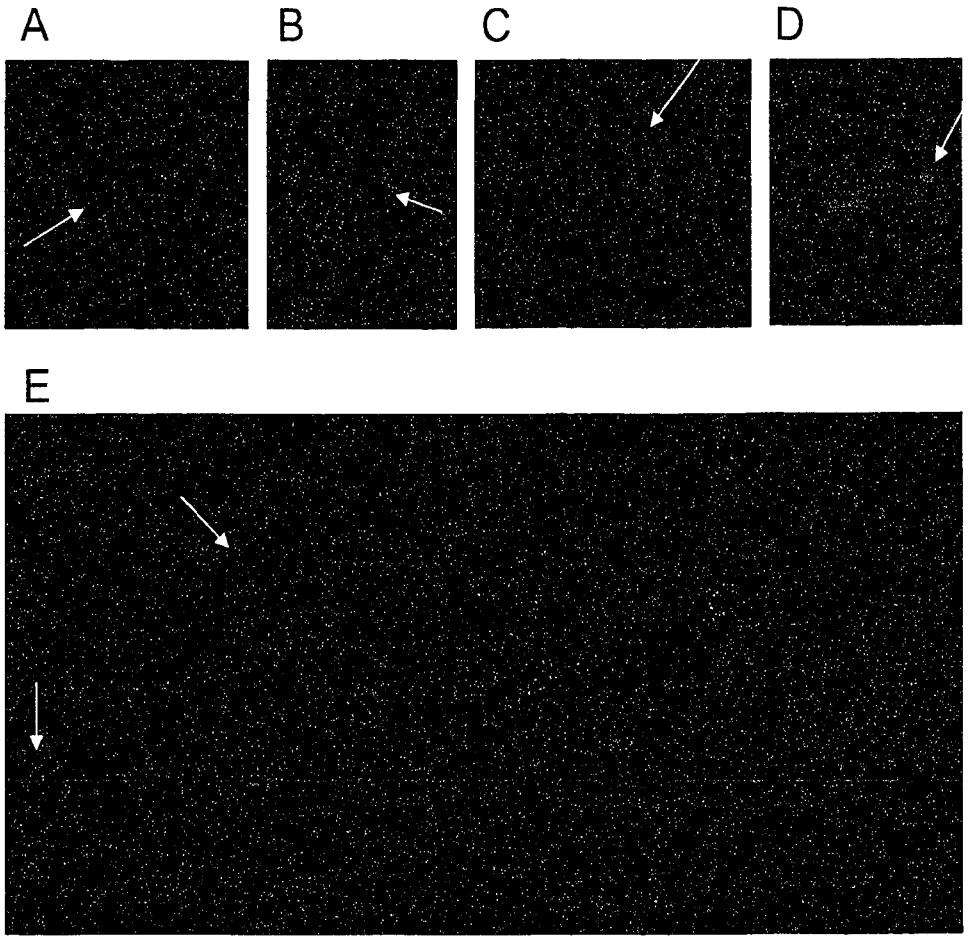
Table 5-1: Chromosomal abnormalities in mouse embryonic fibroblasts[a]

	Metaphases Analyzed	Aneuploidy (%)	Telomere Association[b]	Chromosome Breakage[c]
Msh2$^{-/-}$ MEFs	61	55.7%	5/2744	1/2744
Msh2$^{+/+}$ MEFs	68	16%	No fusion	No chromosome breakage

a. Mouse embryonic fibroblasts were derived from mouse embryo at day 13.5 and were studied for chromosome analysis at passage 4.
b. number of fusions versus numbers of chromosomes analyzed.
c. number of chromosomes with breakages versus numbers of chromosomes analyzed

Figure 5-1: Telomere fusion events in *Msh2*$^{-/-}$ MEFs.

Metaphase spreads of MEFs showing DAPI staining (blue) and telomere fluorescence signals (red). Arrows indicate telomere fusion events (A-C), extra telomere signals (D) and chromosome fragments (E).



To confirm that the changes seen in chromosomal numbers were related to the absence of MMR, standard G-banding of MMR proficient and MMR deficient MEFs was performed and results were scored for chromosome copy number changes. Of the 40 *Msh2*$^{+/+}$ metaphases analyzed, 12 (30%) showed chromosomal number changes (both chromosomal gains and losses) (Table 5-2). In contrast, 35/44 (79.5%) of *Msh2*$^{-/-}$ metaphases analyzed showed chromosomal number changes (Table 5-2). While most chromosomal aberrations seen were in the form of numerical changes, at least one *Msh2*$^{-/-}$ metaphase showed extensive levels of chromosomal fusions and breakage products (Figure 5-2D). Figure 5-2 shows that while the majority of *Msh2*$^{+/+}$ cells retain normal chromosome counts (40, or 80 if cell division has not yet occurred) *Msh2*$^{-/-}$ cells have chromosomal counts that range from 27 to 83 chromosomes.

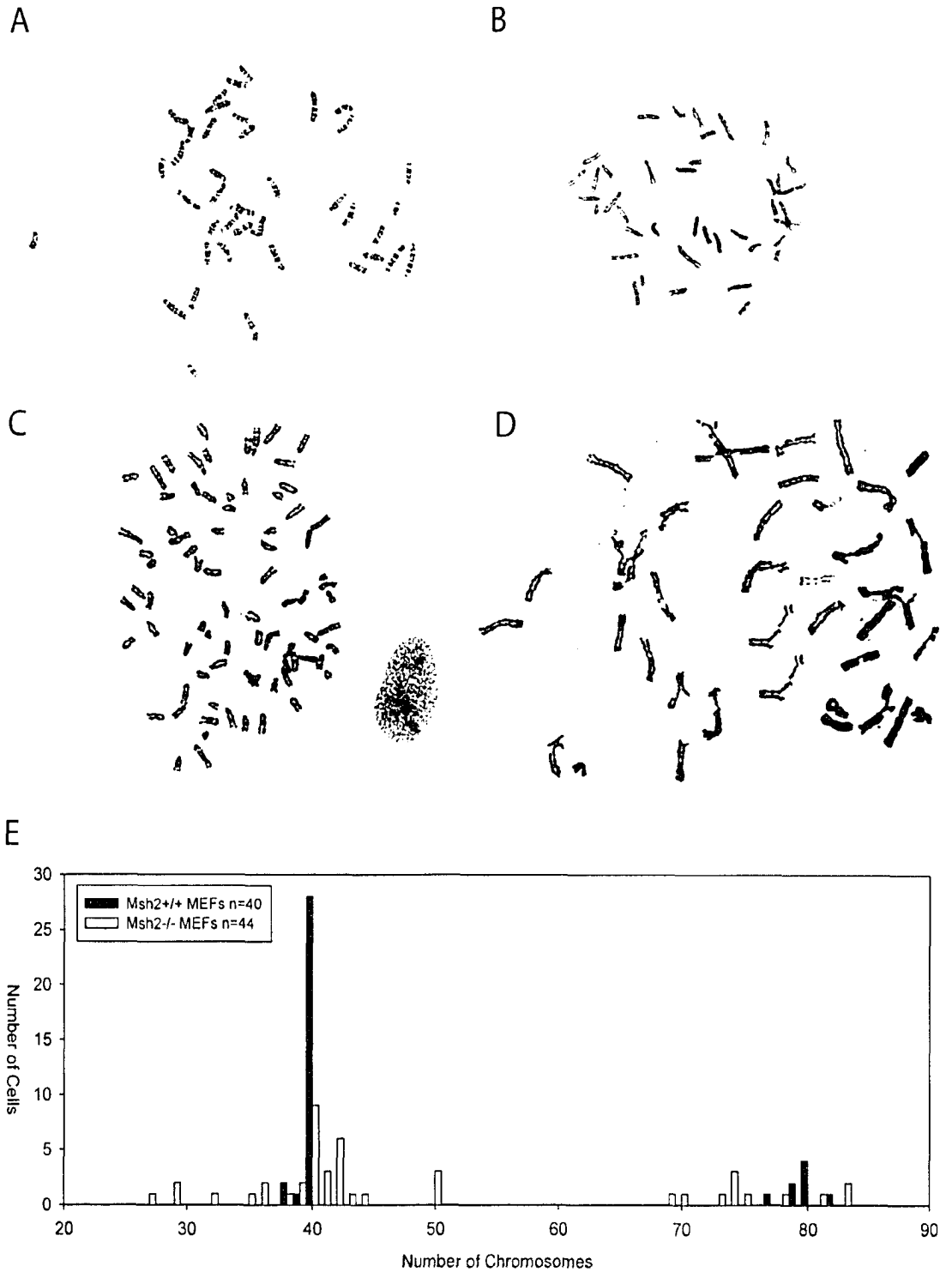
Table 5-2: Increased aneuploidy as seen by G-banding in *Msh2*$^{-/-}$ MEFs compared to *Msh2*$^{+/+}$ MEFs

	# Metaphases	# Aneuploid	# Normal	Percentage
Msh2$^{+/+}$	40	12	28	30%
Msh2$^{-/-}$	44	35	9	79.5%

$p < 0.00005$ by two-sided Fisher exact test

Figure 5-2: Chromosomal Instability in *Msh2*$^{-/-}$ MEFs.

(A-D) Representative photomicrographs of Giemsa-stained metaphase spreads (A) *Msh2*$^{+/+}$ MEFs showing normal chromosomal numbers (n=40). (C) *Msh2*$^{-/-}$ MEF metaphase chromosomes showing a typical aneuploid cell with n=83. (D) *Msh2*$^{-/-}$ MEF metaphase chromosomes showing extensive chromosomal breaks and fusion events. (E) Chromosomal number distribution in *Msh2*$^{-/-}$ and *Msh2*$^{+/+}$ MEFs. A total of 40 *Msh2*$^{+/+}$ metaphase spreads and a total of 44 *Msh2*$^{-/-}$ metaphase spreads were scored. The graph shows that the majority of *Msh2*$^{+/+}$ cells contained a normal number of chromosomes n=40. *Msh2*$^{-/-}$ cells show extensive deviation from the expected normal number of chromosome (n=40). Although *Msh2*$^{-/-}$ cells show a clustering of chromosome counts around the expected 40 or 80 chromosomes *Msh2*$^{-/-}$ cells also showed deviations from this normal. Aneuploidy changes in *Msh2*$^{-/-}$ cells ranged from 23 to 87.



Telomere length analysis: Analysis of telomere lengths in mouse Thymocytes, Splenocytes, and MEFs.

Telomere length was measured in *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ 8 week old murine thymocytes and splenocytes using Flow-FISH analysis (Figure 5-3). No difference in telomere length was observed in thymocytes or splenocytes from *Msh2*$^{+/+}$ compared to *Msh2*$^{-/-}$ mice. To determine whether telomere length alterations were occurring in MEFs in the absence of MMR, Q-FISH was performed on metaphase chromosome spreads from *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ MEFs (Figure 5-4). No difference in the distribution of fluorescence was observed suggesting that there is no difference in telomere length in *Msh2*$^{+/+}$ compared to *Msh2*$^{-/-}$ MEFs.

Telomere repeat amplification protocol (Trap Assay): telomerase activity in the mouse testis

Telomerase activity in *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ mouse testis was tested using the telomere repeat amplification protocol (TRAP assay) (Figure 5-5). No difference in telomerase levels was seen between *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ murine testes.

Figure 5-3: Flow-FISH analysis of telomere lengths in mouse tissues.

Relative telomere length in thymocytes and splenocytes derived from *Msh2*$^{-/-}$ and *Msh2*$^{+/+}$ mouse tissues determined by flow-FISH is shown. In each set, data were pooled from several mice (error bars represent standard deviations). [Thymocyte and splenocyte murine cell extraction was preformed by Marcia Campbell, Flow-FISH was performed by Dr. Liu]

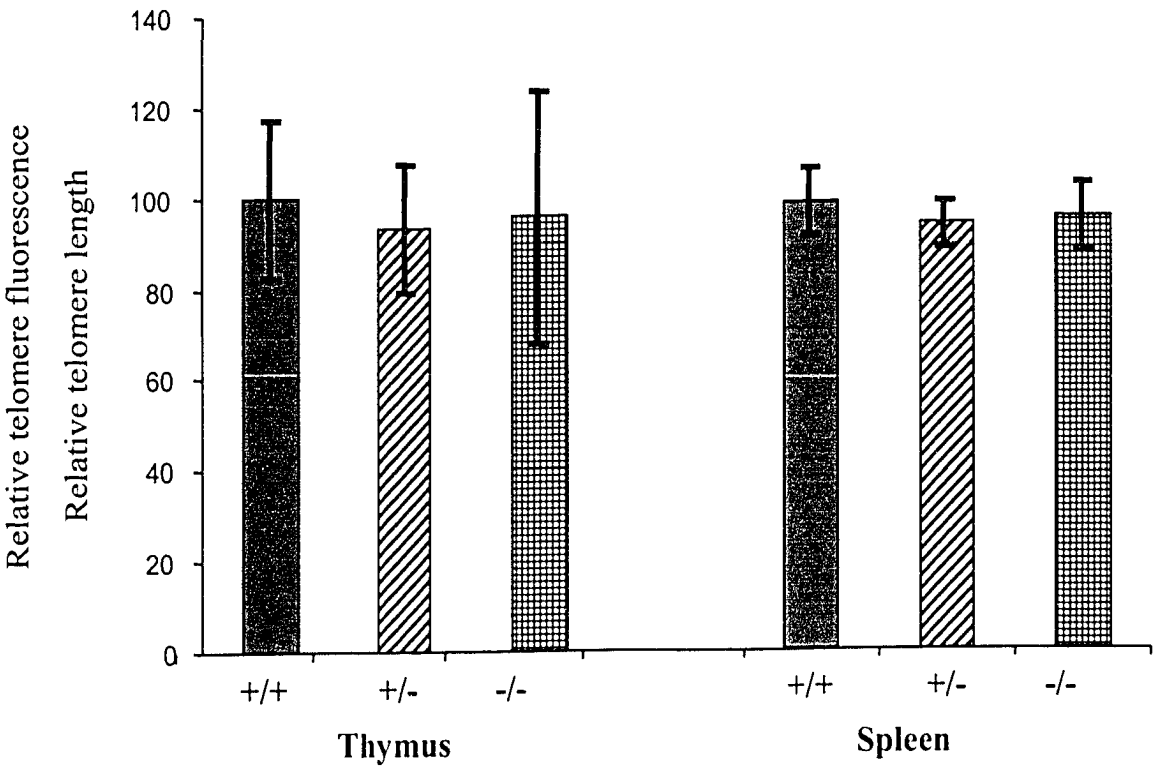


Figure 5-4: Telomere Fluorescence distribution and measurement in *Msh2*$^{+/+}$ and *Msh2*$^{-/-}$ MEFs.

