The role of p53 in virus-induced and multistep lymphomagenesis

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ABSTRACT

It has previously been demonstrated that p53 loss and Moloney murine leukaemia virus (MMLV) infection are weakly collaborative in T cell lymphomagenesis. The work described in this thesis aimed to investigate the role of p53 in MMLV-induced lymphoma and explore the basis of their relatively weak collaboration. The effects of p53 loss in combination with MMLV were explored first in primary fibroblasts. While no viral cytopathology was observed in these cells, it was found that the virus conferred a growth advantage on infected cells that was additive with the effects of p53 loss. MMLV has been reported to induce a preleukaemic phase of apoptosis in the thymus of infected mice and the role of p53 in this phenomenon was investigated next. The response was found to be short-lived, with peak onset varying according to host mouse strain. No evidence of direct induction of p53 was observed in thymus tissue *in vivo*, but evidence on the p53 dependence of the MMLV-induced apoptosis was equivocal, yielding only provisional conclusions.

The role of p53 was then examined at the later stages of MMLV disease and tumours were analysed for loss of heterozygosity of the wild type p53 allele and expression of functional p53 protein with prolonged *in vitro* and *in vivo* passage. Two mouse models were used; a cohort of MMLV-infected mice and also a Runx2/Myc surrogate model which develop tumours after a significantly reduced latent period. The data suggested that whilst p53 loss is obligatory for *in vitro* culture of tumour cells, it is not required for the growth of tumours passaged *in vivo*. The sparing effect was not absolute, however, and there was some evidence of p53 pathway inactivation and allele loss in progressing tumours.

In summary, I conclude that the weak synergy observed between MMLV and p53 loss is principally due to functional overlap, largely driven by activation of MMLV target genes such as Myc and Runx2 that collaborate to suppress p53-dependent pathways *in vivo*.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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DECLARATION

I, Fenella Harriet Aylett Long, do hereby declare that the work carried out in this thesis is original, was carried out by either myself or with due acknowledgement, and has not been presented for the award of a degree at any other university.

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CHAPTER 1

INTRODUCTION

1.1 MULTISTEP CANCER

The development of cancer is a multistep process that involves the mutation of key genes involved in regulating the proliferation, differentiation, survival and invasive properties of cells. Cancer arises when individual somatic cells sustain a series of sequential genetic mutations that provide a growth or survival advantage which allows them to escape from senescence and other growth control mechanisms (Hanahan and Weinberg 2000; Vogelstein and Kinzler 1993). Not only is the activation or over-expression of oncogenes known to be a significant factor in the development of cancer but the loss or inactivation of tumour suppressor genes is now also recognized to be of great significance. Mouse model systems which either overexpress oncogenes or have germline mutations in tumour suppressor genes are an invaluable resource in which to investigate these changes.

1.2 TUMOUR SUPPRESSOR GENES

Tumour suppressor genes are either mutated to an inactive form or transcriptionally downregulated during tumour initiation or progression and, therefore, are presumed to inhibit tumorigenesis when functioning normally (Jacks 1996). Knudson's "two hit" hypothesis for tumour suppressor gene loss during familial and sporadic cancers suggests that loss of heterozygosity (LOH) at the affected locus is a prerequisite for tumour growth (Knudson 1996; Knudson, Jr. 1971). Usually complete loss of function of these genes requires the sequential inactivation of both chromosomal copies since most tumour-associated mutations are recessive. In individuals that carry both wild type alleles of a tumour suppressor gene it would require the loss, usually through mutation or inactivation, of both alleles in order for them to develop tumours, consequently the sporadic occurrence of these tumours is relatively uncommon. By contrast animals that are heterozygous for a tumour suppressor gene are predisposed to tumours because of the increased likelihood of the remaining wild-type allele incurring a mutation.

In the example of human Li-Fraumeni syndrome there is an inherited mutation in one of the p53 alleles and, consequently, only one additional "hit" is necessary to inactivate the

other normal allele. Members of these families tend to develop a variety of tumours by age 30 (Malkin et al 1990; Malkin 1994) and in many of these tumours there is loss of heterozygosity at the p53 allele as is consistent with the “two-hit” hypothesis.

1.3 p53

The role of p53 as a tumour suppressor is observed in mice lacking a functional p53 gene either due to targeted gene knockout (Donehower et al 1992; Jacks et al 1994; Purdie et al 1994) or to transgenic expression of a mutant transgene that exerts a dominant negative effect (Lavigueur et al 1989). These mice are viable but prone to spontaneously develop a range of tumours, in particular lymphomas and sarcomas, primarily before 6 months of age (Donehower et al 1992). Mice that are heterozygous for p53 rarely develop spontaneous tumours before 9 months old (Harvey et al 1993b; Jacks et al 1994; Purdie et al 1994) and these tumours are reported to frequently undergo a deletion in the remaining wild type p53 allele in keeping with loss of p53 being significant in oncogenesis. However some of the tumours retain a functional intact wild type p53 allele implying that, whilst LOH of p53 occurs commonly, it is not a prerequisite for the development of lymphoma (Venkatachalam et al 1998). This suggests that, unlike other tumour suppressor genes, p53 may be an exception to the two hit model in that absence of both p53 alleles is not always necessary for tumour formation and that mere reduction in p53 levels may be sufficient to promote oncogenesis. Studies using carcinogens to induce tumours in p53 heterozygote mice suggest that whether or not LOH of p53 occurs may depend on the carcinogenic agent used and on the tissue targeted (French et al 2001; Venkatachalam et al 2001). For instance LOH of p53 was seen to occur frequently in carcinogen-induced thymic lymphomas and sarcomas but less often in urinary bladder carcinomas (French et al 2001).

1.3.1 Role of p53 in cancer

Approximately 50% of all human tumours carry a p53 mutation (Hollstein et al 1994; Toledo and Wahl 2006) and a proportion of the remaining tumours exhibits increased levels of the negative regulators of p53, for example mdm2 which may be amplified without coexisting p53 mutation. While p53 mutations often occur at an early stage of tumour development, for instance in cervical or squamous carcinomas, in other tumour types, such as colorectal carcinogenesis (Gryfe et al 1997), they occur late or may be selected for during establishment of cell lines *in vitro* (Gaidano et al 1991; Howard et al

1993). The role of p53 in cancer is likely to vary depending on the tissue involved, the chronology of the p53 alteration (early or late), the p53 function targeted (cell cycle or apoptosis) and the nature of any other changes already present in the cell (Soussi and Lozano 2005a)

1.3.2 History of p53

p53 was first identified in rodent cells transformed by the oncogenic Simian virus 40 (SV40) as a cellular protein that forms a stable complex with, and is inactivated by, the large T antigen of this virus (Lane and Crawford 1979; Linzer and Levine 1979) and later it was also found complexed with adenovirus and oncogenic papillomavirus oncoproteins (Sarnow et al 1982; Werness et al 1990). Because p53 is present in high levels in many tumours and tumour cell lines, and also because mutant p53 can immortalise early passage rodent cells, it was initially assumed that it was associated with cellular transformation and it was believed, therefore, that the p53 gene functioned as an oncogene (Eliyahu et al 1984; Hinds et al 1989; Jenkins et al 1984; Lavigueur et al 1989; Rovinski and Benchimol 1988). Later, however, it was realized that, whilst mutant forms of p53 may behave as an oncogene, wild type p53 behaves as a tumour suppressor gene and inhibits oncogene-mediated transformation of cells in culture (Eliyahu et al 1989; Finlay et al 1988; Finlay et al 1989). Further observations, such as the need to prevent expression of functional p53 in the development of murine Friend leukemia virus-induced tumours, support its role in tumour suppression (Ben-David et al 1990; Mowat et al 1985; Rovinski et al 1987). The most conclusive evidence, however, arises subsequent to the generation of p53 null mice and the fact that they are markedly more susceptible to tumour development than wild type mice (Donehower et al 1992).

1.3.3 "Guardian of the genome"

p53 is now known to be a multifunctional protein that is involved in a variety of biological processes although its predominant role is in the regulation of cell proliferation through induction of growth arrest or apoptosis in response to stress signals (Kubbutat and Vousden 1998). The effects of p53 are exerted through both activation and repression of transcription of a series of target genes that are involved in cell cycle regulation (Ho and Benchimol 2003). p53 has been called the "guardian of the genome" because of its capacity to prevent the accumulation of genetic alterations through the regulation of critical checkpoints in response to exogenous cellular insults (Lane 1992). The p53 protein

is stabilised and activated in response to a number of stimuli including exposure of cells to DNA-damaging agents, hypoxia, growth factors and activated oncogenes (Fritsche et al 1993; Graeber et al 1996; Kuerbitz et al 1992; Malkin 2001). Activated p53 can arrest cell cycle progression and allow either the DNA to be repaired or the cell to undergo apoptosis (Figure 1.1).

In tumour cells which harbour mutant p53, the protein is no longer able to control cell cycle regulation, resulting in inefficient DNA repair and the emergence of genetically unstable cells. Whilst the normal p53 protein has a very short half-life and is present within the nucleus at barely detectable levels, mutant forms of p53 exhibit markedly increased stability, have a half-life of several hours and accumulate within the neoplastic cell nucleus (Oren et al 1981; Reich et al 1983; Soussi 2000). These mutations inactivate the negative regulatory function of p53 with respect to cell growth and in many cases also confer a dominant phenotype involved in the maintenance and/or induction of transformation (Levine 1997).

1.3.4 p63 and p73

Two homologues of p53 have been identified relatively recently called p63 and p73. There is marked sequence similarity and conservation of functional domains amongst the three p53 family members. p63 and p73 display transcriptional activation, cell cycle arrest and apoptotic activities akin to those exhibited by p53 (Yang et al 2002). Dissimilarities include the facts that, unlike p53, both genes are necessary for normal development (Mills et al 1999; White and Prives 1999; Yang et al 1999; Yang et al 2000) and that they are rarely mutated in human tumours (Moll 2003).Their role in tumour suppression is somewhat controversial; initial reports suggested that there was no increase in tumour susceptibility in p63 and p73 knockout mice (Moll and Slade 2004; Perez-Losada et al 2005). However, in support of a tumour suppressive role, it has also been shown that mice heterozygote for p63 or for p73 develop a high frequency of malignant tumours and that loss of p63 and p73 cooperate with each other and with p53 loss in tumour suppression (Flores et al 2005).

Figure 1.1: Simplified outline of the p53 pathway

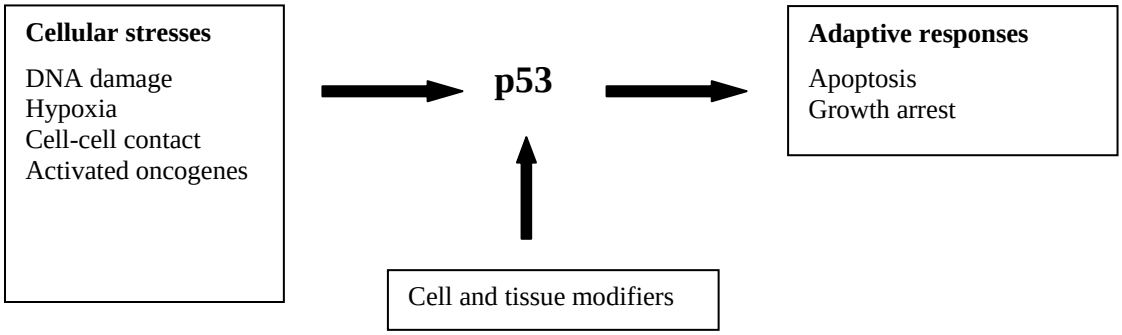


Figure 1.1 represents a simplified summary of upstream stimuli that induce p53 and the subsequent cellular responses to this. These are greatly influenced and modified by local cell and tissue environment.

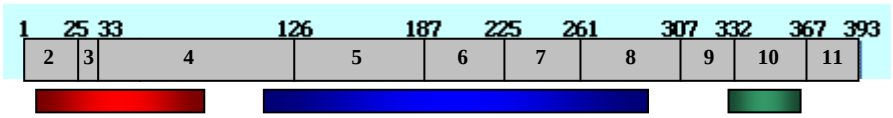
1.3.5 Structure of p53

The human p53 gene is located on chromosome 17 and encodes a protein that is 393 amino acids long. Two p53 genes exist in the mouse, a functional gene which resides on chromosome 11 and an inactive pseudogene on chromosome 14 (Czosnek et al 1984; Rotter et al 1984). The functional mouse p53 gene comprises 11 exons (Figure 1.2), the first of which is non coding, and encodes a protein of 390 amino acids (Bienz et al 1984; Pennica et al 1984). The p53 protein has several regions that have three main distinct, but inter-dependent functions; the central core containing its sequence specific DNA-binding domain, the multifunctional carboxy-terminal domain (C terminal region) and the activation domain (N terminal domain) as shown in Figure 1.2. Disruption of any of these areas leads to inactivation of tumour suppressor function (Ko and Prives 1996).

The p53 core is a region that folds so as to form a domain that interacts with DNA in a sequence-specific manner. 80-90% of p53 mutations have been identified to occur within this region (Ko and Prives 1996). In human cancer, different tumours often display different types of mutation within the p53 gene and this may reflect the different factors contributing to the aetiology of these tumours (Hollstein et al 1991).

The C terminal forms a region that has major regulatory properties. The function of this region is both to control sequence-specific DNA binding and to recognise DNA damage. This region contains the oligomerisation domain, nuclear localisation signals and a region at the extreme C terminus of the protein that is involved in regulation of the sequence–specific DNA-binding function (Kubbutat and Vousden 1998). The C-terminus is responsible for allowing p53 to exist as a tetramer in solution. The DNA binding of p53 is optimal when the protein is in a tetrameric state as a consequence of interactions in the four separated p53 molecules determined by the oligomerisation domain (Jeffrey et al 1995).

Figure 1.2: Simplified structure of the p53 protein



N terminal domain: **Central core:** **C terminal region:**

Transcriptional **Sequence-specific DNA** **Tetramerisation**

Figure 1.2 shows the basic structure of the human p53 protein which comprises several domains which include the central core responsible for sequence-specific DNA-binding, the N terminal region that contains the transactivation domain and the complex, multifunctional C terminal region. Exons are numbered 2-11.

The acidic N-terminal transcriptional activation domain allows p53 to activate the transcription of target genes such as p21 $^{WAF1/CIP1}$ (p21) and other growth regulatory genes (Malkin 2001). This region is also involved in regulating the stability and activity of the p53 protein via interactions with other proteins. The large T antigen of SV40 polyomavirus, the E6 protein of human papillomavirus and the mdm2 protein all bind to the N terminus and negatively regulate the transcriptional activation properties of p53.

1.3.6 Function of p53

p53 is expressed at basal levels in many tissues and is localized to the nucleus. Under normal physiological conditions the half-life of the p53 polypeptide is very short (20 minutes) and it exists in a largely inactive state that is relatively inefficient at binding to DNA and activating transcription (Lakin and Jackson 1999). Activation of p53 leads to a rapid increase, within minutes, in levels of p53 which accumulate in the nucleus. There is also increased ability of p53 to bind DNA and to mediate transcriptional activation of genes that lead to cell cycle arrest, during which time cells may repair DNA damage, or to apoptosis (Giaccia and Kastan 1998; Malkin 2001; Prives 1998).

p53 activation occurs largely as a result of increased stability of the p53 protein (Fritsche et al 1993). The extreme C terminus of the p53 protein controls its sequence–specific DNA binding and transcriptional activity, and these functions can be influenced by covalent and non covalent modifications within the C terminus. Complex post translational modifications of this region include deletion, phosphorylation, glycosylation and acetylation (Giaccia and Kastan 1998; Gu and Roeder 1997; Hickman et al 2002; Hupp et al 1995; Sakaguchi et al 1998). These modifications increase the stability of the protein, which results in its increased half life (hours), and can also activate the DNA-binding function of p53 thus regulating its activity and influencing the expression of target genes. Differences in sequence-specific DNA-binding activity explain the ability of p53 to adopt active or latent forms.

1.3.7 Mdm2

p53 is a very efficient inhibitor of cell growth and, as such, has to be well regulated. Mechanisms which regulate the activity of p53 act by controlling both the level and the location of the p53 protein. The absolute level of p53 is dependent on a balance between protein synthesis and degradation. In normal “unstressed” cells there is rapid turnover of

p53 which is mostly attributed to the protein mdm2 (murine double minute 2) which shuttles p53 from the cell nucleus into the cytoplasm where it undergoes ubiquitin-mediated proteolytic degradation (Bringold and Serrano 2000; Haupt et al 1997; Kubbutat et al 1997). Although some degradation can occur in the nucleus, efficient degradation requires p53 to be exported from the nucleus into the cytoplasm (Balint and Vousden 2001; Malkin 2001). The cellular location of p53 is also a significant factor in terms of p53 function. p53 activates transcription within the cell nucleus whereas cytoplasmic p53 has reduced functional activity.

Mdm2 binds to p53 within the N-terminal transactivation domain and this interaction blocks the ability of p53 to activate transcription and expression of target genes and also promotes the rapid degradation of p53 (Malkin 2001). Cellular insults that lead to p53 activation frequently do so by causing selective changes in its phosphorylation status which decrease its interaction with mdm2 so that p53 levels in the nucleus increase. Different patterns of phosphorylation are seen in response to different insults so that no individual phosphorylation site is responsible for p53 stabilization in response to all signals (Lakin and Jackson 1999; Meek 1998). The multiplicity of these sites of covalent modification and their responsiveness to a wide range of signals suggest that p53 activity is tightly and coordinately controlled in response to stresses and changes in the cellular environment.

Anything that disrupts the interaction between mdm2 and p53, for instance mutations affecting either molecule, will lead to the accumulation of transcriptionally active p53 (Bottger et al 1997; Kubbutat and Vousden 1998; Middeler et al 1997). Furthermore, mdm2–deficient mice are only viable in a p53 null background emphasising the critical role of mdm2 in regulating p53 (Jones et al 1995; Montes et al 1995). An autoregulatory feedback loop exists between p53 and mdm2, whereby p53 activates expression of its own negative regulator. The p53 protein regulates the mdm2 gene at the level of transcription and the mdm2 protein regulates the p53 protein at the level of its activity (Wu et al 1993). This mdm2-p53 feedback loop maintains both p53 and mdm2 at low levels in normal unstressed cells. Mutant p53, such as frequently accumulates within tumour cells, does not bind to mdm2 and is therefore stabilized within the cell. Any p53 that is sequestered within the cytoplasm, unlike nuclear p53, is unable to upregulate the expression of mdm2 and is also stabilized (Kubbutat and Vousden 1998).

1.3.8 INK4a/ARF

The INK4a/ARF locus is second only to p53 in the frequency of its disruption in human cancer (Sherr 2004b). It encodes two unrelated, overlapping tumour suppressor genes $p16^{INK4a}$ and $p19^{ARF}$ ($p14^{ARF}$ in humans) which govern the antiproliferative functions of the retinoblastoma (Rb) and p53 proteins respectively (Haber 1997). Rb, p53, $p16^{INK4a}$ and $p19^{ARF}$ form part of a network that monitors mitogenic signals and restrains aberrant growth-promoting signals from driving cell cycle progression inappropriately. Inactivation of this signaling network occurs in most, if not all, forms of human cancer (Sharpless 2005; Sherr 2004b).

The $p16^{INK4a}$ and the $p19^{ARF}$ genes have their own separate promoters, each of which produces a different transcript. The $p16^{INK4a}$ protein induces G1 arrest through its direct binding to, and inhibition of, cyclin-dependent kinase (CDK) molecules. This growth suppression is mediated by Rb, since over-expression of $p16^{INK4a}$ results in G1 arrest only in cells with functional Rb (Ghebranious and Donehower 1998). $p19^{ARF}$ encodes a second protein which is translated in an alternate reading frame. $p19^{ARF}$ has evolved to trigger p53 activation in response to selective inducers that drive inappropriate cell proliferation, for example following over-expression of oncogenes such as *myc* (Adhikary and Eilers 2005; Gallagher et al 2006; Sherr 2004a; Zindy et al 1998). $p19^{ARF}$ activates p53 by binding directly to mdm2, thereby inhibiting the ubiquitin ligase activity of mdm2 (Ashcroft and Vousden 1999; Lakin and Jackson 1999; Prives 1998) and by sequestering mdm2 within the nucleolus, prevents nuclear export of p53 which is necessary for its degradation (Lowe and Sherr 2003; Sherr 1998; Sherr 2001; Tao and Levine 1999; Weber et al 1999; Zhang and Xiong 2001). The $p19^{ARF}$ protein is involved in suppressing cell proliferation. Over-expression of $p19^{ARF}$ results in stabilisation of p53 and activation of its downstream targets (Bringold and Serrano 2000; Eischen et al 1999). It is, therefore, able to trigger a p53-dependent transcriptional program which leads to cell cycle arrest, both in G1 and G2 (Bringold and Serrano 2000; Kubbutat and Vousden 1998).

Expression of the $p19^{ARF}$ transcript is ubiquitous in post natal tissues, in contrast to the more restricted expression pattern of $p16^{Ink4a}$ (Haber 1997). The normal levels of expression of $p19^{ARF}$ and of $p16^{INK4a}$ are extremely low in most tissues (Serrano 2000) which suggests that, rather than constantly restraining proliferation, they are awaiting activation by inappropriate, hyperproliferative, oncogenic signals. p53 negatively regulates the expression of $p19^{ARF}$ (Stott et al 1998) and, in cells lacking p53, $p19^{ARF}$

levels increase significantly due to interruption of the p53-p19^{ARF} feedback loop. This is readily appreciated at the protein level, especially in cases where mutant p53 and p19^{ARF} accumulate together (Eischen et al 1999). Conversely, the enforced ectopic expression of p53 in such cells can restore p19^{ARF} to its normal levels (Kamijo et al 1998; Stott et al 1998).

1.3.9 p53 and cell cycle arrest

Cell growth normally proceeds through complex signaling pathways that maintain the coordinated sequence of DNA synthesis (S phase) which precedes mitosis in the cell cycle. Cyclin-dependent kinase enzymes (CDKs) determine cell cycle progression and their activation depends on association with a phase-specific cyclin protein. Cyclin-dependent kinases play an important role in mediating the transition through several points in the cell cycle, and anything that inhibits these enzymes may arrest cell growth (Vousden 1995). Damage to DNA activates "checkpoints" that aim to ensure genome integrity. This occurs through the inhibition of CDKs which leads to cell cycle arrest and thus allows DNA repair prior to replication (G1 checkpoint) or mitosis (G2 checkpoint) with apoptosis constituting an alternative pathway of eliminating DNA damaged cells (Gottlieb and Oren 1996). Many cancers display loss of checkpoint function with subsequent replication of damaged DNA.

Although the decision as to whether p53 will induce cell cycle arrest or apoptosis is not fully understood, it is profoundly influenced by cell type and tissue-specific modifiers such as growth factor availability (Bates and Vousden 1996; Gottlieb and Oren 1996; Haupt et al 1996; Lin and Benchimol 1995; Macleod and Jacks 1999; Midgley et al 1995; Oren 2003; Vousden and Lu 2002). An example of this is exhibited by haematopoeitic Baf–3 cells which respond to irradiation by arresting in G1 in the presence of IL-3 but undergo apoptosis in the absence of IL-3 (Canman et al 1995). Another example is seen in mice that undergo whole body gamma irradiation. This results in p53 accumulation in some tissues but not in others which further emphasises the importance of tissue specific factors in controlling the induction of p53. Furthermore only some of those tissues in which p53 is induced undergo apoptosis illustrating significant tissue control over the downstream effects of p53 (Midgley et al 1995). Other variables such as the extent of DNA damage and the levels of p53 also affect the choice between cell cycle arrest and apoptosis (Chen et al 1996; Levine 1997).

p53-mediated upregulation of p21 $^{WAF1/CIP1}$ (p21) leads to cell cycle arrest (el Deiry et al 1993). Through activation of p21, p53 is one of the major regulators of the G1/S and G2/M checkpoints (Bunz et al 1998; Waldman et al 1995). p21 is a cyclin-dependent kinase (CDK) inhibitor that belongs to the Cip/Kip family of CDK inhibitors and negatively modulates cell cycle progression. It can also bind to proliferating cell nuclear antigen (PCNA), a protein significant in DNA synthesis and repair, thereby blocking DNA synthesis (Gartel and Radhakrishnan 2005). Over-expression of p21 results in G1, G2 or S phase arrest and, conversely, p21 deficient cells fail to undergo cell cycle arrest in response to p53 activation after DNA damage (Gartel and Radhakrishnan 2005). The p21-dependent inhibition of CDK complexes functions by activating the retinoblastoma (Rb) protein to repress the E2F-dependent transcription of S phase genes and produce a G1/S arrest (Harper et al 1993). Similarly the Rb protein (pRb) has been shown to function in concert with p21 to reinforce the G2 checkpoint and ensure a blockade in inappropriate DNA replication (Flatt et al 2000; Niculescu et al 1998). Thus p53-mediated maintenance of G2 arrest appears to be dependent on p21 and pRb.

Cells deficient in p21 fail to undergo both G1 and G2 arrest and also fail to couple the processes of DNA synthesis and mitosis. These defects are also present in p53 null cells (Waldman et al 1995; Waldman et al 1996). In the absence of p21, DNA damaged cells arrest in a G2-like state, but then undergo additional S phases without intervening normal mitoses. They thereby acquire grossly deformed polypoid nuclei and subsequently die through apoptosis (Waldman et al 1996). Cells from p21 deficient mice show a normal p53-mediated apoptotic response, illustrating that this function, unlike cell cycle arrest, is not dependent on the activation of p21 (Brugarolas et al 1995; Deng et al 1995).

The p21-induced pause in cell cycling allows cells time to repair DNA. p53 induces the transcription of GADD45 (Growth arrest and DNA damage), a protein involved in DNA nucleotide excision and repair, which also has a role in the G2/M checkpoint (Smith et al 1994; Waldman et al 1996). G2/M cell cycle arrest is complex and is controlled by both p53–dependent and p53–independent mechanisms (Taylor and Stark 2001). p53 regulates this G2/M transition by controlling the cyclin dependent kinase cdc2 which is necessary in order to enter the mitotic phase of the growth cycle In many cells GADD45 is required for a normal p53-dependent G2/M checkpoint in response to certain kinds of DNA damage and this is due to its ability to inhibit cdc2 kinase (Jin et al 2002; Wang et al 1999; Zhan et

al 1999). Cells from GADD45-null mice demonstrate aneuploidy, chromosome aberrations and other features of genomic instability consistent with its role as one of the downstream targets of the p53 pathway involved in maintaining genomic stability (Hollander et al 1999; Hollander et al 2005).

In conclusion, the aim of cell cycle arrest is to allow repair of DNA. If this is successful then p53 activates mdm2 which down regulates p53, thus relieving the cell cycle block (Ko and Prives 1996). If the DNA cannot be successfully repaired then apoptosis is induced.

1.3.10 Rb and p53 network to control cell cycle/apoptosis

The retinoblastoma gene, Rb, was the first tumour suppressor gene to be characterised. Its protein product, pRb, is a nuclear phosphoprotein that also plays a key role in regulating the cell cycle. (Knudson 2001). The Rb protein exists in either an active hypophosphorylated or an inactive hyperphosphorylated state. In its active state pRb serves as a brake on the advancement of cells from the G1 to the S phase of the growth cycle. When the cells are stimulated by growth factors, the Rb protein is inactivated by phosphorylation, the brake is released and the cells traverse the G1-S checkpoint. The p53 and Rb genes are frequent targets of mutation during tumorigenesis and loss of function of both the p53 pathway and the retinoblastoma protein pathway plays a significant role in the development of most human cancers.

The Rb and p53 pathways share some interconnected signaling pathways (Figure 1.3). The active form of Rb blocks entry into the S phase of the growth cycle by binding to and inhibiting members of the E2F family of transcription factors (Sherr and McCormick 2002). The E2F family mediates transcription of genes required for DNA synthesis. The binding of hypophosphorylated pRb to E2F has been shown to inhibit E2F-dependent transcription of S-phase genes and arrests cells at the G1/S transition (Dyson 1998). E2F release during phosphorylation of Rb, stimulates $p19^{ARF}$ expression which in turn results in inhibition of mdm2 and stabilisation of p53 (Bates et al 1998). Activation of p53 induces transcription of p21 which, by inhibiting the cyclin dependent kinases (CDKs), activates Rb and leads to cell cycle arrest.

Cooperation between the p53 pathway and the Rb/E2F pathway is involved in determining the outcome of DNA damage. Loss of Rb function has been associated with loss of G1 arrest after DNA damage. Over-expression of Rb, however, can block p53-dependent apoptosis (Demers et al 1994).

Figure 1.3: Interconnecting pathways between p53 and Rb

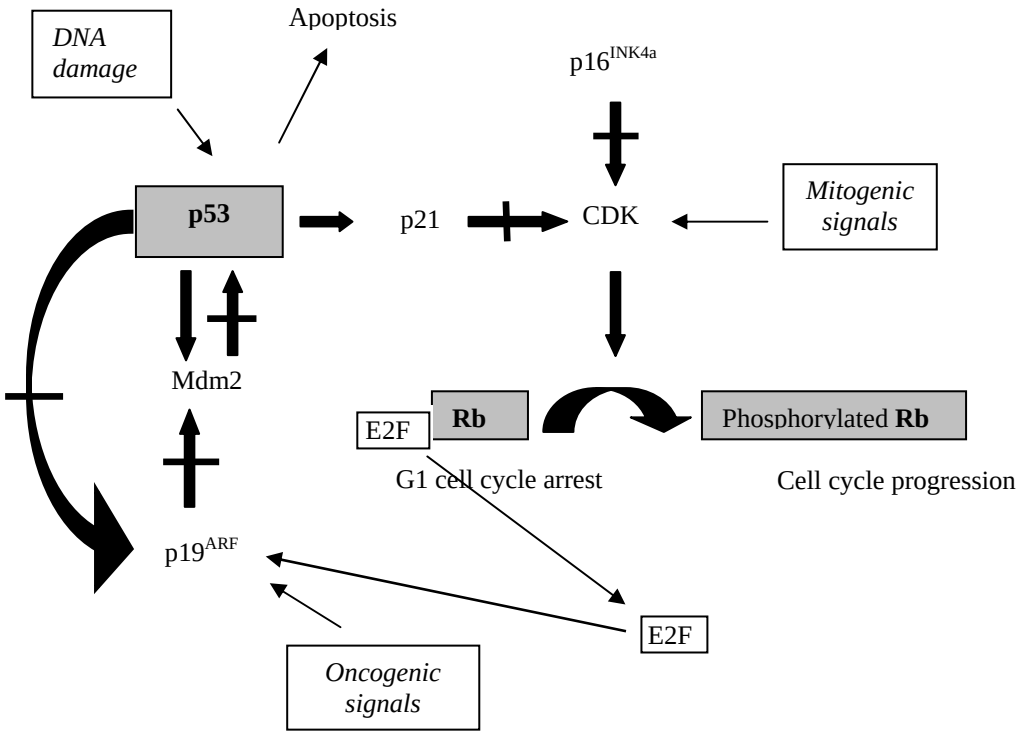


Figure 1.3: Mitotic signals activate cyclin-dependent kinases (CDKs) which phosphorylate Rb. p16 ^{INK4a} inhibits the activity of CDKs to prevent phosphorylation and thereby activate Rb and thus prevents entry into S phase by inhibiting the E2F transcriptional programme. When Rb is phosphorylated it releases E2F which then activates transcription of S phase genes. E2F also activates $p19^{ARF}$ which inhibits mdm2 and thus induces the p53 response; leading either to p53-dependent apoptosis or to induction of the CDK inhibitor p21. Inactivation of CDKs by p21 leads to G1 cell cycle arrest. DNA damage can activate p53 via $p19^{ARF}$ independent signals. p53 negatively regulates the expression of $p19^{ARF}$

1.3.11 p53 and apoptosis

Apoptosis or programmed cell death is a physiological process that is critical for organ development and tissue homeostasis by elimination of defective or potentially dangerous cells without eliciting any associated inflammatory response. Apoptotic cells exhibit characteristic morphological changes that include cell shrinkage, nuclear condensation, DNA fragmentation and plasma membrane blebbing with redistribution of phosphatidylserine to the cell surface (Zimmermann and Green 2001). These changes arise from the activation of intracellular cysteine proteases called caspases (Malkin 2001). p53 both activates and represses many genes that participate in the apoptotic response (Balint and Vousden 2001).

Damage to cellular DNA often has harmful consequences, such as causing gene mutations and malignant transformation, and is therefore usually better removed than tolerated. As discussed previously, this can occur in two ways: either through the repair of DNA (during cell cycle arrest) or by the induction of cell death (apoptosis) and in situations where repair systems are defective, apoptosis is increased (Christmann et al 2003). Although damage to other cellular components can also trigger apoptosis (Tenzer et al 2002), it is damage to DNA that is by far the major initiator (Roos and Kaina 2006). Specific DNA lesions that trigger apoptosis have been identified, the most consistent and lethal of which are double strand breaks (DSBs) in the DNA (Lips and Kaina 2001). DSBs may arise as a primary lesion as in response to ionizing radiation (Teoule 1987), but are also frequently formed following disruption to cell replication due to faulty repair of other primary DNA lesions (Ochs and Kaina 2000). DSBs activate the ATM (ataxia telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3 related) proteins (Ismail et al 2005) which, depending on their cellular environment (ie cell cycle phase and available downstream signaling targets), result in three different possible outcomes: cell cycle arrest, DSB repair or apoptosis via p53 activation (Lavin et al 2005). It is thought that where there are low levels of DSBs, only low levels of p53 are activated by ATM and this upregulates p21 and causes cell cycle arrest. However where there are high levels of DSBs, enough p53 accumulates to activate pro-apoptotic genes such as Bax, Puma and Fas receptor (Roos and Kaina 2006).

The protease (caspase) cascade is the main pathway involved in cell death with the aspartate-specific caspases being the essential mediators of apoptosis (Alnemri et al 1996;

Martin and Green 1995). These act by cleaving cellular proteins via their aspartate residues. By virtue of their proteolytic function, caspases can activate each other, functioning as a powerful activation cascade (Zimmermann and Green 2001) and have, consequently, to be tightly controlled. Because of this they exist latent in the cell cytosol until they are needed at which point they can be rapidly activated (Wolf and Green 1999). Caspases have been grouped into initiator caspases (procaspases 8 and 9) which are involved in upstream regulatory events and effector caspases (3, 6 and 9) which are responsible for cleaving cellular proteins and thus cell death (Zimmermann et al 2001).

There are two main, and fundamentally distinct, pathways which both eventually lead to caspase activation (Wang et al 2005), subsequent cleavage of specific cellular substrates and the resultant morphological and biochemical changes that are associated with apoptosis (Zimmermann et al 2001). The first or intrinsic apoptotic pathway depends on involvement of mitochondria, is receptor-independent and is regulated by members of the Bcl-2 family of proteins (Burlacu 2003; Hinds and Day 2005). The second or extrinsic pathway involves interaction of a death receptor with its ligand. These receptors include tumour necrosis factor receptor-1 and the Fas receptor (Danial and Korsmeyer 2004). The use of transgenic and gene-ablated mice has helped identify the effects of many of the components of the apoptotic pathway (Ranger et al 2001).