Q-FISH was performed on metaphase chromosome spreads from Msh2+/+ and Msh2-/- MEFs. No difference in the distribution of fluorescence was observed suggesting that there is no difference in telomere length in *Msh2*$^{+/+}$ compared to *Msh2*$^{-/-}$ MEFs. In each set, data were pooled from several embryos. [MEF cell generation was performed by Marcia Campbell, Q-FISH assay was performed by Dr. Liu]

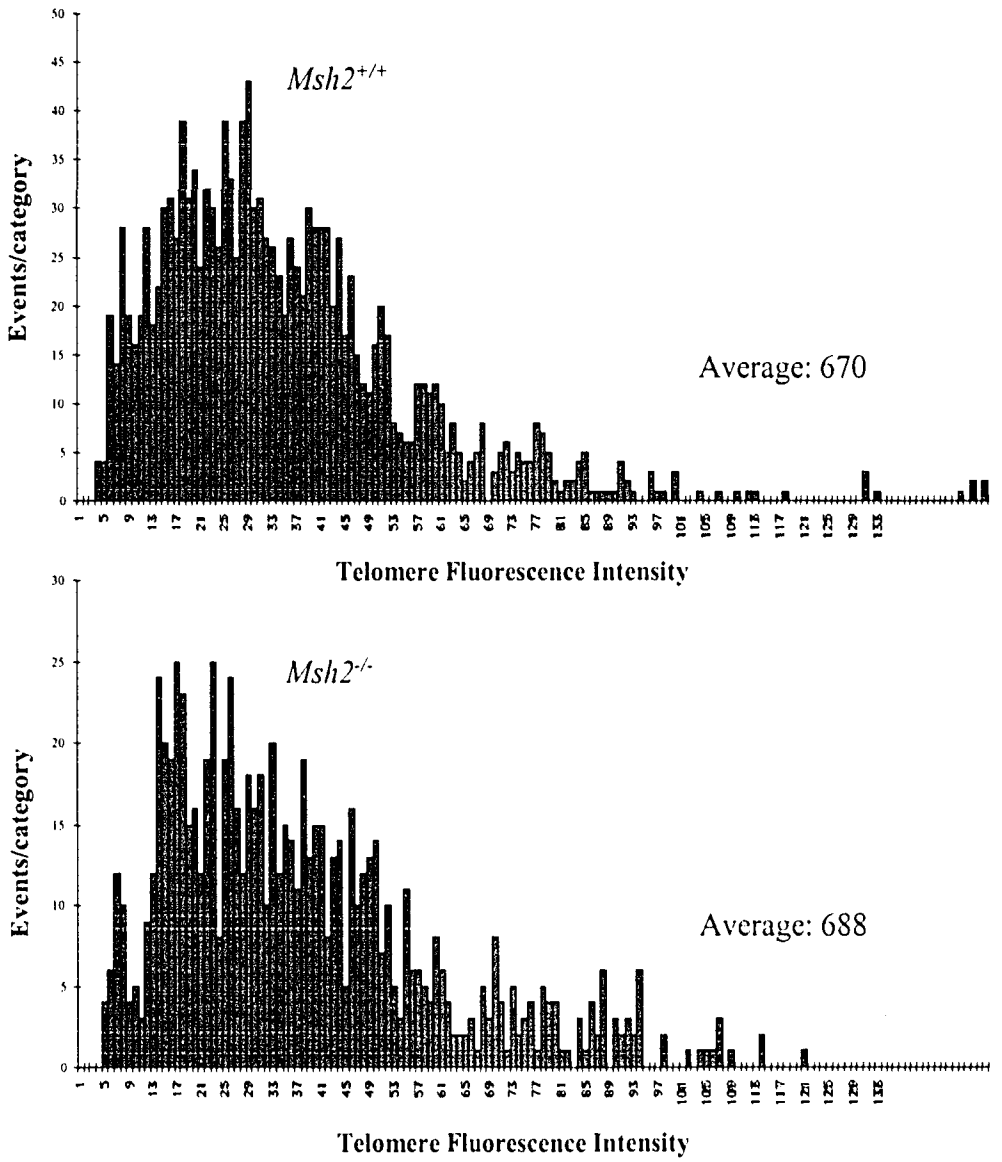
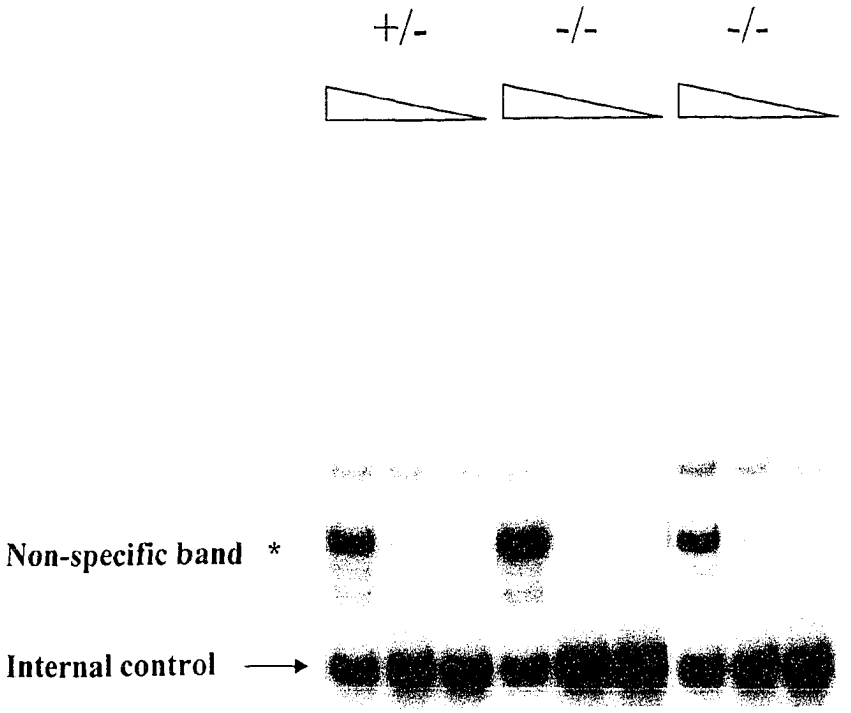


Figure 5-5: Telomerase activity in the mouse testis.

TRAP was performed for 30 PCR cycles on 2.5, 1.25, and 0.625 mg of tissue extracts prepared from *Msh2*$^{+/-}$ and *Msh2*$^{-/-}$ mouse testes extracts. An internal PCR standard for the telomerase repeat amplification protocol is shown at bottom with an arrow. The asterisk (*) indicates a non-specific product from mouse extracts that is resistant to RNase A treatment. [Testis organ extraction was performed by Marcia Campbell, TRAP assay was performed by Dr. Liu]



Centrosome Hyperamplification in Msh2$^{-/-}$ MEFs.

Numerical changes in chromosome number can result from abnormal chromosome segregation during mitotic cell division. To examine whether chromosomal number changes seen in *Msh2*$^{-/-}$ MEFs correlate with centrosome hyperamplification, *Msh2*$^{+/+}$and *Msh2*$^{-/-}$ MEFs were stained with an antibody against the γ- tubulin component of the centrosome.

In addition to the normal distribution of 1-2 centrosomes per cell (Figure 5-6) different patterns of centrosome abnormalities were observed in the *Msh2*$^{-/-}$ cells (Figure 5-6). The majority (88%) of *Msh2*$^{+/+}$ cells displayed a normal number of centrosomes (Table 5-3). In contrast, only 66% of *Msh2*$^{-/-}$ cells were found to have normal numbers of centrosomes. Centrosome hyperamplification (>2 centrosomes) was observed in 34% of *Msh2*$^{-/-}$ cells with 11% of cells having five or more centrosomes. This is significantly different than wild type cells in which only 12% of cells had more than two centrosomes. (p=0.0001)

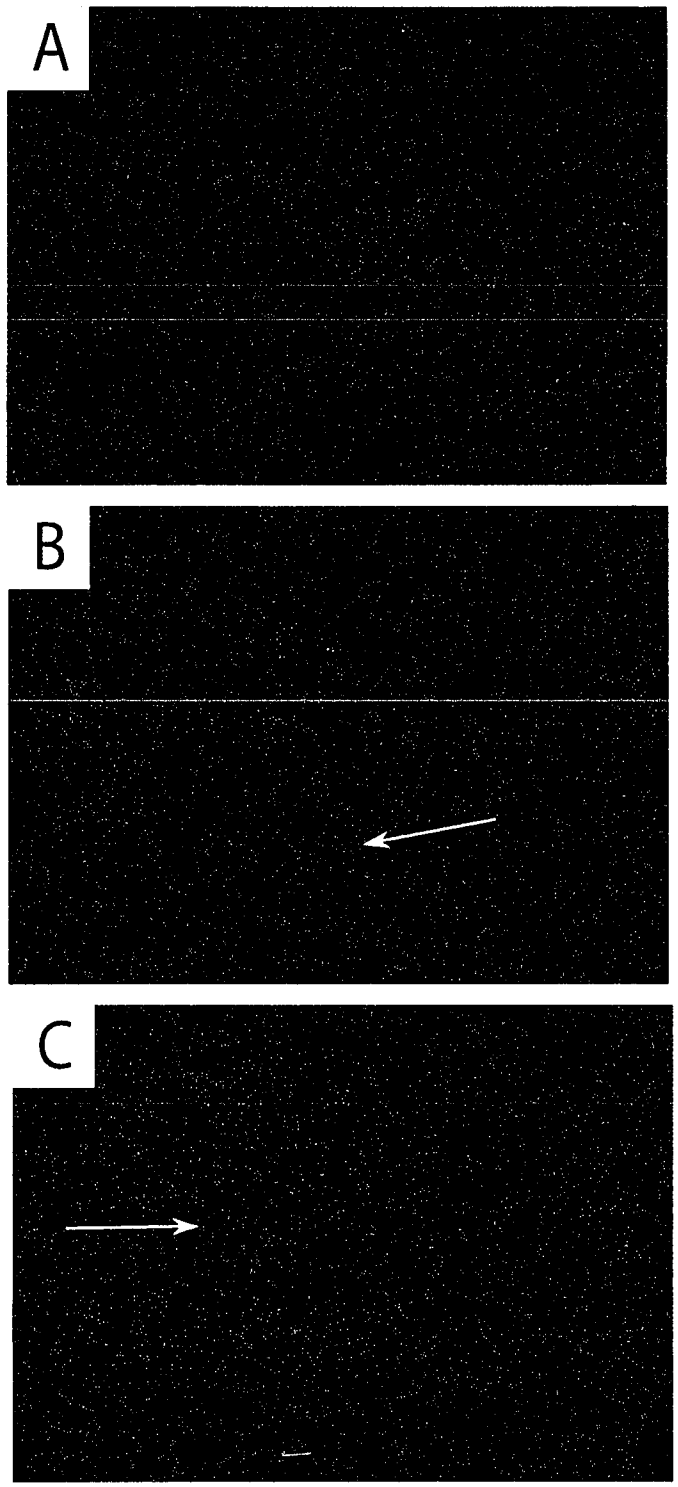
Table 5-3: Centrosome hyperamplification in *Msh2*$^{-/-}$ MEFs

	Number of Centrosomes				
Cells	n = 1	n = 2	n = 3 or 4	n = 5 or 6	n ≥ 7
Msh2$^{+/+}$ MEFs	21.9%	66%	9.9%	1.8%	0.4%
Msh2$^{-/-}$ MEFs	11.3%	54.8%	23.7%	6.6%	3.5%

n indicates the number of centrosomes.
n ranges from 7 -14 in n ≥ 7
for each genotype, three embryos were examined
~ 200 cells were scored or each embryo (ie ~ 600 cells per genotype)

Figure 5-6: Centrosome aberrations in *Msh2*$^{-/-}$ MEFs.

Centrosomes were stained with an antibody against γ-tubulin (red) and the nucleus was counter stained with dapi (blue). (A) An *Msh2*$^{+/+}$ MEF cell with nuclei (blue) and normal staining for two centrosomes (red) as indicated by the arrows (B) An *Msh2*$^{-/-}$ MEF cell with abnormal numbers of centrosomes. Arrow indicating two centrosomes. (C) An *Msh2*$^{-/-}$ cell with hyperamplification of centrosomes. Arrow indicating a cluster of centrosomes.



Discussion

Recent evidence implicates the MMR proteins in cellular immortalization in the absence of telomerase in *S. cerevisiae* (Rizki & Lundblad, 2001). Rizki and Lundblad suggest that MMR's anti-recombination function may inhibit the ALT pathway and that without MMR, the ALT pathway may contribute to cellular immortalization without the need to activate telomerase. I hypothesized that MMR may contribute to suppression of mammalian chromosomal instability through the regulation of telomere function. Using *Msh2*$^{-/-}$ and *Msh2*$^{+/+}$ MEFs, we show that in the absence of MMR there is a significant increase in telomere fusion events, an increase in cellular aneuploidy but no alterations in telomere length. *Msh2*$^{-/-}$ murine testes showed no alterations in telomerase activity. Although MMR does not appear to affect telomere length in mouse cells, MMR appears to affect telomere function, through an as of yet unidentified mechanism. The loss of MMR leads to chromosomal instability, likely increasing the tumorigenic properties of these cells without the need to alter telomerase activity.

I hypothesized that the lack of MMR results in abnormal telomere length leading to an increase in telomere fusion events. In support of this, *Msh2*$^{-/-}$ MEFs showed increased telomere associations compared to *Msh2*$^{+/+}$ MEFs (Table 5-1). Telomere fusions, a form of DNA damage, would be expected to initiate either a cell cycle check point or the engagement of the apoptotic pathway. Without MMR, these pathways may be negatively affected (reviewed in Bellacosa, 2001) contributing to the persistence of these fusion events and the resulting chromosomal instability.

In the absence of MMR, the appearance of telomere fusions can arise by telomeres becoming critically shortened, leading to telomere dysfunction and ultimately telomere associations. Telomere length was compared in MMR proficient and MMR deficient MEFs as well as in murine thymocytes. However, no difference in telomere length was found in either cell type. From this it was concluded that MMR does not affect murine telomere length and a lack of MMR therefore does not lead to an increase in telomere erosion.

However, the increase in telomere size in the mouse (50-200kb) compared to human telomeres (5-80kb) may mean that more extensive telomere loss needs to occur before it can be detected using current methods. Possibly, the young tissues used here (embryonic tissues and 8 week old tissues) have not undergone a significant number of mitoses to demonstrate shortened telomeres and that telomere length changes may occur in later stage development.

The precise role of MMR in telomere function is unknown. Although no difference in telomere length was observed, MMR may affect telomere function in one or more of the following ways, leading to dysfunctional telomeres and telomere fusion events. A loss of MMR may reduce the inhibition of recombination to a level that permits fusion events. Alternatively, an increased mutation frequency in telomere sequence may compromise telomere function. Pickett *et al.* (Pickett *et al.*, 2004) report a correlation between MSI+ and telomere sequence mutations suggesting that an increased mutation frequency from a lack of MMR, leads to telomere mutations and subsequent telomere dysfunction. Increased mutational load may also alter telomere sequence to degree that telomere binding proteins may have altered binding affinities and consequent change in function. Finally, aberrant cell cycle arrest or apoptosis may occur in MMR deficient cells in response to telomere dysfunction. Telomere DNA double strand breaks activate cell cycle arrest and/or apoptosis (Blackburn, 2001). MMR is involved in both cell cycle regulation and the signaling of apoptosis (reviewed in Bellacosa, 2001) and may be required for cell death induced by telomere dysfunction.

Centrosomes are involved in developing the spindle poles necessary for accurate chromosome transmission to daughter cells during mitosis. γ- tubulin is a major component of the centrosome and immunostaining detects centrosomes throughout the cell cycle (Joshi *et al.*, 1992; Stearns *et al.*, 1991; Zheng *et al.*, 1991). Because the centrosome is involved in proper chromosomal segregation it has been hypothesized that centrosome amplification may drive cellular aneuploidy (Carroll *et al.*, 1999; Lingle *et al.*, 2002). Chromosomal number aberrations have been correlated with abnormal numbers of centrosomes (Brinkley,

2001; Carroll *et al.*, 1999; Ghadimi *et al.*, 2000; Lingle *et al.*, 2002; Montagna *et al.*, 2002; Neben *et al.*, 2003; Xu *et al.*, 1999; Zhou *et al.*, 1998). I hypothesized that the chromosomal aneuploidy seen in the absence of MMR may be the result of abnormal numbers of centrosomes. γ-tubulin staining of centrosomes in *Msh2*$^{-/-}$ MEFs demonstrated that a loss of MMR correlates with centrosome abnormalities. To my knowledge this is the first report showing an abnormal number of centrosomes in the absence of MMR. In response to an abnormal number of centrosomes and the extra spindle poles created by extra centrosomes, cells are not known to have a checkpoint that aborts mitosis (Hinchcliffe & Sluder, 2001). In the absence of MMR, abnormal cell cycle regulation may occur leading to inaccurate centrosome duplication and altered centrosome numbers in daughter cells. I have shown here that centrosome hyperamplification is found to be common in cells lacking MMR and thus is likely to be one of the major mechanisms leading to the chromosomal instability observed in MMR deficient MEFs.

In conclusion, loss of mammalian MMR results in abnormal telomere function resulting in telomere fusions. In addition, MMR deficiency is associated with an abnormal number of centrosomes and increased cellular aneuploidy. I suggest that this centrosome hyperamplification leads to the increase in cellular aneuploidy observed. In both humans and mice, a deficiency in MMR results in an increased susceptibility to tumorigenesis arising from an increase in genomic instability. I have shown here that the increase in genomic instability can exist in several forms; telomere instability, centrosome instability and chromosomal instability. Further understanding of how a loss of MMR leads to these forms of instability will help to better understand the pathways underlying tumorigenesis.

Chapter 6 ♦ A lack of DNA mismatch repair on an athymic murine background predisposes to hematological malignancy*

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* *A version of this chapter has been published.*

Marcia R. Campbell, Patrick N. Nation, Susan E. Andrew. (2005)
Cancer Research vol 65 no 7 pp : 2626-35

All of the results presented in this chapter were done by Marcia Campbell. Patrick Nation assisted with the pathological characterization of the tumours

Introduction

Analogous to the mouse models homozygous for a defect in MMR, several human patients with homozygous null mutations in any one of the MMR genes *MLH1*, *MSH2* or *PMS2* have presented with early onset childhood T or B-cell malignancies (Bougeard *et al.*, 2003; De Vos *et al.*, 2004; Gallinger *et al.*, 2004; Ricciardone *et al.*, 1999; Vilkki *et al.*, 2001; Wang *et al.*, 1999a; Whiteside *et al.*, 2002). Although human HNPCC patients primarily develop cancers of the GI tract, lymphomas and leukemias have been observed in certain kindreds (reviewed in Hirano *et al.*, 2002) (Rosty *et al.*, 2000). Hirano *et al.* presented the case of a 52 year old male with a family history consistent with HNPCC. Eight months after undergoing a right hemicolectomy for ascending colon cancer he developed non-Hodgkin's lymphoma involving the ileum and the lungs. Autopsy found no metastasis to the bone marrow, liver, spleen or kidney. Immunohistochemistry of the lymphoma showed it to be T-cell in origin. Microsatellite analysis showed the lymphoma as well as the colon cancer to be unstable, although the *TGFβRII* poly-A repeat was not mutated in the lymphoma. The characteristics of the lymphoma arising in the patient are similar to the lymphocytic tumours found in the Msh2 deficient mice that present with T-cell lymphomas and multiple tissue involvement. This patient manifested a clinical course similar to that observed in the animal models and molecular observations support the inference that the development of lymphoma was specific to a deficiency in MMR and not incidental.

Rosty *et al.* reported the case of a 53 year old male that had a history of colon cancer associated with an inherited mutation in *hMLH1* (Rosty *et al.*, 2000). He presented with duodenal follicular lymphoma staining positive for B-cell markers as well as some reactivity for T-cells. This is similar to what is seen the mice deficient in Msh6 (Edelmann *et al.*, 1997). Interestingly, tumour lymphoid cells stained positive for hMLH1 and hMSH2 suggesting that a second inactivating mutation in *hMLH1* had not occurred and that lymphoid tumour cells still express the wild type allele of *hMLH1*. As well, in contrast to mouse models no metastatic disease was observed. Duodenal biopsies showed the characteristic follicular lymphoma t(14;18)(q32;q21) translocation involving the IgH and

BCL2 locus. However, no microsatellite instability was found consistent with the continued expression of hMLH1 and hMSH2. Rosty *et al.* hypothesize that different molecular pathways leading to tumorigenesis in colon cancer compared to lymphomagenesis may be occurring.

Mouse models genetically engineered to be haploinsufficient for any one of the MMR genes generally do not demonstrate an increased risk of cancer whereas mice that are homozygous null for any one of the MMR proteins Msh2, Msh6, Mlh1 or Pms2, are viable, but are prone to tumorigenesis (Baker *et al.*, 1995; de Wind *et al.*, 1995; Edelmann *et al.*, 1996; Edelmann *et al.*, 1997; Prolla *et al.*, 1998; Reitmair *et al.*, 1995). In contrast to the human tumour spectra of HNPCC patients, mice deficient in Msh2 or Msh6 most commonly develop early onset thymic lymphomas although other tumours such as ovarian and small intestinal tumours occur with a lower frequency (de Wind *et al.*, 1995; Edelmann *et al.*, 1997; Reitmair *et al.*, 1995).

Immunohistochemistry with T and B-cell markers, as well as histology, demonstrated that murine MMR deficient thymic lymphomas are very homogeneous (Lowsky *et al.*, 1997). These lymphomas are predominantly of T-cell origin, characterized by a 'starry-sky' appearance, enlarged nuclei, reduced cytoplasm, and numerous mitotic figures (Reitmair *et al.*, 1995). Hematopoietic development appears normal in the Msh2 null mice (Reitmair *et al.*, 1995). Msh2 deficient thymic lymphomas are thought to represent a single histopathologic entity and the tumour homogeneity suggests specific recurring genetic events may underlie the development of these particular neoplasms. Thymic lymphomas that develop in Msh2 deficient mice have a greatly elevated mutation frequency even when compared to non-tumour tissues, suggesting that a subsequent "hypermutator" phenotype has been acquired in the thymic tumours (Andrew *et al.*, 1997). Subsequent molecular events following loss of MMR likely lead to lymphocyte transformation and expression of a malignant phenotype.

In Msh2 deficient mice the onset of thymic lymphomas begins at two months of age and in Msh6 deficient mice tumour onset begins at nine months; 50% survival times are five

months and nine months respectively (Edelmann *et al.*, 1997; Reitmair *et al.*, 1995). The spontaneous tumour spectrum of Msh2, Msh3, Msh6 and Mlh1 null mice has been altered by breeding these mice to $Apc^{+/-Min}$ mice resulting in increased intestinal tumour incidence, intestinal tumour multiplicity per animal, and reduced survival (Edelmann *et al.*, 1999b; Kuraguchi *et al.*, 2001; Smits *et al.*, 2000). This change in tumour spectrum led to my hypothesis that the spontaneous tumour spectrum of Msh2 and Msh6 deficient mice may be shifted by breeding them to athymic nude mice. I hypothesized that Msh2 and Msh6 deficient mice succumb to thymic lymphomas prior to the development of other cancers such as colonic tumours and are therefore prevented from developing a broader tumour spectrum similar to human HNPCC patients. This hypothesis was tested by breeding Msh2 and Msh6 deficient mice to athymic nude mice, to determine if these mice would develop a tumour spectrum similar to the human HNPCC tumour spectrum and provide us with a novel MMR deficient model.

The nude phenotype is characterized by a loss of function mutation in the *Foxn1* gene (forkhead box N1), a winged helix/forkhead transcription factor, which results in abnormal morphogenesis of the epidermis, hair follicles and thymus (Nehls *et al.*, 1994). Without Foxn1 expression, there is a basic defect in development of the embryonic ectoderm resulting in the absence of a thymus. Without a thymus, these mice cannot attract hematopoeitic precursor cells essential for T-cell development. Consequently, they have severely reduced numbers of immature as well as mature T-cells and are therefore immunocomprimised (Balciunaite *et al.*, 2002). Nude mice do have a minimal amount of extra-thymic T-cell maturation occurring in such organs as the spleen (Palacios & Samaridis, 1991) and they do therefore have a greatly reduced population of circulating mature T-cells.

Here I demonstrate that $Msh2^{-/-}$;$Foxn1^{nu/nu}$ and $Msh6^{-/-}$;$Foxn1^{nu/nu}$ mice develop lymphoblastic lymphomas, predominantly of B-cell origin. Furthermore, the lifespan of Msh6 deficient mice is reduced on a nude background, with 50% survival occurring at nine months in $Msh6^{-/-}$;$Foxn1^{nu/nu}$ mice compared to 11 months in $Msh6^{-/-}$ mice. These findings

support a critical role for the MMR proteins in normal T and B-cell development, and demonstrate that constitutive absence of MMR contributes to development of both T and B-cell malignancy.

Materials and Methods

Genotyping of Mice

Msh2 mice (Reitmair *et al.*, 1995) and Msh6 mice (Edelmann *et al.*, 1997) were bred to nude (*Foxn1*$^{nu/nu}$) mice (Charles River Laboratories) to generate *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$, *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice and *Msh2*$^{+/+}$;*Foxn1*$^{nu/nu}$, *Msh6*$^{+/+}$;*Foxn1*$^{nu/nu}$ mice. *Msh6* genotyping was carried out using earclip DNA as previously described (Edelmann *et al.*, 1997). *Msh2* genotyping was carried out using a PCR assay with earclip DNA and the following three primers: U771 Forward (5'-GCTCACTTAGACGCCATTGT-3') and L926 Reverse (5'-AAAGTGCACGTCATTTGGA-3') amplifying the wild type allele and U771 Forward and Neo Reverse (5'-TGG AAG GAT TGG AGC TAC GG-3') amplifying the targeted allele. The nude phenotype is the result of a single base pair deletion mutation in the *Foxn1* gene that when present in the homozygous state leads to the nude phenotype (Nehls *et al.*, 1994). Genotyping of the *Foxn1* gene was performed using earclip DNA as described (Hirasawa *et al.*, 1998). Briefly, modified PCR primers were used to introduce an artificial restriction site in the *Foxn1* gene PCR product based on the presence or absence of the nude mutation. Subsequently, PCR-RFLP analysis was carried out to distinguish between heterozygous (*Foxn1*$^{+/nu}$) and wildtype (*Foxn1*$^{+/+}$) mice.

Examination of Mice

The mouse experimental protocol was approved by the Health Science Animal Policy and Welfare Committee of the University of Alberta, and all animals cared for according to the guidelines of the Canadian Council on Animal Care. Mice were monitored daily for signs of poor health/grooming or tumorigenesis. Mice were sacrificed by isoflurane overdose upon signs of lethargy, weight loss or physical demonstration of a tumour. Murine tissues were fixed in 10% buffered formalin and embedded in paraffin blocks. Tumour and control brain tissue was frozen at -80°C for DNA analysis. Histological analysis was

performed on 5μm sections stained with hematoxylin and eosin using standard methods (P.N.).