1.3.11.1 Intrinsic pathway

The mitochondria play an important role in apoptosis by releasing factors, most notably, cytochrome c, from the mitochondrial intermembrane space into the cytoplasm (Green and Reed 1998; Kluck et al 1997; Yang et al 1997). Cytochrome c is encoded in the nucleus and it is only when it is imported into the mitochondrion and coupled with a haem group that it becomes active and able to induce caspase activation (Yang et al 1997).

The Bcl-2 family of proteins are divided into groups; the first consists of anti-apoptotic members such as Bcl-2 and Bcl-XL which prevent cytochrome c release from the mitochondria (Kluck et al 1997; Yang et al 1997). Bcl-2 is localized to the mitochondrion which emphasizes the importance of this organelle (Hockenbery et al 1990). The pro-apoptotic members are subdivided into two groups; the Bax subclass (ie Bax, Bak and Bok) and the BH3-only molecules (ie Bid, Bad, Noxa, Puma and Bim) (Nakano and Vousden 2001; Oda et al 2000; Yu et al 2001). The Bax group cause intracellular

membrane disruption and therefore cell death. The BH3-only group act by binding to and inhibiting their anti-apoptotic Bcl-2 counterparts (Cory et al 2003; Danial and Korsmeyer 2004). Cell stress induces pro-apoptotic members of the Bcl-2 family to translocate from the cytosol to the mitochondria. Although pro-apoptotic Bcl-2 family members are found in other locations such as the endoplasmic reticulum and the nuclear envelope, their main effects are in the mitochondria where they promote release of the active form of cytochrome c (Zimmermann and Green 2001).

Cytochrome c is released into the cytoplasm, binds to and catalyses oligomerisation of APAF-1 (apoptotic protease activating factor). Procaspase-9 then forms a complex with them which is called an apoptosome (Chereau et al 2005; Jiang and Wang 2004; Zou et al 1999) which is now able to recruit and activate procaspase-3, which then leads to cascade amplification, activation of other procaspases and apoptosis (Li et al 1997; Zimmermann et al 2001; Zou et al 1999).

There is evidence that the p53 protein itself can directly localize to the mitochondria presenting an additional, less well characterised transcription-independent way of mediating apoptosis (Marchenko et al 2000; Soussi and Lozano 2005). In this system, p53 rapidly translocates to the mitochondria, interacts with Bcl family proteins, induces cytochrome c release and caspase 3 activation. This results in an initial rapid p53-dependent apoptotic response occurring soon after DNA damage that is followed later by an apoptotis response that is dependent on the transcription of p53 target genes (Erster et al 2004).

1.3.11.2 Extrinsic pathway

The second apoptosis pathway involves death receptors of the tumour necrosis factor receptor family (TNFR) which include Fas. Death receptors are linked by domains on “adaptor molecules” that couple them to intracellular procaspases (Wolf and Green 1999). For instance the adapter molecule FADD links the Fas death receptor to procaspase-8 (Ashkenazi and Dixit 1998). In this way binding of death receptors leads to the formation of an intracellular death-inducing signaling complex which couples the death receptor to a cascade of caspases resulting in apoptosis (Balint and Vousden 2001; Dragovich et al 1998; Zimmermann and Green 2001).

In summary, p53-induced apoptosis can occur by several different pathways with the potential to involve many different target genes none of which is the sole principal mediator. The relative contribution of different target genes such as Bax or Fas to p53–mediated apoptosis appears to be cell-type and signal dependent (Dragovich et al 1998; Malkin 2001) so that different tissues appear to exhibit distinct specificity in terms of which apoptotic pathways/p53-regulated genes they employ (Fei et al 2002).

1.3.12 Mutations in p53 in cancer

The p53 protein is a transcription factor that binds a very loose DNA recognition sequence that is found in several hundred genes that are differentially activated depending on the cell type, identity and extent of damage (Soussi and Lozano 2005). p53 gene rearrangements, deletions and mutations are a frequent, but not obligate, part of the pathway to malignant transformation *in vivo.* Furthermore, p53 mutations are also often observed *in vitro* (Ben David et al 1988). Although most tumour suppressor genes are inactivated by mutations that lead to absence of protein synthesis or production of a truncated product, more than 80% of p53 mutations are, in fact, missense mutations that lead to the synthesis of a stable full-length mutant protein that displays either partial or complete loss of its DNA binding activity but is retained and accumulates in the nucleus of tumour cells (Soussi and Lozano 2005). These mutations lead to the loss of some or all wild type functions.

There are 3 ways in which a mutation may affect the function of p53 and predispose to tumorigenesis: Firstly by total loss of wild type function; in many tumours there is a point mutation in the p53 allele and complete loss of the second allele (Greenblatt et al 1994) which results in synthesis of a mutant protein that has lost its tumour suppressor function. Mutant p53, which is transcriptionally inactive, is also often more stable than wild type p53 because of its inability to transactivate mdm2. As a result of lack of mdm2, mutant p53 is not degraded, often has a much longer half life than wild type p53 and accumulates to very high levels within the tumour cells (Kubbutat and Vousden 1998; Shieh et al 1997; Siliciano et al 1997). Secondly, in situations where the other p53 allele is present, this abnormal protein may exhibit dominant negative activity by hetero-oligomerisation with wild type protein expressed by the remaining allele, and thus reduce the number of functional p53 tetramers available in the cell and thereby interfere with the function of any normal p53 protein that is produced (Macleod and Jacks 1999; Munroe et al 1990;

Pavletich et al 1993; Venkatachalam et al 1998). As there is a much greater quantity of mutant p53 than of wild type p53 present in the cell there is likely to be a "squelching" effect by the presence of the mutant protein. Thirdly the mutant may exhibit a gain in oncogenic potential whereby it can activate genes that are generally involved in proliferation or cell survival or it may interact with a large number of cellular proteins and increase tumorigenicity directly (Ko and Prives 1996; Sherr 2004b; Soussi and Lozano 2005). In summary, therefore, it is observed that, although wild type p53 is a tumour suppressor gene and, as such, produces a predictable "loss of function" effect, some mutant p53 proteins may function as an oncogene and, in these instances, p53 is associated with a "gain of function" effect (Dittmer et al 1993; Soussi and Lozano 2005).

1.4 TRANSGENIC MICE

1.4.1 p53 Knockout mice

p53 null mice were developed concurrently in several laboratories with similar observations made by each group (Donehower et al 1992; Jacks et al 1994; Purdie et al 1994). With viable embryos and animals born from a null genotype it was originally assumed that p53 was not required for normal embryonic development (Donehower et al 1992). It is now apparent, however, that the role of p53 in development is not straightforward and a significant rate of abnormalities associated with p53 deficiency have been described which is predominantly, although not exclusively, seen in females (Armstrong et al 1995). There are significantly lower numbers of female p53 null mice compared to p53 null males at weaning, arising secondary to defects in neural tube closure, exencephaly and subsequent embryonic death. Many of these embryos also exhibit a range of craniofacial abnormalities. (Armstrong et al 1995; Sah et al 1995). The defects seen are influenced, to some extent, by mouse strain. p53 deficiency also results in abnormal spermatogenesis, with spermatocytes that are unable to undergo meiotic divisions to generate haploid sperm cells and instead undergo additional replications, giving rise to multinucleated giant cells (Rotter et al 1993).

p53 null mice all develop tumours; predominantly thymic lymphomas and, to a lesser extent, sarcomas; carcinomas occur uncommonly (Donehower et al 1992; Purdie et al 1994). Tumour latency averages about 4-5 months however mouse strain differences can influence both the rate of tumorigenesis and the predominant tumour type (Ghebranious and Donehower 1998). p53 heterozygote mice are also predisposed to the development of

tumours. Tumours in these mice develop after an increased latent period compared to the null mice population and the tumour spectrum is somewhat different also (Purdie et al 1994). These mice predominantly develop sarcomas of varying types. Lymphomas do occur but at a much lower frequency than in the p53 null mice. A small number of adenocarcinomas are also observed. A possible explanation is that by the time loss of the wild type p53 allele has occurred in these heterozygote mice, thymic growth has ceased and the cell populations that are now susceptible to neoplasia are the soft tissues and bone which are growing rapidly (Macleod and Jacks 1999).

Knock out mice have enabled major advances in understanding the action of the p53 gene. Mice subjected to gamma irradiation undergo an acute phase of p53-dependent tissue apoptosis and tissue damage and, at a later stage, develop radiation-induced tumours (Donehower et al 1992; Jacks et al 1994; Kemp et al 1994; Lain and Lane 2003). By contrast the same insult induces minimal apoptosis in tissues of p53 null mice. However 100% of these mice subsequently develop lymphomas emphasizing the role of p53 in apoptosis and in susceptibility to tumour development.

This acute phase of p53-mediated apoptosis was previously considered to be an unfortunate, but inevitable, side-effect of the tumour suppressive effects of p53. Recent work using mice in which p53 status can be switched from knockout to wild type on request (Christophorou et al 2005) has demonstrated that this is, in fact, not the case (Christophorou et al 2006). It was observed that the phase of p53-mediated widespread apoptosis was avoided if the mice were kept p53-deficient during the irradiation insult. Restoration of p53 function after this time period showed that the tumour protective effects of p53 were still present even when the preceding acute DNA-damage response to systemic genotoxic injury had not occurred. It was concluded from these observations that the p53-mediated response to irradiation has no relevance in regards to its subsequent tumour protective effects. This is because the tumour suppressive effects of p53 are mediated by the $p19^{ARF}$ pathway which is activated by aberrant cell proliferation rather than by DNA damage (Zindy et al 2003). The widespread DNA damage that is caused initially by the genotoxic insult leads to an acute phase of p53-mediated apoptosis and tissue damage which is not mediated by $p19^{ARF}$ and is, therefore, distinct from the tumour suppressive pathways. Occasional cells will sustain oncogenic mutations during this phase of DNA damage and those that survive, either unrepaired or misrepaired, will subsequently induce $p19^{ARF}$ and thus lead to p53 activation. It is this latter pathway that is

significant in protection against DNA damage-induced lymphomagenesis, at least in the murine background.

Efeyan et al also demonstrated similar findings (Efeyan et al 2006). They showed that $p19^{ARF}$ null mice respond normally to DNA damage (Kamijo et al 1999; Stott et al 1998) but that they are unable to respond to oncogenic signals irrespective of their p53 status (de Stanchina et al 1998; Palmero et al 1998; Zindy et al 1998) which further emphasizes that that the cancer-protective activity of p53 depends on oncogene signaling via $p19^{ARF}$ rather than on the DNA damage stimulus.

1.4.2 p53 super mice

Aside from the knockout mice, p53 super mice have also been generated which carry supernumerary copies of the p53 gene (Garcia-Cao et al 2002). These mice demonstrate an increased response to DNA damage but are also significantly more resistant to cancer compared to wild type mice. Super p53 mice have also demonstrated p53 to have an antiviral effect (Munoz-Fontela et al 2005). Interestingly, however, super p53 $p19^{ARF}$ null mice succumb to tumours at the same rate as p53 wild type $p19^{ARF}$ null mice once again underlining the role of $p19^{ARF}$ in the tumour protection pathway of p53 (Efeyan et al 2006).

1.4.3 Genetic resistance to chemotherapeutic drugs

Mouse models have also been extensively used to investigate the effects of chemotherapeutic drugs on tumours and to investigate why some tumours are sensitive to chemotherapeutic agents and others are relatively resistant. Tumour-derived cell lines, either *in vitro* or as grafts in nude mice, were previously the main way of evaluating treatment with a drug. Mouse models, however, now enable anti cancer drugs to be assessed in a more valuable way and the potential exists to compare response to treatment of tumours that may differ only in one specific gene (Schmitt et al 2000). Most chemotherapeutic drugs induce genotoxic injury and initiate predictable post damage responses which include apoptosis, cell cycle arrest and senescence (Johnstone et al 2002). Whilst these responses are not considered to be solely responsible for the drug's anti-tumour effects they are thought to contribute and to influence the tumour's sensitivity to treatment. As an extension of this, it might be expected that blocking apoptosis pathways may lead to drug resistance and alter treatment outcome. Lowe et al

demonstrated *in vitro* that the apoptotic phase following genotoxic cellular damage is p53-dependent and suggested that p53 status may have a significant influence on the efficacy of many anticancer drugs (Lowe et al 1993a). p53 mutations have been associated with aggressive tumours, poor prognosis and drug resistance in human patients (Moller et al 1999; Navaratnam et al 1998). It has also been shown that mutated p53 can produce multidrug resistance both *in vitro* and *in vivo* which can be reversed by the reintroduction of wild type p53 into p53 null cells (Wallace-Brodeur and Lowe 1999). Similarly INK4a/ARF mutations have also been shown to increase drug resistance by disabling p53 (Schmitt et al 1999). Needless to say the situation is not clear cut and many other factors such as tumour type and drug used will influence the outcome in each individual case (Herr and Debatin 2001).

1.4.4 p16INK4a/p19ARF knockout mice

Mice with targeted deletions of $p16^{INK4}$ and $p19^{ARF}$ or both have been generated by several labs (Kamijo et al 1997; Serrano et al 1996; Sharpless et al 2001; Sharpless et al 2004). Mice with inactivations of either $p16^{INK4a}$ or $p19^{ARF}$ are tumour prone, but neither is as severely affected as animals lacking both $p16^{INK4a}$ and $p19^{ARF}$ (Ghebranious and Donehower 1998; Sharpless 2005). Both p53 and $p19^{ARF}$ deficient mice spontaneously develop tumours and die of cancers early in life (Jacks 1996; Kamijo et al 1997; Kamijo et al 1999a; Sherr 1998). Perhaps not altogether unexpectedly, $p19^{ARF}$ null animals develop a tumour spectrum more similar to p53 null mice than do either $p16^{INK4a}$ or $p16^{INK4a}$/$p19^{ARF}$ null mice (Kamijo et al 1997; Sharpless 2005). However p53 null mice develop tumours at an earlier age than $p19^{ARF}$ null mice (Moore et al 2003) reflecting the fact that p53 is a more potent tumour suppressor than $p19^{ARF}$ presumably because it is induced by many different forms of cellular stress, including DNA damage and oncogene activation whereas $p19^{ARF}$ is triggered only by the latter (Moore et al 2003).

$p19^{ARF}$ stablilises p53 via inhibition of mdm2 and thus triggers p53-dependent growth arrest in G1 and G2 phases of the cell cycle or, in certain environments, sensitises the cells to apoptosis (de Stanchina et al 1998; Eischen et al 1999; Jacobs et al 1999; Schmitt et al 1999; Zindy et al 1998). Consistent with it being a classic “two hit” tumour suppressor gene, most $p19^{ARF}$ heterozygote tumours exhibit loss of the wild type $p19^{ARF}$ allele (Moore et al 2003). The anti-oncogenic effects of p53 and $p19^{ARF}$ are cooperative and deletion of $p19^{ARF}$ in p53 heterozygote mice significantly shortens tumour latency and

removes any selective pressure to lose the wild type p53 allele (Moore et al 2003). Similarly, both the p53 and the $p19^{ARF}$ alleles are retained in those tumours that develop in p53 heterozygote, $p19^{ARF}$ heterozygote mice.

It has always been considered that the tumour suppressive functions of $p19^{ARF}$ are mediated entirely through p53; double knock out mice lacking both $p19^{ARF}$ and p53 have a similar tumour incidence to those mice lacking p53 which is consistent with this (Weber et al 2000). Also consistent with $p19^{ARF}$ being primarily p53-dependent is the fact that variation in $p19^{ARF}$ dosage has little effect on tumour suppression in the absence of p53 (Moore et al 2003). It has been demonstrated, however, that triple knock out mice lacking $p19^{ARF}$, p53 and mdm2 develop tumours of a broader spectrum and at a higher frequency than double knock out mice lacking mdm2 and p53 or mice that lack p53 alone (Sherr 2006; Weber et al 2000). This suggests that there also exists a tumour suppressor role for $p19^{ARF}$ that is independent of mdm2 and p53 (Weber et al 2000).

1.4.5 Human cancer and p53

Whilst some types of tumours in humans display a very high incidence of p53 mutations (often >75% incidence), in other tumours the incidence may be rare which implies that loss of p53 is not essential for tumour progression in all instances (Calin et al 1999). p53 mutations occur relatively commonly in solid tumours (Harris 1996) whereas they are more infrequent in haematological malignancies (Imamura et al 1994). Nevertheless, alterations in p53 that occur within these tumours are associated with increased malignancy, more drug resistance and poorer patient prognosis (Krug et al 2002; Peller and Rotter 2003; Preudhomme and Fenaux 1997).

Myelodysplastic syndrome (MS) is an example of where the p53 gene is infrequently altered, only about 5-10% of cases exhibit changes, usually point mutations, of the p53 gene (Jonveaux et al 1991; Preudhomme et al 1993; Sugimoto et al 1993; Wada et al 1993) and when p53 gene mutations do occur they tend to be associated with progression to a more malignant tumour phenotype. Similarly p53 mutations occur rarely in the chronic phase of chronic myelogenous leukaemia (CML) but occur relatively often in human patients that develop the myeloid blast crisis of this disease which suggests that alterations in structure and expression of the p53 gene may be involved in the clinical progression of this disease (Ahuja et al 1989; Ahuja et al 1991; Foti et al 1991). In many

human cancers lack of p53 is also associated with increased tumour metastasis (Chen et al 2002; Kastrinakis et al 1995; Silvestrini et al 1996).

1.4.6 p53 and virus-induced cancers

As discussed already, p53 was discovered by virtue of its binding interaction with a viral oncoprotein (SV40 T antigen) (Lane and Crawford 1979; Linzer and Levine 1979). SV40 T antigen also binds to the Rb protein, thereby compromising two major tumour suppressor pathways (Pipas and Levine 2001). Oncogenic DNA viruses have developed a variety of strategies to inactivate or destabilize p53 and/or Rb. For example, the highly oncogenic human papillomavirus subtypes use two viral gene products, E6 and E7 to target p53 and Rb respectively (Hebner and Laimins 2006). DNA viruses, which replicate their DNA in the cell nucleus with the aid of the host machinery, have to overcome the effects of p53 and Rb that would otherwise act as checkpoint inhibitors of viral multiplication. Also, inactivation of these tumour suppressors may serve to pre-empt host cell apoptosis in response to viral infection (Neil et al 1997). Since completion of the lifecycle of DNA tumour viruses normally results in death of the host cell, no tumours are likely to arise and inactivation of tumour suppressors is of little consequence in most cases. However, abortive infection and expression of a limited array of viral early gene products can lead instead to cell transformation. Expression of viral antigens by such transformed cells usually leads to recognition by the host immune system and elimination, but the potent oncogenic potential of the DNA tumour viruses can be manifested in immunocompromised hosts or by infection of non-native, especially rodent, species (Ahmed and Heslop 2006; Collot-Teixeira et al 2004; Kuper et al 2000; Mackey et al 1976).

The interaction of oncogenic retroviruses with p53 is less well understood. Human T-cell leukaemia virus (HTLV) appears to operate similarly to the DNA tumour viruses as it encodes a transforming protein (Tax) which acts indirectly to inactivate p53 (Pise-Masison et al 2005; Pise-Masison and Brady 2005; Tabakin-Fix et al 2006). However, the majority of oncogenic retroviruses lack a transforming gene product such as this. The role of p53 in such cases forms one of the key issues addressed in this thesis. The basic properties of retroviruses and, in particular, the murine leukaemia viruses are now reviewed in some detail.

1.5 RETROVIRUSES

Retroviruses infect many animal species and although they were first discovered because of their association with cancer, not all of them are pathogenic or tumorigenic. (Coffin et al 1997). Many retroviruses have become integrated into the host germ-line and are inherited essentially as Mendelian elements and in many cases have become defective, losing the capacity to replicate or express gene products (Coffin et al 1997). These are termed endogenous retroviruses. The infectious or exogenous retroviruses are currently classified into 7 genera (see http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm) as shown in Table 1.1.

Table 1.1 Classification of Retroviridae

Family	Representative
Orthoretrovirinae	
Alpharetrovirus	Avian leucosis virus
Betaretrovirus	Mouse mammary tumour virus
Gammaretrovirus	Moloney murine leukemia virus
Deltaretrovirus	Human T-cell leukemia virus
Epsilonretrovirus	Walleye dermal sarcoma virus
Lentivirus	Human immunodeficiency virus
Spumaretrovirinae	
Spumavirus	Simian foamy virus

Table 1:1 shows the 7 classes of retrovirus within the subfamilies Orthoretrovirinae and Spumaretrovirinae and gives an example of a virus within each class.

1.5.1 Retrovirus structure and life-cycle

Retroviral particles are 80-100 nm in diameter and are surrounded by a viral envelope that is derived from the host during the process of assembly and budding from the cell membrane. This envelope surrounds a viral capsid (core) that contains the RNA genome (Coffin et al 1997). The viral genome contains the genes *gag,* which encodes internal structural proteins; *pol,* which encodes reverse transcriptase (RT) and integrase (IN) enzymes; and *env* which encodes envelope proteins (SU and TM) (Figure 1.4). This is the

"simple retrovirus" structure which describes the alpha and gammaretroviruses, while other families also encode one or more non-structural regulatory gene products. The retrovirus genome is single stranded RNA (ssRNA) of positive polarity, and the virus particle (virion) contains two such ssRNA molecules in a dimer linkage structure.

Figure 1.4: Schematic representation of a retrovirus

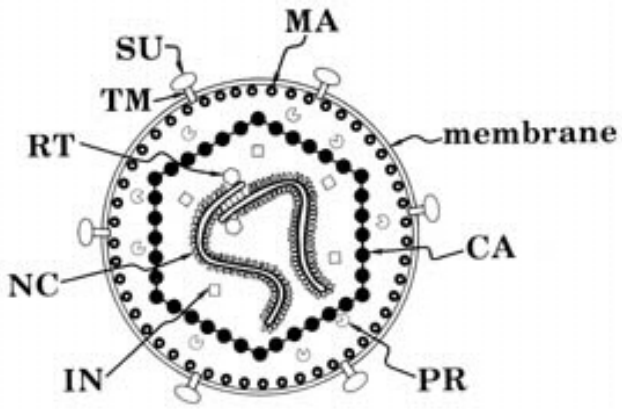


Figure 1.4 illustrates the basic structure of a retrovirus. The following viral proteins are represented: membrane associated protein (MA), capsid (CA), protease (PR), integrase (IN), nucleocapsid (NC), reverse transcriptase (RT), transmembrane (TM) and SU (surface).

The retroviral life-cycle begins with binding of virions to the cell surface and proceeds through uncoating, reverse transcription, integration of proviral DNA into the host genome, expression of proviral mRNA and viral proteins and, finally, assembly and budding of viral particles (Goff 2004). Reverse transcription of the RNA genome to dsDNA occurs in the cell cytoplasm. Reverse transcription results in rearrangement of the termini of the genome and the dsDNA is longer than its template viral RNA by virtue of a repeated sequence at each end, the long terminal repeat (LTR) that contains the proviral U3, R and U5 regions that are ultimately required for expression of viral genes. The viral LTR sequences contain both promoter and enhancer elements as well as a polyadenylation signal (Figure 1.5). Along with virion core proteins, this forms the pre-integration complex (PIC) which then enters the nucleus of the host cell (Jonkers and Berns 1996)

(Goff 2004). Integration of viral DNA is mediated by the viral integrase (IN) protein. Although integration into the host genome is not sequence or site-specific, there is some evidence that gammaretroviruses preferentially integrate near transcriptionally active regions of the host cell DNA (Wu et al 2006). For the simple retroviruses, including murine leukaemia viruses, successful integration requires cell division and passage through mitosis. Cells arrested in G1/G0 or in G2 are consequently not susceptible to infection.

Figure 1.5: Genome structure of a typical retrovirus

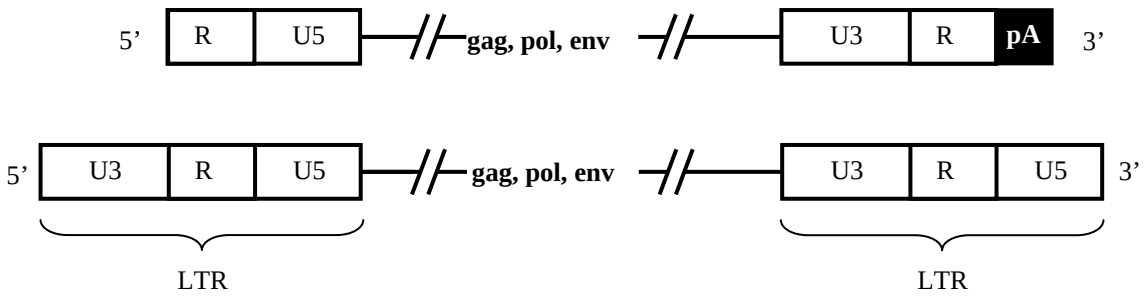


Figure 1.5: The top structure represents the genome of a simple retrovirus with the three viral genes; *gag*, *pol* and *env*. R represents terminal repeat sequences in virion RNA, and pA = polyadenylation signal. The lower structure represents the provirus after reverse transcription has occurred. LTR indicates the proviral long terminal repeat sequences. These are divided functionally into three parts: U3, R and U5. Transcription is initiated from a polII promoter at the 5’ LTR of the provirus at the U3-R junction.

The elements required for initiation, termination and modulation of retroviral transcription reside within the viral LTR sequences, whereas the factors necessary for transcription and translation are supplied by the host cell (Jonkers and Berns 1996). Proviral transcription results in production of copies of viral genomic RNA, which can either be translated to produce viral proteins or packaged by viral proteins to form new core particles. Cores form and mature while budding from the cell surface, during which time they obtain their envelopes consisting of cellular membrane and viral envelope glycoproteins (Coffin et al 1997). Infection by the simple retrovirus is generally non-cytopathic and results in a chronically infected producer cell (Fan 1997).

1.5.2 Murine leukaemia viruses

The murine leukaemia viruses (MLV) belong to the gammaretrovirus genus and conform to the basic structure already outlined. MLV isolates are further divided into 4 categories with respect to their tropism and host receptor use: ecotropic, such as Moloney murine leukaemia virus (infect mouse cells), xenotropic (can only infect cells from species other than mice), amphotropic and polytropic (both infect murine and other species but differ in the host cell receptors used to do so) (Coffin et al 1997). Many MLV isolates have been derived from laboratory or wild mouse strains and these have been found to induce a variety of neoplastic and degenerative diseases (Table 1.2).

Table 1.2: Murine Leukaemia viruses

MLV Virus Isolate	Disease
Akv	B cell lymphoma
Moloney	T cell lymphoma
Friend	Erythroleukaemia
Graffi	Myeloid leukaemia
Cas-Br-E	Spongiform encephalopathy

Table 1.2 shows examples of Murine leukaemia virus (MLV) isolates and the clinical syndromes that they produce.

1.5.3 MLV oncogenesis

While MLV-induced neoplasia is a complex and multi-faceted process, an important and common mechanism involves the ability of the provirus to integrate into host cell DNA and disrupt gene expression. MLV proviruses have strong promoter/enhancer elements, translational initiation signals and other regulatory elements which enable integrated retroviruses to markedly alter the expression of adjacent host genes. Host genes may be inactivated although such events are relatively uncommon, probably due to the diploid state and the low probability of affecting both copies of a tumour suppressor gene. It is much more usual for the provirus to induce high expression of susceptible target host genes such that it gives the host cell a growth advantage and allows cell proliferation and tumorigenesis (Kung et al 1991) (Fan 1997). The first example of this mechanism was discovered with avian leukosis virus (ALV) in which the provirus integrates adjacent to

the *c–myc* proto-oncogene and ultimately leads to the development of avian bursal lymphoma (Hayward et al 1981).

Because proviral integration into the host genome is random, it is likely that there will be multiple rounds of infection and a significant latent period before the provirus inserts adjacent to an appropriate target host gene (Jonkers and Berns 1996). Multiple, consecutive rounds of retroviral insertional mutagenesis yield a full blown tumour in which proviral insertions mark genes that collaborate in tumour development (Jonkers and Berns 1996; Mikkers et al 2002; Mikkers and Berns 2003).

1.5.3.1 Mechanisms of insertional activation

Insertional activation of proto-oncogenes usually involves altered transcription, although altered processing or stability of transcripts may occasionally occur. Depending on the integration site and the transcriptional orientation of the provirus with respect to the cellular gene, viral control elements may result in aberrant initiation, enhanced expression and /or premature termination of host sequences, resulting in high levels of messenger RNAs encoding the intact protein or the production of aberrant transcripts encoding mutant proteins (Jonkers and Berns 1996). Three major mechanisms of enhanced or deregulated transcription have been identified. The first is via 3’ LTR promoter insertion, as occurs with ALV in bursal lymphoma (Neel et al 1981). A similar, but more recently discovered, example is provided by MLV infection of mice carrying a *myc* oncogene where 3’ LTR promoter insertion leads to over-expression of the *Runx1* gene and subsequent T cell lymphomas (Wotton et al 2002). Gene activation by promoter insertion requires the integration of a provirus in the same transcriptional orientation as the target gene and may be associated with large deletions of proviral sequences and derepression of the cryptic 3’LTR (Jonkers and Berns 1996; Kung et al 1991).

The second mechanism is by readthrough activation whereby the viral promoter in the 5’LTR is used to initiate transcription and transcription extends through the viral genome as well as through cellular sequences downstream from the insertion site. Generally the readthrough transcript encompasses both the viral genes and the proto-oncogene (Jonkers and Berns 1996; Kung et al 1991). Avian leucosis virus induces erythroblastosis by readthrough activation of the cellular oncogene *c-erb* (Raines et al 1985).

The third mechanism is that of transcriptional enhancer insertion and this is the most frequent mechanism of gene activation by insertional mutagenesis. Here the enhancer motifs within the retrovirus LTRs act on endogenous promoters within the host genome. The insertion site may be upstream or downstream or at a considerable distance from the activated proto-oncogene. Enhancers are capable of acting over large distances and, in the case of Moloney murine leukaemia virus, can affect target gene expression over several hundred kilobases (Jonkers and Berns 1996). This method allows the most flexibility with respect to proviral orientation and distance between provirus and target gene and thus allows a much larger DNA domain to be an effective target for proviral insertions. Moloney murine leukaemia virus activation of *pim-1* and *c-myc* in T cell lymphoma occurs by this method (Payne et al 1982) (Cuypers et al 1984; Lazo et al 1990b; Tsichlis et al 1990). Transcriptional control sequences in the MLV LTR are important determinants of the oncogenic potential of the virus, and control both the latency period of disease induction and the distinct disease-inducing phenotypes of different viral isolates such as the induction of T-cell or erythroleukaemia by Moloney and Friend MLV respectively (Speck et al 1990; Tsichlis and Lazo 2003).

1.5.4 Oncogene-containing retroviruses

Rare recombinations between retroviruses and the host genome sequences result in the generation of hybrid viruses containing transforming genes. These captured oncogenes (*v-onc* oncogenes) are derived from normal cellular genes known as proto-oncogenes (*c-onc* genes) which are normally involved in growth control (Fan 1997). The resulting v-onc containing retroviruses can induce polyclonal tumours as early as two to three weeks after infection of the host and are often able to transform cells *in vitro* (Jonkers and Berns 1996). Examples from the MLV family include the Abelson murine leukemia virus which expresses *v-abl* (Shore et al 2002).

1.5.5 Retroviral tagging and multi-step oncogenesis

The appreciation that MLV and other simple retroviruses frequently act as insertional mutagens has led to the prospective analysis of tumours for new target genes relevant to cancer at the sites of retroviral insertion (tagging). As these agents integrate randomly with only weak evidence of integration preference, evidence of a common insertion site (CIS) in multiple independent tumours provides prima facie evidence of biological selection for these specific integrations and the proximity of a relevant target gene or

control element (Neil and Cameron, 2002). Many CIS have been identified in different retroviruses and for each virus/host system there is a high frequency of integration within/near a small number of target genes. The genes residing in these CIS are usually related to growth regulation and many possess transforming potential *in vitro* or *in vivo* (Kung et al 1991).

A more advanced application of retroviral tagging has been developed by infecting mice that already carry an active oncogene in the germ-line (Adams and Cory 1992; Jonkers and Berns 1996). These mice generally show accelerated tumour development if infected with MLV early in life, and a clonal pattern of host-virus junction fragments in tumour DNA indicates that insertional mutagenesis has targeted a collaborating gene that can act together with the germ-line oncogene to promote cancer development. A further innovation is the use of Polymerase chain reaction (PCR) based methods, such as inverse PCR or splinkerette PCR amplification, to isolate large numbers of provirus-host DNA junctions from tumours. Combined with the complete mouse genome sequence the location of the common insertion sites can be determined and new oncogenes rapidly identified (Kim et al 2003; Li et al 1999; Mikkers et al 2002). In this way multiple common insertion sites may be identified within any given type of tumour reflecting the fact that generally there are multiple proviral copies inserted independently within the genome of a tumour cell. High throughput screening of proviral integration sites (Li et al 1999; Mikkers et al 2002; Suzuki et al 2002) has markedly increased the identification of genes involved in oncogenesis and permitted identification of many cooperating oncogenic pathways (Uren et al 2005). Uren et al has recently screened mice for genes that collaborate with loss of either p53 or $p19^{ARF}$ (Uren et al 2008).

A similar system, which is utilised in this thesis, involves the analysis of T-cell lymphomas induced by human c-*MYC* linked to a CD2 locus control region (*CD2-MYC*) in mice, where *Runx2* and other members of the *Runx* gene family have been identified as preferred collaborating genes activated by MLV (Stewart et al 1997; Wotton et al 2002). Moreover, CD2-*Runx2* mice display the reciprocal pattern with preferred MLV insertions at *c-myc* and *N-myc* (Blyth et al 2001), illustrating the potent synergy between Myc and Runx family oncogenes (Blyth et al 2006). Another variation on this theme is “complementation tagging” which can be used in compound mutant mice to identify genes that can complement for the loss of other genes ie genes that act downstream from, or in parallel to, the mutated gene (Berns et al 1999; Jonkers and Berns 1996). A

complementation group includes genes that can mutually substitute for each other, and that are either functionally related or closely genetically linked. For instance, individual lymphomas will have an insertion at only one locus from each complementation group, indicating that a single hit to each pathway is likely to be sufficient for tumour development (Scheijen et al 1997).

1.5.6 Pathogenesis of Moloney murine leukaemia virus

As studies with Moloney murine leukaemia virus (MMLV) infection constitute a significant component of this thesis, its biology and pathogenesis are considered in greater detail. MMLV is a highly pathogenic retrovirus that induces T cell lymphoma with a 100% incidence and a latency of between 3-9 months post infection depending on strain of virus and of mouse (Tsichlis and Lazo 1991; van Lohuizen and Berns 1990). The lymphomas that develop consist of T cells, however the T cell phenotype can be variable (Lazo et al 1990a). Neonatal infection is the most efficient method of inducing leukemia since infection of adult mice often does not cause disease, presumably due to immune controls (Fan 1997; Lazo et al 1990a; Tsichlis and Lazo 2003). Inoculation of newborns causes infection of lymphocytes during a phase when T cell precursors are undergoing extensive division and proliferation. This is relevant since the MMLV provirus can only integrate into the genome of cells that are dividing, it also enables the number of infected cells to increase dramatically and allows production of large amounts of virus progeny (Mikkers and Berns 2003).