General of Kaplan Meier Survival Curves and their Statistical Analysis

Msh2$^{-/-}$*;Foxn1*$^{nu/nu}$, *Msh6*$^{-/-}$*;Foxn1*$^{nu/nu}$ and *Msh2*$^{+/+}$*;Foxn1*$^{nu/nu}$ *Msh6*$^{+/+}$*;Foxn1*$^{nu/nu}$ mice were sacrificed and necropsies were performed to determine cause of lethargy and/or to excise tumour samples. Time of death and gross pathology was recorded for each mouse. Using the R statistical analysis software package version 1.5.1. and the 'survival library' function, we generated Kaplan Meier survival curves and compared time of death due to tumorigenic causes in wild type mice versus MMR deficient mice (http://www.R-project.org) (Ihaka & Gentleman, 1996).

Immunohistochemistry

Tumour sections (5-7 μm) were dewaxed in xylene, incubated in block solution and stained with the following antibodies: CD3 (DakoCytomation), B220 (BD·PharMingen), Pax5 (BD·PharMingen), Ki67(BD·PharMingen), CD45 (Research Diagnostics Inc).

PCR Assay for D_HJ_H Rearrangement Status

D_HJ_H rearrangement analysis was performed as previously described (Chang *et al.*, 1992). Briefly, two forward primers were used that are immediately 5' of the D_H elements (DSF: 5'-AGGGATCCTTGTGAGGGATCTACTACTGTG-3' and Dq52: 5'-GCGGAGCACCACAGTGCAACTGGGAC-3'). These primers are based on a consensus sequence and together recognize all of the D_H elements. The reverse primer (J_H4: 5'-AAAGACCTGCAGAGGCCATTCTTACC-3') is immediately 3' of the J_H4 element. The DSF/J_H4 primer pair amplifies recombination products between DSP and DFL D_H gene elements and all of the J_H elements. The Dq52 and J_H4 primer pair amplifies recombination products between Dq52 and all J_H4 elements. If rearrangement of the D_HJ_H locus has occurred, one of four PCR product sizes (0.12kb to 1.3 kb) will be observed depending on which J_H element was used. DNA from NIH-3T3 cells was used as a germline control and mouse spleen DNA was used as a positive control.

Microsatellite Instability Analysis

Matched normal brain samples and tumour biopsies were harvested from five *Msh2*$^{-/-}$;*Foxn1Foxn1*$^{nu/nu}$ mice were analyzed for microsatellite instability (MSI+). DNA was extracted using Qiaquick DNA extraction columns (Qiagen Inc.). Microsatellite loci were amplified using fluorescently labeled Licor PCR primers and products were analyzed on a Licor electrophoresis gel system (Licor-Biosciences). Ten loci were investigated for microsatellite status using primers as follows: 1) Five primer pairs used amplified mononucleotide repeats: JH101, JH102, JH103, JH104 (Edelmann *et al.*, 1997) and U12235 (5' GCTCATCTTCGTTCCCTGTC-3' and 5'-CATTCGGTGGAAAGCTCTGA-3') (Edelmann *et al.*, 2000). 2) Three primer pairs amplified dinucleotide repeats: D1mit83, D7mit17, D7mit91 (Whitehead Institute: www-genome.wi.mit.edu/cgi-bin/mouse/sts_info). 3) One primer pair, Tcrb, amplified a trinucleotide repeat $(GCT)_{12}$, [Tcrb Forward 5'-AGTTTTAGGCTATAGGTT-3' and Tcrb Reverse 5'-TGATCTAGAGAAAGGGTAGGTCTA-3'] (Hearne *et al.*, 1991) and 4) one primer pair, Cyp1a2, amplified a tetranucleotide repeat $(CAAG)_{10}$, [Cyp1a2 Forward 5'-TGGCAGGACTGCACCTAAGCT-3' and Cyp1a2 Reverse 5'-ACTGGAACCTTAGAGCATGAG-3'] (Hearne *et al.*, 1991).

Results

Msh2*$^{-/-}$*;Foxn1*$^{nu/nu}$ *and Msh6*$^{-/-}$*;Foxn1*$^{nu/nu}$ *Mice Have a Significantly Reduced Lifespan

Msh2 and Msh6 mice were bred to athymic nude mice to generate *Msh2*$^{-/-}$*;Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$*;Foxn1*$^{nu/nu}$ mice. *Msh2*$^{+/+}$*;Foxn1*$^{nu/nu}$ and *Msh6*$^{+/+}$*;Foxn1*$^{nu/nu}$ mice were bred as control mice. A survival curve was generated to investigate differences in rates of tumorigenesis between *Msh6*$^{-/-}$*;Foxn1*$^{nu/nu}$ mice and *Msh6*$^{-/-}$ mice; (Figure 6-1A) (*Msh6*$^{-/-}$ survival data from (Edelmann *et al.*, 1997)). *Msh6*$^{-/-}$ mice live significantly longer than *Msh6*$^{-/-}$*;Foxn1*$^{nu/nu}$ with a median survival time to tumorigenesis of 11 months compared to nine months in the *Msh6*$^{-/-}$*;Foxn1*$^{nu/nu}$; p= 0.007. At nine months of age only 46% of the *Msh6*$^{-/-}$*;Foxn1*$^{nu/nu}$ mice were still alive whereas 60% of the *Msh6*$^{-/-}$ mice were still alive. By 12 months of age only 12.5% of the *Msh6-/-;Foxn1*$^{nu/nu}$ mice were alive, however, 35%

of the *Msh6*$^{-/-}$ mice were still alive. Although the genetic background of the two mouse models was not identical, (the *Msh6*$^{-/-}$ mice were a C57BL/6 background and the *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice were on a C57BL/6/Balb/C background) different genetic backgrounds of *Msh6*$^{-/-}$ mice had previously been determined to have no effect on spontaneous survival of the mice (Edelmann, personal communication).

Second, a Kaplan Meier survival curve was generated comparing death due to tumorigenic causes in *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice with *Msh6*$^{+/+}$;*Foxn1*$^{nu/nu}$ mice (Figure 6-1B). Of the 64 *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice studied, 44 (68.8%) developed tumours compared to 4 of the 28 (14.3%) *Msh6*$^{+/+}$;*Foxn1*$^{nu/nu}$ control mice (Figure 6-1B). *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice developed tumours at a significantly higher frequency than did the *Msh6*$^{+/+}$;*Foxn1*$^{nu/nu}$ mice; p=0.005. Of the 44 *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice that developed tumours, 39 (88.6%) mice developed lymphoblastic lymphomas. Of the four *Msh6*$^{+/+}$;*Foxn1*$^{nu/nu}$ mice that developed tumours, all developed lymphoblastic lymphomas; with three of the four mice concurrently presenting with *Pneumocystis carinii* pneumonia. During the course of this study a *Pneumocystis carinii* infection within the colony caused the development of pneumonia and death in several *Msh6*$^{+/+}$;*Foxn1*$^{nu/nu}$ mice, likely associated with the immunocompromised state of the nude background.

Thirdly, a Kaplan Meier survival curve was generated comparing death due to tumorigenic causes in *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice with *Msh2*$^{+/+}$;*Foxn1*$^{nu/nu}$ mice (Figure 6C). The *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice developed tumours at a significantly higher frequency than did the *Msh2*$^{+/+}$;*Foxn1*$^{nu/nu}$ control mice; p= 0.00005 Of the 14 *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice studied, eight (57%) developed lymphoblastic lymphomas, one developed a basal cell skin tumour, four died prematurely and were not available for post mortem analysis and only one mouse died of pneumonia. Median survival time of *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice was 5.54 months. By seven months only 31% of the mice were alive and by nine months only 15.5% of mice were alive. To investigate the time to tumorigenesis in the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ versus the time to tumorigenesis in the *Msh2*$^{-/-}$ mice a Kaplan Meier survival curve was generated using survival data from the *Msh2*$^{-/-}$ colony housed at the University of Alberta (Figure

6D). Time to tumorigenesis in *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ compared to *Msh2*$^{-/-}$ mice was not significantly different;p=0.36.

Msh2$^{-/-}$;*Foxn1*$^{nu/nu}$ *and Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ *Mice Develop Lymphoblastic Lymphomas*

Several of the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice presented with clearly visible subcutaneous tumors appearing often on the neck and chest region (Figure 6-2).

Figure 6-1: Survival of *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice.

Lifespan of the mice and cause of death was recorded and Kaplan Meier survival curves were generated using the R statistical analysis software package version 1.5.1. For all curves, death due to tumorigenic causes was recorded and used in calculating the survival curves.

A. *Msh6*$^{-/-}$ mice survival compared to *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice survival; p=0.007. (*Msh6*$^{-/-}$ survival data obtained from (Edelmann *et al.*, 1997)

B. *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice survival compared to *Msh6*$^{+/+}$;*Foxn1*$^{nu/nu}$ mice survival; p=0.005.

C. *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice survival compared to *Msh2*$^{+/+}$;*Foxn1*$^{nu/nu}$ mice survival; p=0.0005.

D. *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice survival compared to *Msh2*$^{-/-}$ mice survival; p=0.36.

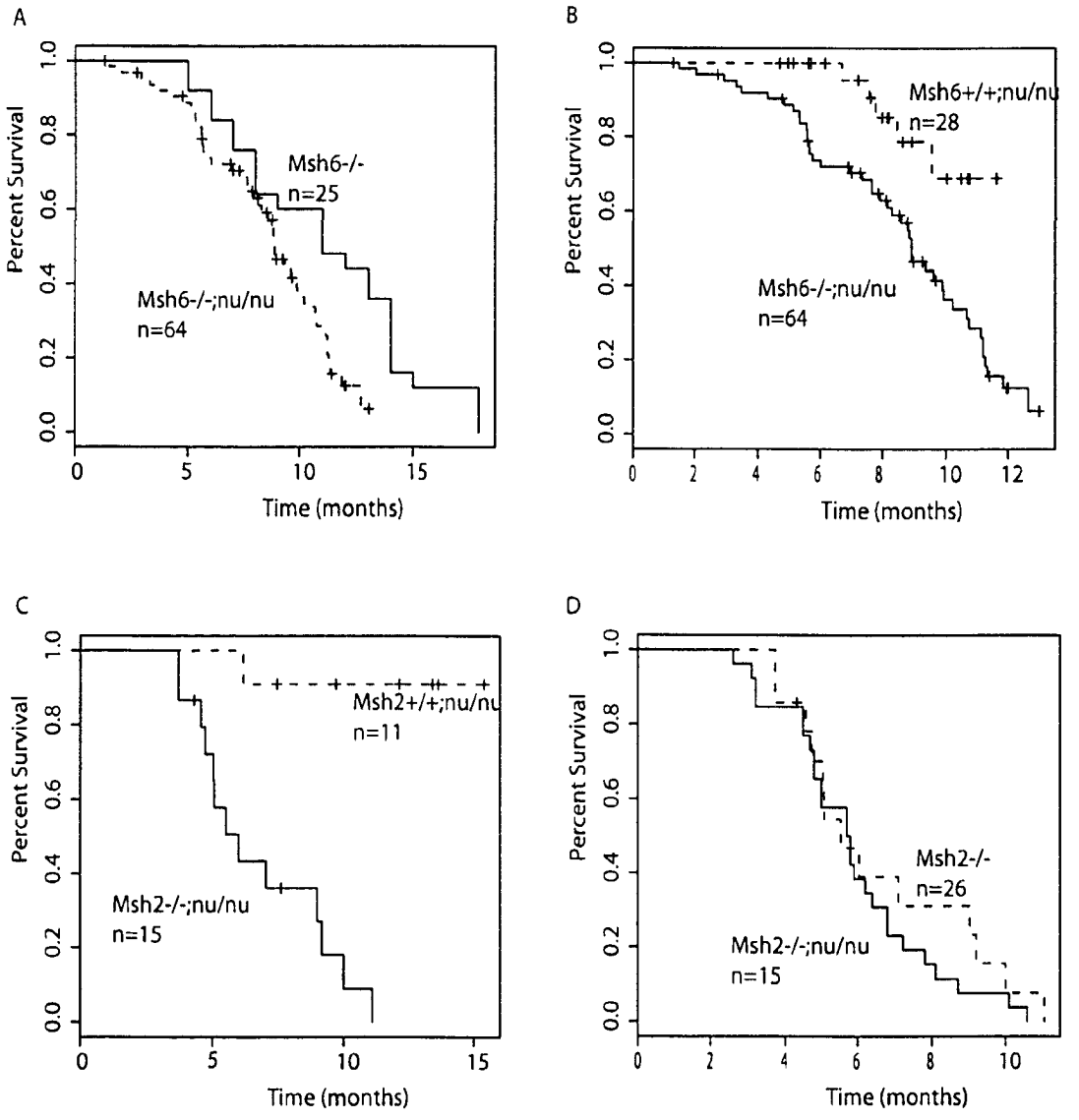


Figure 6-2: Lymphoblastic lymphoma from an *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mouse.

Tumor presentation is often seen as a subcutaneous mass on the head and/or neck region of the mouse. **A**. A superficial cervical lymph node may be involved in this mouse **B**. The same mouse as in **A**. This tumor mass was clearly visible and measured 1 cm in diameter.

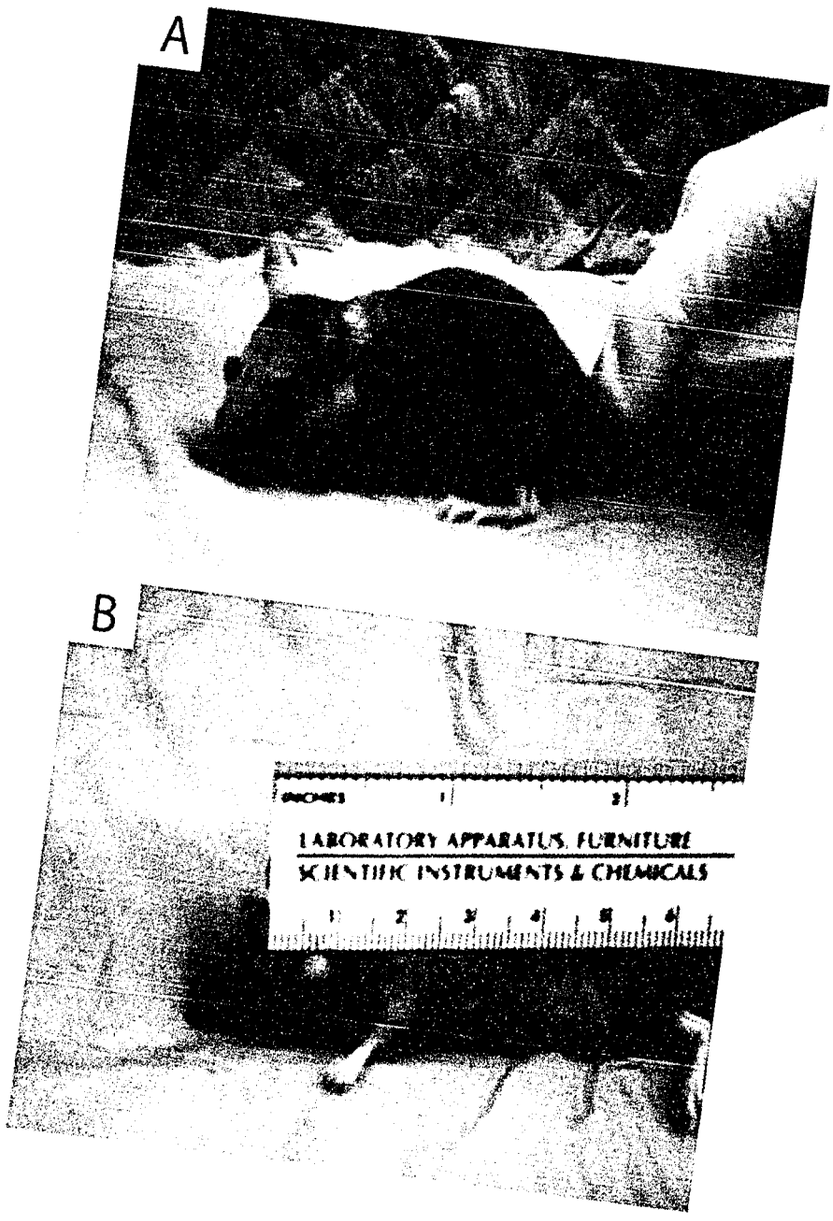


Table 6-1:Histopathological Findings in *Msh6*$^{-/-}$*Foxn1*$^{nu/nu}$ mice

Mouse ID	Age (Mnths)	Histology	Lymphnode	Colon	Spleen	Liver	Other tissues
6N565	2.0	1. lymphoblastic 2. Salvary gland tumors	+	-	+	-	
6N658 *	3.3	lymphoblastic	+	-	+	+	Skin: normal
6N619	3.5	1. Basal cell tumor 2. Papilloma	NI	-	-	-	
6N347	5.1	lymphoblastic	+	NI	+	+	
6N643 *	5.3	lymphoblastic	+	+	+	+	
6N593 *	5.5	lymphoblastic	+	NI	+	+	
6N641 * **	5.6	lymphoblastic	+	-	+	-	
6N646 *,**	5.6	lymphoblastic	+	-	+	+	
6N627 *	5.6	lymphoblastic	NI	-	+	-	
6N877 **	5.7	lymphoblastic	NI	+	-	-	Lung: *Pneumocystis carinii* infection
6N621 *	5.7	lymphoblastic	+	-	+	+	Skin: lymphoma infiltration
6N877	5.7	lymphoblastic	+	-	-	NI	Bone marrow: normal
6N870 **	7.0	lymphoblastic	NI	+	+	+	
6N626	7.4	lymphoblastic	-	-	+	+	
6N605 *	7.7	lymphoblastic	NI	-	+	+	
6N608	7.7	lymphoblastic	+	-	-	-	
6N410	8.1	lymphoblastic	+	NI	+	+	
6N348	8.3	1.basal cell tumor 2. sebaceous adenoma	+	-	-	+	
6N649 **	8.6	lymphoblastic	NI	NI	+	+	
6N497	8.8	lymphoblastic	+	NI	NI	NI	
6N665	8.9	lymphoblastic	+	-	NI	+	Lung: Pneumocystis carinii infection
6N666	8.9	lymphoblastic	NI	-	+	+	Lung: normal
6N718	9.9	lymphoblastic	NI	NI	NI	NI	
6N629	10.2	disseminated lymphoma	-	+	-	-	
6N597	10.8	lymphoblastic	+	-		+	
6N722 **	11.1	lymphoblastic	+	-	+	+	
6N555	11.2	Bronchoalveolar tumor	NI	-	-	-	Lung: Bronchoalveolar tumor Lung: *Pneumocystis carinii* infection
6N559	11.2	lymphoblastic	+	-	+	+	Lung: Pneumocystis carinii infection
6N645	11.3	lymphoblastic	+	NI	+	+	
6N653	11.8	lymphoblastic	+	-	+	+	Lung: lymphoma infiltrate Bone marrow: normal
6N501 **	12.7	Basal cell tumor	NI	-	-	-	Skin: Basal cell tumor Lung: *Pneumocystis carinii* infection

NI = not investigated; + = positive for lymphoma infiltration; - = negative for lymphoma infiltration

* has been immunophenotyped with CD3 and PAX5 (Table 6-2)

** has been investigated for the presence of D_HJ_H gene rearrangements (Table 6-3)

H&E staining of sections from tumours arising in *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice was performed and tumours were examined histologically. Of the tumours arising in the *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice, the majority were lymphoblastic lymphomas (88.6% of tumours) (Table 6-1). Of the nine mice that developed tumours in the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ colony, eight mice developed lymphoblastic lymphomas (88.9% of tumours) (Table 6-1). Both *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas appear histologically as monotonous sheets of poorly differentiated lymphoid cells with macrophages scattered throughout giving a 'starry-sky' appearance. This parallels what is seen in *Msh2*$^{-/-}$ thymic lymphomas. In addition to the 'starry-sky' appearance, both MMR deficient thymic lymphomas and MMR deficient lymphoblastic lymphomas have cells with enlarged nuclei, reduced cytoplasm and numerous mitotic figures.

Table 6-2: Immunohistochemistry of *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas

		Marker					Tumour origin
Mouse ID	Tumor Type	CD3	B220	PAX5	Ki67	CD45	T or B-cell
6N586	Lymphoma	-	-	+	+	-	B-cell
6N593	Lymphoma	-	-	+	+	-	B-cell
6N621	Lymphoma	-	-	+	+	-	B-cell
6N627	Lymphoma	-	+ (low)	+	+	-	B-cell
6N641	Lymphoma	-	+	+	+	-	B-cell
6N643	Lymphoma	-	-	+	+	-	B-cell
6N646	Lymphoma	-	-	+	+	-	B-cell
6N658	Lymphoma	-	-	+	+	+ (low)	B-cell
6N605	Squamous skin tumor	-	-	-	-	-	unknown

- negative staining in the tumor
+ positive staining in the tumor
+ (low) <10% of cell staining positive staining in the tumor

Lymphoblastic Lymphoma Characterization

Several of the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice presented with clearly visible subcutaneous tumours commonly appearing on the neck and chest region. H&E staining showed many of these tumours to be lymph nodes that had undergone general infiltration and replacement by malignant lymphocytes. However, in several cases the precise tissue of origin could not be identified due to the destruction of tissue structure by malignant cells (Figure 6-2). Widespread tumour infiltration into major organs was found to have occurred in the majority of mice. Organs commonly infiltrated by tumour cells include the liver, spleen, kidney, heart and colon. In the liver and spleen there was sinusoidal tumour infiltrate as well as perivascular infiltrate (Figure 6-2C). Dissemination to the heart occurred in several mice as well, and although the myocardium itself was normal, there was an infiltrate of tumour cells in the adventitia of the aorta, and associated nerves. Tumour masses were composed of a uniform population of quite immature lymphocytes and presented with the signature 'starry sky' appearance due to the scattered tingible body macrophages (Figure 6-2B). As well, mitoses and aberrant mitotic forms were present at a high rate. Three *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice were investigated for the presence of malignancy in the bone marrow. In all cases scattered malignant lymphocytes were present but it was not clear whether there was indeed bone marrow origin of tumour cells or whether this was simply an infiltration of metastasis.

Cellular Changes in Colonic Tissues

I hypothesized that in the absence of a thymus *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice may develop primary tumours of the gastrointestinal epithelium similar to human HNPCC patients. However, mice began to develop lymphoid tumours with no indication of primary intestinal tumour formation. Of note, colons from several mice did show lymphoid infiltration of the lamina propria of intestinal villi and the presence of lymphoid follicles in the mucosa with increased prominence of submucosal lymphoid follicales. To further investigate the possibility of early stages of tumour formation in the colon, I undertook a hyperproliferation study of the colon hypothesizing that early cellular changes may be occurring that are not seen macroscopically. *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice were

sacrificed at 8weeks, 24 days and 3 months old and each colon was divided into three sections. Three mice for each age group were used to account for individual variation between mice. In all colon sections examined there were no unusual cellular changes or thickening of the epithelial cell wall. We found no evidence of cellular hyperproliferation or pre-cancerous lesions in the colon and conclude that the colon is not susceptible to tumour formation in these mice.

Lymphoblastic Lymphoma Immunohistochemistry Characterization

To further characterize the tumours arising in the *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice I performed immunohistochemistry using several antibodies on eight lymphoblastic lymphomas and one squamous cell tumour (Tables 6-1 and 6-2). The *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ tumours were stained with CD45 (leukcocyte common antigen). CD45 is a marker found on hematopoietic cells with higher expression in lymphocytes then leukocytes (Kurtin & Pinkus, 1985). One of the eight *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas tested showed weak positive staining (<10% of cells staining positive). The remaining seven tumours tested were negative (Figure 6-3E). Negative staining for CD45 suggests that the lymphoblastic lymphomas arising in these mice are arrested at a very early stage in differentiation, having arisen prior to the expression of CD45. Tumours were stained with Ki67. Positive Ki67 staining in all nine tumours tested indicates a high proliferation index leading us to conclude these tumours were rapidly growing (Figure 6-3F). To determine if the lymphoblastic lymphomas were T- or B-cell in origin next we stained them with CD3 (T-cell specific marker) and B220 (B-cell specific marker) (also known as CD45R).None of the tumours tested stained positive for CD3 (Table 6-2). Only two were positive for B220 (Table 6-2). Tumours were then stained with Pax5, a very early marker specific for the B-cell lineage (Nutt *et al.*, 1999). The Pax5 protein (also known as B-cell-specific activator protein; BSAP) is a transcription factor expressed in the earliest B-cell precursors and once activated causes a cascade of other genes to be turned on and the cell to develop along the B-cell lineage (Nutt *et al.*, 1999). All of the *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas tested were Pax5 positive, indicating these tumours were early B-cell in origin (Figure 6-3F

and 6-G). The squamous cell skin tumour did not stain positive for either of the T- or B-cell markers.

Figure 6-3: Histology of *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ murine lymphoblastic lymphomas and tissue infiltration.

A. *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ murine lymphoblastic lymphoma stained with H&E (200X). Note the destruction of normal lymph node structure by the infiltrating malignant cells. **B.** *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ murine lymphoblastic lymphoma stained with H&E (500X). Note the monotonous sheet of neoplastic cells interlaced with macrophage cells giving rise to the 'starry sky' appearance. The arrows denote mitotic figures. **C.** *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mouse demonstrating infiltration of malignant lymphocytes around the portal veins of the liver (50X). **D.** Enlargement of the area of malignant infiltration around the portal veins in the liver (400X). Note the very similar appearance of the malignant infiltration in the liver to that of the malignant lymph node in B.