There are recognized sequential stages in the development of MMLV-induced T cell lymphoma. An early preleukemic stage seen within 40-50 days of inoculation is characterised by polyclonal proliferation of “potentially leukaemic” haematopoeitic precursor cells in the bone marrow and spleen (Fan 1997; Tsichlis and Lazo 2003). This suggests that early events take place in thymocyte precursor cells (bone marrow cells or splenocytes) rather than in the target thymocytes where the tumour ultimately develops. The late stages of T cell leukemogenesis appear to arise as a consequence of proviral integrations and proto-oncogene activation, leading to transformation and outgrowth of usually one or a few independent subclones. The tumour finally becomes rapidly growing and may metastasize (Jonkers and Berns 1996; Tsichlis and Lazo 2003).

The early changes in MMLV-induced leukemogenesis include defects in bone marrow haematopoeisis, splenomegaly and thymic atrophy. Early bone marrow infection is essential for efficient leukemogenesis by MMLV. The first cells that are infected after intraperitoneal inoculation of neonatal mice appear to be osteoclasts or osteoclast progenitors (Okimoto and Fan 1999). It is unlikely, however, that terminally differentiated osteoclasts are the primary target for infection and is more likely that the virus targets osteoclast precursor cells such as peritoneal macrophages/monocytes which subsequently migrate to the bone marrow where infection then spreads to haematopoietic progenitor cells such as lymphoid precursors that later migrate to the thymus. Splenomegaly may be explained by hyperplasia of multiple haematopoietic cell lineages (including erythroid and myeloid) (Fan 1997; Li and Fan 1990). It has been suggested that this process represents splenic extramedullary haematopoeisis arising to compensate for decreased bone marrow haematopoeisis which occurs secondary to viral infection and inhibition of bone marrow stromal cells.

Thymic atrophy is associated with an increased rate of thymocyte apoptosis in MMLV-infected mice as compared with uninfected mice and is considered to be important for efficient leukemogenesis (Bonzon and Fan 1999). However the mechanism by which this is achieved not clear. It is possible that apoptosis demands increased recruitment of cells into the thymus from the pool of “potentially leukaemic” lymphoid cells within the bone marrow or spleen. It is also thought that progression of thymocytes to become fully malignant may require the inheritance of multiple genetic mutations, some of which enable the cell to survive this apoptotic crisis (Yoshimura et al 2000).

A cell has to undergo multiple cycles of MMLV infection in order to induce the leukaemic process efficiently (Fan 1997). Early infection in the bone marrow or spleen leads to expansion of a population of hyperplastic, lymphoid progenitors which migrate to the thymus where they undergo further differentiation. Rounds of subsequent infection of these cells, either by MMLV or variants with altered host range, are required thereafter for the insertional activation of cellular proto-oncogenes necessary for tumorigenesis.

A later event occurring in MMLV leukemogenesis is the appearance, *in vivo*, of mink cell focus-inducing (MCF) viruses which are so called due to their ability to cause cytopathic foci on mink lung fibroblasts (Hartley et al 1977). These are *env* gene recombinants between the infecting ecotropic murine leukemia virus and endogenous polytropic murine

leukemia virus proviruses. The recombinant MCF virus particles contain chimeric envelope glycoproteins, generated through recombination with endogenous sequences, which can interact with surface receptors that are distinct from those used by ecotropic murine leukemia viruses and thus overcome this barrier to re-infection (Mikkers and Berns 2003; van Lohuizen and Berns 1990). This potentially allows additional rounds of viral infection and integration at key sites within the host cell genome. MCF viruses may also contribute to leukaemogenesis via envelope glycoprotein mediated growth factor receptor stimulation in the early preleukemic, hyperplastic phase (Jonkers and Berns 1996).

1.6 p53 AND MMLV

Retroviral oncogenesis usually involves activation of oncogenes. Less commonly, retroviruses operate by inactivating tumour suppressor genes (Kung et al 1991). However, it is notable that inactivation of the p53 gene by Friend MLV proviral insertion was one of the key observations that led to the understanding that wild-type p53 is a tumour suppressor rather than an oncogene (Johnson and Benchimol 1992).

A high proportion of Friend virus induced murine erythroleukemia cells lines demonstrate abnormalities within the p53 gene often arising due to proviral insertion into the p53 gene (Ben-David et al 1990; Ben David et al 1988). Cells are frequently homozygous for these p53 abnormalities which include point mutations, deletions and insertions (Ben-David et al 1990; Johnson and Benchimol 1992). In some cases p53 expression is completely abolished, but in many instances high levels of mutant, often truncated, non-functional p53 proteins are induced (Ben David et al 1988; Mowat et al 1985; Munroe et al 1990). The high frequency with which rearranged p53 genes are detected in Friend erythroleukemia clones (Howard et al 1993), the finding that cell clones with one rearranged allele frequently lose the remaining wild type allele and the fact that p53 null mice succumb to the disease more rapidly than p53 wild type littermates (Wong et al 1999) suggest that functional inactivation of the p53 gene confers a selective growth advantage on transformed erythroid precursors during the course of Friend disease, however the precise role of p53 loss in the progression of Friend erythroleukemia is unclear and is a relatively late event (Prasher et al 2001).

p53 inactivation appears to also be important in the induction of B-cell leukaemia by Abelson murine leukemia virus (AbMLV). However, it should be noted that this is a rapidly-transforming retrovirus that transforms cells by expressing the protein tyrosine kinase *v-Abl* oncogene (Mostecki et al 2000). Oncogenesis involves an early phase of polycolonal proliferation which is followed by a phase of "apoptotic crisis", the onset of which is associated with critical changes in levels of $p19^{ARF}$ expression and with changes in localization of $p19^{ARF}$ within the nucleus (Zimmerman and Rosenberg 2008). Cells that survive this crisis emerge as fully transformed malignant cells and these cells have often acquired p53 mutations or down-regulated $p19^{ARF}$ expression (Thome et al 1997). AbMLV-infected cells that lack p53 or $p19^{ARF}$ escape this crisis phase (Radfar et al 1998; Thome et al 1997). To further support the importance of p53 in this viral system, it is seen that establishment of *v-Abl*-expressing p53 heterozygote primary cells in culture is accompanied by loss of the remaining p53 allele (Shore et al 2002). Also p53 null mice succumb to AbMLV-induced leukemia with a rate almost twice that of either wild type or heterozygote animals (Unnikrishnan et al 1999).

In view of the findings from the Friend and Abelson MLV systems, the role of p53 in Moloney MLV-induced T-cell lymphoma was of interest particularly since inactivation of p53 in the germline predisposes mice to tumours similar in phenotype to those induced by MMLV (Donehower et al 1992; Purdie et al 1994) (Fan 1997) and p53 heterozygote mice tend to develop T cell lymphomas which commonly show loss of the wild type p53 allele (Harvey et al 1993; Jacks et al 1994; Purdie et al 1994). By contrast, when MMLV-induced T cell lymphomas were screened for loss of heterozygosity it was concluded that tumour suppressor gene inactivation was not a significant factor in MMLV leukemogenesis (Lander and Fan 1997). Furthermore, MMLV-induced lymphomas from p53 heterozygote mice indicate a low frequency of inactivation of the remaining p53 allele which suggests that, unlike with Friend leukemia virus, loss of the p53 pathway is not a prerequisite for MMLV leukemogenesis (Baxter et al 1996). Moreover, in those MMLV-induced lymphomas where partial loss of the wild type p53 allele did occur, there was no evidence of gene rearrangements to suggest that this was caused by proviral insertion. It appeared, instead, to have been lost by a non-viral mechanism such as chromosomal nondisjunction. *In vitro* culture of these tumours, however, invariably led to loss of functional p53 (Baxter et al 1996).

MMLV infected mice that are p53 null develop tumours significantly faster than those that are heterozygote which, in turn, develop faster than in p53 wild type mice (Baxter et al 1996). This suggests that there is some cooperation between the effects of MMLV and p53 loss in thymic lymphoma development and this is further substantiated by the fact that MMLV infected p53 null mice develop thymic lymphomas significantly faster than do uninfected p53 null mice (Baxter et al 1996). Although this effect is significant, the degree of synergy is weak compared to the very marked synergistic effect that exists between MMLV infection and a *c-myc* transgene (Stewart et al 1993; van Lohuizen et al 1991) or between the p53 null genotype and the c-*myc* transgene (Blyth et al 1995) and this has been proposed to be due to a partial functional overlap between MMLV infection and p53 loss in T lymphoma development. Further investigations have shown no differences in the MMLV hit rates at a number of common insertion sites (e.g. *c-myc*) in p53 wild type and null mice (Baxter et al 1996) suggesting that the synergistic effect of p53 loss and MMLV infection is not explained by any influence that the p53 null genotype may have on the spectrum of proto-oncogenes targeted by MMLV. However, it was noted that the proviral copy number was reduced in p53 null lymphomas suggesting that p53 loss might reduce the number of hits required and that this might affect an, as yet unknown, subset of MMLV target genes (Baxter et al 1996).

Runx2 and *Myc* are both common insertional targets of MMLV in T cell lymphomagenesis (Stewart et al 1997). These two genes are powerful collaborating genes that induce rapid T cell lymphomas in uninfected mice (Vaillant et al 1999). Interestingly tumours developing in *Runx2/Myc* transgenic mice have also been found to retain p53 *in vivo* although, in all cases, growth of thymic tumour cells *in vitro* led to loss of p53 function (Blyth et al 2006). As with MMLV-induced tumours the *Runx2/Myc* combination appears, therefore, to override the need to lose p53 *in vivo*. This is reminiscent of observations that have been made when the two oncogenes *ras* and *myc* were combined in a prostate cancer model (Lu et al 1992). Alone, the *ras* oncogene leads to prostatic hyperplasia and p53 mutations are present within the cells. However, no such mutations are seen in prostatic carcinoma cells that arise from *ras* and *myc* acting together, suggesting that activated *ras* and *myc* together are also able to bypass the need for p53 mutation and may neutralise the tumour suppressor activity of p53 in some other way.

1.7 AIMS OF THE THESIS

The central aim of the work in this thesis was to investigate the interaction between MMLV and p53 in T-lymphoma development. I set out to determine whether collaboration between p53 inactivation and MMLV *in vivo* operates at the level of (1) virus-induced cytopathology, (2) host cell immortalisation and transformation, (3) progression and metastasis, or a combination of these stages. For parts of this work I examined the interaction between p53 loss and the *Runx2/Myc* oncogene combination as a functional surrogate for the T-cell lymphoma programme activated by MMLV.

CHAPTER 2

MATERIALS AND METHODS

2.1 ANIMALS

All animal work was carried out under Home Office regulations in line with the Animals (Scientific Procedures) Act 1986.

Mice deficient for a functional p53 tumour suppressor gene were derived by homologous recombination in murine embryonic stem cells by L. Donehower and colleagues (Donehower et al 1992). These animals have been backcrossed for 20 generations onto an inbred C57Bl/6 strain. Unless stated otherwise, experimental cohorts used in this thesis were derived by mating heterozygous p53 mice from this inbred background to generate homozygous ($p53^{-/-}$), heterozygous ($p53^{+/-}$) and wild type ($p53^{+/+}$) littermate controls.

The generation of CD2-*MYC* and CD2-*Runx2* transgenic mice by pronuclear microinjection has been described previously (Stewart et al 1993; Vaillant et al 1999). These cohorts are maintained on a C57Bl/6 x CBA/Ca background strain.

2.1.1 Challenge of mice with MMLV

MMLV clone 1A supernatant was isolated from replication-competent virus infected Mov 3T3 cells. These were a gift from Dr. A. Berns from cells that were originally derived from David Baltimore's laboratory from MMLV infected mouse cells. Aliquots of 1ml of filtered supernatant were frozen at -70°C until required. Neonatal animals were inoculated intraperitoneally with 0.1ml of supernatant within 24 hours of birth.

2.1.2 Clinical examination and post mortem investigation

Experimental cohorts and breeding stocks of mice were maintained for defined periods of time and the health of animals checked at least three times weekly. The development of lymphoid neoplasia in the transgenic animals presented as cachexia and tachypnoea. Animals were humanely killed by cervical dislocation when clinical signs first became evident.

Post mortem examination was routinely undertaken immediately after euthanasia and pathological findings were recorded. Particular attention was paid to the lymphoid organs, especially the thymus, spleen and mesenteric lymph nodes. Evidence of neoplastic involvement of non-lymphoid organs was also noted. Tissues were frozen in cryotubes (Invitrogen) in liquid nitrogen (BOC) for DNA analysis, fixed in 10% neutral buffered formalin for histopathological examination and placed in complete RPMI medium (Invitrogen) for preparation of single cell suspensions.

2.1.3 Mouse tail biopsy

Transgenic/null animals were identified from DNA analysis carried out on tail biopsy. Potential transgenic animals were weaned from parents and separated by sex at 3-4 weeks of age. At 4-6 weeks of age, animals were anaesthetized in a halothane chamber and a 0.5-1cm biopsy of tail excised. The wound was cauterized and the animal marked using an ear nicking identification system.

2.1.4 Cell transplantation

For *in vivo* passage of tumour cells, <0.5ml of cell suspensions in sterile phosphate buffered saline were injected intraperitoneally into immunocompromised (MF1 nude, Harlan, UK) or histocompatible (C57Bl/6) hosts using a sterile insulin syringe with a 29 gauge needle (Dunwood). Animals were monitored and culled at presentation of clinical disease or at a predetermined end point if no clinical disease was apparent. Immunocompromised animals were housed in filter-top sterile cages.

2.2 SOUTHERN HYBRIDISATION ANALYSIS

2.2.1 Buffers

2.2.1.1 Lysis buffer

100mM Tris-HCL (Fisher Scientific), pH 8.5; 5mM EDTA, pH 8.0 (Sigma); 0.2% sodium dodecyl sulphate (SDS) (Fisher Scientific) and 200mM NaCl (Fisher Scientific) were mixed together and made up in distilled water.

2.2.1.2 Loading dye

0.25% Bromophenol blue (Sigma), 0.25% Xylene Cyanol FF (Sigma), 30% glycerol (Fisher Scientific) and 10mM EDTA (Sigma) were made up in distilled water.

2.2.1.3 1 x TAE buffer

40mM Tris-acetate and 2mM EDTA (Sigma) were made up in distilled water.

2.2.1.4 20x SSC

3M NaCl and 0.3M Trisodium citrate were made up in distilled water.

2.2.1.5 Denaturation buffer

1.5M NaCl (Fisher Scientific) and 0.5M NaOH (Fisher Scientific) made up in distilled water.

2.2.1.6 Neutralisation buffer

1.5M NaCl (Fisher Scientific) and 0.5M Tris (Fisher Scientific) were made up in distilled water, pH 7.4.

2.2.1.7 TE buffer

10mM Tris (Fisher Scientific), pH 8.0, 1mM EDTA (Sigma) were made up in distilled water, pH 8.0

2.2.2 Isolation of genomic DNA from mouse biopsy

Fresh tail tissue was placed in an eppendorf tube (Elkay) in 0.5ml of lysis buffer. Proteinase K (Invitrogen) was added to a final concentration of 0.5mg/ml and the samples incubated at 55°C for approximately 16 hours. Samples were then centrifuged in a microfuge at 13000 rpm for 10 minutes to obtain a firm pellet. The supernatant was then added to 0.5ml isopropanol (BDH) in an eppendorf tube and the tube inverted several times until precipitation was complete. Samples were then centrifuged at 13000 rpm for 10 minutes in a microfuge so that the DNA sank to the bottom of the tube. The supernatant was discarded and the sample allowed to air dry. The DNA was then resuspended in distilled water and agitated gently for several hours at room temperature until it dissolved. DNA concentration of the sample was measured using a spectrophotometer (Beckman Du^{R} 640).

2.2.3 Isolation of DNA from tumour tissues

Frozen tumour tissue was ground in liquid nitrogen and DNA was isolated using the Nucleon® II genomic DNA extraction kit, soft tissue protocol (Tepnel Life Sciences PLC) according to manufacturer's instructions.

2.2.4 Separation of genomic DNA

Enzymatic restriction digests were set up using BamH1 (Invitrogen) to examine the p53 gene and a cocktail of BglII and EcoRI (Invitrogen) to examine the CD2-Myc transgene. 50µl digests were set up with 3µl of enzyme, 5µl of the appropriate enzyme buffer (Invitrogen), 1µl spermidine (Sigma), 10µg sample DNA and distilled water to make up the volume. Digests were set up at 37°C overnight. 5µl of loading dye was added to each digested sample. DNA fragments were separated on a 0.8% agarose (Invitrogen) gel overnight in 1x TAE buffer at 20V. DNA gels were then stained with 50mg/L ethidium bromide (Sigma) in 1x TAE buffer for 20 minutes. They were viewed on an ultraviolet transilluminator and photographed using a Fisher Scientific system to confirm the presence of DNA in the lanes. The gel was then placed in denaturation buffer and gently agitated for 30 minutes. It was then placed in neutralisation buffer and gently agitated for another 30 minutes. The DNA was then transferred to Hybond ^{TM}N membrane (Amersham Biosciences) in 20x SSC by the Southern Blotting technique as described by Sambrook et al (Sambrook J, Fritsch E. F, and Maniatis T, 1989, Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press). DNA was immobilised to the nylon membrane by UV crosslinking using a UV Stratalinker XL1500 (Spectronics Corporation). Fragments of interest were detected by hybridisation with radiolabelled probes.

2.2.5 Radiolabelled probes

The p53 null allele was detected using a polymerase chain reaction (PCR)-generated p53 exon 4 probe (primers 5'-CCATCACCTCACTGCATGG-3' and 5'-CGTGCACATAACAGACTTGGC-3'), which also reveals wild-type and pseudogene alleles of p53. The CD2-Myc transgene sequence was detected using an exon 3 probe generated from a PCR fragment derived from the pMC41 plasmid (Stewart et al 1993). Rearrangement of the T-cell receptor (TCR) ß-chain gene was determined using a 496-bp PCR-derived fragment of the $C_ß$ gene, derived from the 1.2-kb fragment of clone 86T5

(Baxter et al 2001). Fragments used in the generation of probes were kindly supplied by the Molecular Oncology Laboratory, University of Glasgow.

Using the Rad Prime DNA labelling system (Invitrogen, Life Technologies), probes were radiolabelled to generate dsDNA probes. 20ng of fragment was made up to 20µl with ultra-pure water, boiled for 5 minutes and immediately chilled on ice. 1µl of kit dATP, 1µl of kit dTTP, 1µl of kit dGTP, 20µl 2.5x random primers solution (Rad Prime) and 2.5µl α 32P dCTP (Amersham) were added and the volume made up to 49µl with sterile water. 1µl of Klenow fragment was then added and the mixture incubated at 37°C for 10 minutes. The labelled fragment was then eluted through a nick column (Pharmacia) with TE buffer and stored at –20 °C for up to 2 weeks. A 2µl aliquot of the probe was taken to measure counts per minute using a scintillation counter (Perkin Elmer Life Sciences).

2.2.6 Hybridisation of DNA blots

Before hybridisation, DNA blots were pre-incubated with 10-20ml of Rapid-Hyb^{TM} (Amersham International) hybridisation solution in a roller bottle (Hybaid) at 65°C for 30 minutes. Radiolabelled ds DNA probe and salmon sperm (Sigma) were boiled for 5 minutes and chilled on ice. 100µl of salmon sperm and 5 x 10^5 –1 x 10^6 counts per minute of radiolabelled ds DNA probe were added to the pre-hybridised blots. The blots were then hybridised for 2-3 hours at 65°C using Rapid-Hyb^{TM} (Amersham International plc) hybridisation solution as per supplier's instructions. Blots were rinsed once in 2 x SSC and then washed three times for 20 minutes; each wash at 60°C with 0.5% SSC and 0.5% SDS. Blots were then set up for fluorescence imaging (using a Storm 840 imager).

2.3 TISSUE CULTURE TECHNIQUES:

All centrifugations, throughout these studies, were done either in a bench-top centrifuge of 30cm diameter or in a microfuge of 16cm diameter unless otherwise stated.

2.3.1 Cell Lines

Cell lines used are depicted in Table 2.1.

Table 2.1: Cell Lines

Cell Line	
NIH 3T3	American Type Culture Collection, Virginia
SV3T3	Kind gift from Professor Ken Parkinson. These are 3T3 cells immortalised by SV40 in which p53 is stabilised.
Mov 3T3	Kind gift from Dr Anton Berns. These are MMLV infected 3T3 cells.

Table 2.1. shows the cell lines used in this thesis and from where they were obtained.

2.3.2 Media

All media preparation was carried out under aseptic conditions.

2.3.2.1 Complete RPMI for lymphocytes

RPMI 1640 medium (Invitrogen) was supplemented with 10% heat inactivated foetal calf serum (Perbio), 100U/ml penicillin, 100μg/ml Streptomycin, 2mM L-glutamine (all from Invitrogen) and 5 x 10^{-5}M 2-mercaptoethanol (BDH).

2.3.2.2 Cell freeze down medium for lymphocytes

Complete RPMI was supplemented with 10% dimethyl sulphoxide (Sigma-Aldrich) and 20% foetal calf serum (Perbio) and filter sterilised through a 0.20μm filter (Sartorius).

2.3.2.3 Complete DMEM for fibroblasts

Dulbecco's Modified Eagle Medium (DMEM, Invitrogen) was supplemented with 10% heat inactivated foetal calf serum (Perbio), 100U/ml penicillin, 100μg/ml Streptomycin, 2mM L-glutamine (all from Invitrogen) and 5 x 10^{-5}M 2-mercaptoethanol (BDH).

2.3.2.4 Cell freeze down medium for fibroblasts

10% dimethyl sulphoxide (Sigma-Aldrich) and 50% foetal calf serum (Perbio) was made up in complete DMEM medium (as above) and filter sterilised through a 0.20μm filter (Sartorius).

2.3.3 Single cell lymphocyte preparation

Tumour/thymus tissue was "pushed" through a 70μm cell strainer (BD Falcon) into a 60mm Petri dish (Nunclon) using the barrel of a 1ml syringe (BD Plastipak^{TM}) in complete RPMI medium to yield a single cell preparation suspended in 5-10ml of complete RPMI medium. A total cell count was carried out by trypan blue (Invitrogen) exclusion on a haemocytometer (Sigma-Aldrich). In those cases where only viable cells were required (ie for *in vitro/in vivo* passage) then lymphocytes were isolated in a Ficoll-Paque (Pharmacia) density gradient at 3000rpm for 10 minutes in a 15 ml centrifuge tube (Falcon). The interphase layer containing the live lymphocytes was washed in 10ml complete RPMI medium and centrifuged at 1500rpm for 5 minutes. The supernatant was discarded; the cells resuspended in 5-10ml of complete RPMI medium and the cells counted by trypan blue exclusion. Cells were frozen to -70°C in 1.5ml cryotubes (Invitrogen) at a concentration of 5-10 x 10^6 cells/ml in freeze down medium, using isopropanol (BDH) filled controlled rate freeze down tubs (Sigma-Aldrich) and stored in liquid nitrogen (BOC).

2.3.4 UV irradiation of lymphocytes

A single cell preparation was made from tissue/tumour cells. 2 x 10^6 cells were harvested, washed in phosphate buffered saline (PBS) and then placed into a 10cm petri dish. Cells were exposed to 60 J/m^2 UVC irradiation using a UV Stratalinker XL 1500 (Spectronics Corporation). Cells were centrifuged, resuspended in 10 ml complete RPMI medium and incubated in a 10cm petri dish at 37°C in an atmosphere of 5% CO_2 in air. After 24 hours the cells were washed in PBS and assessed for apoptosis as described in Section 2.4.1.

2.3.5 Culture and establishment of lymphocytic tumour cell lines (in vitro passage)

Tumour cells were prepared, as described above, to give a single cell preparation. Cells were cultured in 25cm^2 or 75cm^2 tissue culture flasks (Corning) at a concentration of 2.5 x 10^6 cells/ml in complete RPMI medium at 37°C in an atmosphere of 5% CO_2 in air. Cell cultures were passaged every 4-7 days into fresh medium and maintained at a density of 5 x 10^5–5 x 10^6 cells/ml.

2.3.6 Preparation of lymphocytic tumour cells for in vivo transplantation (in vivo passage)

A single cell preparation was made from the tumour. 2 x 10^7 cells were harvested, washed in PBS and resuspended in a final volume of <0.5ml PBS and kept at 4°C. This was injected by intra-peritoneal route into the recipient mouse.

2.3.7 Preparation of mouse embryonic fibroblasts (MEFs)

A pregnant female mouse was sacrificed at day E13.5, where the day of plug is taken as day 0.5. The uterus was removed and washed in sterile PBS (Invitrogen). Under aseptic conditions, the foetuses were isolated from the uterus. The head and liver were discarded from each foetus and the body was placed in 2ml sterile PBS in a small petri dish and minced using scalpels (Fisher Scientific). 1ml 0.1% trypsin/EDTA (Invitrogen) was added and left for 10-15 minutes at 37˚C. This was pipetted up and down occasionally in order to disperse the cells. 10ml complete DMEM was then added and the suspension was allowed to settle for a few seconds. The supernatant was removed (leaving behind any large "lumps") and placed in a 15ml centrifuge tube (Falcon) and centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the material resuspended in 5ml complete DMEM, put into a 25cm^2 tissue culture flask (Corning) and kept at 37°C. Cells were passaged at confluence from a 1 x 25 cm^2 flask to 2 x 75 cm^2 flasks (Corning) and then to 2 x 162 cm^2 flasks (Corning) when they were harvested by standard trypsinisation and frozen in cryotubes (Invitrogen) at 1-5 x 10^6 cells per ml as passage 3, and stored in liquid nitrogen (BOC).

2.3.8 Fibroblast cell culture

Mouse embryo fibroblasts (MEFs) were cultured in complete DMEM in 25cm^2 or 75cm^2 tissue culture flasks (Corning) at 37°C in an atmosphere of 5% CO_2 in air. Cell cultures were passaged every 5-7 days just before the cells became completely confluent. Culture medium was removed and discarded, and the cell layer briefly rinsed with PBS. 1-3ml of Trypsin/EDTA solution (Invitrogen) was added to the flask and the cells observed until the cell layer was dispersed (~5 minutes). Cells were washed in 10ml complete DMEM medium and centrifuged at 1000rpm for 5 minutes. The supernatant was discarded and the cells resuspended in an appropriate volume of complete DMEM.

2.3.9 Virus infection of fibroblasts

Mouse embryonic fibroblasts (MEFs) were plated at 8 x 10^5 cells in 10ml of complete DMEM medium in a 10cm petri dish and incubated overnight at 37°C in an atmosphere of 5% CO_2 in air. The medium was then removed and replaced with 10ml filtered viral supernatants prepared from cultures of Mov3T3 cells in the log phase of growth supplemented with 4μg/ml polybrene (Sigma). The MEFs were incubated 24 hours at 37°C. The viral supernatant was then removed and replaced with normal medium and the cells incubated at 37°C for another 24 hours. The cells were now ready for use in growth curve, or other, experiments.

2.3.10 Fibroblast growth curve studies

Fibroblast samples were plated in triplicate in 12 well plates (Becton Dickinson) at 2.5 x10^4 cells in 2ml of complete DMEM medium and cultured at 37^{o}C in 5% CO_2. Individual plates were set up for each time-point, at which time cells were harvested by trypsinisation and counted by trypan blue exclusion on a haemocytometer. At harvesting, all media and rinses were kept to enable a live/dead count to be evaluated. Media changes were carried out at days 4, 7, 8, 12, 15 and 18 after set up to replenish exhausted media.

2.3.11 UV Irradiation of MEF cultures

Cells were set up into 12 well plates (5x 10^4 cells in 2mls of medium). The medium was removed from all wells 24 hours later and the wells were rinsed with prewarmed PBS (Invitrogen). Cells were exposed to UV-C irradiation at 20 J/m^2 using a UV Stratalinker XL 1500 (Spectronics corporation). Fresh medium was then put into all wells. Control plates also had their medium changed at this time. Cells were harvested and counted over the next 10 days. “Conditioned” media changes were made at 4 and 8 days after set up into the 12 well plates, whereby 1ml of the old media was removed from the well and replaced by 1ml of fresh media.

2.4 FLOW CYTOMETRY

All flow cytometry was carried out on a Beckman Coulter Epics XL and data analysed using the Expo32^{TM} software package.

2.4.1 Assessment of apoptosis

Thymic lymphocytes were stained, according to manufacturer's instructions, with Annexin-V fluorescein and propidium iodide using the Annexin-V-Fluos staining kit (Roche); in summary, 10^6 cells were harvested, washed in sterile PBS (Invitrogen) and resuspended in 100µl of staining solution containing 20µl annexin-fluorescein labelling reagent and 20µl propidium iodide made up to 1000µl in HEPES buffer. The cells were then incubated for 10-15 minutes at 15-25˚C and analysed.

2.4.2 Immunophenotype Analysis

2.4.2.1 Flow cytometry buffer

0.1% bovine serum albumin (BSA, Sigma) and 0.01% sodium azide (Sigma) in PBS (Invitrogen) were filter sterilised through a 0.20 µm filter (Sartorius) and stored at 4°C.

2.4.2.2 Annexin binding buffer

10mM Hepes (Gibco)/NaOH (Fisher Chemicals) pH 7.4, 140mM NaCl (Fisher Scientific) and 5mM $CaCl_2$ (BDH) were made up in distilled water and stored at 4°C.

Labelling of rodent cells for immunophenotyping was carried out on ice. Cells were harvested and counted, then centrifuged in 15ml Falcon tubes and the supernatant discarded. The sample was washed in 5ml flow cytometry buffer and centrifuged at 1200 rpm for 5 minutes. Cells were adjusted to 1 x 10^7 cells per ml and 2 x 10^6 cell aliquots used for each antibody reaction. The sample was centrifuged in a microfuge at 7000 rpm for 3 minutes and the supernatant removed. The pellet was resuspended in the remaining small volume. 5µl CD4-Cy5 (Cy-Chrome^{TM}–conjugated rat antimouse CD4 monoclonal antibody (BD Biosciences Pharmingen) was added to the sample and vortexed. 10µl CD8-FITC (Rat antimouse CD8 alpha: FITC, Serotec) was added and the sample vortexed. Samples were placed in the dark for 30 minutes on ice. 200µl flow cytometry buffer was

added to the sample and vortexed. The sample was centrifuged at 7000 rpm for 3 minutes, washed in 200µl flow cytometry buffer, centrifuged and resuspended in 100µl Annexin binding buffer and put into a 5ml round–bottomed Falcon tube (Becton Dickinson). 5µl Annexin PE (Annexin V-Phycoerythrin, BD Pharmingen) was added and the samples incubated in the dark at room temperature for 15 minutes. 400µl Annexin binding buffer was added and the samples analysed immediately by flow cytometry.

2.4.3 Cell cycle analysis

2.4.3.1 Sample buffer

1g glucose (Sigma) and 1 litre PBS (Invitrogen) were filtered through 0.20µm filter (Sartorius) and stored at 4°C.

2.4.3.2 20x Propidium Iodide (PI) stock solution

100mg propidium iodide (Sigma) in 100ml distilled H_2O was filtered through 0.20µm filter (Sartorius) and stored at 4°C in a dark container.

2.4.3.3 PI staining solution

0.5 ml 20 x PI stock solution (final concentration 50 µg/ml) and 1000 K units Rnase A (100U/ml final) (Sigma) were added per 10ml sample buffer and mixed together just before use.

Cells were harvested into 15ml falcon tubes, centrifuged 10 minutes at 1000 rpm, resuspended in 10-12 ml sample buffer and kept on ice. Cells were centrifuged further at 1000 rpm for 10 minutes and the sample buffer wash repeated twice more. The cells were then counted and cell concentration was adjusted to 1-3 x 10^6 cells/ml in sample buffer. 1ml of cell suspension was taken and centrifuged for 10 minutes at 1000 rpm and the supernatant discarded. The sample was briefly vortexed in order to resuspend the pellet and ice-cold 70% ethanol was added drop-by-drop to the pellet whilst continuing to vortex. Samples were fixed for at least 18 hours at 4°C (for up to several weeks).

For staining, samples were vortexed briefly and centrifuged for 5 minutes at high speed (3000 rpm). The ethanol was poured off without disturbing the pellet and the sample then gently vortexed in order to resuspend the cells in the residual ethanol. 1ml PI staining solution was added to 5x 10^5 cells and the sample placed into a 5ml round–bottomed

Falcon tube (Becton Dickinson). Tubes were gently rocked in the dark at room temperature for a minimum of 30 minutes, then stored at 4°C and analysed within 24 hours by flow cytometry.

2.5 WESTERN IMMUNOBLOTTING

2.5.1 Buffers

2.5.1.1 SDS gel-loading sample buffer

50mM Tris/HCl (pH 6.8) (Fisher Scientific), 100mM dithiothreitol (BDH), 2% SDS (Fisher Scientific), 0.1% bromophenol blue (Sigma) and 10% glycerol (Fisher Scientific) were mixed together and stored at –20°C.

2.5.1.2 Whole cell lysis buffer

20mM HEPES pH 7.0 (Sigma), 5mM EDTA (Sigma), 10mM EGTA (Sigma), 0.1μg/ml Okadeic acid (Sigma), 5mM Sodium fluoride, 1mM DL-dithiothreitol (Sigma), 0.4M Potassium chloride (BDH), 0.4% Triton-X-100 (Sigma), 10% Glycerol (Fisher Scientific) were mixed together and made up to 10 ml with distilled water and divided into 2ml aliquots which were then stored at -20°C. The following reagents are added to each 2ml aliquot on the day of use and mixed for 5-10 minutes at 4°C before use: 5μg/ml Pepstatin A (Sigma), 5μg/ml Leupeptin hemisulphate (Sigma), 1mM Benzamidine (hydrochloride hydrate) (Sigma), 5μg/ml Aprotinin (Sigma), 50μg/ml Phenylmethanesulphonylfluoride (PMSF) (Sigma)

2.5.1.3 Tris Buffer Saline Tween (TBST)

1.21g Tris (Fisher Scientific), 8.765g NaCl (Fisher Scientific), 0.5ml Tween-20 (BDH Organics) were mixed together and made up in 1 litre of distilled water and brought to a pH of 8.0.

2.5.1.4 Electrophoresis "running" buffer

25mM Tris (Fisher Scientific), 250mM glycine (Fisher Scientific), pH 8.3 and 0.1% SDS (Fisher Scientific) were made up in distilled water.

2.5.1.5 Transfer buffer (BioRad mini-protean system)

0.192M glycine (Fisher Scientific) and 25mM Tris (Fisher Scientific) were made up in distilled water. 15% methanol was added last to the mixture.

2.5.1.6 Dry blot buffer

5.8g Tris (Fisher Scientific), 2.92g Glycine (Fisher Scientific), 0.37g SDS (Fisher Scientific) and 200ml methanol (Fisher Scientific) were made up to 1 litre in distilled water.

2.5.2 Preparation of cells for Western blotting

20 x 10^6 cells were harvested, washed in 5ml ice-cold phosphate buffered saline (PBS), (Invitrogen) and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded, the cells resuspended in 500µl ice-cold PBS and transferred to a screw cap eppendorf. This was then centrifuged in a microfuge at 6500 rpm for one minute at 4°C. The supernatant was removed and the pellet stored at -70 °C.