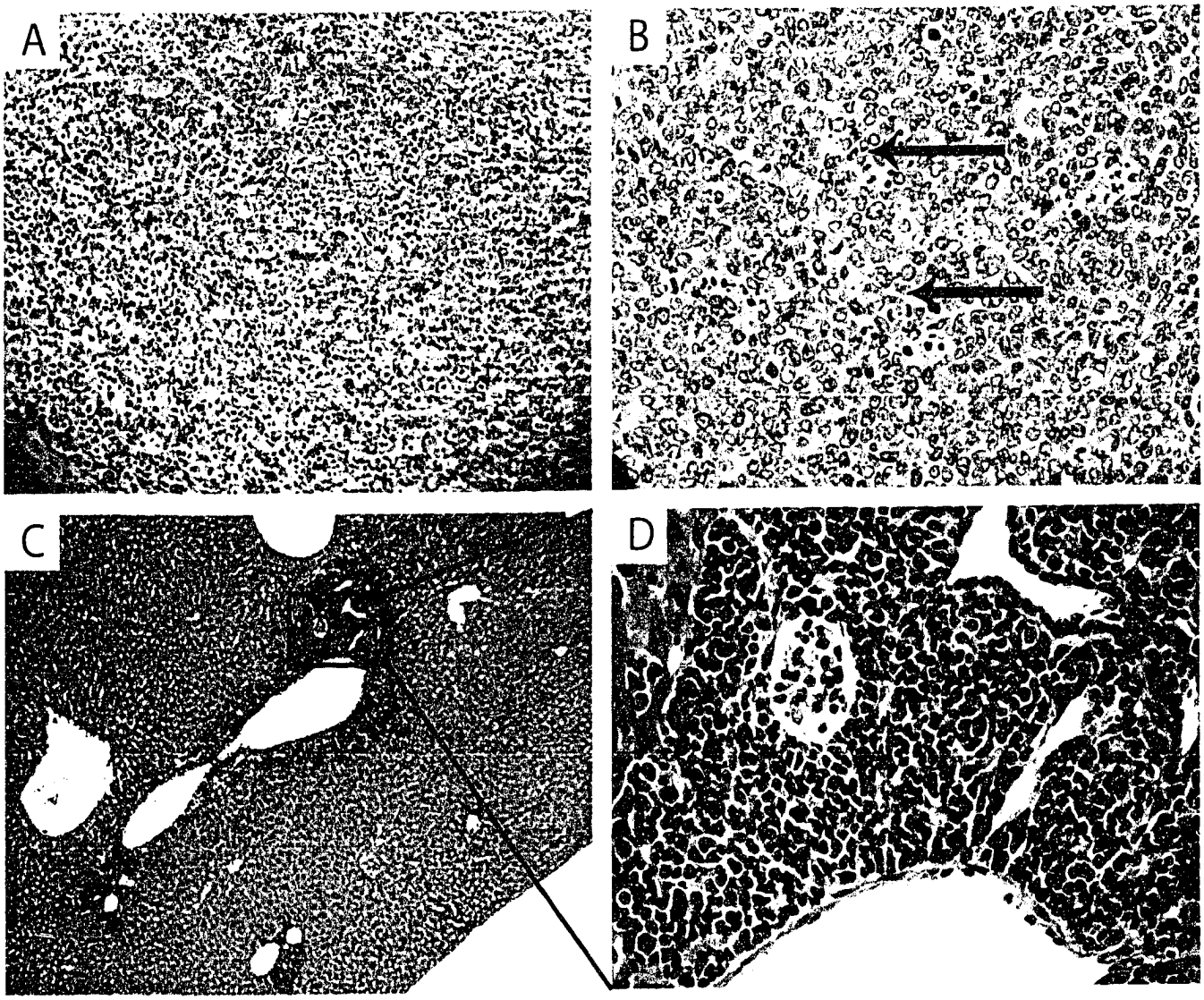
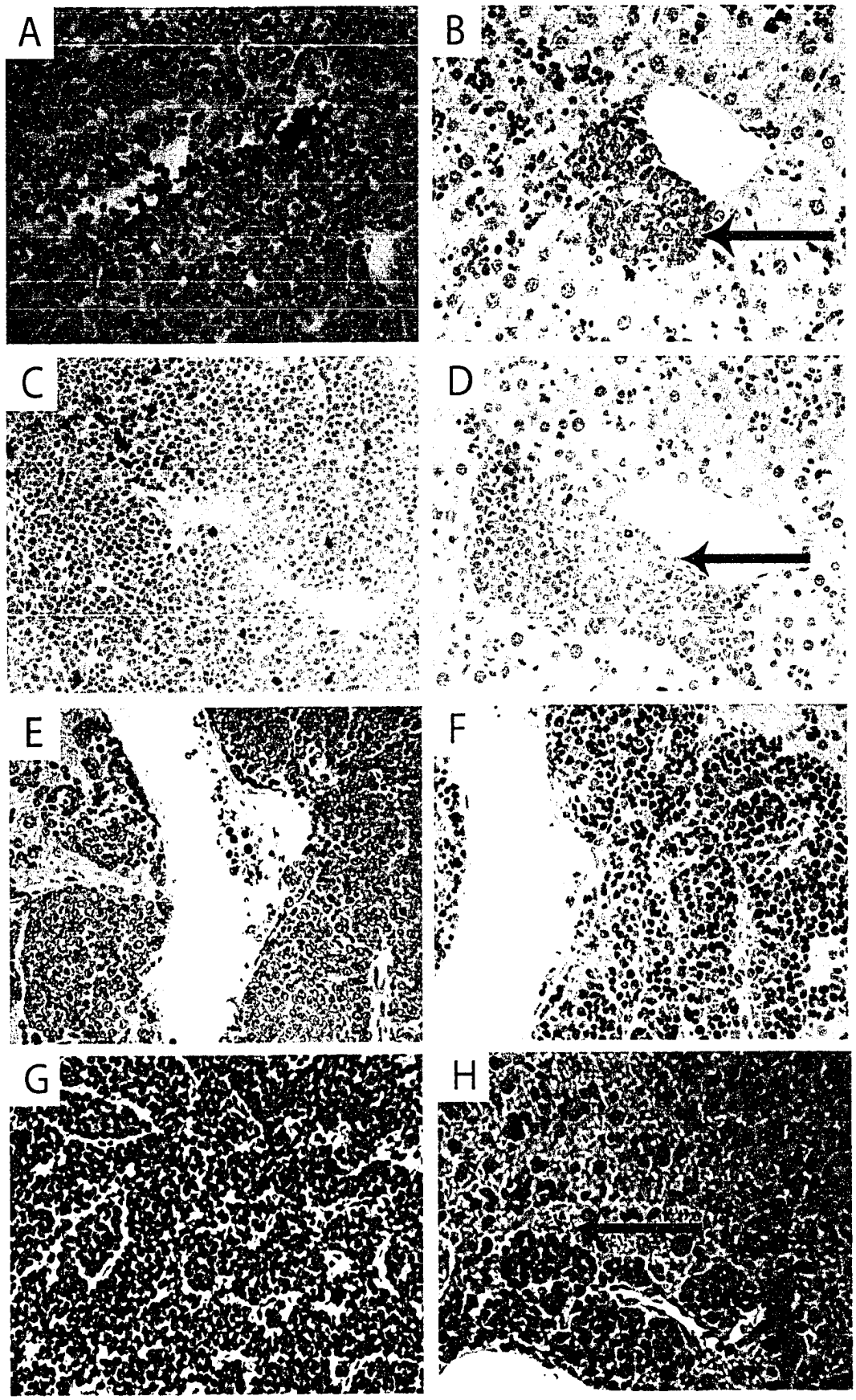


Figure 6-4: Immunohistochemical staining of *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ murine lymphoblastic lymphomas.

A. Lymphoblastic lymphoma staining negative for B220 (400X). Note the positively staining normal cells but a lack of staining in the malignant cells. **B**. Lymphoma infiltrated liver tissue with the malignant infiltrate staining negative for B220 (arrow) (400X). **C**. Lymphoblastic lymphoma staining negative for CD3. Note the occasional normal T-cell staining positive for CD3 (400X). **D**. Lymphoma infiltrated liver tissue with malignant infiltrate staining negative for CD3 (arrow) (400X) **E**. Lymphoma infiltrated liver tissue with malignant infiltrate staining negative for CD45 (400X). **F**. Lymphoma infiltrated liver tissue with malignant infiltrate staining positive for Ki67 (400X). **G**. Lymphoblastic lymphoma staining positive for Pax5 (400X). H. Lymphoma infiltrated liver tissue with malignant infiltrate staining positive for Pax5 (400X).



D_HJ_H Gene Rearrangement Analysis of Lymphoblastic Lymphomas

To further elucidate the stage of B-cell development I analyzed D_H to J_H gene rearrangements in *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas. Six of the eight *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphoma tumours tested were B220 negative but seven of the eight tumours tested were Pax5 positive. This led me to hypothesize that these tumours were arrested early in B-cell development and would likely not have undergone D_H to J_H gene rearrangements. I looked for the presence of D_H to J_H gene rearrangements in seven *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas. Of the seven tumours analyzed, six showed D_H to J_H gene rearrangements at at least one locus (Table 6-3). On average, tumours displayed two (of a possible four) D_H to J_H gene rearrangement products. Four of the seven tumours investigated had matched normal brain control DNA. Interestingly, three of the four matched normal brain samples showed the same D_H to J_H gene rearrangement products found in the corresponding tumour tissue leading us to leading us to hypothesize that tumour metastases to the brain has occurred, as the brain is not expected to have undergone D_H to J_H gene rearrangement.

In addition, the presence of D_H to J_H gene rearrangements in six lymphoblastic lymphomas arising in the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice was assessed (Table 6-3). All six of the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas tested showed D_H to J_H gene rearrangements. Three of the six tumours had rearrangement products of four, three and two products respectively. Five of the six tumours had matching normal brain control DNA. Three of the five brain control samples had rearrangement products corresponding to those found in the tumour, leading me to suggest that metastasis has occurred to the brain in the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas as well as the *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas.

Table 6-3: D_HJ_H gene rearrangements in lymphoblastic lymphomas from *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice

		D_HJ_H rearrangements per tumor									
		DSF/JH4 primer pair					DQ52/JH4 primer pair				
		DJH1	DJH2	DJH3	DJ4	germline	DJH1	DJH2	DJH3	DJ4	germline
Msh2$^{-/-}$;*Foxn1*$^{nu/nu}$											
tumor	40	■									
tumor	2N109					■				■	
tumor	2N134	■	■	■	■		■	■	■	■	■
tumor	2N283			■	■						■
tumor	2N319		■	■			■				■
tumor	2N346		■								■
Msh6$^{-/-}$;*Foxn*$^{nu/nu}$											
tumor	6N501					■					■
tumor	6N641			■	■						■
tumor	6N646			■							
tumor	6N649				■			■	■		■
tumor	6N722		■	■	■						■
tumor	6N870			■							■
tumor	6N877	■	■	■	■			■	■		

■ represents D_HJ_H gene configurations that were present

Microsatellite Instability Analysis of Lymphoblastic Lymphomas

Two dinucleotide repeat microsatellite markers were previously used to assay the levels of microsatellite instability in six *Msh2*$^{-/-}$ mice(Reitmair *et al.*, 1995). While normal tissues from the *Msh2*$^{-/-}$ mice were stable, all lymphomas and tumour infiltrated organs displayed novel allele sizes at one or both of the loci tested (Reitmair *et al.*, 1995). From this I therefore hypothesized that *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas would also display high levels of microsatellite instability. Using five *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas from five different mice we investigated the stability of microsatellite sequences at 10 loci. Five primer pairs analyzed mononucleotide repeats, three primer pairs analyzed dinucleotide repeats, one primer pair analyzed a trinucleotide repeat and one primer pair analyzed a tetranucleotide repeat. The size of the PCR product from tumour DNA was compared to the size of the PCR product from normal matched brain DNA. Tumours were considered microsatellite unstable low (MSI-L) if one or two markers out of 10 tested showed instability. Tumours were considered microsatellite unstable high (MSI-H) if three or more markers out of ten tested showed instability. All tumours tested showed instability at at least one marker tested and were classified as microsatellite unstable. Two of five tumours tested showed instability at one or two markers and were classified as MSI-L, where as three of five tumours tested showed instability at greater than three markers tested and were classified as MSH-H. Overall, there were 14/50 unstable reactions, corresponding to 28% instability.

Discussion

Since MMR is thought to have a ubiquitous role in DNA repair and mutation avoidance, the tissue specificity of tumorigenesis in human HNPCC patients as well as in MMR deficient mice is unexpected. In contrast to the GI tumours that develop in human HNPCC patients, MMR deficient mice develop predominately thymic lymphomas.

I hypothesized that MMR deficient mice succumb to tissue specific lymphomagenesis too early to allow for the development of a tumour spectrum similar to human HNPCC

patients. If mice are prevented from developing lymphomas they may develop GI tumours similar to human HNPCC patients and therefore present as a model with which to study MMR deficient GI tumour development. We wanted to test this hypothesis by breeding Msh2 and Msh6 deficient mice that are prone to spontaneous development of thymic lymphomas, to athymic nude mice. Removal of a thymus and reduction of the number of T-cells will prevent thymic lymphomagenesis and may therefore may result in an altered spontaneous tumour spectrum.

Breeding Msh2 and Msh6 deficient mice onto an athymic nude background therefore removing the organ prone to tumorigenesis, promoted the development of predominantly B–cell lymphoblastic lymphomas. Moreover, these lymphomas arise from a pre-B cell lineage at an early age, starting at 6.5 weeks of age. Thus, mice lacking MMR appear destined to develop hematological malignancy, even with the removal of the thymus and a greatly reduced T-cell component.

The median time to tumorigenesis in the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice, 5.54 months, was not altered compared to the *Msh2*$^{-/-}$ mice. This is in contrast to the original expectation that mice would live longer when prevented from developing thymic lymphomas. I conclude that the time to develop tumours in the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice was not altered in the absence of a thymus. I propose that MMR plays a crucial role in the development of hematological cells and that without MMR, these cells are prone to malignant transformation. In contrast to the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice, the *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice have reduced survival time to tumorigenesis (median survival 8.9 months) compared to the *Msh6*$^{-/-}$ mice (median survival 11 months). The earlier age of onset of *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ malignancies may be due to differences in genetic background between the *Msh6;nude* and *Msh6* animals although work by Edelmann (personal communication) would suggest that background differences have no effect on the survival of *Msh6*$^{-/-}$animals. In addition, the loss of the FOXN1 transcription factor may contribute to neoplasia in the absence of MMR, as it is expressed and important for cells other than B and T- cells. However, nude mice themselves do not show an increase in spontaneous tumorigenesis and this is unlikely. The

early onset of B-cell malignancies in *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice demonstrates no survival advantage to mice lacking a thymus and thus a T-cell component . No primary tumours of other organs such as intestine, endometrium and GI occur when the onset of thymic lymphomagenesis is reduced in the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ or *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice. MMR deficient mice treated with genotoxic exposure have accelerated onset of lymphogenesis (Colussi *et al.*, 2001; Jansen *et al.*, 2000; Qin *et al.*, 1999) also supporting the idea that in the constitutive absence of MMR, mice are poised to develop predominantly hematological malignancies, whether spontaneous or induced.

Msh2$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice are susceptible to lymphomagenesis. Although not seen macroscopically, early lesions in the colon may be occurring. This would indicate that cellular changes are in fact altering the epithelium in the colon but that mice are succumbing to lymphomagenesis prior to further development of the colonic lesions. I saw no cellular differences suggestive of a hyperproliferative or altered state in any of the colons studied irregardless of age or region of colon investigated leading us to suggest that MMR deficient nude mice are not prone to colonic lesions. The continued development of lymphomas and the absence of intestinal malignancies in *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice supports the idea that MMR is involved in the maintenance of hematological cells.