2.5.3 Preparation of protein extract from cell pellet

Cell pellets were gently resuspended in 200µl whole cell lysis buffer and gently rotated at 4°C for 15 minutes. The lysate was spun in a microfuge at 4 °C for 20 minutes at 14000 and the supernatant removed to a fresh pre-cooled eppendorf and stored at –70 °C.

2.5.4 Protein concentration estimation

A standard curve for protein estimation was prepared using different concentrations of Biorad protein dye reagent at spectrophotometer wavelength 595 nm. A known dilution of the protein sample was placed in the spectrophotometer (Beckman Du^R 640) and the protein concentration measured against this graph.

2.5.5. Separation of proteins and Western transfer

The BioRad minigel, the Atta (GRI technologies) and the NuPage® (Invitrogen, UK) systems were used to electrophoretically separate 15-20µg (minigel and NuPage® systems) or 45µg (Atta system) protein extracts. Protein samples were prepared with distilled water and 10µl 2xSDS gel-loading buffer to give a final volume of 20µl (minigel) or 60µl (Atta), heated to 100°C to denature the proteins and then loaded. A denaturing

SDS polyacrylamide (BioRad) gel was used in electrophoresis buffer. Gels were run at 110V for one and a half hours (minigel) or 250V for 2 hours (Atta). Samples were then transferred to Hybond ECL (Amersham Biosciences). With smaller gels the BioRad mini-protean system was used and transfer carried out in transfer buffer at 80V for 45 minutes. With larger gels the semi-dry blotting transfer system (Bio-Rad Transblot^{R} SD system) with dry blot buffer was used. Completion of transfer was assessed by prestained molecular weight markers (BioRad).

2.5.6 Detection of proteins

Following transfer of proteins, the membrane was incubated overnight on a shaker at 4°C in 5% non fat dry milk (Marvel) made up in TBST. It was then washed 3x 10 minutes in TBST on the shaker. The membrane was then incubated with primary antibody at the recommended dilution made up in 5% Marvel/TBST and agitated gently for 4 hours at room temperature or overnight at 4°C. Membranes were again washed 3x 10 minutes and then incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse, anti-rabbit or anti-goat all from Sigma) at the recommended dilution in 5% Marvel/TBST for one hour at room temperature. The membrane was washed 3x 10 minutes. Detection of proteins was carried out by enhanced chemiluminescence (ECL), using the ECL-plus detection kit (Amersham) according to manufacturer's instructions. Membranes were exposed to Hyperfilm^{TM} (Amersham) and exposed for variable lengths of time (5 seconds to 1 hour). Antibodies and controls used in these procedures are shown in Table 2.2 and 2.3.

Table 2.2: Antibodies used for Western analysis

Antibody	Antibody dilution	Manufacturer	Secondary antibody*
p53(1C12) monoclonal	1:2000	Cell Signaling Technology	Anti mouse-HRP
$p19^{ARF}$ polyclonal	1: 100	Abcam (ab80)	Anti rabbit-HRP
p21 polyclonal (M19)	1: 500	Santa Cruz Biotechnology (sc-471)	Anti mouse-HRP
ß actin (I-19)	1: 1000	Santa Cruz Biotechnology (sc-1616)	Anti goat-HRP

Table 2.2 shows the antibodies used in this thesis along with their dilution, manufacturer and appropriated secondary antibody. *Secondary antibodies purchased from DAKO

Table 2.3: Controls applied in Western analysis

Antibody	Positive control	Negative control
p53	Wild type MEFs irradiated with UVC SV3T3 cells	p53 null MEFs
$p19^{ARF}$	SV3T3 cells	NIH 3T3 cells
p21	UVC irradiated wild type MEFs	NIH 3T3 cells

Table 2.3 shows the positive and negative controls used in Western analysis. SV3T3 cells are fibroblasts that are transformed by the SV40 virus and express high levels of p53 (Lane and Crawford 1979; Linzer and Levine 1979; Oren et al 1981). NIH 3T3 cells are a widely used mouse fibroblast cell line.

CHAPTER 3

MOLONEY MLV INFECTION IN VITRO

Moloney murine leukaemia virus (MMLV) induces T cell lymphomas in mice (Fan 1997), an event that can be accelerated by pre-existing genetic lesions, most notably the presence of transgenic oncogenes (Stewart et al 1993; van Lohuizen et al 1991). Mice null for the p53 tumour suppressor gene also have a strong predisposition to develop T-cell lymphomas (Donehower et al 1992) of a similar phenotype to that found in the virus-induced tumours. It has been shown that, together, p53 loss and MMLV are weakly synergistic in T cell lymphomagenesis, however the mechanism underlying this cooperation is not known (Baxter et al 1996). Does the collaboration between p53 loss and MMLV operate at the level of virus replication and host cell viability and would one therefore expect loss of p53 to render the host cells resistant to virus-induced growth suppression or cytopathic effects? Or does loss of p53 synergise with MMLV in promotion of cell immortalisation and enable cells to bypass replicative crisis? Furthermore, it is possible that MMLV in some way influences or induces the expression of p53, possibly by triggering a DNA damage response, which may manifest as changes in levels of apoptosis? In this case, loss of the host cell defence mechanism in cells lacking p53 could alter the dynamics of MMLV-induced cellular effects.

Previous *in vitro* studies involving various murine leukaemia virus (MLV) strains and p53 have given contrasting results: The *in vitro* cytopathic effect of a leukaemogenic mink cell focus-forming (MCF) MLV isolate in mink fibroblasts was found to be independent of p53 (Nanua and Yoshimura 2004) whereas the apoptotic crisis caused by Abelson MLV infection in primary pre-B cells was seen to be mediated by p53 (Unnikrishnan et al 1999). Abelson MLV (Mostecki et al 2000; Thome et al 1997; Unnikrishnan and Rosenberg 2003)and Friend leukaemia virus (Ben-David et al 1990; Ben David et al 1988; Johnson and Benchimol 1992) have both been reported to select for inactivation of p53 *in vitro*. There have not, however, been any previous *in vitro* studies that have focused on Moloney MLV and p53. This chapter sets out to investigate the *in vitro* interaction of MMLV with host cells using a primary fibroblast cell system, in particular, looking at any collaborative effects with p53 loss.

3.1 EXPERIMENTAL PROTOCOLS

All experimental approaches were carried out to the specifications described in Chapter 2 except where stated as below.

3.1.1 Infection of mouse embryonic fibroblasts (MEFs)

Wild type (passage 3) and p53 null (passage 3) MEFs were recovered from liquid nitrogen and expanded for five days *in vitro*. MEFs at passage 4 were harvested and set up in 10cm petri dishes each containing $8x10^5$ cells/10ml of medium. Cells were then infected with virus. Growth curves were set up in 12 well plates, each well containing 2.5 $x10^4$ cells in 2ml of medium and cultured at 37˚C at 5% carbon dioxide. Each sample was set up in triplicate in the following groups; wild type (wt) MEFs, wild type MEFs infected with MMLV (wt/MMLV), p53 null MEFs (p53KO), p53 null MEFs infected with MMLV (p53KO/MMLV).

Cells were trypsinised, harvested and counted daily for 7 days starting from 2 days after set up. They were also counted at days 15 and 22 post set up. Both live and dead cells were counted where all washes and medium were included in the cell counts. Cell death was assessed by Trypan Blue exclusion. Media was replenished in all wells at 4, 7, 8, 12**,** 15 and 18 days after set up.

3.1.2 Adapted 3T3 protocol

12 well plates were set up as described for the growth curve, containing triplicate wells for the following groups; wt MEFs (wt), wt MEFs with MMLV infection (wt/MMLV), p53 null MEFs (p53KO), p53 null MEFs with MMLV infection (p53KO/MMLV). Each well contained 2.5 x 10^4 cells in 2ml of medium. Every three to four days the cells were trypsinised, harvested and counted. Each well was then set up again with 2.5 $x10^4$ of the harvested cells in 2ml medium. At each time point the cell count was recorded. The mean cell count of the triplicate wells for that cell population was calculated. The “gain” (ie increase or sometimes decrease) in cell number was calculated and the “cumulative gain” (ie the increase/decrease from the starting count of 2.5 $x10^4$) recorded for each cell population over a period of 78 days (a further 22 passages).

3.1.3 Effects of low serum and UVC irradiation on MEFs

12 well plates were set up as described above for the growth curve. Each well contained $5x10^4$ MEFs in 2ml medium. For each of the following groups triplicate wells were prepared; wt MEFs; wt MEFs/MMLV p53 null MEFs; p53 null MEFs/MMLV.

3.1.3.1 Low serum treatment of MEF cultures

24 hours after set up in culture, complete media was replaced with media containing 0.5% serum. Cells were maintained in low serum throughout the experiment while control wells were maintained in normal 10% serum. The cells were then rinsed with PBS, trypsinised, harvested and counted. Both live and dead cells were counted where all rinses and medium were included in the cell counts. Counts were carried out daily over 5 days. The medium was replenished in all wells 4 days after set up.

3.1.3.2 UV irradiation of MEF cultures

MEFs were irradiated with UVC irradiation. “Conditioned” media changes were made at 4 days after set up into the 12 well plates, whereby 1 ml of the old media was removed from the well and replaced by 1ml of fresh media. Cells were counted over an 8 day period.

3.1.3.3 Propidium iodide staining

Cells exposed to UV irradiation and low serum were fixed and stained with propidium iodide and analysed by flow cytometry. The subG1 cell population was measured as an indication of the percentage of apoptotic cells. When this was carried out, all media and washes were kept during the harvesting of the cells.

3.1.4 Western blot analysis

A protocol was set up to investigate the effect of MMLV infection on p53 expression. Wild type MEFs (passage 4) were infected with MMLV viral supernatant prepared from exponentially growing Mov3 3T3 fibroblasts. Infected and uninfected MEFs were harvested from petri dishes at 2, 6, 10 and 24 hours after infection. At each of these time points, the cells were pelleted and frozen at -70°C in preparation for Western blot analysis of p53 expression.

3.1.5 Statistical analysis

All graphs were plotted on Sigmaplot; with error bars representing standard deviation. P values were calculated using Student's t test.

3.2 RESULTS

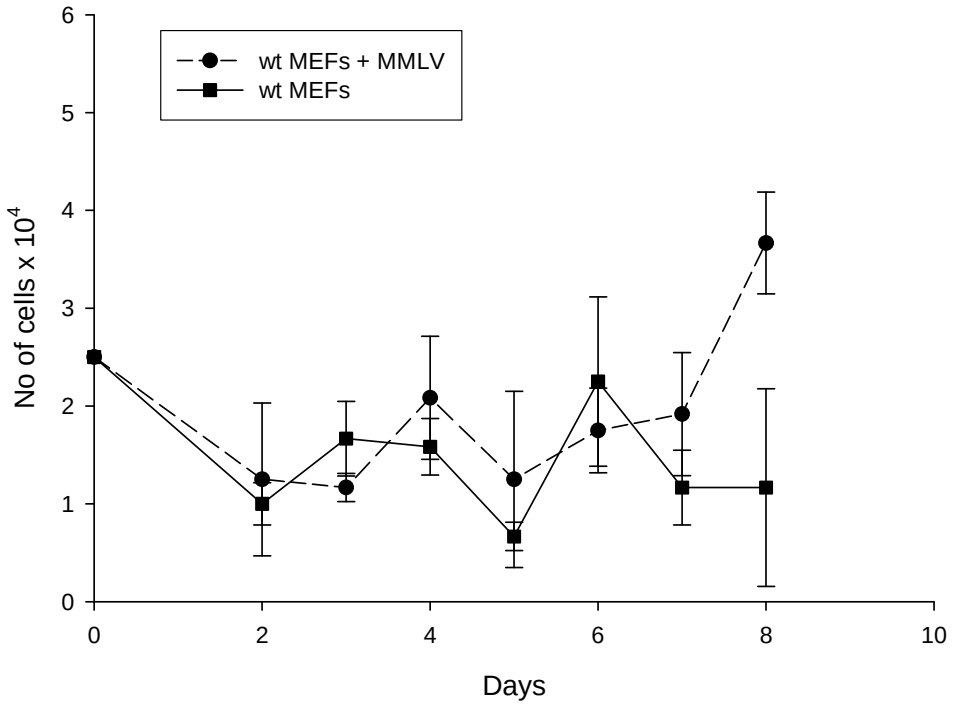
3.2.1 MMLV enhances growth of MEFs in vitro and does not induce p53 expresssion.

Growth characteristics of MEFs can be influenced by the mouse strain from which they are derived, age of the foetus at time of preparation and on the passage of the cells (Busuttil et al 2006; Zhang et al 2003) (Blyth, unpublished). It is not clear what effects Moloney MLV has on *in vitro* cultured cells. Growth curves were set up to determine live and dead cell numbers over a 3 week period in order to compare the performance of wild type controls with those infected with MMLV and to assess the effects of infection (Figure 3.1). The results show that there was a minimal increase in growth of wild type cells, with less than a doubling in cell number over the 22 day observation period. Infection with MMLV appeared to have no effect on cell number in the first 7 days of the curve. However after this time wt/MMLV cells appeared to perform better than uninfected controls, albeit this difference was not significant (except at day 8 where $P<0.002$). The dips in cell numbers seen in both the infected and uninfected populations can be reconciled with media changes where it would be expected that cell growth would be perturbed.

The cell population reflects a dynamic culture of dividing and dying cells. Assessment of cell number does not distinguish between these two cell processes; for instance if cell death balances mitosis, an increase in live cell number will not be apparent by this assay. Trypan blue uptake by cells can be used as a determinant of cell death whereby the disrupted membrane permeability of late apoptotic and necrotic cells will allow uptake of the vital dye (Freshney 1987; Martin and Cotter 1996). Dead cell numbers of wt cells +/- MMLV, as assessed by Trypan blue, are tabulated in Table 3.1. As can be seen there was no appreciable difference in cell death, as assessed with Trypan blue staining, between wild type MEFs +/- MMLV. Indeed, no death was observed within the first 4 days in any of the groups. Within such small numbers of dead cells it is difficult to determine if the increased numbers of dead cells seen at day 15 in the wt culture without virus is a significant finding considering that by day 22 there was no difference. This increase in dead cells may however help to explain why there appears to be more cells in Figure 3.1 within the MMLV infected group.

Figure 3.1: Growth curve of wild type MEFs +/- MMLV

A



B

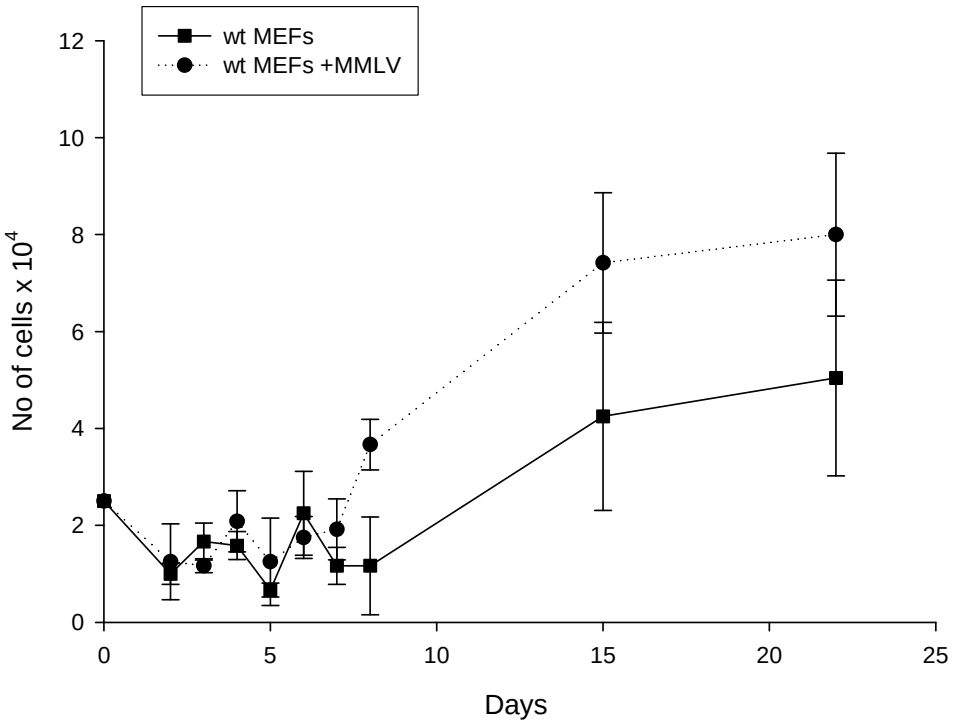


Figure 3.1: Growth curve of wild type MEFs as assessed by live cell counts. Graphs are shown for MMLV infected and uninfected MEFs. Figure A shows the count over the first 8 days and Figure B depicts the full 22 days. ($P<0.002$ at day 8).

Table 3.1 : Number of dead cells in wild type MEFs populations

Days	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**15**	**22**
Wild type (wt)	0	0	0	0	0	0	0	3.75	1.25
wt	0	0	0	0	2.5	0	0	2.5	1.25
wt	0	0	0	5.0	0	0	0	2.5	1.25
Average	**0**	**0**	**0**	**1.7**	**0.83**	**0**	**0**	**2.9**	**1.25**
wt +MMLV	0	0	0	0	0	2.5	0	1.25	1.25
wt + MMLV	0	0	0	2.5	0	2.5	0	1.25	1.25
wt + MMLV	0	0	0	2.5	2.5	0	0	1.25	1.25
Average	**0**	**0**	**0**	**1.7**	**0.83**	**1.7**	**0**	**1.25**	**1.25**

Table 3.1: This shows the number of dead cells x 10^3 as assessed by Trypan blue uptake. MMLV infected and uninfected populations of wild type MEFs are compared. The average value for each group is also shown. Each line represents replicate counts.

MMLV has been shown to induce apoptosis in pre-leukaemic thymocytes (Bonzon and Fan 1999) and p53 is a known inducer of apoptosis (Balint and Vousden 2001; Levine 1997; Vousden and Lu 2002). An assessment was, therefore, carried out to ascertain if p53 might be induced in cells following infection with MMLV. Western blot results (results not shown) did not demonstrate any difference in levels of p53 expression between infected or uninfected wild type MEFs detectable by this method. It is possible, however, that the situation may be different in different cell types and, in later chapters, levels of p53 expression are also assessed in thymocytes, both with and without MMLV infection.

3.2.2 MMLV and p53 loss contribute independently and additively to enhance growth of MEFs.

Although there is information available from work in other laboratories on the characteristics of p53 null MEFs (Harvey et al 1993c; Hermeking and Eick 1994; Jones et al 1996; Tomicic et al 2005), it was necessary for the purposes of this experiment to establish how these cells would perform *in vitro* in the culture conditions of our laboratory. In this experiment cell viability was assessed in p53KO and wild type MEFs over a 3 week period and the effects of MMLV infection were assessed.

As can be seen from Figure 3.2, p53 null MEFs had a considerable growth advantage over passage matched wild type controls during the growth period. Whereas wild type MEFs maintained a steady cell number over the course of the graph, p53 null MEFs grew at a steady rate, doubling within a 24 hour period over the first 3 days of the growth curve. Whilst this rate of growth then slowed down a little, at the end time point these p53KO cells had increased in cell number by about ten times. As before, media changes affected cell viability immediately after replenishment.

As with the wt MEFS, MMLV infection of p53 null MEFs had no effect on cell number within the first 8 days post infection (Figure 3.3). Uninfected p53KO cells appeared to perform better at later stages of the growth curve than p53KO/MMLV MEFS however this difference was not significant with a high degree of variation occurring between wells as can be seen from the error bars. Once again, media changes affected cell viability immediately after replenishment.

Figure 3.2: Growth curve of p53 null MEFs

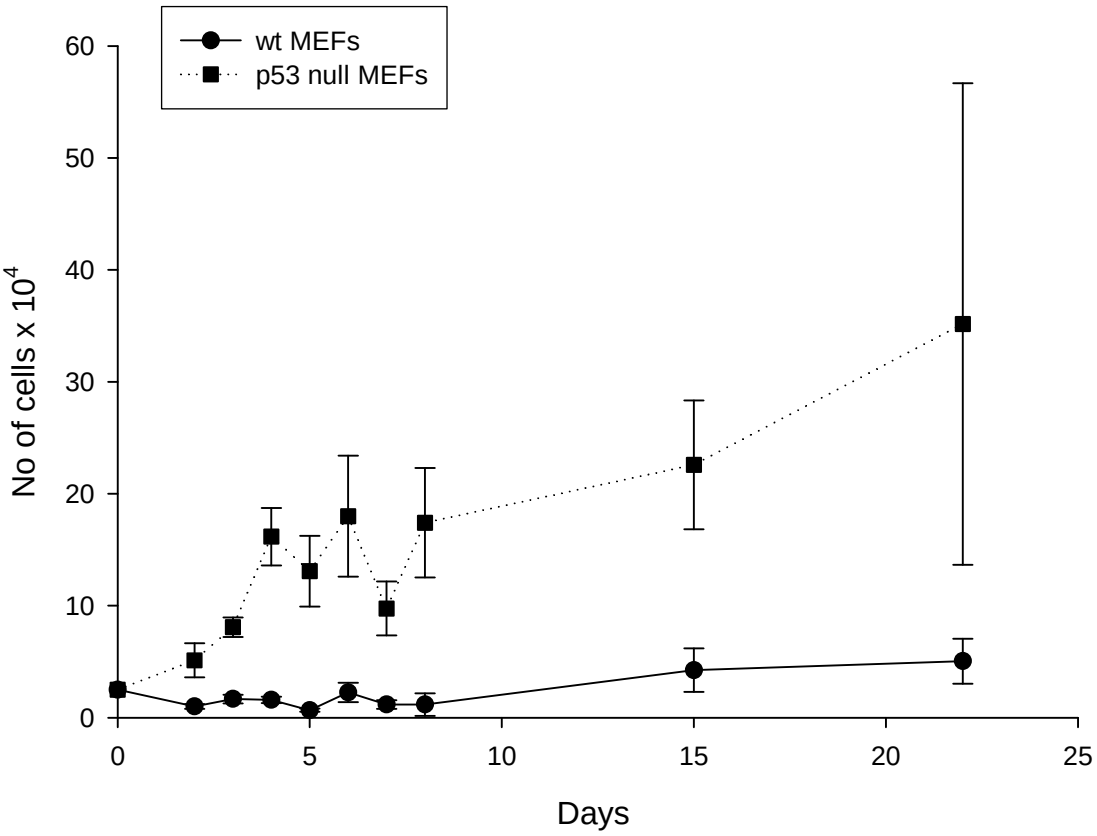
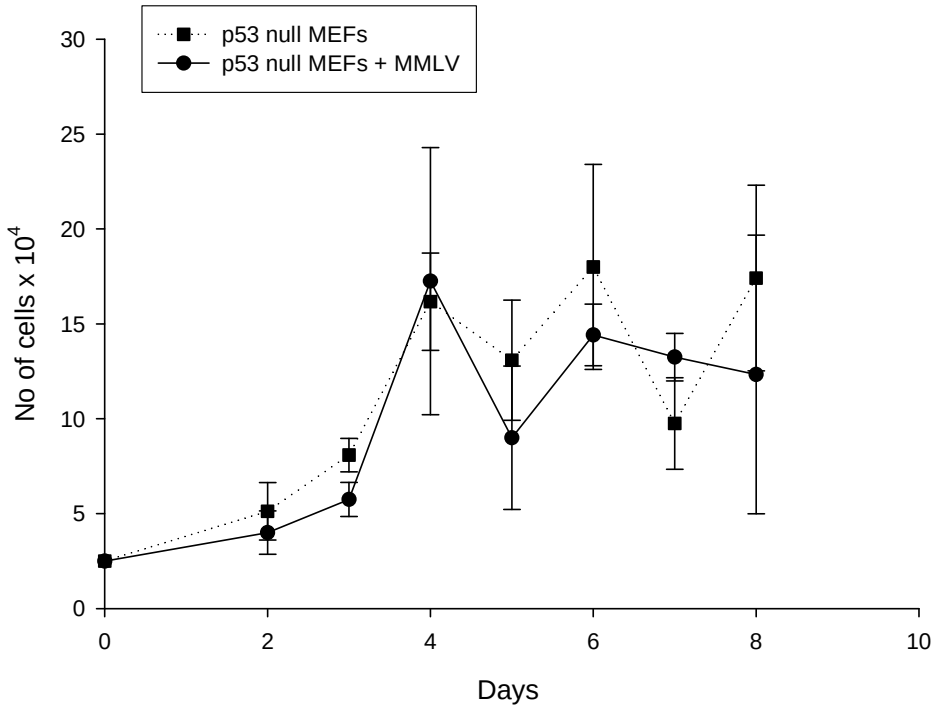


Figure 3.2: Cell growth of wild type and p53 null MEFs over a 22 day period

Figure 3.3: Growth of p53 null MEFs +/- MMLV

A



B

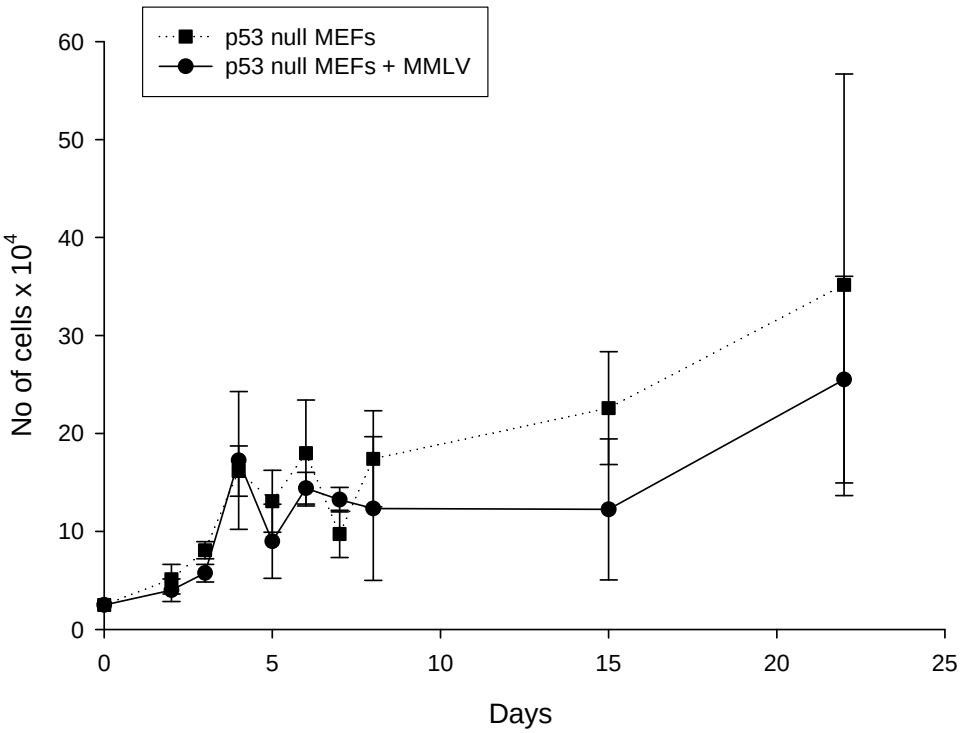


Figure 3.3: Growth curve of p53 null MEFs as assessed by live cell counts. Graphs are shown for MMLV infected and uninfected MEFs. Figure A shows the count over the first 8 days and Figure B depicts the full 22 days.

p53 null populations were stained with Trypan blue and cell death investigated (Table 3.2). There was no difference in the level of Trypan blue positive cells during the early stages of growth post-infection (2-4 days) however with the exception of day 15, there was generally less cell death in the p53KO/MMLV group from day 5.

In general there were higher numbers of dead cells in the p53 null population compared to in the wild type population (Table 3.2 versus Table 3.1). This may reflect the fact that there are significantly greater numbers of total cells in the p53 null cultures compared to in the wild type cultures. In fact, dead cell count as a percentage of total cell count is actually higher in the wild type population which would be expected when one considers the role of p53 in mediating growth arrest and apoptosis.

3.2.2.1 3T3 assay

Cells in culture undergo a limited number of cell divisions and then arrest in a quiescent, yet viable, state known as replicative senescence (Hayflick 1965; Hayflick and Moorhead 1961; Lundberg et al 2000; Wynford-Thomas 1999). In the approach to replicative senescence, cell populations show a gradual decline in the proportion of dividing cells, the timing of this decline varies between cell types and between sister clones, until eventually the culture consists entirely of senescent cells and growth stops (Thomas et al 1997). Cellular senescence is irreversible and is thought to be a key restraint on the progression of tumours (Smith and Kipling 2004) and, whilst normal cell populations will eventually demonstrate replicative senescence with prolonged passage in culture, cells that become transformed tend to bypass senescence and can proliferate indefinitely (Lundberg et al 2000). In human somatic cells replicative senescence occurs due to shortening of telomeres and lack of expression of telomerase (Espejel and Blasco 2002; Ramirez et al 2001). This contrasts with the situation in the mouse where MEFs possess telomerase activity and have long telomeres and senescence occurs by, predominantly, telomere-independent mechanisms. It is suggested that senescence of MEFs only occurs when cells undergo stressful conditions such as high oxygen tension or “culture shock” (Parrinello et al 2003; Ramirez et al 2001; Sherr and DePinho 2000).

Table 3.2: Number of dead cells in p53 null MEFs populations

Days	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**15**	**22**
p53 null	0	0	0	2.5	2.5	0	2.5	2.5	5.0
p53 null	0	5.0	0	5.0	5.0	5.0	5.0	0	5.0
p53 null	0	0	0	2.5	2.5	2.5	2.5	5.0	0
Average	**0**	**1.7**	**0**	**3.3**	**3.3**	**2.5**	**3.3**	**2.5**	**3.3**
p53 null +MMLV	0	2.5	0	0	0	0	2.5	5.0	2.5
p53 null + MMLV	0	2.5	0	2.5	2.5	2.5	2.5	2.5	2.5
p53 null +MMLV	0	0	0	0	0	2.5	2.5	7.5	2.5
Average	**0**	**1.7**	**0**	**0.83**	**0.83**	**1.7**	**2.5**	**5.0**	**2.5**

Table 3.2: This shows the number of dead cells x 10^3 that stained with Trypan blue. MMLV infected and uninfected populations of p53 null MEFs are compared. The average for each group is also shown. Each line represents replicate counts.

Assessing the time to replicative senescence is a useful measure of cell lifespan ie how many passages it will undergo, before senescence occurs and an adapted 3T3 assay is a standard protocol that is used to investigate this. There are many regulatory proteins that are involved in mediating the entrance of the cell into senescence but p53 is known to have a significant role and loss of functional p53 usually enables cells to escape senescence (Bond et al 1994), (Rogan et al 1995; Wynford-Thomas 1999). MEFs enter a state of growth arrest after a relatively short time in culture; generally about 10-15 population doublings (Smith and Kipling 2004) or 7 passages in culture (Park et al 2004) and, in murine cells, senescence requires a functional $p19^{ARF}$-p53 pathway (Bond et al 1994), (Kamijo et al 1999; Rogan et al 1995; Serrano et al 1996; Wynford-Thomas 1999). Consequently disruption of p53 function, either by mutation of p53 or by interference with genes that regulate p53 such as $p19^{ARF}$ or mdm2, allows MEFs to bypass senescence; p53 null MEFs do not, therefore, exhibit senescence and are able to retain a high proliferative potential in culture (Kamijo et al 1997).

The results from the 3T3 assay were repeatable in two independent experiments (Figure 3.4) and demonstrated that p53 null MEFs grew cumulatively faster than wild type MEFs. MMLV infection of both wt and p53 null MEFs offered no advantage over the first 5 passages. However after this time MMLV infection resulted in significant growth advantage in both wt and p53 null cultures. This became apparent from about 22 days post set up and continued until the end of the experiment. Wild type cells infected with MMLV could not match the growth potential of p53 null cells which implies that although MMLV appears to give some advantage to the cells, it is not performing the same role as p53 loss. This observation is further strengthened by the fact that p53 null and MMLV together are seen to give a much better advantage to the cells. These cells (p53KO/MMLV) showed a 400 fold increase in cumulative growth over the 80 day period (22 passages).

Uninfected wt MEFs did not grow as anticipated during the early passages and were relatively static in cell number. A possible explanation for this is that the cells, at passage 5 by the time they were set up into the 12 well plates, had already entered replicative senescence. However after approximately 11 passages (about 40 days), this population also started to show some increase in growth, albeit at a much slower rate than those cells infected with MMLV. This suggests the possibility that the cells may have acquired some growth-advantageous genetic mutation that is MMLV-independent.

Figure 3.4: 3T3 passage of MEFs +/- MMLV

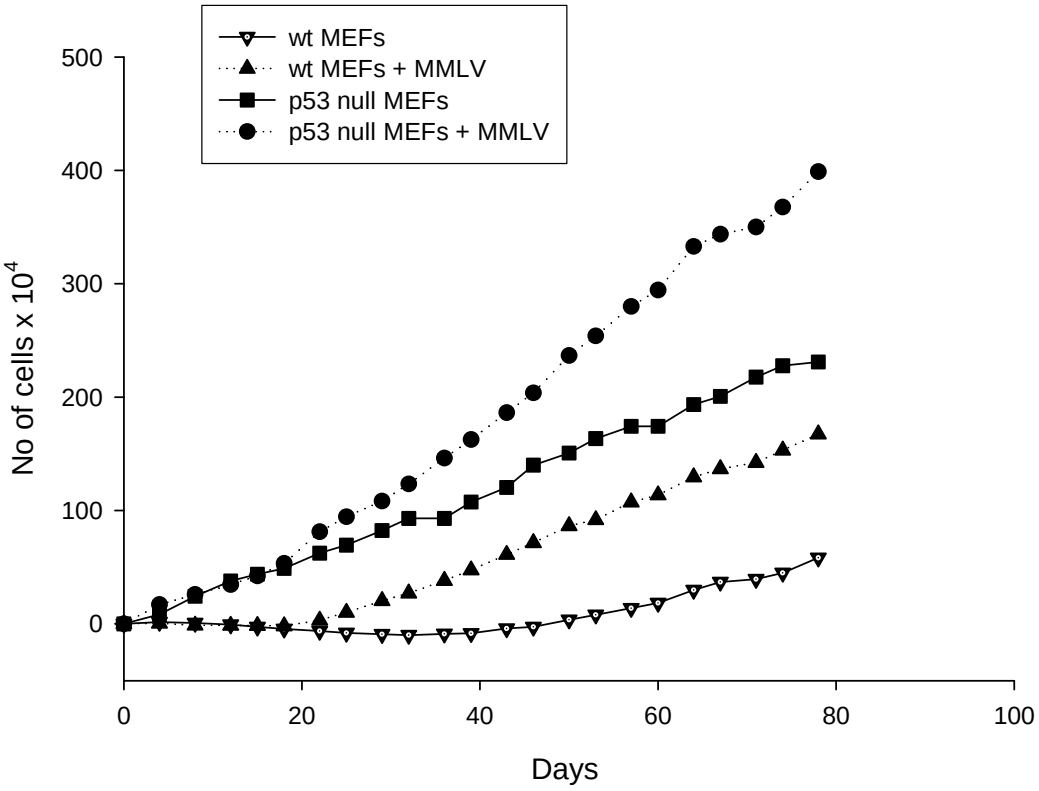


Figure 3.4: This graph depicts the cumulative growth of wild type and p53 null MEFs +/- MMLV as cultured under adapted 3T3 protocol conditions. Cultures were harvested and counted every 3-4 days and the cumulated growth plotted. A decrease in cell number, as observed at early passage, represents a "negative gain" and this appears as a value less than zero on the graph.

3.2.3 Increased growth of MEFs in low serum is conferred by p53 loss but not MMLV infection.

Cells in culture ultimately cease to grow due to nutrient deprivation and contact inhibition (Campisi 1997; Fagotto and Gumbiner 1996; Lundberg et al 2000; Marcotte and Wang 2002). Cells that are "transformed", for instance by oncogenic viruses, may be less influenced by these normal growth constraints and may exhibit changes in growth phenotype that can be identified by plotting growth curves (Fagotto and Gumbiner 1996). Cells that are placed in medium deficient/low in serum will rapidly exit the cell cycle and either enter into the quiescent state or may undergo apoptosis (Dulbecco 1970; Evan et al 1992; Huang and Cidlowski 1999; Preston et al 1994). Different cell populations may respond differently to low serum conditions depending on cell type and the serum concentration (Chattopadhyay et al 2001). For instance, transformed cells may be anticipated to have reduced serum requirements compared to normal cells.

MMLV infected and uninfected cells were cultured in low serum and the effects of MMLV infection were investigated. As expected, wild type MEFs exhibited a rapid and progressive decrease in cell viability when cultured in low serum conditions (Figure 3.5). The graph suggests that low serum conditions may be able to support a low number of viable cells in culture and it would have been interesting to continue the growth curve for a longer time period. MMLV infection, however, did not seem to have any significant influence on cell viability in low serum conditions.

Compared to the wild type MEFS, p53 null MEFs, in general, fared much better under low serum conditions. Although an initial decrease in cell number (both infected and uninfected p53 null cells) was observed at 2-3 days after cells were placed in low serum, cell number then started to increase and parallel the growth characteristics of cells in normal serum, albeit the cell number was consistently lower (Figure 3.6). All p53 null MEF populations exhibited a marked drop in cell number at day 5 in response to the medium change at day 4. However whilst p53 null cells in normal serum started, once again, to increase in number, those in low serum started to level out. The addition of MMLV in low serum conditions resulted in a reduction in cell number at this time.

Figure 3.5: Growth of wild type MEFs in low serum

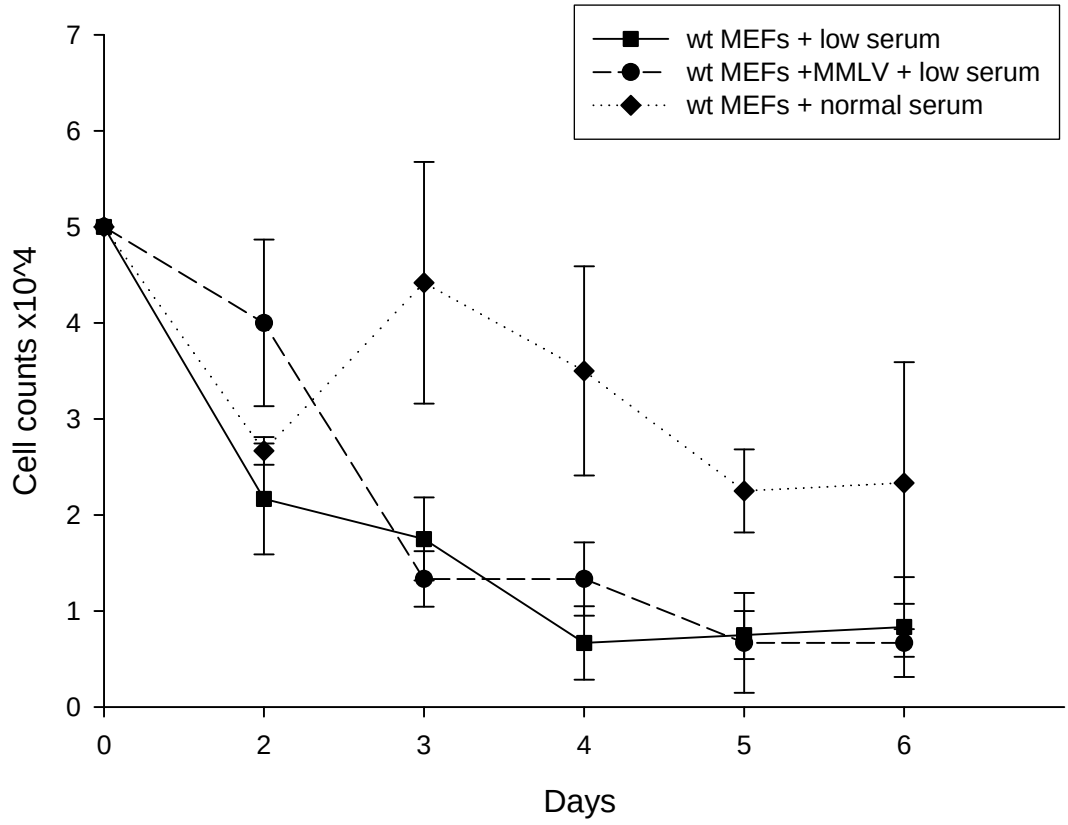


Figure 3.5: Cells were allowed to settle in wells for 24 hours before media was replaced with low serum (0.5%) at time 0 in the graph. Cultures were maintained in low serum throughout. The graph depicts the cell number of wild type MEFs, +/- MMLV, when cultured in low (0.5%) serum conditions.

Figure 3.6: Growth of p53 null MEFS in low serum

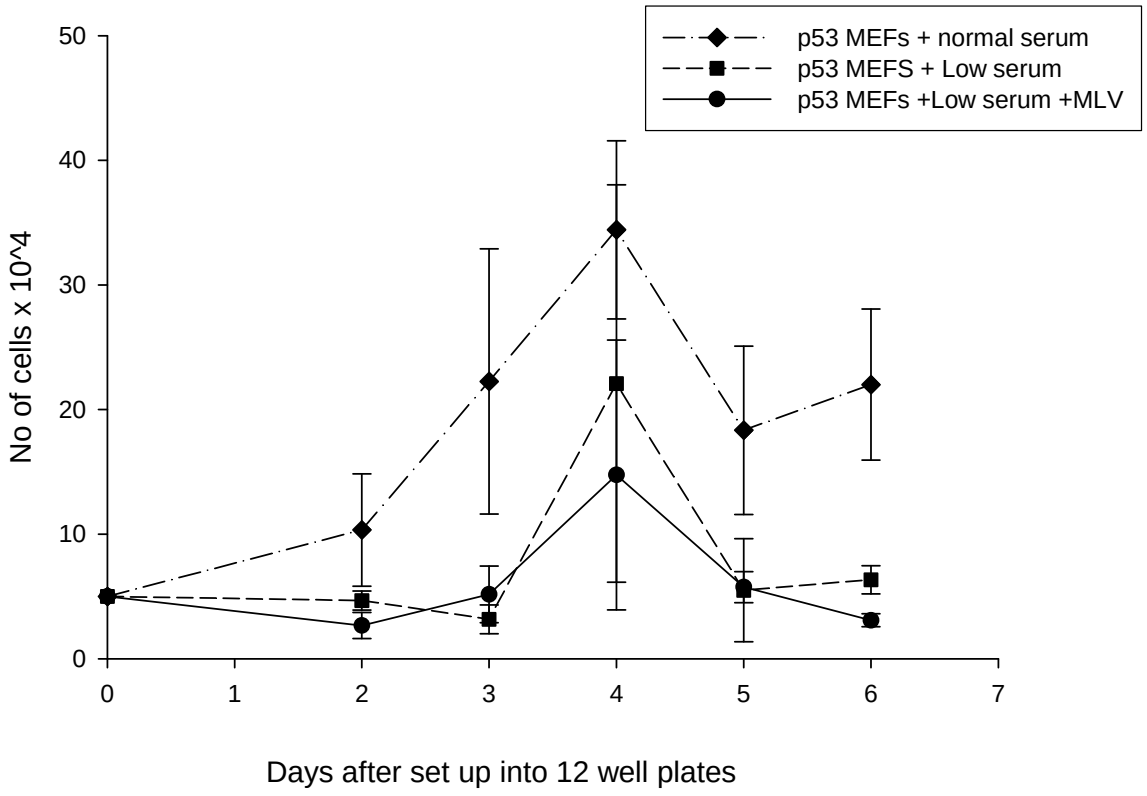


Figure 3.6: Cells were allowed to settle in wells for 24 hours before media was replaced with low serum (0.5%) at time 0 in the graph. Cultures were maintained in low serum throughout. The graph depicts the cell number of p53 null MEFs, +/- MMLV, when cultured in low (0.5%) serum conditions.

Overall, the results show that low serum (0.5%) medium produces a dramatic decrease in the cell viability of wild type MEFs which is not influenced by MMLV infection. p53 loss markedly alleviates the effects of low serum on cell growth. This is not unexpected since p53 null or mutant cells are less subject to cell cycle check points compared to p53 wild-type cells and are more tolerant of adverse conditions, such as exhaustion of growth factors in the culture medium, than are wild type cells (Lane 1992; Levine 1997).

3.2.4 Loss of p53, but not MMLV, leads to increased resistance to UV irradiation in MEFs.

Infected and uninfected MEFs were exposed to UVC irradiation as a means of causing DNA damage/cellular injury. The influence of MMLV infection and p53 status of the cells was observed in response to this cellular insult. Cells were set up into the 12 well plates at day 0 and irradiated at day 1. Each of the wild type MEF populations demonstrated an initial decrease in cell number (Figure 3.7). Irradiated cells never recovered and continued to progressively decrease in number in contrast to the unirradiated cells which increased in cell number from day 2. The addition of MMLV to the cultures made no difference to the irradiated cells; cell numbers were comparable in the infected and uninfected wild type MEFs following irradiation.

In this experiment wild type MEFs achieve higher cell numbers by day 12 (around 14 x 10^4 in Figure 3.7) than do those in the earlier growth curves (around 2.5 x 10^4 in Figure 3.1). This is attributed to the fact that the cells in this experiment were initially set up at a much higher cell density in order to cope with the subsequent anticipated drop in cell numbers. The resultant changes in cell to cell contact, cell contact-dependent signalling and concentrations of growth factors are considered to be significant in encouraging cell growth (Fagotto and Gumbiner 1996)**.**

MEFs null for p53 were subjected to the same conditions. p53 null MEFs, both with and without irradiation, grew better than did wt MEFs throughout the experiment (Figure 3.8). UVC-irradiated p53KO/MMLV MEFs were consistently lower in cell number than irradiated p53KO cells throughout the experiment. The differences were statistically significant at 2 days post set up ($P<0.03$) and at 6 days ($P<0.02$). Unlike in previous graphs where replenishment of the medium is associated with a drop in cell numbers, in this case there was an increase in cell numbers in all MEFs groups when the media was

changed at day 4. This is presumably due to the fact that this was a “conditioned” medium change whereby certain, influential growth enhancing factors remain in the “old” serum that is not replaced.

UV irradiation causes damage to cellular DNA and, since p53 is often involved in DNA damage-induced apoptosis, it was interesting to evaluate levels of apoptosis occurring in wild type and p53 null irradiated populations. To determine apoptotic percentages, cell cycle analysis was carried out using propidium ioidide (PI) staining and the subG1 population assessed (Table 3.3).

The results demonstrate that, as expected, UVC irradiation increased levels of apoptosis in all populations, especially soon after irradiation (time points 2, 3 and 4 days). Although the numbers of dead cells counted by Trypan blue staining were also increased (Table 3.4*),* actual dead cell numbers were still relatively low and did not vary much between the different cell populations which emphasises the observation that Trypan blue staining has limitations and only reveals cells in the late apoptotic stages. Interestingly, the degree of UVC induced apoptosis was considerably less in the p53KO MEFs than in the wt MEFs at 6-8 days suggesting the possibility that p53 loss may confer some protection against UVC irradiation later in the culture period. p53 null MEFs tolerated UVC irradiation much better than the wt cells and were able to grow (Table 3.3). This is unsurprising since p53 null MEFs are not subject to p53-mediated apoptosis in response to irradiation. p53 independent factors, however, still remain operational and this would explain why cell viability was slightly reduced in these cells compared to non-irradiated cells.

At all time points there were higher numbers of apoptotic cells within MMLV infected unirradiated MEFs (Table 3.3), both wt and p53KO, than within uninfected counterparts. Within the p53 null population, MMLV infection increased apoptosis of irradiated cells at all time points except day 3. This is consistent with Figure 3.8 where MMLV infected irradiated cells were lower in number than uninfected irradiated cells. MMLV infection did not, however, have any consistent influence on levels of UVC induced apoptosis in wild type MEFs. Overall, therefore, the results suggest that UVC irradiation, and possibly MMLV infection (this is not corroborated by Table 3.1 or Table 3.2), cause increased apoptosis in wild type and p53 null MEFs. Less clear cut is the possibility that MMLV exacerbates the levels of UVC induced apoptosis within p53 null cells.

Figure 3.7: Growth of wild type MEFs +/- UVC

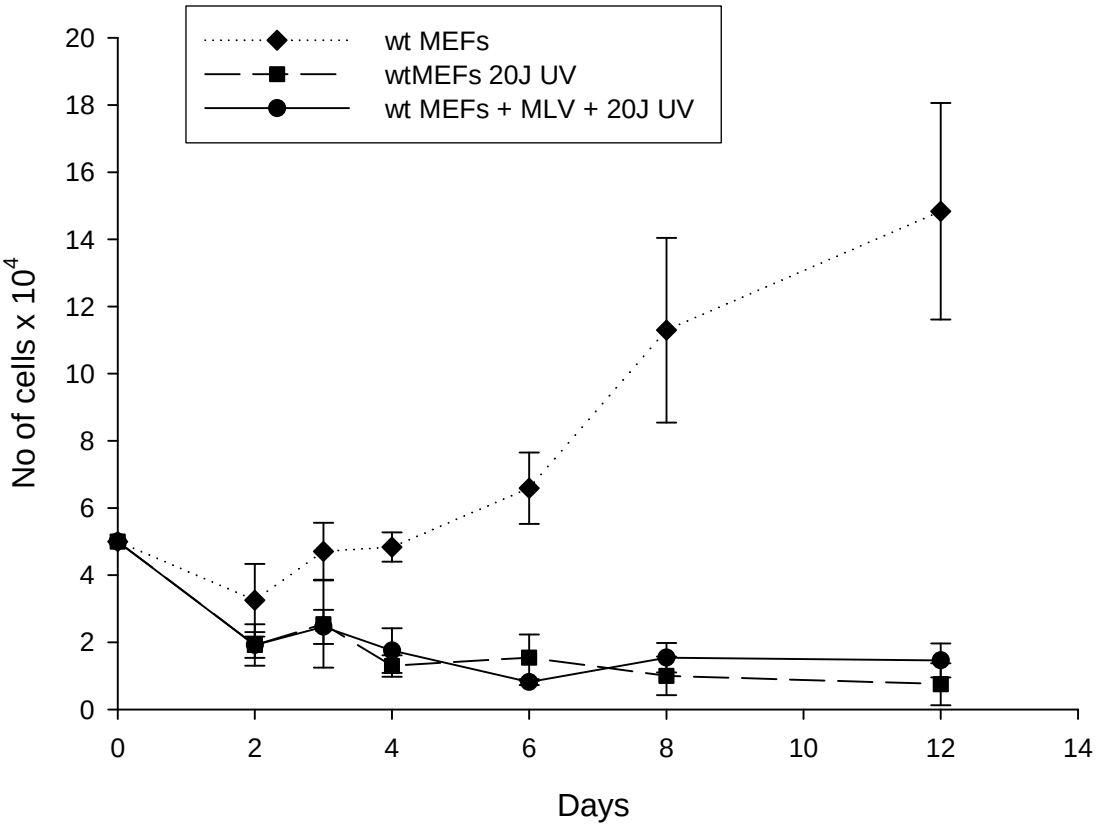


Figure 3.7: This graph represents the growth of MEFs +/- MMLV following exposure to UVC. Cells were plated at day 0, irradiated at day 1 and counted at daily intervals from day 2.

Figure 3.8: UVC irradiation of p53KO MEFs +/-MMLV

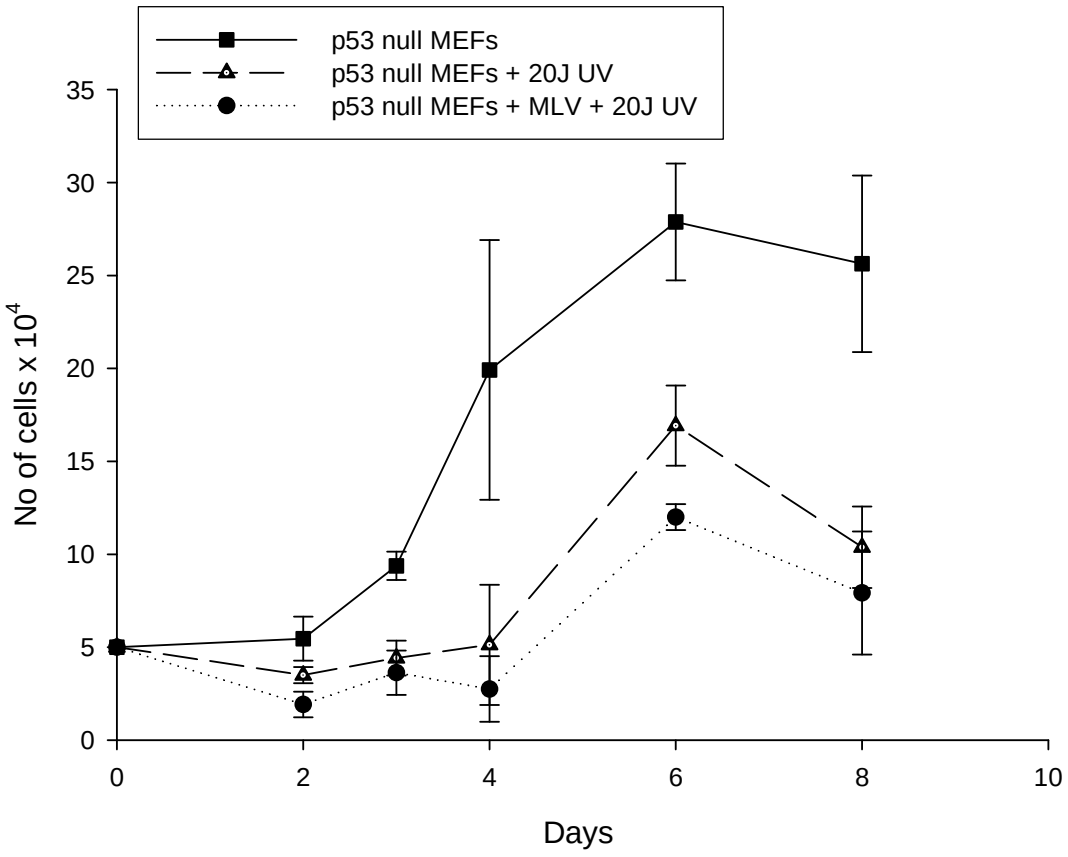


Figure 3.8: This graph represents the growth of p53 null MEFs +/- MMLV following exposure to UVC. Cells were plated at day 0, irradiated at day 1 and counted at daily intervals from day 2.

Table 3.3 Assessment of Apoptosis in UVC treated MEFs

Days	2	3	4	6	8
Wt	1.9	2.0	1.2	0.9	0.6
Wt +UVC	10	3.8	7.9	3.1	5.7
Wt + MMLV	2.7	2.6	1.9	3.1	1.4
Wt + MMLV + UVC	9.7	3.1	7.5	4.1	6.7
p53 null	0.6	0.9	0.6	1	0.7
p53 null + UVC	11.1	7	4	1.6	1.5
p53 null + MMLV	1.5	1.1	1.1	2.1	1.9
p53 null + MMLV + UVC	12.6	5.9	7.4	2.5	3.1

Table 3.3: Cell cycle analysis of fixed MEFs was carried out by flow cytometry and an assessment of the subG1 population as an indication of apoptosis was determined. The table depicts the % of cells in the sub G1 phase following irradiation with 20 J/m^2 UVC irradiation.

Table 3.4: Number of dead cells in irradiated MEFs populations

Days	2	3	4	6	8
wt	2.1	2.1	2.5	2.1	2.5
wt+UVC	3.3	3.3	3.8	5.8	2.9
wt+MMLV	2.9	2.5	3.3	2.9	2.5
wt+ MMLV+UVC	2.5	1.25	3.8	2.9	2.9
p53 null;	1.7	2.1	2.1	2.5	2.5
p53 null+UVC	2.9	2.9	2.5	3.3	3.0
p53null+MMLV	1.25	2.5	2.5	2.9	2.9
p53null+MMLV+UVC	2.5	2.9	3.8	3.3	2.9

Table 3.4: This shows numbers of dead cells $x10^3$ as assessed by Trypan blue uptake within cell populations following exposure to $20J/m^2$ UVC irradiation. MMLV infected and uninfected populations of wild type and p53 null MEFs are compared. Each result represents the average value for replicate counts.

3.3 DISCUSSION

This chapter investigates the role of MMLV on cell viability and growth *in vitro* and explores whether or not the p53 gene can influence the effects of MMLV on cell behaviour. For this purpose, mouse embryonic fibroblasts (MEFs) which are primary, undifferentiated cells that do not harbour any oncogenic lesions and have the potential for *in vitro* passage were used. Although thymocytes are the *in vivo* target cell for MMLV, primary thymocytes are poorly viable in culture. Foetal thymic organ culture would, however, be a consideration for future *in vitro* experiments by way of providing a three dimensional environment which parallels the *in vivo* system more closely than two dimensional cell culture systems and major advantages are that it allows thymocytes to interact with stromal cells and is able to support a full range of T cell precursor differentiation (Jenkinson and Anderson 1994; Jenkinson and Owen 1990).

Cell death ie apoptosis has been shown to be a significant step in the pathogenesis of many retroviruses (Meyaard et al 1994; Rojko et al 1996) including the oncoretroviruses Avian leukosis virus (ALV) and Moloney MLV (Bonzon and Fan 1999; Weller and Temin 1981). *In vitro* studies have shown cell death to be associated with accumulation of unintegrated viral DNA, representing viral superinfection, which correlates with viral cytopathogenicity (Keshet and Temin 1979; Weller et al 1980; Weller and Temin 1981). Retroviral integration into the host chromosome causes damage to the host DNA which, if repaired, results in stable integration of the provirus. If, however, the DNA is unrepaired it triggers the cellular DNA damage response, which may occur via p53 dependent or independent pathways, and apoptosis ensues (Norbury and Zhivotovsky 2004; Skalka and Katz 2005). *In vitro* infection of mink cells with mink cell focus-forming (MCF) MLV is one example where cell death occurs independently of p53 activation (Nanua and Yoshimura 2004).

The mechanism by which the p53 pathway impacts on MMLV infection is not known. One hypothesis is that MMLV may exert some of its effects by altering levels of p53 expression. It is suggested that MMLV may induce p53 by one of two distinct pathways: either by causing genotoxic injury and eliciting a $p19^{ARF}$-independent DNA damage response as has been reported to occur with other retroviruses (Skalka and Katz 2005) or by activating proto-oncogenes and inducing a $p19^{ARF}$-mediated tumour suppressive

response (Christophorou et al 2006; Efeyan et al 2006). However in this study there is no evidence to suggest that the virus induces expression of p53 although it is possible that higher multiplicities of infection and therefore higher levels of unintegrated DNA may give a different outcome. Further experiments would need to be done to confirm this.

Although the initial 22 day study does not demonstrate a significant growth advantage with MMLV infection, the results of the 3T3 assay show that MMLV infection bestows on both wt and p53 null cells a significant growth advantage over uninfected cells which only becomes apparent from about 22 days post set up ie 24 days post infection. The results do not exclude the possibility that MMLV infected cell populations exhibit an initial period of cell loss/apoptosis which is later followed by the more growth advantageous effects of the virus. This would mimic the *in vivo* situation in which there is an initial phase of apoptosis that is followed eventually by tumour development (Bonzon and Fan 1999).

Overall, the results of this chapter suggest that both MMLV infection and p53 loss are able to enhance the growth potential of MEFs although by apparently different mechanisms since p53 loss confers some resistance to UVC whereas MMLV does not. The 3T3 assay demonstrates the growth advantage conferred by MMLV infection and reinforces the observation that this appears to be independent of p53 loss. Whilst the mechanism is not known, one potential explanation is that this is due to retroviral insertional mutagenesis leading to selection of cells with enhanced growth properties. Follow- up analysis of integration sites in long term MEFs cultures may reveal further information about such preferential growth-promoting lesions.

It is also possible that insertional mutagenesis is not involved and that a gene product of MMLV may be exerting a direct effect on cell growth as is the case for the *Env* gene of Jaagsiekte Sheep Retrovirus (JSRV) which is capable of directly transforming cells (Caporale et al 2006; Wootton et al 2006). To explore this further one would need to transduce MEFs with vectors expressing one or more viral gene products (ie Gag, Env).

In summary, this chapter investigates interactions between MMLV infection and p53 within the limitations of an *in vitro* environment. The results suggest that MMLV infection confers a growth advantage on wild type MEFs that appears not to be p53 dependent and that becomes evident after fairly prolonged culture. p53 loss seems to

further enhance this growth advantage although the mechanism by which this is achieved is unclear.

CHAPTER 4

IN VIVO INFECTION OF MMLV- THE ROLE OF P53 IN THE PRE-LEUKAEMIC THYMUS

When inoculated neonatally, Moloney murine leukaema virus (MMLV) induces T cell lymphomas in 100% of animals following a reported latency period of 3 to 9 months (Tsichlis and Lazo 1991; van Lohuizen and Berns 1990). This manifests as a marked enlargement of the thymus with frequent, but variable, involvement of the spleen, lymph nodes and sometimes of non lymphoid organs. It has been reported that during the early, preleukaemic stages of MMLV infection there is a marked decrease in thymic size compared to uninfected mice (Davis et al 1986). This has been shown to be due to an increased rate of thymocyte apoptosis occurring 4-10 weeks post inoculation (Bonzon and Fan 1999) and it is considered that this is a significant factor in MMLV leukaemogenesis although the mechanism by which it influences tumour development is unclear. One theory postulates that thymic atrophy leads to increased recruitment of potentially preleukaemic prothymocytes from the bone marrow and spleen in order to repopulate the thymus, and that MMLV-induced lymphomas originate from these recruited cells (Bonzon and Fan 1999). The pathways used by MMLV to induce thymocyte apoptosis during this phase of the infection process are also not clear.

The p53 tumour suppressor gene is an important mediator of apoptosis and is induced by a variety of genetic insults (Balint and Vousden 2001; Vousden 2000). It is, therefore, interesting to question whether p53 has a role, or indeed is required, during this preleukaemic phase of the infection process. Although it has been proposed that this wave of apoptosis facilitates the pathogenesis of viral infection, it is not unreasonable to suggest that it may provide a protective mechanism against transformation by MMLV. If so, perhaps this would explain why tumour onset is accelerated in MMLV infected mice that lack functional p53 (Baxter et al 1996). In this chapter I set out to investigate the early apoptotic phase occurring in the thymus of MMLV infected mice as reported by Fan *et al* (Bonzon and Fan 1999) and to test the importance of functional p53 for this aspect of MMLV-induced infection.

4.1 EXPERIMENTAL PROTOCOLS

All experimental approaches were carried out to the specifications described in Chapter 2 except where stated as below.

4.1.1 Animal procedures

Groups of wild type mice (referred to as “cohort A”), infected with MMLV were used in order to establish and verify the existence of a preleukaemic phase of apoptosis (Bonzon and Fan 1999) in the mouse strains available in our laboratory. An F1 hybrid of C57Bl/6 and CBA/Ca was used in this study. Standard groups of 4 or more mice were used at each sampling time. Mice were culled by recognised procedures at weekly intervals between 3 and 10 weeks of age. In every case the sex of the mouse, date of birth and death were recorded. The mouse and thymus were weighed, and thymic weight as a percentage of body weight recorded. Tail biopsy was taken for genotyping and a post mortem examination was conducted to assess whether there were any gross differences between the infected and uninfected groups. A small sample of thymic tissue was placed in neutral buffered formalin (10%) for subsequent histological examination. The rest of the thymus was put into complete RPMI medium on ice from which a single cell suspension was prepared. From this, cells were prepared for flow cytometry and also pelleted and stored at -70˚C for subsequent Western blot analysis.

Inbred C57Bl/6 mice heterozygote for the p53 null allele were bred together to generate experimental groups of sibling mice. These litters, hereafter referred to as “cohort B”, contained a mixture of strain-matched p53 null, p53 heterozygote and wild type mice. The mice were examined between 3 and 8 weeks old. Mice were culled and tissues examined in the same way as for ’’cohort A’’ described above.

4.1.2 Flow cytometry

Assessment of apoptosis and immunophenotyping was carried out as described in Chapter 2.

4.1.3 Histological examination

Thymic tissue was explanted, fixed in 10% neutral buffered formalin, prepared for histological examination (1-2 μm thick sections) and stained with haematoxylin and eosin

by Mr Iain MacMillan and staff. The number of apoptotic cells recognized in 5 high power fields (x400) was counted and recorded for 4 animals in each of the following groups; MMLV infected and uninfected wild type mice (cohort A) at 3, 4, 5 and 6 weeks old.

4.1.4 Statistical analysis

All graphs were plotted using Sigmaplot software; with error bars representing standard deviation. P values were calculated using Student's t test.

4.2 RESULTS

4.2.1 Assessing preleukaemic effects following MMLV infection of C57/CBA wild type mice

It has previously been shown that MMLV induces a preleukaemic phenotype in virally infected mice manifesting as thymocyte apoptosis occurring between the ages of 4-10 weeks (Bonzon and Fan 1999). To confirm that this preleukaemic stage was also evident in our standard laboratory mouse strain (C57/CBA F1; cohort A), the analysis of Fan and colleagues was repeated. To this end, neonatal C57/CBA pups were infected with MMLV supernatant within 24 hours of birth and culled at predetermined time points at which time the thymus was collected and analysed. Non-infected controls, of the same genetic strain and housed in the same facility, were also assessed as a comparison. For each time point at least 4 animals were collected.

4.2.1.1 Thymic size is not changed following MMLV-infection in young C57/CBA wild type mice

MMLV-infected and non-infected controls were sacrificed at weekly intervals between the ages of 3 and 10 weeks. As a first assessment of thymic abnormality a gross pathological examination was carried out at necropsy. At this time thymic weights were recorded and samples collected. In none of the groupings analysed was there evidence of significant gross changes in thymus size or shape between infected animals and non infected controls of the same age. Nor was there evidence of changes in any other major organs. Although this assessment indicates that none of the animals at these ages presented with evidence of tumour it does not rule out the possibility of microscopic precancerous lesions. Thymic weight was measured on a balance to 4 decimal places and related to body weight. Thymic weight was recorded as a percentage of total body weight as a more accurate assessment of thymic size (Table 4.1). The percentage thymic weight (T%) was generally seen to decrease with increasing mouse age in both infected and uninfected groups and within both males and females. Although the values in all cases do not conform exactly to this rule, outliers tend to represent cases with higher standard deviations, as in the 6 week infected mice, or where females are compared to males (uninfected 9 week mice compared to 6, 7 & 8 weeks). The decreasing thymic size is consistent with the fact that thymic involution normally begins to occur at about the time

of sexual maturity which, histologically, corresponds to the fact that the thymus effectively ceases to generate T cells (Montecino-Rodriquez et al 2005) at this time.

Although decreased thymic size has been reported to occur with MMLV infection during the preleukaemic period (Davis et al 1986), the differences in T% between infected and uninfected mice of similar ages were marginal and T% was not usually lower in the infected mice (Table 4.1 and Figure 4.1). Furthermore T% was significantly higher in the infected cohort at 7 weeks old ($P<0.001$) and at 8 weeks old ($P<0.01$). Numbers of mice within each group, particularly within the 7-10 week olds, are relatively low, however, and higher numbers, with a better representation of sex, would be needed to allow a more accurate comparison. It is also interesting to note that the T% for male mice is generally lower than that for female counterparts, which would not be unexpected since males tend to have higher body weights than females of the same age. This is relevant because in some of the infected to uninfected comparisons the cohorts comprise solely of one gender. Thus the comparison of infected female mice at 7 & 8 weeks is made against uninfected males which could account for the higher T%s. Interestingly, the actual thymic weight was also generally higher in the infected females compared to in age matched uninfected males at 7 and 8 weeks (Table 4.2).

4.2.1.2 No histological differences in levels of thymic apoptosis between MMLV infected and uninfected mice at 3-6 weeks old

Histological examination of thymic tissue was undertaken as an attempt to quantify levels of apoptosis induced after MMLV infection. This method has the advantage that it may be possible to localise apoptotic activity to a specific anatomical region within the thymus. During their normal maturation process thymocytes undergo the stages of beta selection, positive selection and negative selection in which cells that fail to be selected undergo apoptosis in the thymic cortex (beta selection and positive selection) and in the medulla (negative selection) (Zhang et al 2005). This implies that in the normal uninfected thymus one may expect to see apoptosis occurring throughout the thymus. The histological results did not show any significant difference in the number of apoptotic cells present in the thymus of MMLV infected compared to uninfected mice (Table 4.3). Nor was it possible to identify any anatomical region within the thymus in which apoptosis was preferentially concentrated.

Table 4.1: Percentage thymic weight in wild type mice (cohort A) ± MMLV

Age	MMLV infected						Uninfected controls					
	All	**nA**	**Females**	**nF**	**Males**	**nM**	**All**	**nA**	**Females**	**nF**	**Males**	**nM**
3	0.80±0.08	8	0.79±0.09	6	0.87±0.03	2	0.80±0.14	13	0.8±0.11	2	0.8±0.14	11
4	0.58±0.09	8	0.56±0.10	7	0.59±0	1	0.53±0.06	7	N/A	0	0.53±0.06	7
5	0.49±0.07	8	0.51±0.07	7	0.39±0	1	0.41±0.11	8	0.45±0.08	3	0.38±0.12	5
6	0.30±0.15	8	0.47±0.02	3	0.2±0.04	5	0.30±0.06	8	N/A	0	0.30±0.06	8
7	0.39±0.04	4	0.39±0.04	4	N/A	0	0.21±0.04	4	N/A	0	0.21±0.04	4
8	0.39±0.09	4	0.39±0.09	4	N/A	0	0.19±0.05	4	N/A	0	0.19±0.05	4
9	0.25±0.11	4	N/A	0	0.25±0.11	4	0.37±0.03	4	0.37±0.03	4	N/A	0
10	0.15±0.07	4	0.24±0	1	0.12±0.04	3	0.19±0.11	4	N/A	0	0.19±0.11	4

Table 4.1 shows the mean thymic weight as a percentage of total body weight with standard deviation. This is shown for MMLV infected and uninfected mice, males and females together as well as separately; at different ages (weeks). N/A = not applicable and refers to mouse groups in which there were mice of only one sex. n= number of mice in group. A= males and females together. F= females. M= males.