MMR deficient thymic lymphomas have been shown to comprise a single histopathologic entity and to be derived from normal hematopoietic progenitors (Edelmann *et al.*, 1997; Reitmair *et al.*, 1995). Thymic lymphomas from *Msh2*$^{-/-}$ mice closely resemble human precursor T-cell lymphoblastic lymphomas (LBLs) (Lowsky *et al.*, 1997). Histological examination of lymphomas from both *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice revealed characteristics similar to *Msh2*$^{-/-}$ thymic lymphomas including a 'starry-sky' appearance and numerous mitotic figures. Histologically, *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ tumours are practically indistinguishable from *Msh2*$^{-/-}$ thymic lymphomas. From this, I suggest that similar underlying molecular pathways leading to tumorigenesis likely occur in these MMR deficient mouse models resulting in very histologically similar

tumours. This supports a strong role for MMR in maintaining the genomic integrity of hematological cell types, and suggests that the absence of MMR is associated with accumulation of mutations in key tumour suppressor and oncogenes and the development of hematological malignancies.

Of the 23 *Msh2*$^{-/-}$ thymic lymphomas in the literature that have been immunophenotyped, 22 of 23 express CD3 demonstrating their derivation from precursor T- cells (Reitmair *et al.*, 1995) (Lowsky *et al.*, 1997). Of the eight *Msh6*$^{-/-}$ lymphomas that have been immunophenotyped, five of eight are B-cell in origin and only three of eight are T-cell in origin (Edelmann *et al.*, 1997). This may suggest a propensity for mice lacking Msh6 to develop B-cell malignancies while mice lacking Msh2 develop T-cell malignancies. If mice lacking Msh6 are indeed prone to develop B-cell malignancies then it is not surprising that *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ rapidly develop B-cell lymphoblastic lymphomas.

Only one of the *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas stained positive for the marker CD45, confirming it was lymphoid in origin. Negative staining in seven of the eight tumours tested, however, suggested that these tumours were very early in development and did not yet present with the CD45 marker. B-cell development is characterized by the rearrangement and expression of immunoglobulin heavy and light chain genes. The first Ig gene rearrangement (occurring during the early-pro-B-cell stage) is the joining of D_H (diversity) gene elements to J_H (joining) gene elements followed in the late-pro-B-cell stage by V_H (variable) to D_HJ_H joining. B220 (known as CD45R in humans) is a cell surface B-cell marker expressed in early pro-B-cells prior to or in conjugation with D_H to J_H gene rearrangement. After staining of the *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ tumours found them to be B220 negative as well as CD3 negative, I investigated for the presence of Pax5. Negative staining for B220 while staining positive for Pax5 indicates that the majority of lymphoblastic lymphomas arising in *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice are very early B-cell in origin. I hypothesized that these tumours are likely to be arrested in B-cell development prior to D_H to J_H joining. Surprisingly, all but one of the tumours showed some degree of D_H to J_H rearrangements suggesting that B-cell development is abnormal in these tumours. It is

possible that in the absence of MMR, B-cell immunoglobulin gene rearrangement is incorrectly regulated, resulting in recurring mistakes at this stage of development leading to tumorigenesis. It was observed, however, that no one particular class of D_H or J_H elements were over represented, suggesting that no particular element is responsible for the abnormal regulation. Further investigation for the presence of V_H to DJ_H rearrangements may help narrow down the stage of development at which these lymphoblastic lymphomas arise.

I hypothesized that *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas would display a high degree of MSI+, an indication of high levels of overall genomic instability. All tumours tested showed microsatellite instability. Instability was seen almost exclusively in mono- and dinucleotide repeats; instability was negligible in the trinucleotide marker and nonexistent in the tetranucleotide marker tested. Particular mononucleotide (JH101, JH103 and U12235) and dinucleotide markers (D1mit83 and D7mit17) showed greater levels of instability than comparable markers; suggesting that some and not all microsatellites are informative when assaying for MSI. In addition, the length of a microsatellite may play a role in its susceptibility to instability. In *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ lymphoblastic lymphomas, instability was not seen in the shorter mononucleotide repeats (JH102 and JH104, 8 and 10 base pairs respectively), but was seen in the longer mononucleotide repeats (JH103 and U12335, 13 and 24 base pairs respectively). Due to the lack to microsatellite instability seen in *Msh6*$^{-/-}$ tumour cells (Edelmann *et al.*, 1997), likely due to the redundancy of Msh6 and Msh3 in forming a functional MutL heterodimer, MSI was not tested in tumours arising in *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice.

In contrast to HNPCC patients with heterozygous MMR mutations, patients with homozygous mutations in MMR genes present with early onset T or B-cell malignancies. From this I suggest that the effect of a loss of MMR is highly dependent upon when in development loss of MMR occurs. For example, constitutive loss of MMR in either mice or humans leads to a predisposition to develop hematological cancers. Alternatively, in HNPCC, loss of the wildtype allele that results in loss of MMR occurs in specific tissues

later in life. Those tissues, such as the colon, may be those with higher exposure to exogenous DNA damaging agents and may therefore result in a different tumor spectrum.

Previous reports have suggested that thymic lymphomas predominate in MMR deficient mice due to the high turnover of developing T-cells in the thymus and subsequent accumulation of mutations in key downstream genes (de Wind *et al.*, 1995; de Wind *et al.*, 1998). The findings presented here that MMR deficient mice develop hematological malignancies even in the absence of a thymus leads me to suggest that malignant transformation is independent of cell turnover rate. Altered MMR gene expression, MMR gene mutation and/or MSI+ has been observed in varying proportions of human hematological neoplasms, consistent with the idea that loss of MMR has a significant role in human hematological malignancy (Gu *et al.*, 2002; Kotoula *et al.*, 2002; Matheson & Hall, 1999; Seedhouse *et al.*, 2003; Wada *et al.*, 1994).

The Andrew laboratory has previously reported that constitutive loss of MSH2 in a human patient leads to T-cell acute lymphocytic leukemia and features consistent with Neurofibromatosis type 1 (Whiteside *et al.*, 2002). Recently, this patient developed B-cell lymphoma (Dr R MacLeod personal communication). This parallels the B-cell malignancies seen in the *Msh2*$^{-/-}$;*Foxn1*$^{nu/nu}$ and *Msh6*$^{-/-}$;*Foxn1*$^{nu/nu}$ mice and further strengthens the conclusions that MMR plays an important role in the development of T and B-cells. These results support the hypothesis that MMR has a key role in developing T and B-cells. MMR is implicated as an important repair mechanism in normal T and B-cell development.

Chapter 7 ♦ Discussion

In this thesis I have evaluated the role of MMR in the generation of genetic instability leading to the development of tumorigenesis in the mouse. Mice that are homozygous null for any one of the MMR proteins Msh2, Msh6, Mlh1 or Pms2, are prone to tumorigenesis (Baker *et al.*, 1995; de Wind *et al.*, 1995; Edelmann *et al.*, 1996; Edelmann *et al.*, 1997; Prolla *et al.*, 1998; Reitmair *et al.*, 1995). Mice deficient and proficient in the MMR proteins Msh2 and Msh6 were used to determine how loss of MMR results in tissue specific tumorigenesis, with a different tissue spectrum in mouse compared with man. I hypothesized that in the absence of MMR high levels of genetic instability and the dysregulation of cellular processes resulting in thymic lymphomas, which are the predominant tumours arising in MMR null mice. My work demonstrates that MMR plays an important role in centrosome formation and aneuploidy, normal telomere function, T and B cell development, as well as molecular instability. Thus, the absence of MMR contributes to tumorigenesis through multiple pathways

Molecular characterization of murine *Msh2*$^{-/-}$ thymic lymphomas

Secondary gene identification

When this thesis began, characterization of the thymic lymphomas arising in the *Msh2*$^{-/-}$ mice was limited. To better understand the molecular pathways that underlie tumorigenesis in *Msh2*$^{-/-}$ thymic lymphomagenesis, I undertook molecular characterization of the tumours. In chapter 2 I investigated whether similar genes were becoming mutated in MMR deficient mouse models as were known to be mutated in human MMR deficient colorectal tumours. Notably, the *TGFβRII* gene contains a coding repeat that is mutated in more than 90% of human HNPCC tumours. The corresponding repeat is interrupted in the murine sequence and I showed that it is not prone to mutation in the *Msh2*$^{-/-}$ thymic lymphomas. I found that many of the coding repetitive tracts mutated in human colorectal tumours were not present in murine sequence or were not mutated in murine MMR deficient tumours. This is strongly suggestive that different genetic pathways leading to tissue specific tumorigenesis are occurring in each species: and the nature of the genes containing the

repetitive coding tracts contributes to the different tissue susceptibilities observed in each species. I hypothesized that different genes are susceptible to mutagenesis in the absence of MMR in the mouse compared with humans.

Thymic lymphomas from *Msh2*$^{-/-}$ mice showed exclusive mutation patterns consisting of a greatly elevated mutation frequency and multiple mutations within short regions of DNA (Baross-Francis *et al.*, 1998). I hypothesized that in the absence of MMR a mutation had occurred in a mutator gene leading to the elevated levels DNA aberrations. A gene(s) associated with the replication machinery might have become altered during tumorigenesis. When I began this project very little mouse DNA sequence data was available, however, the catalytic subunit of mouse DNA pol δ had been sequenced and was available in GenBank. The gene contained five exons with a total of six coding repeats consisting of 5 base pairs or longer. I hypothesized that these repeat sequences could be targeted for mutation in the absence of MMR. Studies from *S. cerevisiae* demonstrated that strains with mutations in DNA pol δ displayed a mutator phenotype and double *Msh2*$^{-/-}$*/Polδ* mutations have hyper-mutation frequencies above either mutation alone suggesting Msh2 and DNA pol δ act synergistically (Tran *et al.*, 1999). A mutation in DNA pol δ could explain the hyper mutator phenotype seen in the *Msh2*$^{-/-}$ thymic lymphomas. In chapter 3 I sequenced the catalytic subunit of the DNA pol δ gene in nine *Msh2*$^{-/-}$ thymic lymphomas. Comparative sequence analysis allowed me to identify seven sequence locations that differed between published cDNA and gDNA, likely polymorphisms. As well, I identified a previously unknown nucleotide polymorphism in the murine DNA pol δ sequence that differed from the published sequence available at the time of this project. Notably, the six coding mononucleotide repeat sequences were not mutated, suggesting that polymerase slippages, in the absence of MMR, do not result in mutations of the mononucleotide tracts of DNA pol δ. I was able to exclude DNA pol δ as a target gene mutated in MMR deficient murine lymphomagenesis and I identified four novel polymorphisms in the murine DNA pol δ gene.

Future Directions

Broader searches for downstream candidate genes mutated in the absence of MMR are needed to successfully identify larger numbers of target genes. I suggest several approaches that screen large numbers of genes be used.

1) DNA sequencing of candidate genes is labour intensive and time-consuming, and larger numbers of downstream genes can be more efficiently screened at the RNA level. Intragenic frameshift mutations resulting from a lack of MMR result in an altered and unstable transcript. Unstable transcripts allow for the detection of mutations at the RNA level. Using cDNA from *Msh2*$^{-/-}$ thymic lymphomas compared with normal thymus and non-tumour control tissues, expression arrays would be useful in determining genes that are differentially expressed in MMR deficient tumours.

2) Mononucleotide repeats containing greater than 5 base pairs are known to be mutated in the absence of MMR. However, identifying genes that contain such coding repeats is difficult. Doing BLAST searches of mRNA sequences, using 5-10 base pair repeats, inevitably retrieves polyadenylation signals as well as repetitive non-coding sequence. I would use a bioinformatics approach retrieving only coding sequence from the data base and then analyze that sequence for coding repeats. Using the Ensembl database in combination with the Perl programming language, all exon sequence (i.e. coding sequence excluding 5′ and 3′ UTRs, poly (A) tails etc.) can be retrieved from the Ensembl data base, reassembled into whole gene structures and then parsed for coding repeats. This approach would allow for the identification of coding repetitive sequences that may be targets for mutation in the absence of MMR.

Spontaneously arising murine Msh2$^{-/-}$ *thymic lymphomas show lower than expected levels of microsatellite instability*

At the start of this thesis, microsatellite instability (MSI) analysis had been performed on a few types of Msh2 deficient murine cells using only dinucleotide repeat markers (de Wind *et al.*, 1995; Reitmair *et al.*, 1995). However, the frequency and/or degree of instability were not properly assessed. Microsatellite status had been analyzed in 24 *Msh2*$^{-/-}$ embryonic stem (ES) cell lines showing a relatively high degree of MSI+ with 25%- 33% demonstrating MSI+, at dinucleotide repeats (de Wind *et al.*, 1995). Hypothesizing that

Msh2$^{-/-}$ thymic lymphomas would display even higher levels of MSI+, I assayed ten *Msh2*$^{-/-}$ thymic lymphomas tumours for microsatellite instability at ten loci. I used mono, di, tri and tetra-nucleotide microsatellite markers and tested for the presence of MSI+. *Msh2*$^{-/-}$ thymic lymphomas exhibited lower than expected levels of instability at the sequence level. This was surprising and led me to hypothesize that murine MMR deficient tumorigenesis may develop along different molecular pathways than in humans.

UVB induced murine Msh2$^{-/-}$ skin tumours show no microsatellite instability

I hypothesized that both spontaneous and induced MMR deficient tumours would display high levels of MSI+. Using UVB induced *Msh2*$^{-/-}$ deficient murine skin tumours, I assayed for the presence of microsatellite instability (Chapter 2). No instability was seen in the *Msh2*$^{-/-}$ mice or wild type mice, for any of the microsatellite markers tested. UVB induced tumours have a longer latency until development (compared with *Msh2*$^{-/-}$ thymic lymphomas). The longer length of time to tumorigenesis would suggest that UVB induced tumours might have had more time to acquire microsatellite mutations. Even with the longer time to tumorigenesis, my results suggest that UVB induced skin tumours do not acquire microsatellite mutations. This is suggestive that different molecular pathways leading to tumorigenesis are occurring in spontaneous compared with induced MMR deficient tumorigenesis. UVB may induce DNA damage such as 6-4 photoproducts that are not detected by microsatellite analysis but may be recognized by MMR and result in MMR mediated cell regulation.