Figure 4.1: Percentage thymic weight of MMLV infected preleukaemic mice (cohort A)

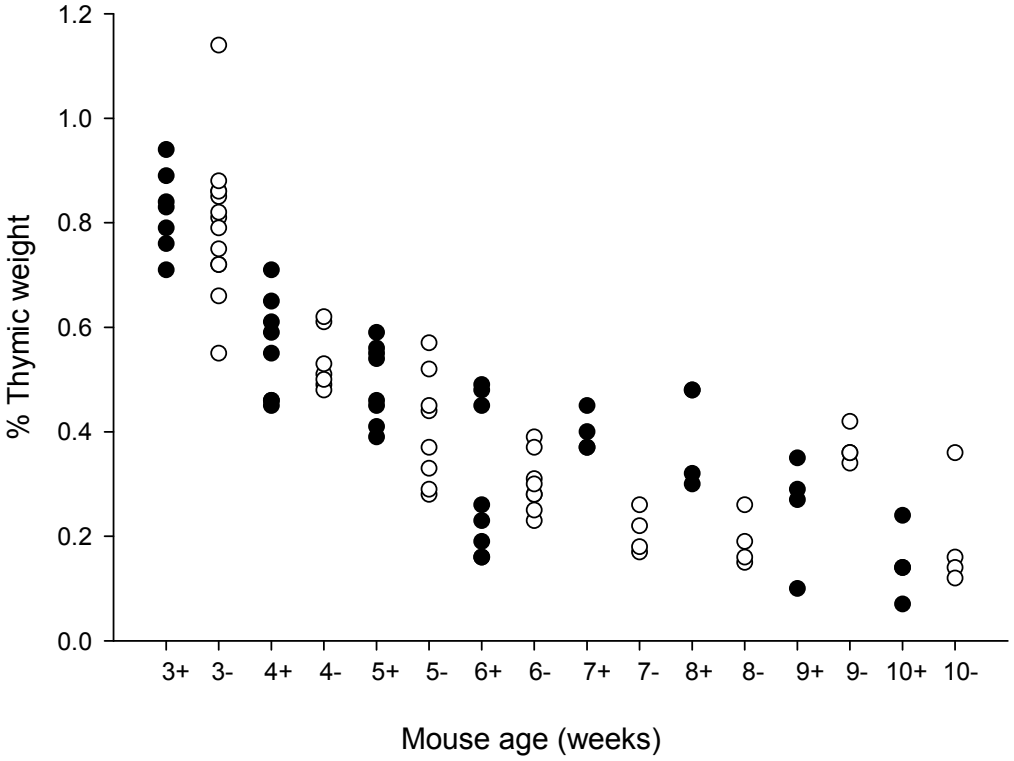


Figure 4.1: Thymic weight as a percentage of body weight in wild type (C57/CBA) mice between the ages of 3 and 10 weeks. Circles represent individual animals: + denotes infection with MMLV. – denotes uninfected mice.

Table 4.2: Actual thymic weight in wild type (cohort A) mice

Age	MMLV infected			Uninfected controls		
	All	**Females**	**Males**	**All**	**Females**	**Males**
3	80+/-10	80+/-10	80+/-10	86+/-20	79+/-3	87+/-20
4	80+/-20	80+/-20	112+/-0	95+/-10	N/A	95+/-10
5	90+/-10	90+/-10	83+/-0	79+/-15	85+/-8	75+/-18
6	60+/-20	80+/-10	50+/-10	69+/-10	N/A	69+/-10
7	80+/-10	80+/-10	N/A	55+/-10	N/A	55+/-10
8	80+/-20	80+/-20	N/A	50+/-10	N/A	50+/-10
9	70+/-30	N/A	70+/-30	72+/-10	72+/-10	N/A
10	40+/-10	40+/-10	40+/-10	56+/-30	N/A	56+/-30

Table 4.2 shows the mean thymic weight (milligrams) with standard deviation. This is shown for MMLV infected and uninfected mice, males and females together as well as separately; at different ages (weeks). N/A = not applicable and refers to mouse groups in which there were mice of only one sex. Cohort numbers as in Table 4.1.

Table 4.3: Visual assessment of apoptotic cells within the thymus of young MMLV infected and non-infected C57/CBA mice (cohort A)

Age (weeks)	MMLV infected	Non-infected
3	15.5±2.0	15.75±2.2
4	18±4.9	22±2.0
5	15.75±0.9	17±2.9
6	18.25±2.9	16±4.5

Table 4.3: Total number of apoptotic cells as assessed by microscopic visualisation counted within 5 randomly chosen high powered fields (x400) in MMLV infected and non-infected mice at 3, 4, 5 and 6 weeks old. Each result represents the mean value of four mice +/- standard error

In vivo, apoptosis is a dynamic process whereby dead cells are phagocytosed and where gross histological visualization only detects those cells considered to be in the late stages of apoptosis. For this reason a method to detect cells in earlier stages of apoptosis was applied. Annexin V is a more specific marker of apoptosis and is able to also recognise cells that are in early stages of apoptosis (Homburg et al 1995).

4.2.1.3 Annexin V staining reveals a transient increase in apoptosis in the thymus of MMLV infected wild type mice at 3-6 weeks old.

In order to establish that the reported phase of preleukaemic apoptosis (Bonzon and Fan 1999) occurs in our laboratory mouse strain (C57/CBA), levels of thymocyte apoptosis were investigated using Annexin V staining to identify potential differences between MMLV infected and uninfected populations. Annexin V is a calcium and phospholipid binding protein that selectively binds to negatively charged phospholipids. It has a high affinity for phosphatidylserine (PS) (Vermes et al 1995). At an early stage of apoptosis there is translocation of PS from the inner side of the plasma membrane to the outer side. PS is, therefore, exposed on the outer cell surface where it is bound by annexin-V and this can be detected by flow cytometry. In this way annexin is a marker for apoptosis (Homburg et al 1995; Koopman et al 1994; Verhoven et al 1995). Annexin V staining revealed significant differences in the amount of apoptosis between MMLV infected and uninfected mice at 3, 4, 5 and 6 weeks of age (Figure 4.2). In each case there was significantly increased apoptosis in the infected mice compared to the uninfected group based on the percentage of cells which gave a positive result with annexin V antibody. By contrast, there was no significant increase in the levels of apoptosis in mice older than 6 weeks old, although it should be noted that these groups consisted of smaller cohort sizes.

4.2.1.4 Surface marker immunophenotyping reveals total and apoptotic thymocyte populations to be similar in MMLV infected and control mice.

CD4 and CD8 are surface T cell markers that are used to phenotype T lymphocytes. CD4 and CD8 are routinely used to identify T-cell populations at different stages of maturity. CD4/CD8 staining was used here in order to try and identify more closely the apoptotic thymocyte population as observed in the MMLV-infected cohorts. An assessment was made of the T-cell subpopulations after gating on the annexin positive cells in order to establish if the apoptotic cells (as seen in Figure 4.2) were restricted to one particular subpopulation of T-cells.

Figure 4.2: Annexin V staining in MMLV infected wild type mice (cohort A)

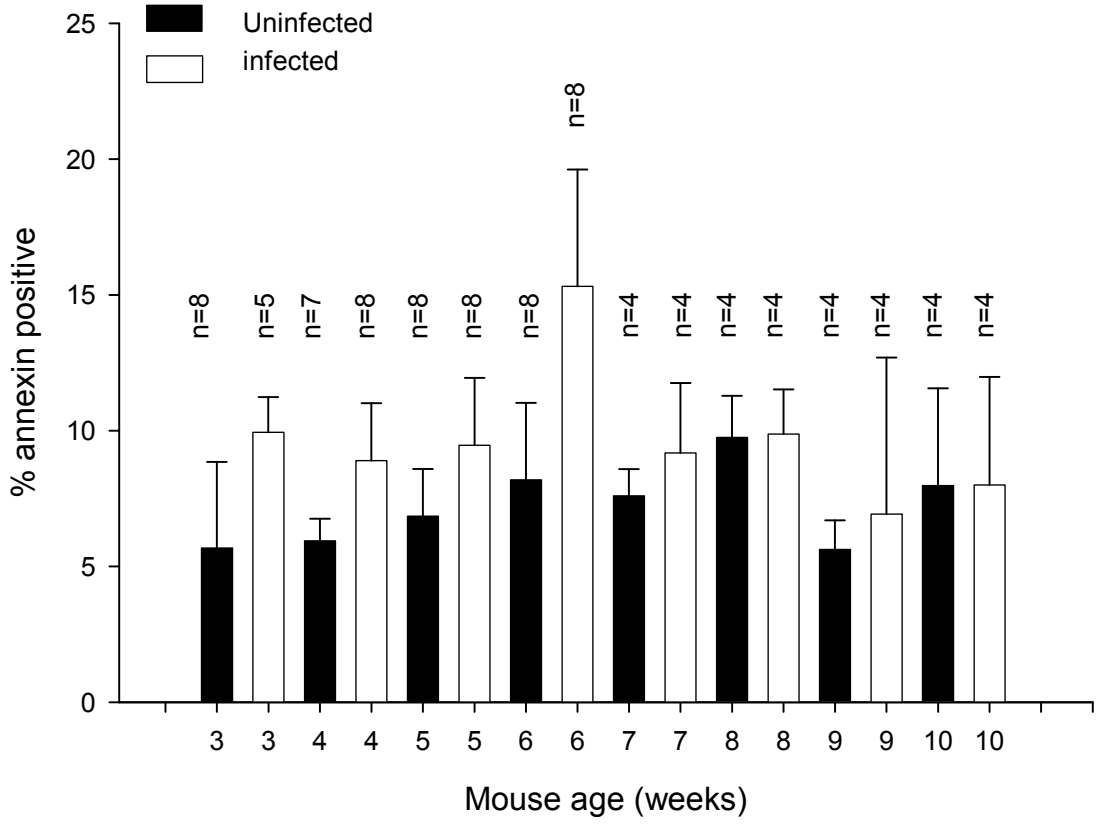


Figure 4.2: Annexin staining in MMLV infected and non-infected control mice between the ages of 3 and 10 weeks. n= number of mice within each group. Results expressed as a percentage of apoptotic cells over total cell number. P values are as follows: 3 weeks: $p<0.02$, 4 weeks: $p<0.004$, 5 weeks: $p<0.03$, 6 weeks $p<0.001$.

When the total cell population was examined it was seen that there was no difference in the T-cell sub-populations between MMLV-infected and uninfected mice showing that viral infection does not skew the T-cell phenotype of the young adult mouse (Table 4.4). Neither was there a discernible difference in the T-cell phenotype when gating on the apoptotic (annexin-V positive) cell population. When comparing the annexin-V positive cells against the total cell population it was apparent that there were noticeably more CD8 single positive cells in the apoptotic fraction. Nonetheless this was not significantly altered when comparing between the infected and non-infected mice. There were also significantly less double negative cells in the apoptotic cell population versus the total thymus, again affecting both infected and uninfected groups in a similar fashion. These results suggest that the dying cells are not double negative and that CD8 single positive are more prone to undergo thymocyte apoptosis than CD4 single positive cells in these age groups. However, as there were no significant differences in T cell phenotypes between those mice infected with MMLV and those that had not been infected, these observations are not directly related to MMLV infection but rather represent a more general feature of thymocyte apoptosis.

4.2.1.5 Western blot analysis reveals no increase in p53 expression following MMLV infection.

Annexin-V staining demonstrated the presence of significantly increased levels of apoptosis in 3-6 week old MMLV infected (cohort A) mice compared to uninfected mice (Figure 4.2). The hypothesis was then put forward that this may occur due to upregulation of p53 and Western blot analysis was carried out to investigate this. Western blot analysis of thymocytes from wild type C57/CBA mice, was performed at 3, 4, 5 and 6 weeks of age in order to establish whether MMLV infection increases expression of endogenous p53 protein. No difference was observed in p53 levels between infected and uninfected mice suggesting that MMLV infection does not cause any change in levels of p53 protein expression detectable by this method (Figure 4.3 shows the results at 3 and 4 weeks).

Although there was no difference in levels of p53 expression, it is possible that the p53 that is expressed is altered and non functional. In order to assess this, levels of the p53 target gene p21 were analysed. Western blot analysis for p21 expression was performed but gave disappointing results. There was no appreciable p21 expression in any of the groups investigated despite the fact that the positive control worked well. The reason

behind this is not clear and time constraints did not allow it to be repeated. It is possible that the result may be related to the fact that p21 is not always expressed by T lymphocytes. Different stages of T cell growth, such as quiescence and proliferation, can greatly influence p21 expression (Coats et al 1999; Firpo et al 1994).

4.2.2 Investigating the requirement for p53 in MMLV-induced preleukaemic apoptosis

It has been shown above and by Fan and colleagues (Bonzon and Fan 1999) that MMLV infection induces a phase of preleukaemic apoptosis in young mice after infection. It is known that p53 is an important mediator of apoptosis (Balint and Vousden 2001; Vousden 2000) therefore it was reasonable to ask if a functional p53 pathway is required for apoptosis induced following MMLV infection. Whilst no induction of p53 was observed at the protein level (Figure 4.3), a more direct approach was to assess MMLV induced apoptosis in thymocytes lacking p53. For this purpose, a cohort of p53 null neonates (cohort B) was infected with MMLV and sacrificed at various ages to determine the levels of *in vivo* apoptosis and compared to age and strain-matched controls.

4.2.2.1 Thymic weight/size is not changed in MMLV infected mice (cohort B)

As with cohort A, the thymus was weighed at necropsy and thymic weight was recorded as a percentage of total body weight. This cohort comprised a mixture of p53 wild type, p53 heterozygote and p53 null littermate matched individuals. Results are tabulated for each genotype separately (Table 4.5 & Figure 4.4). Interestingly all of the p53 null mouse groups contained less females than males. This is consistent with reports that there is a significant rate of developmental abnormalities occurring in mice, predominantly affecting females, in association with p53 deficiency and consequently significantly less females than males survive to weaning (Armstrong et al 1995; Hu et al 2007; Sah et al 1995).

As with cohort A, there was a general decrease in thymic weight (T%) with increasing mouse age in both infected and uninfected groups and within both males and females where available. Within the wild type population there was no significant difference in T% between infected and uninfected mice in any of the age groups. Within the p53 null mice, although the infected mice often had higher T% than their uninfected counterparts, this was not significant except at 6 weeks old ($P<0.05$).

Table 4.4: CD4/CD8 staining of thymocytes in MMLV infected mice

	Total cell population					
	Infected			Uninfected		
	3wks	4wks	5wks	3wks	4wks	5wks
CD4-8-	8.4±2.7	19±7.3	15.7±13.3	8.6±3.8	10.2±5.2	13.1±3.5
CD4+8+	77.7±2.5	67.3±6.1	70.3±10.9	79.6±2.9	78.4±7.8	73.5±4.6
CD4-8+	5.2±0.9	4.5±0.8	5.6±0.5	3.9±0.9	3.9±0.9	5.7±1.8
CD4+8-	8.6±2.6	9.1±1.4	8.3±2.2	7.4±2.3	7.6±2.2	7.7±0.3
	Apoptotic cell population					
	Infected			Uninfected		
	3wks	4wks	5wks	3wks	4wks	5wks
CD4-8-	3.9±1.3	5.1±0.9	4.0±0.6	4.2±1.7	5.1±2.5	4.8±2.3
CD4+8+	75.9±4.3	71.8±8.8	72.3±0.7	73.4±6.1	74.1±7.4	72.9±6.1
CD4-8+	13.9±2.9	15.2±6.1	17.6±2.2	17.2±5.9	13.6±4.8	15.4±5.9
CD4+8-	6±1.6	7.7±4.2	6±1.5	5.3±1.2	7.1±1.9	6.9±0.4

Table 4.4 shows the mean percentage of cells in each T-cell sub-population and within the total and apoptotic (annexin positive) population at different ages (weeks). Standard deviation is also shown. n= 8 (3 weeks), n=4 (4 weeks) & n=4 (5 weeks) for the MMLV infected cohort and n=9 (3 weeks), n=3 (4 weeks) and n=4 (5 weeks) for the uninfected cohort where n= number of mice within each group.

Figure 4.3: Western analysis of thymocytes from MMLV infected C57/CBA mice

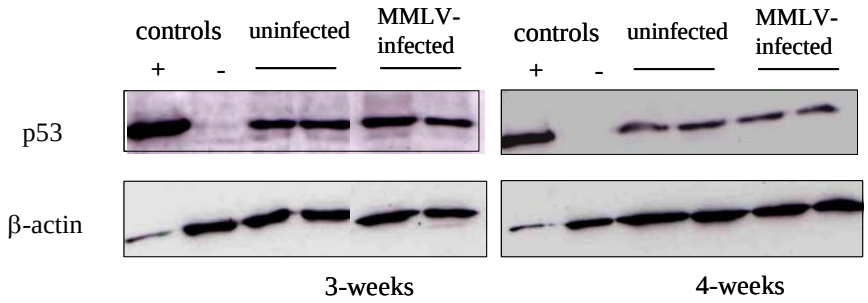


Figure 4.3: Thymocytes from 3 and 4 week old mice were assessed for levels of p53 expression by Western blot analysis. Levels of p53 (top) and ß actin (bottom) as a loading control are shown. Uninfected and MMLV-infected mice at 3 weeks (left) and 4 weeks (right) of age, p53 positive control and negative controls are illustrated in this figure.

Table 4.5: Percentage thymic weight in cohort B mice with MMLV

A: Wild type mice

Age	MMLV infected						Uninfected controls					
	All	nA	Females	nF	Males	nM	All	nA	Females	nF	Males	nM
3	0.95+/-0.23	13	0.97+/-0.19	6	0.92+/-0.28	7	0.88+/-0.21	4	0.72+/-0.04	2	0.74+/-0.33	2
4	0.62+/-0.16	15	0.69+/-0.22	5	0.59+/-0.12	10	0.79+/-0.13	5	0.72+/-0.13	2	0.77+/-0.08	2
5	0.61+/-0.21	10	0.64+/-0.23	7	0.52+/-0.17	3	0.47+/-0.11	8	0.71+/-0	1	0.43+/-0.06	6
6	0.49+/-0.11	10	0.54+/-0.10	6	0.44+/-0.09	4	0.54+/-0.13	10	0.58+/-0.10	8	0.38+/-0.02	2
7	0.44+/-0.05	4	0.45+/-0.08	2	0.48+/-0.05	2	0.30+/-0.06	7	N/A	0	0.30+/-0.06	7
8	0.37+/-0.12	11	0.48+/-0.07	5	0.28+/-0.05	6	0.32+/-0.15	5	0.40+/-0.14	3	0.19+/-0	2

B: Heterozygote mice

Age	MMLV infected						Uninfected controls					
	All	nA	Females	nF	Males	nM	All	nA	Females	nF	Males	nM
3	0.89+/-0.15	22	0.97+/-0.19	8	0.86+/-0.12	14	0.83+/-0.13	16	0.83+/-0.13	10	0.81+/-0.14	6
4	0.62+/-0.12	24	0.67+/-0.12	12	0.57+/-0.13	12	0.59+/-0.08	8	0.58+/-0	1	0.60+/-0.09	7
5	0.49+/-0.10	17	0.53+/-0.11	4	0.47+/-0.09	13	0.52+/-0.08	8	0.6.+/-0.07	4	0.44+/-0.05	4
6	0.46+/-0.08	10	0.53+/-0.09	3	0.43+/-0.05	7	0.38+/-0.09	11	0.49+/-0	1	0.37+/-0.08	10
7	0.46+/-0.11	12	0.54+/-0.06	7	0.35+/-0.03	5	0.35+/-0.13	6	0.51+/-0.03	2	0.27+/-0.05	4
8	0.34+/-0.11	7	0.47+/-0.12	2	0.28+/-0.07	5	0.42+/-0.09	7	0.46+/-0.05	5	0.31+/-0.04	2

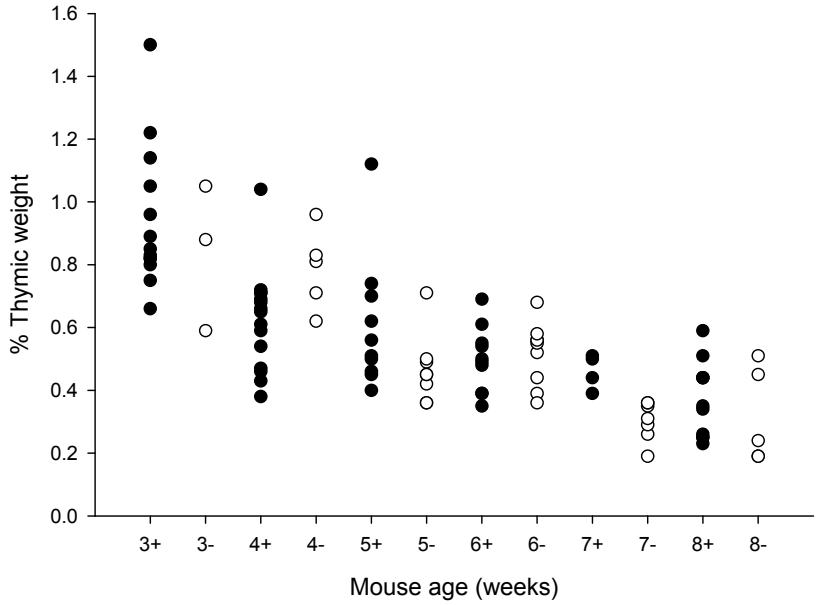
C: p53 null mice

Age	MMLV infected						Uninfected controls					
	All	nA	Females	nF	Males	nM	All	nA	Female	nF	Males	nM
3	0.87+/- 0.04	5	0.88+/-0.01	2	0.87+/-0.05	3	0.74+/-0.07	4	N/A	0	0.74+/-0.07	4
4	0.56+/-0.11	4	0.69+/-0	1	0.52+/-0.09	3	0.70+/-0.12	5	N/A	0	0.70+/-0.12	5
5	0.68+/-0.19	4	0.67+/-0	1	0.68+/-0.23	3	0.48+/-0.06	4	N/A	0	0.48+/-0.06	4
6	0.53+/-0.15	8	N/A	0	0.53+/-0.15	8	0.37+/-0.06	6	N/A	0	0.37+/-0.06	6
7	0.46+/-0.11	4	0.54+/-0	1	0.43+/-0.12	3	0.29+/-0.03	4	N/A	0	0.29+/-0.03	4
8	0.38+/-0.24	3	0.25+/-0	1	0.44+/-0.29	2	0.30+/-0.09	4	N/A	0	0.30+/-0.09	4

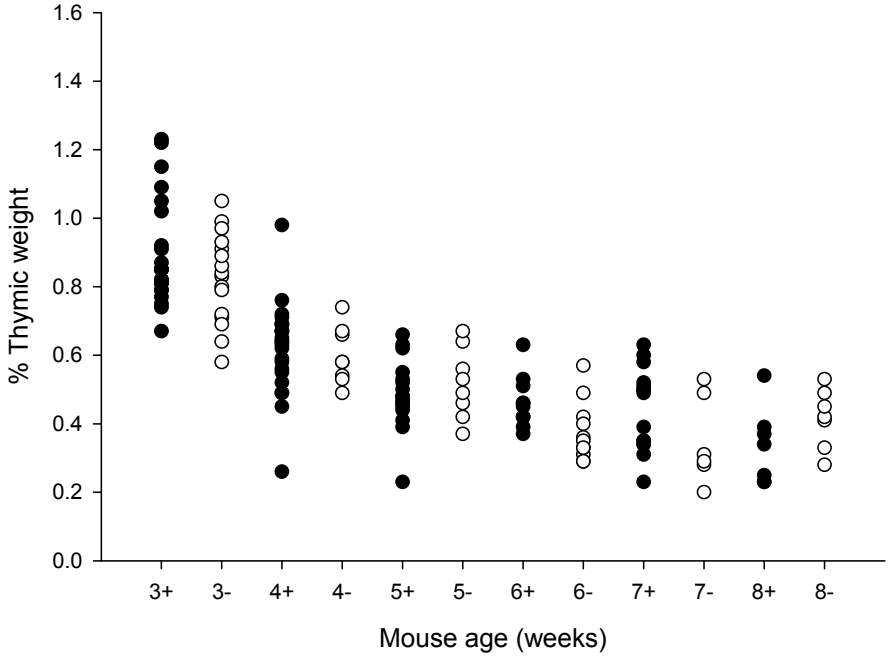
Table 4.5 shows the mean thymic weight as a percentage of total body weight with standard deviation. This is shown for wild type, p53 heterozygote and p53 null mice within cohort B. Results are also shown for MMLV infected and uninfected mice, males and females together as well as separately at different ages (weeks). n= number of mice in group. A= males and females together. F=females. M=males.

Figure 4.4: Percentage thymic weights of MMLV infected preleukaemic mice (cohort B)

A: Wild type mice



B:Heterozygote mice



C: p53 null mice

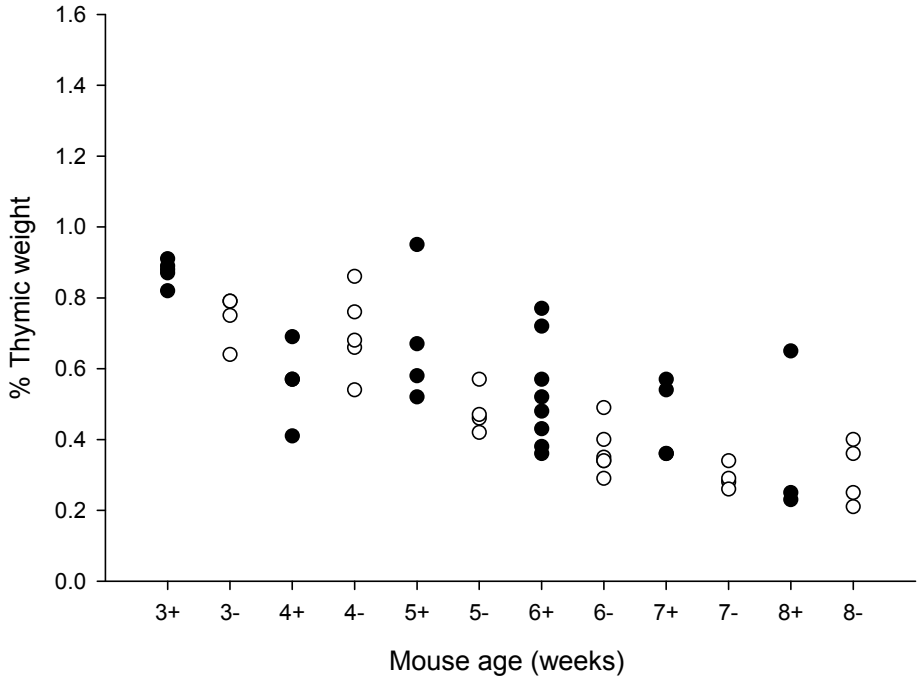


Figure 4.4: Thymic weight as a percentage of body weight in mice from cohort B, between the ages of 3 and 8 weeks. Results are shown for wild type, p53 heterozygote and p53 null mice. Circles represent individual animals: + denotes infection with MMLV. – denotes uninfected mice.

4.2.2.2 Annexin V staining reveals a transient increase in apoptosis in the thymus of MMLV infected mice

Having established the existence of a preleukaemic phase of apoptosis within the C57/CBA (cohort A) mice, thymocyte apoptosis was then investigated on a p53 null background. p53 null mice, p53 wild type and p53 heterozygote inbred (C57Bl/6) siblings (cohort B) were derived to avoid effects of mouse strain differences. Annexin V staining and flow cytometric analysis revealed a trend to increased apoptosis in infected wild type mice compared to uninfected mice in all age groups (Figure 4.5) similar to that observed on the C57/CBA background (cohort A). The results suggest, however, that significant induction of cell death may only be occurring at 6-7 weeks of age in this cohort. This is in contrast to cohort A, where it occurred at 3-6 weeks old. The age difference observed between cohort A and cohort B may reflect mouse strain differences. However it is also possible that the large error bars present in Figure 4.5 (cohort B) may be obscuring significant differences in apoptosis that are also present within the other age groups. It is interesting that the levels of apoptosis, both with and without MMLV infection, appear to be higher in cohort A mice (Figure 4.2) than in cohort B wild type mice (Figure 4.5).

After establishing that C57Bl/6 inbred mice (cohort B) demonstrate significantly elevated levels of apoptosis at 6 and 7 weeks post-MMLV infection, it was decided to concentrate on this age group when assessing the influence of p53 status. Therefore MMLV infected and uninfected mice were more closely examined within the 6 and 7 week old groups. At both 6 (Figure 4.6) and 7 weeks (Figure 4.7) of age, p53 wild type and p53 heterozygote mice (despite large error bars in the 6 week old heterozygotes) showed significantly greater levels of apoptosis when infected with MMLV. Interestingly those animals without functional p53 showed no significant difference in levels of apoptosis after MMLV infection.

Only 4 mice were present in each of the 7 week old p53 null groups and the range of values in the infected group has lead to a very large error bar so that, although the graph suggests that there may be a difference between infected and non-infected null mice, this is not statistically significant. Thus, the results depicted in Figure 4.6 and Figure 4.7 suggests that functional p53 may be needed for MMLV induced apoptosis.

Figure 4.5: Thymocyte apoptosis in MMLV-infected wild type C57Bl/6 mice

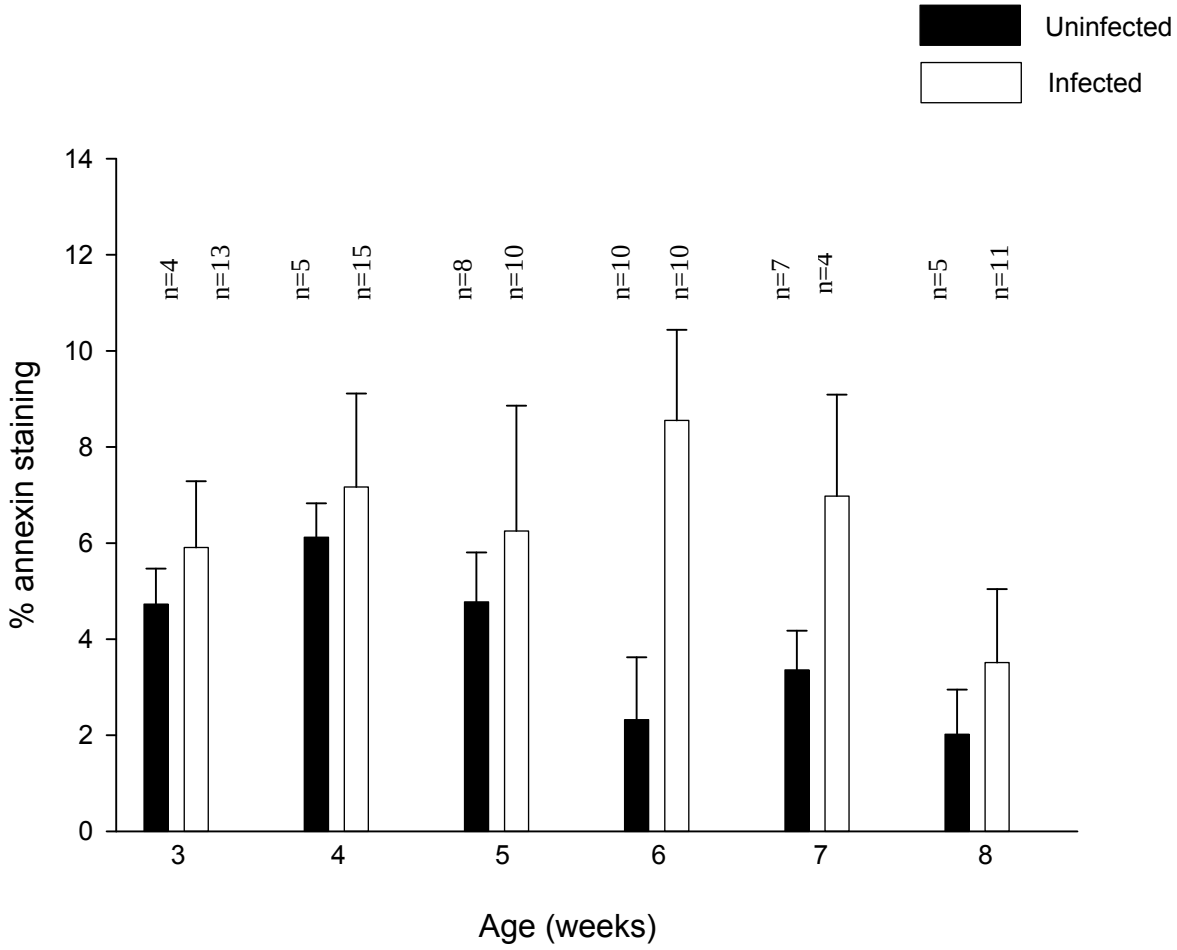


Figure 4.5: This figure shows the percentage of thymocytes staining positive for Annexin-V in MMLV infected and non-infected C57Bl/6 inbred mice between 3 and 8 weeks old. Animals in this cohort were wild type littermate controls to the p53 null mice described below. Age in weeks is given along the X-axis. P values are as follows: 3 wks: p=0.13, 4 wks: p=0.26, 5 wks: p=0.15, 6 wks: p<0.001, 7 wks: p=0.002, 8 wks: p=0.067. n is the number of mice within each group.

Figure 4.6: Thymocyte apoptosis in 6 week MMLV-infected mice with and without endogenous p53 alleles.

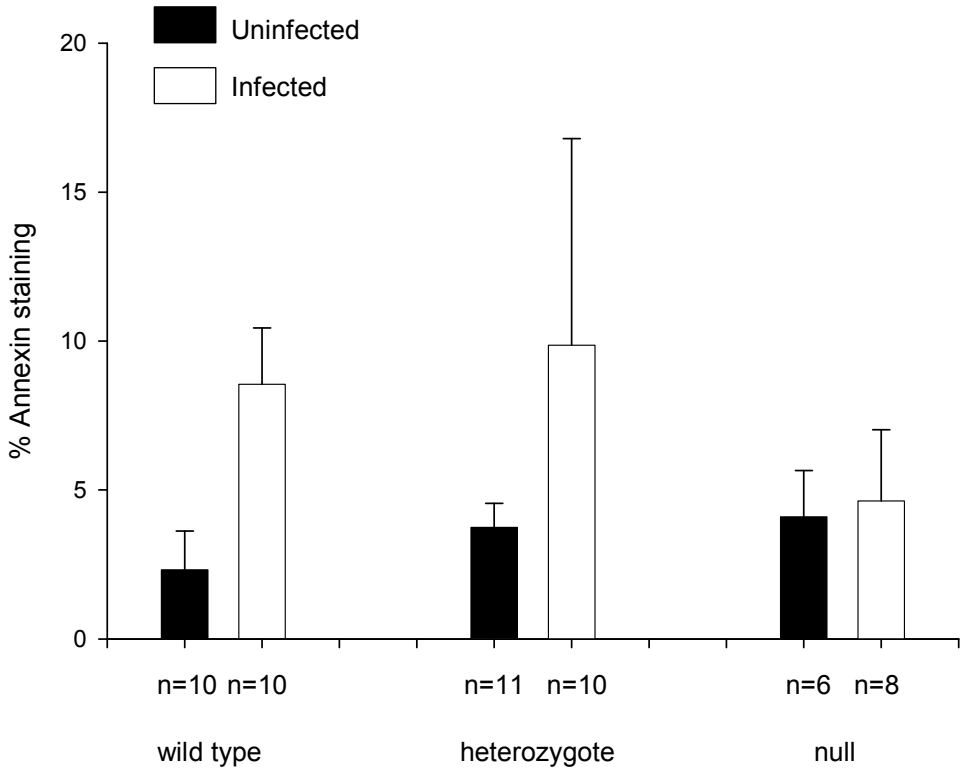


Figure 4.6: This figure depicts the percentage of apoptotic cells as scored positive for annexin staining in thymocytes from MMLV infected and uninfected mice at 6 weeks old, where n refers to numbers of mice within each group. Results are shown for wild type, p53 heterozygote (one p53 allele) and p53 null mice. P values of infected versus non-infected are as follows: wild type: $p<0.001$, heterozygote:$p=<0.01$, null: $p=0.64$

Figure 4.7: Thymocyte apoptosis in 7-week old MMLV-infected with and without endogenous p53 alleles.

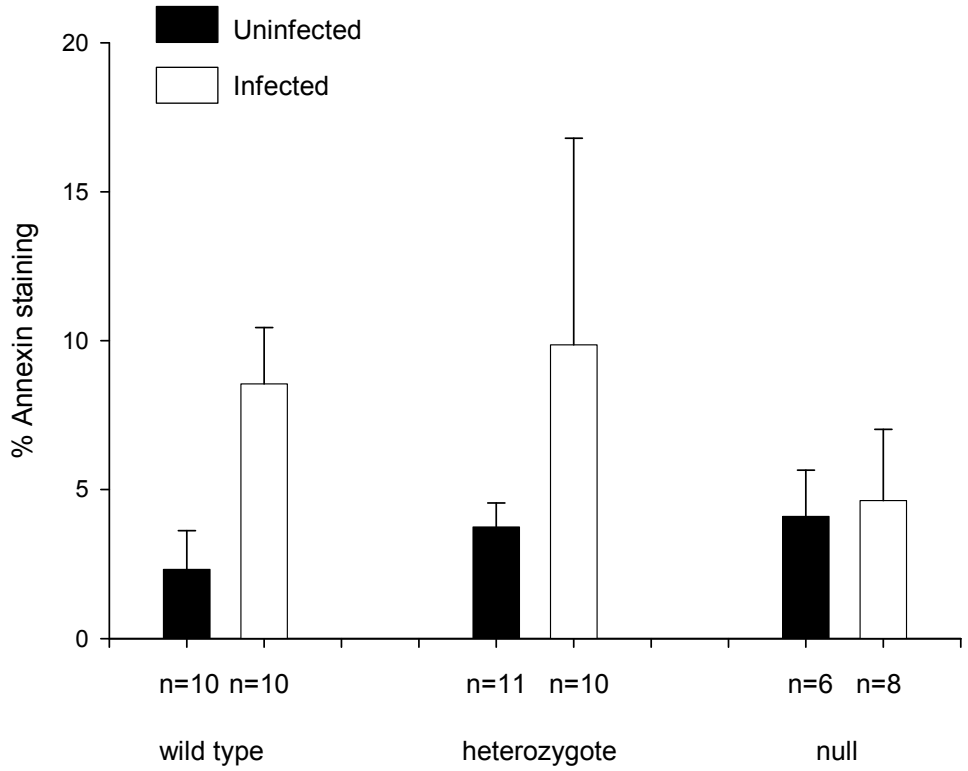


Figure 4.7: This figure depicts the percentage of apoptotic cells as scored positive for annexin staining in thymocytes from MMLV infected and uninfected mice at 7 weeks old, where n refers to numbers of mice within each group. Results are shown for wild type, p53 heterozygote (one p53 allele) and p53 null mice. P values of infected versus non-infected are as follows: wild type: p=0.002, heterozygote:p=0.002, null: p=0.19

In summary, the results comparing MMLV-infected and non-infected mice suggest that loss of p53 may interfere with the phase of MMLV-induced apoptosis. Following on from this, one would expect there to be significantly less apoptosis occurring in infected nulls compared to infected wild types. In order to further investigate this, levels of apoptosis were compared between p53 wild type, p53 heterozygote and p53 null mice within the infected cohort B mice (Figure 4.8). Unexpectedly the results only demonstrated a significant difference in levels of apoptosis between null and wild type infected mice at 6 weeks old (P=0.001). Whilst this was consistent with the results in Figure 4.6, the difference in apoptosis between null and wild type mice at 7 weeks old was not significant and therefore did not appear to support the results in Figure 4.7. The number of null mice within most of the groups was fairly low and the large range of values within any one group has generated large error bars which may offer some explanation as to why it is difficult to extract significant conclusions from these results.

4.2.2.3 MMLV-infection does not detectably increase p53 expression in C57Bl/6 mice (cohort B)

Western blot analysis of thymocytes from wild type mice on a C57Bl/6 background (cohort B) was performed at 3, 4, 5, 6 and 7 weeks old to see if MMLV infection had any influence on p53 expression. Figure 4.9 shows the results at 6 weeks old. As with the mice from cohort A (Figure 4.3) there was no difference observed in p53 levels between infected and uninfected mice confirming that MMLV infection does not cause any change in levels of p53 expression, at least detectable by this method.

Figure 4.8: Effects of p53 gene dosage on thymocyte apoptosis following MMLV infection.

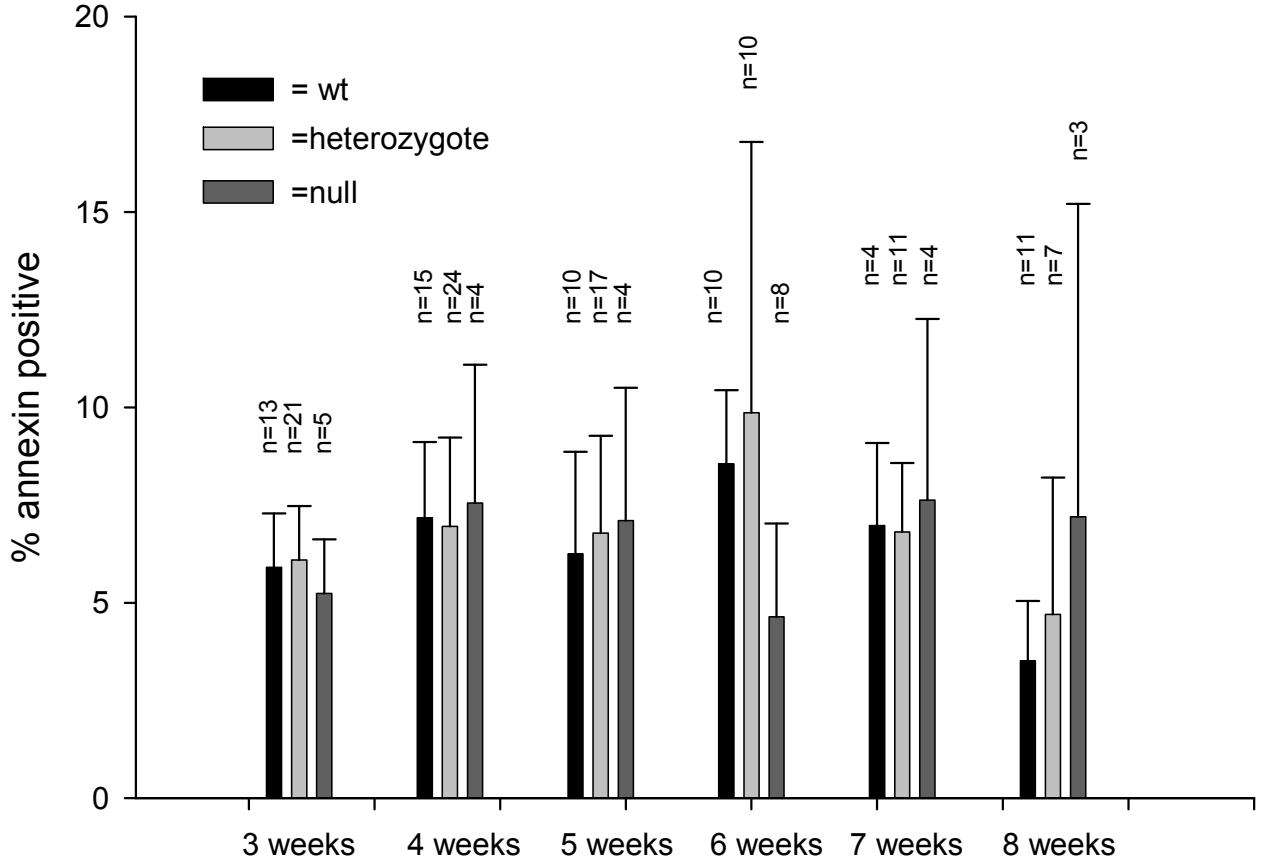


Figure 4.8: % annexin staining in thymocytes from cohort B mice at 3, 4, 5, 6, 7, and 8 weeks old. Results are shown for wild type, p53 heterozygote and p53 null mice that were all neonatally infected with MMLV. n=number of mice within each group. When values for wild type and null mice were compared statistically: At 3 weeks, p=0.37, 4 weeks p=0.77, 5 weeks p=0.62, 6 weeks p= 0.001, 7 weeks p=0.81, 8 weeks p=0.14.

Figure 4.9: Western blot analysis of thymocytes from MMLV-infected C57Bl/6 wild type mice (cohort B)

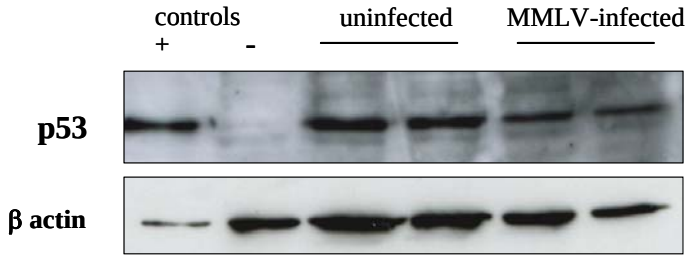


Figure 4.9: Thymocytes from 6 week old wild type mice were assessed for levels of p53 expression by Western blot analysis. Levels of p53 (top) are shown. ß actin is included as a loading control. p53 positive and negative controls are shown and two examples of 6 week old uninfected and 6 week old MMLV infected mice.

4.3 DISCUSSION

Historically, the different theories put forward to explain the pathogenesis of MMLV infection could lead one to anticipate certain outcomes with regards to changes in thymic size. One early proposal was that MMLV-induced leukaemia relies on the development of a preceding phase of chronic cellular immune stimulation during which interleukin 2 (IL-2) levels are elevated, leading indirectly to proliferation of thymocytes and thus an increase in thymic size (Lee and Ihle 1981). A later theory proposed that the envelope glycoprotein gp70, which is encoded by the Moloney murine leukaemia mink cell focus-forming virus (MMLV MCF), activates receptors of the Epo-R IL-2 family causing prolonged cellular proliferation, potentially leading to leukaemogenesis (Li and Baltimore 1991). This theory would also suggest an increase in thymic size during the preleukaemic stage. However it has been reported that 4-10 weeks post inoculation with MMLV there exists a preleukaemic period which manifests as a decrease in thymic size (Davis et al 1986) and is characterised by significantly increased thymocyte apoptosis (Bonzon and Fan 1999). The premise of this chapter was to further investigate this reported preleukaemic phase and to explore, in particular, the involvement of the p53 tumour suppressor gene in this.

In this study there was a general trend for thymic weight as a percentage of body weight (T%) to decrease as the mice got older which is consistent with the fact that the thymus is normally largest in young animals and then regresses as animals age ((Montecino-Rodriquez et al 2005)). The explanation as to why the T%s were increased with MMLV infection in some of the mouse groups when Davis *et al* (Davis et al 1986) had reported the opposite is not clear. Davis used NIH Swiss mice and, although assessment of “thymic size” is not described, refers to work by Storch (Storch et al 1985) who document decreased thymic weight in infected mice from about 4 weeks old using NFS/N mice. It is possible that the different mouse strain used in this PhD may be a significant factor. It is also likely that differences in inoculated strains/titres of MMLV virus may also contribute to this discrepancy. T% is generally lower in males than in females and this may also influence the results in those age groups where single and opposite sex groups were compared. It should be noted that the levels of apoptosis recorded in this work may not be as dramatic as those observed by others and therefore a significant difference in thymus size may not be expected. Individual weights varied over a wide range within each age group, and it is possible that larger numbers of mice within each group may have clarified

these results and perhaps yielded significant differences between infected and uninfected animals at other ages.

It was hoped that histological examination of the thymus would provide one approach by which to examine differences in numbers of apoptotic cells between infected and uninfected mice. Whilst no obvious differences were observed, it should be noted that counting numbers of apoptotic cells by this method is a fairly inaccurate and crude method and was only ever expected to demonstrate gross differences between different treatments. This method is hampered further by the fact that thymic sections taken from individual mice are likely to include different proportions of cortex and medulla. This may be a significant factor if apoptosis occurs more in one region compared to another. It was however difficult to identify any particular anatomical area of increased apoptosis from the small tissue sections examined. Another more accurate method of identifying apoptotic cells within tissue sections is TUNEL, Terminal transferase-mediated UTP nick end-labeling (Gavrieli et al 1992; Heatwole 1999; Otsuki et al 2003; Sgonc and Gruber 1998). This involves enzymatic in situ labelling of DNA strand breaks which occur early during apoptosis and is a technique used to give information relating cellular apoptosis to histological localisation or cell differentiation. TUNEL has been effective at discerning levels of apoptosis *in vivo* particularly in tumours (Hall et al 1998). Nonetheless TUNEL does have its own limitations. Because *in vivo* apoptosis is a very dynamic process with macrophage engulfment rapidly clearing dying cells in the thymus (Fadok et al 1992; Savill et al 1993), it can be difficult when very low levels of apoptosis are occurring to identify subtle changes in the levels between different groups (K. Blyth, personal communication).

Annexin staining, which enables identification of early and late apoptotic cells (Homburg et al 1995; Koopman et al 1994; Verhoven et al 1995) allowed quantitative assessment of preleukaemic thymocyte apoptosis in MMLV infected C57/CBA and C57Bl/6 inbred mice. Within the control C57/CBA (cohort A) population, apoptosis was significantly increased at 3, 4, 5 and 6 weeks old in MMLV infected mice compared to non-infected mice. Whereas in the inbred C57Bl/6 (cohort B) group, significantly increased apoptosis was seen at 6 and 7 weeks old. This is in contrast to that reported in the literature where apoptosis occurs between 4 and 10 weeks post inoculation (Bonzon and Fan 1999). Since NIH/Swiss mice were used in the work of Bonzon and Fan it is presumed that this discrepancy arises largely due to mouse strain differences that affect the timing of peak

virus replication in thymus. The apoptotic values reported by Bonzon *et al* were generally higher than those observed in this thesis despite similar latent periods for tumour development, again emphasizing probable mouse strain differences.

Apoptosis is programmed cell death which is essential in the development of multicellular organisms and in the maintenance of tissue homeostasis. During normal thymic development T cells that are not "selected" for further development are eliminated by apoptosis (Benoist and Mathis 1997; Chao and Korsmeyer 1998; Nikolic-Zugic 1991) and several apoptosis related molecules play essential roles at different stages in T lymphocyte development (Zhang et al 2005). CD4/CD8 staining of thymocytes was performed with a view to identifying whether or not a specific cell type is targeted during the preleukaemic phase of apoptosis or whether apoptosis was affecting all thymocyte populations equally. The results suggest that, at 3-5 weeks old, the predominant population of apoptotic thymocytes were CD8 single positive T lymphocytes and that MMLV infection has no influence on this apoptotic cell population. Therefore, it is possible that MMLV may increase apoptosis in a population of T cells that is already primed for apoptosis, presumably as a part of a normal, physiological process ie T cell selection.

In order for a tumour to grow, cell growth must outpace cell loss. How then is the phase of preleukaemic apoptosis that is seen following MMLV infection overcome in order for tumour development to occur? When MMLV-induced tumours were investigated it was observed that there was down regulation of pro-apoptotic *Fas* and up regulation of anti-apoptotic *Bcl-2* in infected tumour cells when compared to uninfected cells (Bonzon and Fan 2000). This suggests that the preleukaemic episode involves both the intrinsic and extrinsic pathways of apoptosis and that it is necessary to attenuate both in order for tumour outgrowth to occur. However it has also been shown that the development of MMLV-induced tumours is not accelerated in mice in which *Fas* is mutated (Cameron et al 2000).

The molecular mechanism by which MMLV manifests the preleukaemic phase of increased apoptosis has not been described and this study set out to explore the possibility that p53 may have a role in this and, if so, whether p53 is induced by MMLV (either directly or as a DNA damage response)? Western analysis, however, did not show any difference in p53 expression between MMLV infected and uninfected cells to support this theory. Assuming, therefore, that there are no subtle changes in p53 levels that are beyond

the level of detection by Western analysis, the results suggest that the p53 protein is not upregulated by MMLV infection.

Loss of p53 is known to cooperate with MMLV in leukaemogenesis (Baxter et al 2001) albeit not as potently as other oncogene partners (Stewart et al 1993; van Lohuizen et al 1991). Does p53 loss do this by sparing thymocyte apoptosis and thus accelerating tumour development? Bonzon and Fan's observations suggest that this would be unlikely since it is considered that the preleukaemic phase of apoptosis is a significant factor in facilitating efficient leukaemogenesis (Bonzon and Fan 1999). In order to assess whether the observed preleukaemic phase of thymocyte apoptosis is able to occur in the absence of p53, mice null for endogenous p53 were infected with MMLV. The extent of thymocyte apoptosis in infected and uninfected young adult mice was examined.

When all the age groups were looked at within the infected, cohort B mice, there was no apparent consistent difference in levels of apoptosis between mice with two (wild type), one (heterozygous) or no (null) endogenous p53 alleles (Figure 4.8) and it would, therefore, appear that MMLV may enforce its phase of preleukaemic apoptosis irrespective of the p53 status of the mouse. However when the results were more closely looked at, it was observed that at 6 weeks old there was a significant increase in apoptosis in wild type mice compared to null mice implying that a functional, intact p53 pathway may, in fact, contribute to, the MMLV induced phase of apoptosis. The significant increase in levels of apoptosis in infected compared to uninfected mice observed in p53 wild type and heterozygote, but not in p53 null, groups at 6 and 7 weeks old (Figure 4.6 and Figure 4.7) also supports this observation. Higher numbers of mice, particularly p53 null individuals, within each age group would need to be investigated in order to confirm this.

In summary; p53 loss and MMLV are known to exhibit weak synergy in T cell leukaemogenesis although the mechanism behind this is unknown. In this study possible interactions between p53 and MMLV at an early stage of the disease were investigated. The results herein will need further clarification, however it cannot be ruled out that p53 may play a subtle role in the preleukaemic phase of apoptosis that occurs in MMLV infected thymocytes and that this phase is attenuated in p53 null mice. It is difficult to make definitive conclusions from the current data set due to the equivocal results between the different analyses. However these results pave the way for further analysis with a larger cohort set. The results do not offer an explanation for the cooperation between p53

loss and MMLV infection in lymphomagenesis although it is unlikely, at least solely, to be occurring at this early stage of the disease. It is perhaps more likely that MMLV targets endogenous genes for retroviral activation which form the basis of collaboration on the background of p53 loss.

CHAPTER 5

THE ROLE OF P53 IN DISSEMINATED T-CELL LYMPHOMA

Whilst many tumours are known to lose the p53 tumour suppressor gene *in vivo*, this is not an absolute requirement for tumour growth (Calin et al 1999; Imamura et al 1994; Preudhomme et al 1993; Sugimoto et al 1993; Wada et al 1993). By contrast, tumour cell line establishment *in vitro* is almost always accompanied by loss or inactivation of this gene or its associated pathways (Hietanen et al 1995; Howard et al 1993; Van Leeuwen et al 1996) and this is consistent with reports that p53 null cell lines grow readily in culture (Rogan et al 1995; Tsukada et al 1993).

Studies of retroviruses such as Friend MLV and Abelson MLV, have shown that functional inactivation of p53, which includes both complete loss of p53 expression and also expression of mutant non-functional p53 proteins, is advantageous for progression of neoplastic disease (Ben-David et al 1990; Ben David et al 1988; Howard et al 1993; Mowat et al 1985; Munroe et al 1990; Prasher et al 2001; Shore et al 2002; Thome et al 1997). It has also been shown that with Friend MLV, Abelson MLV and Moloney murine leukaemia virus (MMLV) infection, p53 null animals succumb to malignant disease significantly faster than do either wild type or heterozygote animals (Baxter et al 1996; Unnikrishnan et al 1999; Wong et al 1999) implying that loss of p53 promotes the development/progression of these diseases.

However, in MMLV induced T cell lymphomas of $p53^{+/-}$ mice, tumour cells do not always exhibit loss of heterozygosity of p53 *in vivo*, although the wild-type allele is invariably lost when tumours are grown *in vitro* (Baxter et al 1996). It has been hypothesized that those cellular targets that are preferentially activated by MMLV circumvent the need to lose the p53 allele *in vivo* (Baxter et al 2001). This hypothesis has been explored further in transgenic mice expressing two MMLV target genes, *Myc* and *Runx2*, in the T-cell compartment (Blyth et al 2006) for which loss of p53 is not selected *in vivo*.

Targeted ectopic expression of the *Runx2* and *Myc* oncogenes to the T cell lineage with a CD2 promoter results in lymphoma development (Stewart et al 1993; Vaillant et al 1999). The combined expression of these genes significantly accelerates lymphomagenesis

whereby bi-transgenic animals develop tumours at a much reduced latency and increased frequency compared to parental groups (Blyth et al 2001; Vaillant et al 1999). Although these events are potent collaborating genetic lesions, they are insufficient by themselves to induce tumours, consistent with the multistep cancer hypothesis (Hanahan and Weinberg 2000). An obvious collaborating candidate lesion would be loss of p53 which has been shown to cooperate independently with *Myc* and *Runx2* to induce T cell lymphomas (Blyth et al 1995; Blyth et al 2001). Furthermore Runx2/Myc transgenic offspring with a germline p53 heterozygote allele develop lymphomas with reduced latency compared to those with a wild type p53 configuration (Blyth et al 2006). However, primary tumours from these animals retain a functional p53 allele suggesting that this gene combination is able to bypass the requirement for genetic loss of p53, at least in the primary tumours. In contrast, *in vitro* culture of these tumours leads to rapid loss of the wild-type p53 allele (Blyth et al 2006).

This invites speculation as to whether loss of p53 may be selected during tumour progression and, in particular, in tumours undergoing metastasis, which conceivably generates stresses similar to those operating on cells cultured *in vitro.* For example, tumour growth in new tissue niches might force cells to adapt to limiting concentrations of specific growth and survival factors, or to altered oxygen tension. In this regard, metastasis to non-lymphoid tissues might generate a different set of selective pressures compared to peripheral lymphoid organs. In this chapter, the role of p53 loss in tumour metastasis was examined during disseminated T-lymphomagenesis. Runx2/Myc transgenic animals have been used as a genetic surrogate for MMLV infection. Figure 5.1 shows the rationale for this study.

Figure 5.1: The role of p53 in tumour metastasis

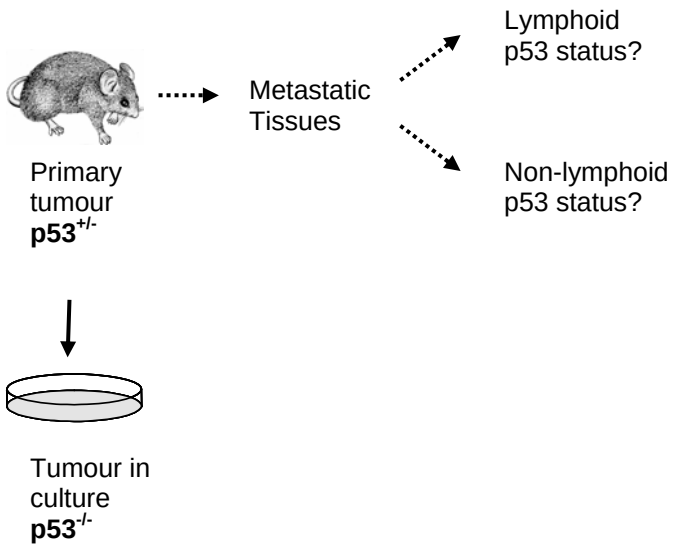


Figure 5.1: Primary tumours developing in Runx2/Myc p53 heterozygote mice retain p53 heterozygosity but the wild type p53 allele is lost with *in vitro* culture. This study aims to explore p53 expression in metastatic/disseminated tumour foci, both in lymphoid and non-lymphoid tissues.

5.1 EXPERIMENTAL PROTOCOLS

All experimental approaches were carried out to the specification as described in Chapter 2 except where stated below.

5.1.1 Animal Procedures

A highly tumour prone transgenic model was generated as a surrogate for the MMLV oncogenic programme, in which animals carried the CD2-Runx2 (hereafter Runx2) and CD2-MYC (hereafter Myc) transgenes and were heterozygous for an inactivated p53 allele. This was achieved by firstly breeding $Myc^{tg/tg}$ animals with p53 null ($p53^{-/-}$) mice to achieve $Myc^{tg/-}p53^{-/-}$ offspring (where tg represents presence of the transgene). Males with this genotype were subsequently bred to $Runx2^{tg/tg}$ females in order to generate offspring of the required genetic make up, $Runx2^{tg/-}Myc^{tg/-}p53^{+/-}$, which are hereafter designated RMP. Seven RMP mice were investigated in this study. Direct investigation of a $Runx2^{tg/-}$ $Myc^{tg/-}$ $p53^{-/-}$ cohort is not possible since the parental strains required do not survive to breeding age.

As soon as mice developed signs of illness or obvious tumours they were killed by cervical dislocation. Date of birth and death were recorded and a postmortem examination was conducted. Distribution of tumours throughout the body and any other abnormalities were recorded. A small sample of tumour tissue was snap frozen in liquid nitrogen for molecular analysis. Single cell suspensions were prepared, cells pelleted and frozen at -70° C for subsequent Western and Southern analysis.

5.2 RESULTS

This chapter investigates p53 loss during the disseminated spread of T cell lymphoma. For this purpose one defective p53 allele was provided in the germ line whilst the fate of the remaining allele was followed during tumour cell evolution.

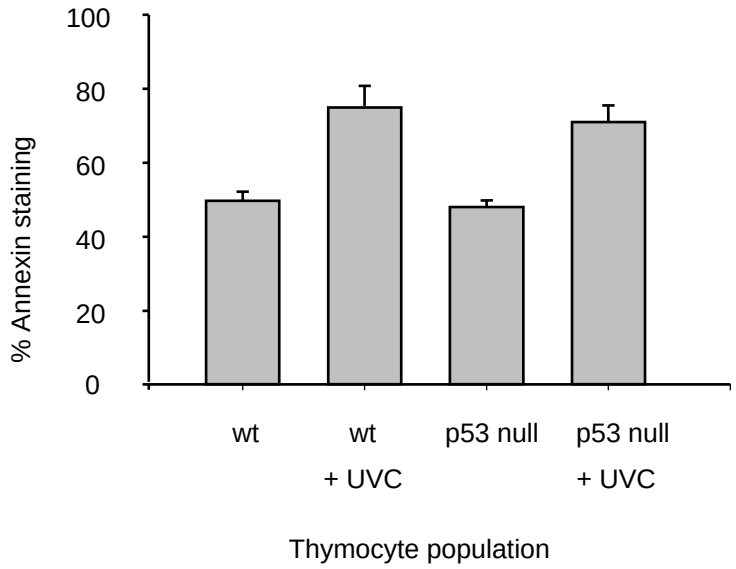
5.2.1 Testing the functionality of p53

In $p53^{+/-}$ mouse tissues, Southern blot analysis is convenient to reveal loss of the wild-type allele, but cannot be used to demonstrate that the remaining wild-type allele is functional. It was therefore important to corroborate the function of the residual p53 allele by a further test. One surrogate marker is the resistance of cells lacking p53 to radiation-induced apoptosis (Lowe et al 1993b). Cells which have functional p53 will respond to gamma irradiation by apoptosis and this has previously been used to assess the presence of wild type p53 in T-lymphoma cells using flow cytometry detection methods (Baxter et al 2001). Cells that lack p53 or have mutant p53 are refractory to this stimulus and do not exhibit any increase in apoptosis. Unfortunately I had no access to a gamma irradiation source at the time that this study was carried out. For this reason, I decided to test the sensitivity of tumour cells to UVC radiation as a potential alternative/additional test of p53 functionality.

The response to UVC irradiation is not always predictable and depends on cell type, degree of cell proliferation and differentiation and on UVC dosage (Sabapathy et al 1997). UVC irradiation damages DNA through the induction of pyrimidine dimers and this may lead to p53-dependent DNA repair, to p53-dependent apoptosis (Smith et al 2000; Van Sloun et al 1999) or to activation of p53–independent G1 and G2 arrest responses depending on the circumstances (Attardi et al 2004). Since UVC irradiation was readily available for use in the laboratory, a trial study was set up in order to assess this method as a possible test of p53 functionality in T lymphocytes (Figure 5.2A). The results demonstrated, however, that thymocytes subjected to non-lethal doses of UVC irradiation were sensitive to apoptosis regardless of their p53 status and that p53 was not required for UVC induced apoptosis in murine thymocytes. The test was, consequently, deemed unsuitable as a test of p53 functionality for this experiment.

Figure 5.2: Surrogate markers for p53 function

A. Apoptosis of murine thymocytes exposed to UVC irradiation



B. $p19^{ARF}$ and p53 expression

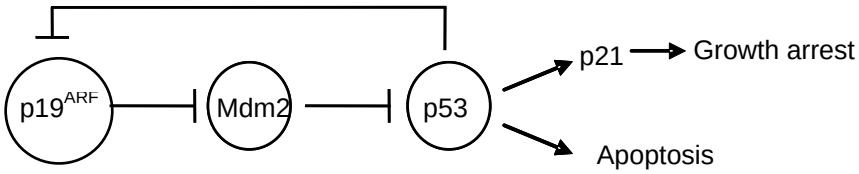


Figure 5.2A shows levels of apoptosis, as assessed by annexin-V staining and flow cytometry, within populations of wild type and p53 null thymocytes with and without exposure to 60 J/m^2 UVC irradiation. 5.2B shows the interactions between p53 and $p19^{ARF}$ and the feedback repression loop by which intact p53 represses transcription of $p19^{ARF}$ (Sherr and Weber 2000).

Alternative assays which could be applied to test for functional p53 include detection of p21 expression by western analysis. p21 is a downstream transcriptional target of p53 and is induced in wild-type p53-containing cells by exposure to DNA damaging agents, but is not induced in mutant p53-containing cells (el Deiry et al 1994; Venkatachalam et al 1998). The gene is activated in a p53-dependent manner following gamma radiation. Several attempts to discern the p21 protein status in the RMP samples were disappointingly unsuccessful and uninformative where antibodies did not detect protein in the test samples.

Finally, Western blot analysis of $p19^{ARF}$ expression can be used as an indication of the presence or absence of functional p53 (Baxter et al 2001; Eischen et al 1999). A feedback mechanism exists between these molecules (Sherr 1998; Sherr and Weber 2000) whereby lack of functional p53 allows upregulation of $p19^{ARF}$ to occur (Figure 5.2B). Similarly, high levels of p53 expression depress $p19^{ARF}$ expression. Concurrent expression of $p19^{ARF}$ and p53 proteins, therefore, can occur when p53 is mutant and non functional, and $p19^{ARF}$ expression can be used as a sensitive biomarker for the loss or impairment of p53 function.

5.2.2 Tumour dissemination throughout multiple tissues in a model for murine T cell lymphoma

A cohort of $Runx2^{tg/-}$ $Myc^{tg/-}$ $p53^{+/-}$ (RMP) mice was monitored on a daily basis and mice were culled at the first signs of ill-health. Both the *Runx2* and the *Myc* transgenes are targeted to the T cell lineage with the CD2 locus control region and single transgenics develop predominantly thymic lymphosarcomas with variable degrees of tumour infiltration in other lymphoid and non lymphoid organs (Stewart et al 1993; Vaillant et al 1999). The mice typically presented with dyspnoea, distended abdomen and altered gait. Interestingly, the severity of clinical signs did not always reflect the degree of tumour burden. All animals were killed between 36 and 44 days of age with an average latency of 40 days. This is representative of the latency period for a larger cohort of genetically similar RMP mice (Blyth et al 2006). Gross enlargement of organs at necropsy, as compared to a control age-matched animal, was used as an indication of those organs involved in tumour development. In almost every case the thymus, mesenteric and prescapular subcutaneous lymph nodes were very enlarged (Table 5.1), although in the case of mouse #123, organ enlargement was less marked. One mouse had an enlarged

kidney. The spleen was enlarged in 4 of the other mice, 2 of which also presented with an enlarged liver.

These results represent only gross pathological observation and do not rule out the possibility that microscopic changes were present in the tissues scored as not enlarged. The primary tumour site in every mouse examined was presumed to be the thymus for two reasons. Firstly, this was usually the largest tumour in the body and secondly, the transgenes, Runx2 and Myc, are both targeted to the T cell compartment and are expressed throughout T-cell development (Blyth et al 1995; Vaillant et al 1999).

5.2.3 p53 status remains heterozygote in disseminated lymphoma

Previous studies, using p53 heterozygote mice, have shown that different mouse models differ in their propensity to lose the p53 wild type allele during tumour development (Baxter et al 1996; Baxter et al 2001; Blyth et al 1995; Harvey et al 1993; Jacks et al 1994; Purdie et al 1994; Venkatachalam et al 1998). The mice used in this study were genotypically heterozygote for the p53 null allele and a selection of their primary and secondary (disseminated) tumour tissues was assessed for the presence of the wild type allele by Southern blot analysis (Table 5.2).

Occasional tissues did not yield an interpretable result due to poor or insufficient DNA extraction. DNA samples from two of the tumour series (#125 and 126) were, unfortunately, extracted using a different extraction kit, resulting in such a poor DNA yield that analysis was not possible. Nonetheless it was possible to get a result of their p53 status as assessed by western analysis (Table 5.3).

It was evident that all tissues examined maintained the knockout and wild type alleles in more or less equal proportions suggesting that the normal allele had not been selected against during tumour evolution. However the presence of the allele does not discount the possibility that the p53 protein is mutated or non functional. Although the wild type p53 allele has been confirmed to be functional in other populations of p53 heterozygote mice where loss of heterozygosity does not occur (Baxter et al 2001; Venkatachalam et al 1998), a direct analysis of the protein was carried out for the complete set of RMP primary tumours and for a selection of other tissues from these animals (Table 5.3). Western blot results showed that all tumours examined, both at the primary (thymus) and secondary

sites, expressed the p53 protein (Figure 5.3) which is consistent with the Southern blot results. In addition to those tissues shown in the table (Table 5.3) a limited number of spleen and subcutaneous lymph nodes were also tested and were also positive for expression of p53.