Inter simple sequence-PCR can be used to evaluate genomic instability in the mouse

Inter-simple sequence PCR has been proven to be a fast and reproducible technique for quantitation of amplifications, deletions, translocations, and insertions in human sporadic colorectal cancer (Anderson *et al.*, 2001; Basik *et al.*, 1997; Stoler *et al.*, 1999). This technique uses degenerate primers to assay inter-microsatellite sequences for genomic instability. Prior to this, inter-SSR PCR had not been used to assay for instability in the mouse. In collaboration with the Conti laboratory, we were successful in adapting inter-SSR PCR for the analysis of genomic instability in mouse tumours (Benavides *et al.*, 2002). This is the first report using inter-SSR PCR to assay genomic instability levels in

Msh2$^{-/-}$ thymic lymphomas. We showed that 25% of thymic lymphomas were unstable. I have used a novel technique to demonstrate that various forms of genomic instability are driving MMR deficient lymphomagenesis.

Despite finding genomic instability in the *Msh2*$^{-/-}$ thymic lymphomas, as assayed using a candidate gene screen, microsatellite instability assay and inter-SSR PCR, I found lower than expected levels of instability at the sequence level. This led me to hypothesize that other types of genetic instability may be present, driving the early onset of thymic lymphomas observed in the MMR deficient mouse models. To test this hypothesis, I assayed *Msh2*$^{-/-}$ thymic lymphomas for the presence of chromosomal instability that may co-exist with the already identified genomic instability to drive tumorigenesis (Chapter 4).

Future Directions

As discussed earlier, our findings that spontaneously developing *Msh2*$^{-/-}$ murine tumours show low levels of MSI+ as compared with no microsatellite instability seen in the UVB induced tumours, was unexpected. I hypothesize that other induced tumours will be microsatellite stable as well. Further investigation of this phenomenon (prior to testing on mouse models) might include analysis of MMR deficient cells for MSI+, after irradiation treatment or drug treatment.

Moderate levels of chromosomal instability in murine *Msh2*$^{-/-}$ thymic lymphomas and *Msh2*$^{-/-}$ mouse embryonic fibroblasts

In the absence of MMR, DNA alterations at the sequence level, as reflected by MSI+, is well characterized (reviewed (Peltomaki, 2001b). However, given that my experiments showed low levels of MSI+ in *Msh2*$^{-/-}$ murine lymphomagenesis and that human lymphomagenesis and leukemiagenesis often coincides with the presence of chromosomal instability (CIN), I hypothesized MMR deficient murine lymphomas may present with CIN, as well as MSI+. Spectral karyotype analysis of *Msh2*$^{-/-}$ thymic lymphomas revealed that the majority of tumours present with translocations and rearrangements as well as whole chromosomal gains and losses (Chapter 4). Chromosomal instability has never

before been investigated in MMR deficient murine lymphomas. This novel finding shows that MSI+ and CIN may be functioning together to generate sufficient genetic instability that tumorigenesis is induced. My results suggest that CIN and MSI+ do not have to be mutually exclusive pathways, but that both types of genomic instability can arise and contribute to tumorigenesis. In addition, CIN may arise as a direct result of MSI+, through mutations occurring in genes regulating spindle pole movement in a targeted manner, if such genes contain coding mononucleotide runs. No single chromosomal translocation or rearrangement, identifying a chromosomal location of a critical gene in lymphomagenesis, appears to be occurring in the *Msh2*$^{-/-}$ thymic lymphomas. The common occurrence of multiple chromosomal alterations in each lymphoma suggests that in *Msh2*$^{-/-}$ thymic lymphomas chromosomal aberrations are an important means of cellular dysregulation. I propose that the chromosomal instability seen in the *Msh2*$^{-/-}$ thymic lymphomas is a 'dynamic' or 'continuous' form of instability that is constantly changing, giving rise to new mutations. Prior to work presented in this thesis, tumours showing MSI+ were thought to show little or no chromosomal instability and karyotype analysis performed on cell lines deficient in MMR suggested these cell lines were diploid or near-diploid (Abdel-Rahman *et al.*, 2001; Melcher *et al.*, 2002; Tsushimi *et al.*, 2001). The microsatellite status of tumours is gaining importance in clinical treatment as MSI+ has been reported in ~15% of sporadic colon tumours and numerous other cancers and is associated with resistance to various chemotherapeutic agents (Atkin, 2001). It will become critical to know the MMR states of all tumours, not only CIN tumours.

Future Directions

Are the existences of CIN and MSI+ independent from one another or possibly, a consequence of the other phenomenon? CIN may arise due to mutations in genes involved in spindle assembly or other processes governing chromosomal stability. If the loss of MMR is the first step in the tumorigenic pathway, then the resulting increase in mutation frequency may allow for mutations in coding repeats and the subsequent loss of chromosomal stability. Using bioinformatics approaches such as those discussed previously, only coding exon sequence from the Ensembl data base could be retrieved and

then analyzed using the Perl programming language. Gene names and functions could be parsed so that only exons from genes with relevant function are retrieved and parsed for coding repeats. This approach would allow for the identification genes involved in the maintenance of chromosomal stability that contain coding repetitive sequences that may be targets for mutation in the absence of MMR.

Constitutive loss of MSH2 in a rare human patient results in chromosomal translocations and rearrangements involving the telomeres

Our laboratory identified a 24 month old human patient with a novel homozygous mutation in the *MSH2* gene. Genetically, this patient recapitulates our Msh2 mouse model, lacking MMR activity from conception. He was successfully treated for T-cell acute lymphocytic leukemia by chemotherapy. Following chemotherapy, a lymphoblastoid cell line, from normal cells, was derived from this patient. With this cell line, I used spectral karyotype analysis (SKY) to assay for chromosomal instability in this rare human patient. Although, these cells were not from a tumour sample, I hypothesized that chromosomal instability was occurring for several reasons. i) constitutive loss of MMR in human patients has been shown to result in hematological malignancy (Bougeard *et al.*, 2003; De Vos *et al.*, 2004; Gallinger *et al.*, 2004; Ricciardone *et al.*, 1999; Vilkki *et al.*, 2001; Wang *et al.*, 1999a; Whiteside *et al.*, 2002). Thus, the lymphoblastoid cell line was created from a cell type that is prone to tumorigenesis in the absence of MMR (further discussed in Chapter 6) and an underlying level of ongoing instability may be occurring. ii) CIN is a common feature of hematological malignancy, iii) our laboratory found no evidence of MSI+ using DNA from this patient suggesting that other forms of instability are functioning to drive tumorigenesis (Whiteside *et al.*, 2002), and iv) chromosomal instability analysis on murine MMR deficient lymphomas indicates that CIN may be a feature of MMR deficient hematological malignancies and may be an important mechanism generating genomic instability in the pathway to tumorigenesis. SKY analysis of the cell line derived from this patient revealed

that almost one quarter (5/21) of the metaphase analyzed demonstrated abnormal karyotypes with four of the five abnormal metaphases presenting with translocations and rearrangements. Of the 21 metaphases examined, dicentric chromosomes were observed with high frequency suggesting chromosomal breakage and improper re-annealing has occurred. As well, all of the aberrations involved the telomeres. This is suggestive of a role for MMR in the maintenance of telomere function and in its absence, loss of telomere function may allow for improper telomere fusions and subsequent chromosomal instability. I have shown here for the first time, telomere associations in mammalian cells that lack MMR. This is discussed in more detail in Chapter 5. Since this work has been completed, this patient has relapsed, presenting with B-cell lymphoma. I hypothesize that the CIN detected in normal cells, post chemotherapy, was ongoing and made this cell type prone to chromosomal translocations and rearrangements in the absence of MMR. These types of alterations may have contributed to the development of the B-cell lymphoma in this patient.

Future Directions

The development of another malignancy in this patient may provide us with further material with which to study the progression of tumorigenesis in patients who lack MMR from conception. Previously, we have used non-tumour tissue from this patient for analysis (DNA, lymphoblastoid cell line). Access to a tumour sample would allow me to compare the levels of CIN in the normal lymphoblastoid cell line to that of the B-cell lymphoma tissue. Does the tumour contain any of the same chromosomal rearrangements found in normal lymphoblastoid cells suggesting that selective pressure as selected for those changes

A lack of DNA mismatch repair results in an increase in telomere fusions in mouse embryonic fibroblasts

The chromosomal alterations involving telomeres detected by SKY analysis of a rare human patient deficient in MSH2 as well as previous work in *S. cerevisiae* suggest that MMR may play a role in the maintenance of telomere function (Bechter *et al.*, 2004; Rizki & Lundblad, 2001). I hypothesized that MMR proteins may function to prevent

recombination of telomeres and maintain genomic stability in mammalian cells. Using *Msh2*$^{-/-}$and *Msh2*$^{+/+}$ MEFs, we show that in the absence of MMR there is a significant increase in telomere fusion events (Chapter 5). This suggests that MMR functions in mammalian cells to maintain telomere function. How does MMR affect telomere function? Telomere length may be altered in the absence of MMR resulting in abnormal telomere function. I compared telomere lengths in MMR proficient compared with MMR deficient cells. Using murine thymocytes, splenocytes and MEFs, I showed there to be no difference in telomere lengths. Although MMR does not appear to affect telomere length in mouse cells, MMR may affect telomere function in other ways or our assay is not sensitive enough to pick up subtle changes in telomeres lengths that may be biologically relevant.

DNA mismatch repair is involved in the regulation of centrosomes

The increase in telomere fusions seen in *Msh2*$^{-/-}$ MEFs suggests that the lack of MMR is leading to an increase in chromosomal instability. This is consistent with the results of Chapter 4. In Chapter 5 I further investigated the levels of chromosomal instability in *Msh2*$^{-/-}$ MEFs using G-banding techniques. I showed that in the absence of MMR, chromosomal aneuploidy was present. Centrosomes are critical in maintaining proper chromosome segregation during mitosis and centrosome aberrations have been implicated in the development of tumour aneuploidy. Hypothesizing that chromosomal gains and losses may be arising due to aberrant centrosomal proteins, I used immunofluorescence techniques to analyze the numbers of centrosomes present in MMR proficient and MMR deficient MEFs. I found that in the absence of MMR there was a significant increase in the number of centrosomes. This has never been shown to be occurring in the absence of MMR. This is an important finding as centrosome dysregulation suggests a novel role for the MMR proteins. Interestingly, other DNA repair proteins have been shown to be involved in centrosome regulation. Specifically, the loss of BRCA1 in MEFs results in multiple functional centrosomes leading to unequal chromosome segregation and aneuploidy (Xu *et al.*, 1999). BRCA1 is part of the BASC complex, (BRCA1-associated genome surveillance complex) composed of tumour suppressors, DNA damage repair

proteins and signal transducing proteins (Wang *et al.*, 2000). Msh2 in known to be part of this BASC complex. If, without BRACI you get centrosome hyperamplification, it is possible that another protein that is part of the same complex may produce the same phenotype if absent. I suggest that this centrosome hyperamplification seen in the absence of MMR leads to the increase in cellular aneuploidy observed and this may be contributing to the development of tumorigenesis.

Future Directions

1) I have shown that, in the absence of MMR, hyperamplification of centrosomes is seen. Centrosomes are known to be part of the microtubule organizing center and, as such, produce functional spindle poles that attach to chromosomes and pull them apart during cell division. The next step would be to show that the increase in the numbers of centrosomes results in functional centrosomes, producing multiple spindle poles. This would give further evidence that the absence of MMR results in a change of centrosome function.

2) To further prove that the hyperamplification of centrosomes that we see is a direct result of a loss of MMR, recombinant protein assays could be used. Using immortalized MEFs deficient in Msh2 (and demonstrating centrosome hyperamplification), I would reintroduce Msh2 into the cells. I would allow them to grow for several generations to see if Msh2 would be able to rescue the hyperamplification phenotype showing the MMR plays a direct role in centrosome regulation.

Generation of a novel mouse model to study hereditary non-polyposis colorectal cancer

Mice that are homozygous null for Msh2 or Msh6 are prone to tumorigenesis and commonly develop early onset thymic lymphomas (de Wind *et al.*, 1995; Reitmair *et al.*, 1995). I hypothesized that Msh2 and Msh6 deficient mice succumb to thymic lymphomas prior to the development of other cancers such as colonic tumours and are therefore prevented from developing a broader tumour spectrum similar to human HNPCC patients. To further understand the tissue specificity of the T-cell lymphomas arising in the Msh2

and Msh6 deficient mice, I decided to use genetic means to create a novel animal model: I bred *Msh2*$^{-/-}$ and *Msh6*$^{-/-}$ mice to athymic nude mice to determine if tumours would arise in other tissues in the absence of a thymus. These mice continued to develop hematological malignancies, developing lymphoblastic lymphomas, predominantly of B-cell origin. The continued development of hematological malignancy even in the absence of the thymus suggests that MMR plays an important role in the maintenance of genomic stability in hematological cell types and in its absence these cell types are prone to instability and malignancy. This is a novel role for the MMR proteins and I have developed a new animal model that can be used for future studies to elucidate possible roles for MMR in T and B cell development. In conclusion, I have furthered our understanding of how tumorigenesis arises in a background lacking MMR.

Analysis of DNA mismatch repair protein expression in the mouse using western blot techniques

Previous RNA expression analysis showed Msh2 expression to be ubiquitous (Varlet *et al.*, 1994). However, thymus was not included in this experiment and the experimental results for the spleen were unclear. Using normal mouse protein extracts, I showed high levels of Msh2, Msh6 and Pms2 MMR protein in murine thymus and spleen. I suggest that this high level of expression is due to high levels of MMR proteins in T-cells. This would help explain why the thymus is prone to tumorigenesis in mouse models. High levels of MMR protein expression suggest a strong role for MMR in that tissue and removing MMR has drastic consequences. I suggest there is a particular requirement for MMR in the maintenance of B and T-cell stability.

Conclusions

MMR is involved in several cellular processes that contribute to tumorigenesis. Using several techniques, I investigated the levels of genetic instability in MMR deficient murine lymphomas leading to tumorigenesis. As well, I've exploited mouse models to further understand the role of MMR in B and T-cell function. I've furthered our understanding of

the multi-functional roles that the MMR proteins have within a cell. I've shown that MMR influences many different cellular pathways and I've provided evidence for its role in microsatellite instability, chromosomal instability, telomere function, and centrosome function.

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