5.2.4 Examination of p19^{ARF} status to assess functionality of p53

The western blot results for these tumours are summarized in Table 5.3 and the expression of functional p53 is summarized in Table 5.4.

The results show that 5 of the seven primary tumours arising in the thymus of the RMP mice express p53 but not p19^{ARF} (Table 5.4, Figure 5.3) which suggests that the retained wild type p53 is functional. The result for the mesenteric lymph node from tumour 120 (120 mln) is difficult to interpret since there is artefactual distortion of the gel however the blot suggests that p19^{ARF} is negative whilst p53 is positive.

Two tumours (121 and 122) express p53 and p19^{ARF} concurrently suggesting that p53 is not functional and that it may be mutated. However, this was a feature of the primary tumours rather than a de novo event occurring during dissemination. Interestingly these were the earliest mice to succumb to tumours which allows one to conjecture that those tumours sustaining a mutation in the p53 allele may arise faster than those tumours in which p53 remains functional. More tumours would need to be examined in order to further explore this suggestion. Another possible explanation for the apparent co-expression of p53 and p19^{ARF} is that this arises from different subsets of the tumour cell population. Thus, a minor proportion of cells that have lost the wild-type allele would contribute p19^{ARF} expression while the remaining cells continue to express p53.

Of the 5 primary tumours expressing functional p53, 3 retained functional p53 in their tumour infiltrated tissues (metastases). Tumour 126 is interesting in that p53 expression is stronger for the thymus (126 thymus) than for the mesenteric lymph node (126 mln) despite strong actin bands for both (Figure 5.3). This implies that p53 expression may be reduced at this focus. Secondary (disseminated) tumours 123 and 125 also displayed evidence of loss of p53 function as assessed by p19^{ARF} expression in the presence of detectable p53 protein.

Table 5.1: Assessment of tumour dissemination in RMP mice

Mouse	thymus	spleen	mln	sln	kidney	liver	Latency
120	+++	++	+++	+++	-	-	37 days
121	+++	++	+++	++	-	+	36 days
122	++++	+	+++	++	-	-	36 days
123	+	+	+	+	-	+	38 days
124	+++	-	++	++	+	-	44 days
125	+++	-	+++	+++	-	-	44 days
126	+++	-	++	-	-	-	44 days

Table 5.1 summarises the gross postmortem changes in the RMP mouse cohort and depicts which organs were enlarged. - = no gross pathological enlargement. + = extent of enlargement of organs with + < ++ < +++. mln = mesenteric lymph node. sln = subcutaneous lymph node. Latent period for tumour is also shown.

Table 5.2: p53 status of tumours by Southern blot analysis

Mouse	thymus	mln	sln	liver
120	+/-	+/-	n/d	n/d
121	n/c	+/-	n/d	+/-
122	+/-	+/-	+/-	n/d
123	+/-	+/-	+/-	n/d
124	+/-	+/-	n/d	n/d

Table 5.2: A selection of tissues from mice within the RMP cohort was analysed by Southern blot analysis for their DNA status. +/-= presence of the wild type (+) and null (-) p53 alleles. n/c = not conclusive. n/d = not done. mln = mesenteric lymph node. sln = subcutaneous lymph node.

Table 5.3: p53 and $p19^{ARF}$ protein expression in tumours in RMP mice

Mouse	Thymus		mln		Liver	
	p53	**$p19^{ARF}$**	**p53**	**$p19^{ARF}$**	**p53**	**$p19^{ARF}$**
120	+	-	+	-	n/d	n/d
121	+	+	+	+	+	+
122	+	+	+	+	n/d	n/d
123	+	-	+	+	+	+
124	+	-	+	-	n/d	n/d
125	+	-	+	+	n/d	n/d
126	+	-	+	-	n/d	n/d

Table 5.3: A range of tissues from mice 120-126 was assessed for levels of p53 and $p19^{ARF}$ protein expression by western blot analysis. + = presence of protein. n/d = not done. Mln = mesenteric lymph node.

Table 5.4: Tumours expressing functional p53

	Tumours expressing functional p53	Tumours with non functional p53
Primary (RMP) tumours	120, 123, 124, 125, 126	121, 122
Disseminated tumours	120, 124, 126	121, 122, 123, 125

Table 5.4 shows the tumours expressing functional p53 as determined by lack of concurrent $p19^{ARF}$ expression. RMP represents primary tumour arising in the thymus. Disseminated tumours represent secondary sites in lymph nodes and liver.

Figure 5.3: Western analysis of RMP tumours

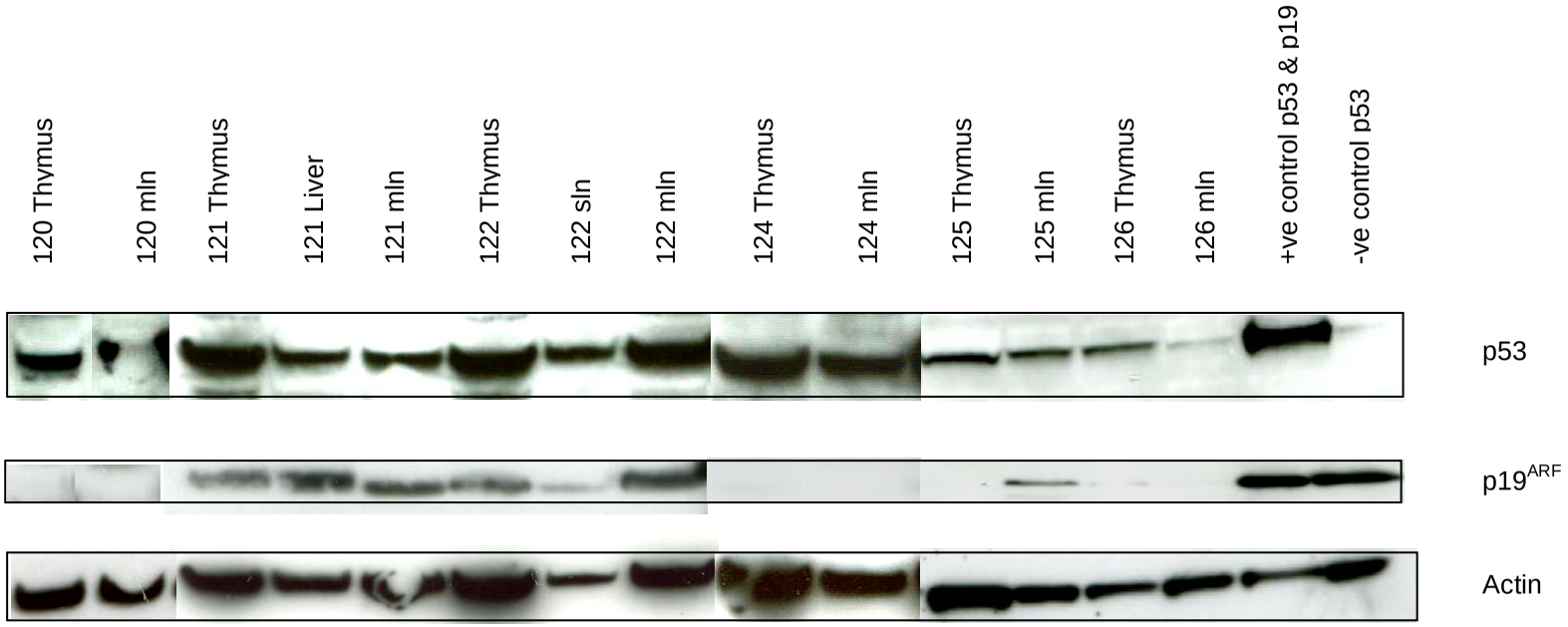


Figure 5.3: A range of tissues explanted from mice 120, 121, 122, 124, 125 and 126 was assessed for levels of protein expression by Western blot analysis. Levels of p53 (top) and $p19^{ARF}$ (middle) are shown. Actin (bottom) is included as a loading control. mln=mesenteric lymph node. sln=subcutaneous lymph node.

5.3 DISCUSSION

The work in this chapter sought to investigate the role of p53, and more particularly the requirement to lose p53, during the disseminated spread of T cell lymphoma in mice. In this study Runx2/Myc transgenic animals were used as a surrogate, highly tumour-prone model for MMLV-induced lymphomagenesis.

Whilst the need to lose p53 is usually avoided in MMLV-induced lymphomagenesis, there are occasional primary tumours that do exhibit loss of the wild type allele (Baxter et al 1996) and it is probable that this loss depends on host genes that are activated within individual tumours. By contrast, loss of the p53 allele was not seen in any of the RMP primary tumours examined which is consistent with previous reports (Blyth et al 2006), and most of these tumours retained functional p53 expression suggesting that loss of functional p53 is not necessary for initiation of tumour development. Recent evidence demonstrates that Runx2 and Myc collaborate by neutralizing the negative growth effects of each other and this perhaps enables them to bypass the need to lose p53 in the primary RMP tumours (Blyth et al 2006).

Whilst expression of the wild type p53 allele was also retained in all disseminated foci, it was sometimes associated with co-expression of $p19^{ARF}$. It is conceivable that these cells were displaying early induction of $p19^{ARF}$, overriding the suppressive effect of p53. However, in view of the strong negative correlation between p53 and $p19^{ARF}$ in previous studies (Blyth et al 2006) it seems more likely that these observations reflect the presence of cells with non-functional p53, presumably due to mutation. If so, it would appear that dissemination is, to some extent, selecting for loss of p53 and hence that p53 expression can restrict the ability of cells to grow at secondary tumour sites. Interestingly, Stewart et al (Stewart et al 2007) recently conducted a retroviral mutagenesis screen to look for progression genes in Runx2/Myc lymphomas. MMLV infection of these mice lead to accelerated tumour onset and increased tumour dissemination. Intriguingly, the target genes identified in this screen included D-cyclins and Pim kinases, which both impinge on cell cycle regulation through antagonism of p21. These findings provide a parallel with my results since loss of functional p53, as observed in some of the disseminated tumours, would also be expected to result in loss of expression of p21 (el Deiry et al 1994; Venkatachalam et al 1998). Unfortunately, the limited involvement of non-lymphoid

organs in the current RMP mice made it difficult to draw any clear conclusion about the relative importance of p53 in lymphoid and non-lymphoid dissemination.

CHAPTER 6

P53 AND IN VIVO TUMOUR PROGRESSION

In Chapter 5, I demonstrated that loss of the wild type p53 allele was not a common, or required, attribute of $Runx2^{tg/-}Myc^{tg/-}p53^{+/-}$ (RMP) primary tumours. Nonetheless, Blyth and colleagues reported that loss of the wild-type p53 allele was strongly selected during *in vitro* culture of these tumour cells, yet it was retained on tumour transplantation *in vivo* (Blyth et al 2006). This observation was somewhat surprising in view of the findings described in chapter 5 which showed that some metastatic tumours of RMP mice express $p19^{ARF}$, implying at least partial loss of p53 function during tumour dissemination. To investigate this further I wished to test if serial *in vivo* transplantation would ultimately result in loss of p53. The strategy underlying these experiments is depicted in Figure 6.1. Depending on the immune competence of the host and the route of inoculation, transplantation presents a novel set of selective pressures as tumours have to adapt to non-physiological sites and *in vivo* conditions.

As discussed previously, RMP mice have been used here as a genetic surrogate for MMLV-induced tumours. Therefore I wished to establish whether the findings for RMP tumours with respect to retention of the p53 allele during transplantation, would also hold for Moloney murine leukaemia virus (MMLV) induced T cell lymphomas of $p53^{+/-}$ mice. As with the RMP tumours, these tumour cells do not often exhibit loss of heterozygosity of p53 *in vivo*, although the wild-type allele is invariably lost when tumours are grown *in vitro* (Baxter et al 1996). Retention of the p53 allele during *in vivo* passage of these tumours has not been formally tested to date and I was interested to investigate the pressures on the p53 pathway during transplantation on this tumour background.

Figure 6.1: Selection for loss of p53 function in tumour transplantation *in vivo*

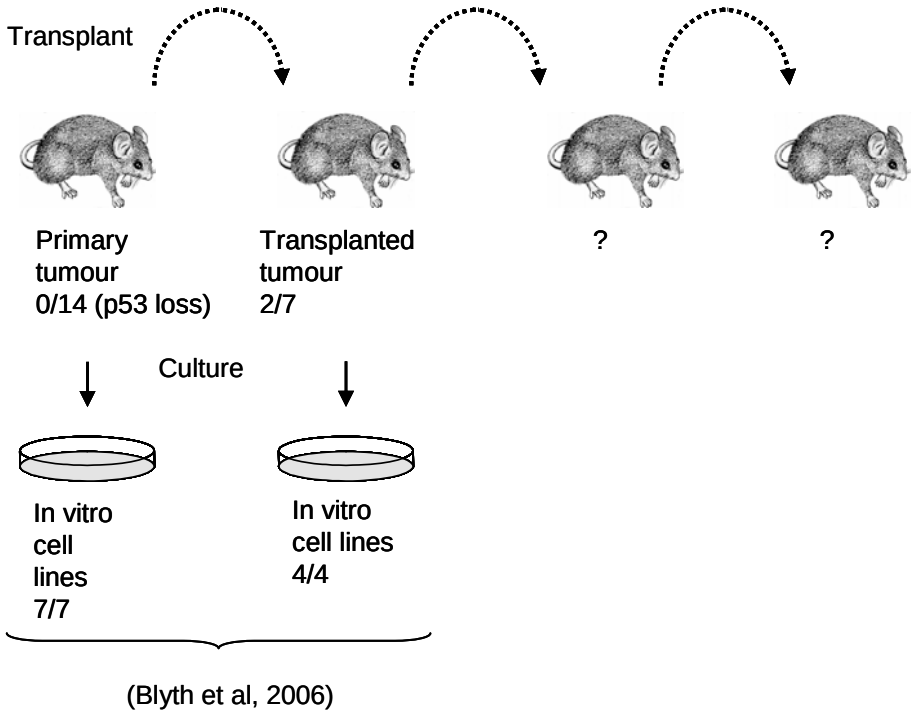


Figure 6.1: Blyth et al have shown previously that 0 out of 14 tumours that developed in $Runx2^{tg/-}Myc^{tgt/-}p53^{+/-}$ (RMP) mice exhibited loss of the p53 wild type allele (Blyth et al 2006). 2 out of 7 tumours that were transplanted *in vivo* lost the wild type allele. All tumours lost the allele with *in vitro* growth. This study aims to investigate whether the p53 wild type allele is lost with further *in vivo* transplantation of the tumour.

6.1 EXPERIMENTAL PROTOCOLS

All experimental approaches were carried out to the specification as described in Chapter 2 except where stated below.

6.1.1 Animal Procedures

Two different murine models of lymphoma, both genetically heterozygous for functional p53, were investigated for their propensity to lose the wild type p53 allele during the *in vitro* and *in vivo* passaging of tumour cells Firstly, eight animals from a viral induced T-lymphoma model, in which inbred C57Bl/6 mice heterozygous for the inactivated p53 allele (Donehower et al 1992) were infected with MMLV within 24 hours of birth, were used in this study. Secondly, six RMP mice were studied. The breeding of this cohort is described in Chapter 5.

6.1.2 In vitro culture

Tumour cells were cultured in complete RPMI and incubated at 37°C. Cell pellets were harvested at various different passages and stored at -70°C for subsequent Western and Southern analysis.

6.1.3 In vivo transplants

Tumour cells (2×10^7 cells) were inoculated intraperitoneally into either MFI *nu/nu* immunocompromised (Harlan, UK) or C57Bl/6 immunocompetent recipient mice. Recipient mice were all female and were between 6 and 8 weeks old at the time of transplant. All recipient mice were killed as soon as any sign of illness developed, usually abdominal enlargement, dyspnoea or depression, (in accordance with Home Office regulations). Any mice still alive 12 weeks after the initial inoculation were killed and examined at postmortem. Tumour cells from the MFI *nu/nu* immunocompromised mice were inoculated into a second and, in one case, into a third *nu/nu* recipient. These mice were denoted N1, N2 and N3 respectively. When recipient mice developed tumours they were killed. Post mortem examinations were conducted and tumour cells prepared for Western analysis, *in vitro* culture and further *in vivo* passage where appropriate.

6.2 RESULTS

This Chapter investigates p53 loss during *in vivo* transplantation and *in vitro* growth of virus induced T cell lymphoma. For this purpose one defective p53 allele was provided in the germ line whilst the fate of the remaining allele was followed during tumour cell evolution. A cohort of MMLV-infected p53 heterozygote animals was studied for this analysis and, as a surrogate for virus-induced lymphoma, a Runx2/Myc oncogene model was also used which had a significant advantage in that tumours develop after a much shorter latent period than in the viral infected group.

6.2.1 Investigating the role of p53 in MMLV-induced lymphoma .

MMLV and p53 loss are collaborative in T cell lymphomagenesis (Baxter et al 1996) although this collaboration is weak compared to other MMLV models (Stewart et al 1993; van Lohuizen et al 1991). MMLV-induced tumours on a p53 heterozygote background generally do not lose the allele *in vivo,* nonetheless allele loss is inevitable when the cells are cultivated *in vitro* (Baxter et al 1996). *In vivo* passaging of these tumour cells has not been investigated previously. In order to address this, a cohort of p53 heterozygote animals on an inbred C57Bl/6 strain was infected with MMLV. This cohort of animals was found to develop lymphomas between 62 and 134 days of age. On post mortem examination, all of the mice exhibited neoplastic enlargement of multiple tissues. Tissues most commonly affected were thymus, mesenteric and subcutaneous lymph nodes, liver and spleen. The kidney was less frequently involved (Table 6.1).

Primary tumour cells were then transplanted into recipient animals. Immunocompetent C57BL/6 mice were initially tested as recipients for the MMLV infected mice. Since the recipient mice were of a similar genetic background to the donor mice it was hoped that the tumour cells would not be rejected. However after 12 weeks these recipient mice were all still clinically normal. They were killed (according to Home Office requirements) and post mortem examination revealed no abnormalities. It was assumed that the lack of tumour development in the C57BL/6 recipient mice was due to the fact that these animals were immunocompetent. Thus, an alternative immunocompromised model was chosen for subsequent transplants. Tumour cells from two mice, # 419 and # 420, were inoculated into MFI *nu/nu* immunocompromised mice (N 419 and N 420). The nude recipients developed overt illness and tumours within 20 days (N 419) and 28 days (N 420) of inoculation. In each case post mortem examination revealed tumour involvement of the

liver, spleen, kidneys, mesenteric and subcutaneous lymph nodes. Organ enlargement was much more marked in nude recipient N 420 than in N 419. The left ovary was also hugely enlarged in N 420.

6.2.1.1 Southern blot analysis reveals retention of wild type p53 allele with in vivo passage

Tumours (primary and transplants where appropriate) from the MMLV infected cohort were analysed for their p53 status. In 7 out of the 8 cases, the primary tumours remained heterozygote for p53 by Southern analysis. The exception was the primary tumour 392 which was p53 null (Figure 6.2). This finding is consistent with previous reports that occasional MMLV-induced $p53^{+/-}$ tumours lose the wild-type p53 allele (Baxter et al 1996). Tumour cells from mice 419 and 420 were also analysed after *in vivo* passage through a MF1 *nu/nu* immunocompromised mouse. In both instances tumour cells harvested from the recipient mouse (N 419 and N 420) were p53 heterozygote by Southern analysis (Figure 6.2).

The results for *in vitro* culture were less straightforward (Table 6.2). Of four primary tumours that were analysed by Southern blot, three exhibited early *in vitro* loss of the p53 allele. Primary tumour 419, however, retained the p53 allele at early passage, although Figure 6.2 demonstrates that this retention is not complete and that a degree of loss of the allele is already apparent. Loss of the allele was seen to be complete by late passage of this tumour. Tumours derived from transplanted mice were also cultivated *in vitro*. Both N 419 & N 420 transplanted tumours successfully established *in vitro*. Allelic loss was evident at early passage of N 420 (passage 2). By contrast, transplanted tumour N 419 remained p53 heterozygote despite prolonged *in vitro* passage as tested at passage 10.

6.2.1.2 Western blot analysis reveals loss of functional p53 expression with in vitro passage

To confirm and extend the Southern blot results, tissue from primary tumours, transplanted tumours and their cell lines were assessed for p53 and $p19^{ARF}$ protein expression by western analysis. Western blot results are shown in Figure 6.3. Results for tumour 420 demonstrated p53 expression by the primary tumour. The associated absence of $p19^{ARF}$ suggests that this protein is functional. *In vitro* passage of cells from both the primary and the transplanted tumours is associated with loss of the p53 allele by Southern

analysis and, in accord with this observation, p53 is absent and $p19^{ARF}$ is expressed (western blot), albeit weakly in the early passage of the primary tumour.

The Western blot results for tumour 419 are also shown in Figure 6.3. As can be seen, despite retention of the wild type p53 allele (as confirmed by Southern analysis) with early *in vitro* culture, there is no expression of p53 which is corroborated by the concurrent expression of $p19^{ARF}$. This would imply that the p53 allele may be a non-expressing mutant (Blyth et al 2006; Hietanen et al 1995; Van Leeuwen et al 1996) which strongly suggests that inactivation of p53 is advantageous or even essential for tumour cell lines to successfully establish in culture. Nonetheless, its disappearance from late passage cells indicates that it did not confer a strong advantage to those cells expressing it.

Table 6.1: Assessment of tumour dissemination in MMLV infected $p53^{+/-}$ mice

Mouse	thymus	spleen	Mln	sln	kidney	liver	Latent period	Recipient
385	+	++	+	-	-	+	91	C57Bl/6
386	+	++	++	+	+	+	91	C57Bl/6
392	+	++	+	+	-	+	123	C57Bl/6
393	+	+	+	-	-	+	62	C57Bl/6
395	++	++	++	++	-	+	134	C57Bl/6
399	++	+	++	+	-	+	91	C57Bl/6
419	++	++	++	++	+	+	128	MFI *nu/nu*
420	++	+	+	+	+	+	127	MFI *nu/nu*

Table 6.1 summarises the gross postmortem changes in the MMLV infected mouse cohort and depicts which organs were enlarged. - = no involvement, + = extent of gross enlargement of organs (+ < ++ < +++). Latent period is in days. mln=mesenteric lymph node, sln= subcutaneous lymph node. Recipient mice are either C57Bl/6 or MFI nu/nu mice.

Figure 6.2: Southern blot analysis of p53 status in MMLV induced tumours

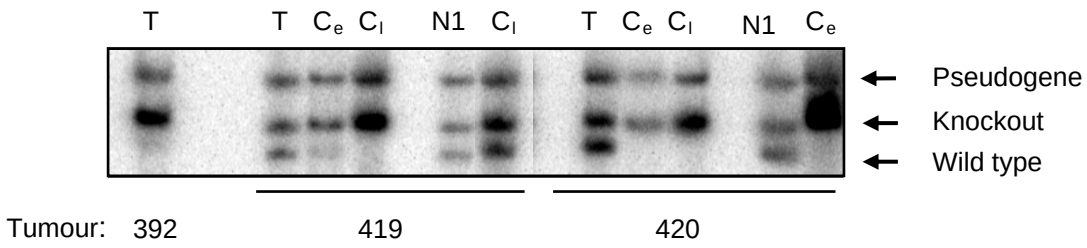


Figure 6.2: Southern blot results for p53 status of primary and transplanted MMLV infected tumours. Results are also shown for *in vitro* passage of tumours. T: Primary tumour, N1: Tumour from first transplant, Ce and C_l refer to early and late passage cell lines established from tumours in adjacent lane. The respective p53 alleles are indicated by arrows.

Table 6.2: p53 status of MMLV infected tumours as assessed by Southern blot analysis

	Primary tumour			*Post transplant (nude recipient)*		
		***In vitro* passage**			***In vitro passage***	
	T	**E**	**L**	**T_1**	**E**	**L**
385	+/-	-/-	-/-	n/a	n/a	n/a
393	+/-	-/-	-/-	n/a	n/a	n/a
419	+/-	+/-	-/-	+/-	+/-	+/-
420	+/-	-/-	-/-	+/-	-/-	-/-

Table 6.2 shows the p53 status of MMLV infected tumours as assessed by Southern blot analysis. Results are shown for both the primary tumour (T) and the transplanted tumour (T_1 =post transplant nude recipient). Results are also shown for *in vitro* passage of tumour cells. E=early passage. L=late passage. n/a: not applicable.

Figure 6.3: Protein expression in MMLV-induced tumours and cell lines

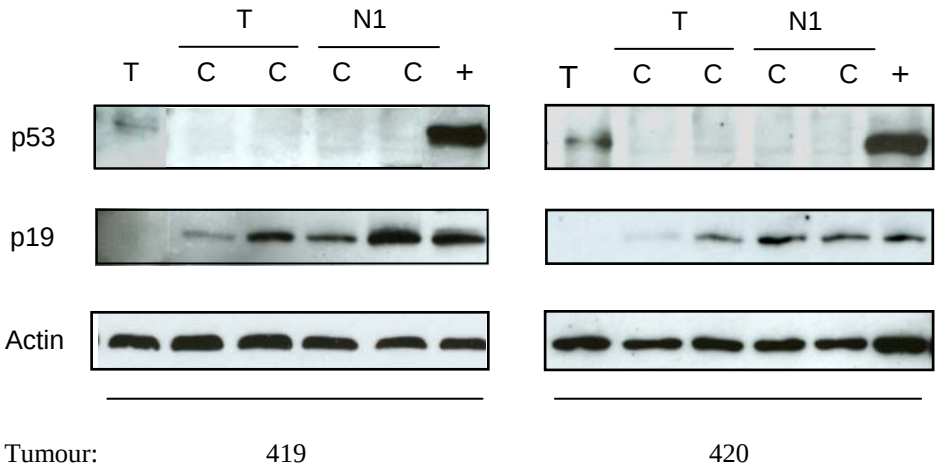


Figure 6.3: Tumours # 419, 420 were assessed for levels of protein expression by Western blot analysis. Levels of p53 (top) and p19 (middle) are shown. Actin (bottom) is included as a loading control. Results are shown for the primary (T) tumours and for early and late *in vitro* passaged cell lines (Ce, Cl) derived from the primary and transplanted (N1) tumours. There was insufficient tissue for analysis of the primary transplanted tumours. + denotes positive control for p53 and $p19^{ARF}$ expression.

6.2.2 Investigating the role of p53 during transplantation of Runx2$^{tg/-}$Myc$^{tg/-}$p53$^{+/-}$ (RMP) lymphomas.

A cohort of 6 Runx2$^{tg/-}$Myc$^{tg/-}$p53$^{+/-}$ (hereafter RMP) transgenic animals was used in this study. These animals succumbed to tumours between 26 and 36 days of age with an average latency of 29 days consistent with previous reports (Blyth et al 2006). For each individual, post mortem examination was consistent with thymic lymphoma. The thymus was markedly enlarged by a firm, white mass. In 4 of the mice there was also some enlargement of mesenteric and subcutaneous lymph nodes. In 2 cases there was slight enlargement of other tissues notably liver and spleen (Table 6.3).

The RMP mice tended to develop tumours after a much shorter latent period than the MMLV infected mice and the tumours generally were less disseminated throughout the body. Only one of the RMP mice (#119) exhibited gross involvement of a non-lymphoid organ (Table 6.3) whereas all of the MMLV-infected mice showed involvement of the liver and some also showed involvement of the kidney. One possibility is that the increased latent period observed in the MMLV-infected mice allows time for the tumour to metastasise further to different sites in the body.

Tumour cells were prepared from the thymic mass from each of the RMP mice. These cells were then passaged *in vivo* and *in vitro* to determine the fate of the p53 allele in tumour cells undergoing transplantation. Figure 6.4 serves to illustrate the design of this experiment and is a diagrammatic summary of the events for one of the mice (#114). It shows which tissues were harvested and stored and also depicts the *in vitro* and *in vivo* passages that were undertaken and whether or not these were successful.

6.2.2.1 In vivo passage of RMP tumours yields disseminated abdominal tumours in recipient mice

For each of the RMP mice, thymic tumour cells were inoculated intraperitoneally into MFI *nu/nu* immunocompromised mice that were then monitored for tumour development. Five of the recipient MFI *nu/nu* mice (N1 mice) developed tumours 10-25 days after inoculation. The sixth recipient MFI *nu/nu* mouse inoculated with primary lymphoma, #119, did not become unwell or develop neoplasia. It was killed 12 weeks post inoculation and post mortem examination did not reveal any abnormalities. The explanation for this is not known, it may simply be that the transplantation was unsuccessful, however one

cannot eliminate the possibility that the tissue harvested from the donor mouse was not neoplastic.

Each of the other 5 MFI *nu/nu* mice exhibited enlargement of the mesenteric lymph node by dense white tumour tissue. In 4 of the mice there was also massive neoplastic enlargement of the uterus and ovaries. In 2 cases there was development of a subcutaneous ventral abdominal mass in addition to the intra-abdominal abnormalities (Table 6.4). In 2 cases (N1 113 and N1 115) there were "strands" of white, neoplastic tissue "free" within the abdomen that were not obviously associated with any particular organ. There was an anterior abdominal tumour mass present in N1 114 although it was unclear in which tissue it had originated.

Apart from the two mice that had subcutaneous masses, tumour tissue was always restricted to the abdomen. The subcutaneous masses occurred around the intraperitoneal injection site and were presumed to represent infiltration of the subcutaneous tissues with tumour cells. There was never any involvement of the thorax and, since *nu/nu* mice do not have a thymus, thymic involvement is excluded.

At the time of postmortem, tumour tissue was harvested from the primary recipient mice (N1) for further *in vitro* and *in vivo* passage. In each case tumour tissue was harvested from a lymphoid source where possible. For instance, tumour involving the mesenteric lymph node was chosen in preference to tumour involving the uterus since there would be less risk of non-tumour/non lymphoid cells interfering with subsequent *in vitro* and *in vivo* passage. Mice were killed as soon as clinical signs of discomfort/illness appeared. In quite a few cases, the organs were so mildly enlarged by the tumour that it was difficult to be absolutely sure that the tissue harvested was truly neoplastic rather than normal/hyperplastic lymphoid tissue.

Tumour tissue from the MFI *nu/nu* recipients was processed and then transplanted into secondary MFI *nu/nu* nude recipients (N2). These 5 N2 mice developed tumours or became clinically ill 11-18 days after inoculation. On post mortem examination there was enlargement of the mesenteric lymph node in all cases. This was often only slightly enlarged which meant that it was sometimes not possible to be able to carry out all downstream analyses due to limiting amounts of material. In two instances, # N2 114, # N2 113, there was accumulation of white firm material, presumed to be neoplastic tissue,

within the anterior abdominal cavity. In 2 cases there was enlargement of the uterus. Details of individual pathologies are given in Table 6.5.

Tissue was harvested from all the mice and, wherever possible, was stored for subsequent DNA analysis, western analysis and *in vitro* passage. Tissue from one N2 mouse (N2 114) was further transplanted into a tertiary recipient mouse (N3 114). This mouse (N3 114) developed a subcutaneous mass 1 cm in diameter on the ventrocaudal abdomen. It was killed 16 days after inoculation. On post mortem examination there was enlargement of the mesenteric lymph node. Tumour was taken, prepared and stored for further Southern and Western analysis.

It is interesting to observe that, whereas in the RMP mice the primary tumours involved the thymus with other tissues being less consistently, and variably, involved, the tumour distribution in the primary transplant (N1) mice was quite different. The thorax was never involved in these N1 mice which is likely to reflect the fact that tumour cells were inoculated into the peritoneum and that the host animals were athymic. The mesenteric lymph node was consistently and, often, most obviously involved with variable involvement of other lymphoid and non lymphoid organs. Tumour distribution in the secondary transplant (N2) mice was similar to the N1 mice although the number of tissues involved in individuals was frequently less than in the N1 mice. This finding appeared to coincide with those N2 mice that had a decreased latent period compared to their N1 counterparts and presumably, therefore, less time available for the neoplasm to disseminate. Although not a feature of the primary tumours arising in the RMP mice, the frequent involvement of the uterus and ovaries in the MFI *nu/nu* animals, both N1 and N2, is an interesting observation and suggests that the genital tract may be a receptive environment for lymphoma spread and development in these animals. Table 6.6 summarises the tissues which were harvested from each animal (original RMP mouse and recipients) and either passaged or kept for downstream analyses.

Table 6.3: Assessment of tumour dissemination in RMP mice

Mouse	thymus	spleen	mln	sln	kidney	liver	Latent period
112	+	+	+	+	-	-	26
113	+++	-	+	+	-	-	27
114	+++	-	-	-	-	-	27
115	+++	-	+	+	-	-	27
118	+++	-	-	-	-	-	29
119	+++	-	++	++	-	+	36

Table 6.3 summarises the gross postmortem changes in the RMP mouse cohort and depicts which organs were enlarged. + = extent of gross enlargement of organs where – is no obvious involvement and + < ++ < +++. Latent period to tumour development in days is also shown. mln= mesenteric lymph node. sln= subcutaneous lymph node.

Figure 6.4: Experimental design for tumour passaging for RMP 114

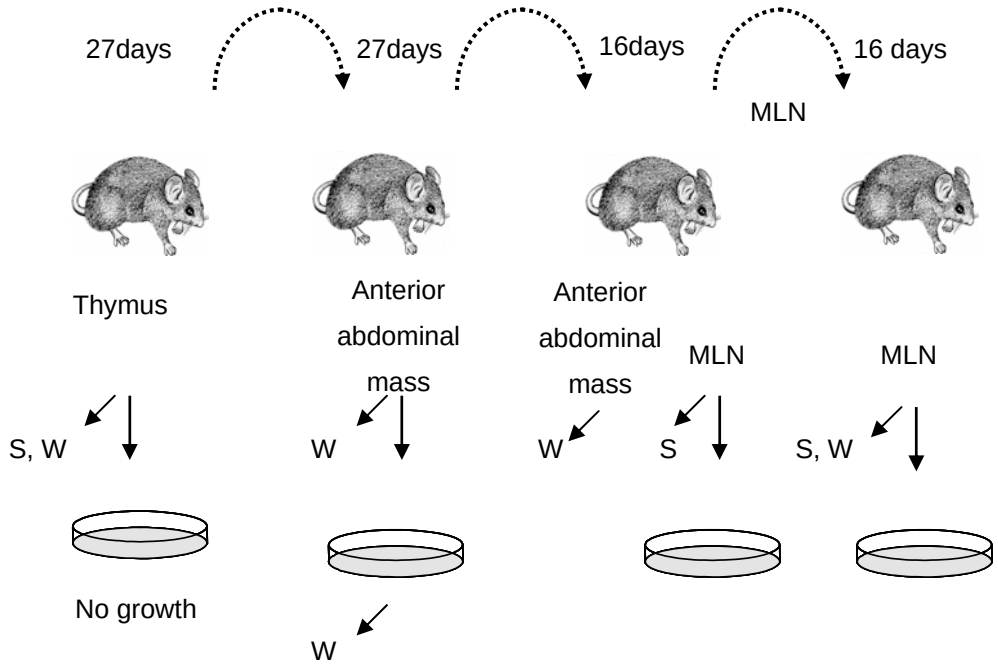


Figure 6.4 depicts the experimental design for *in vitro* and *in vivo* sampling in an example tumour (#114) demonstrating those tissues that were taken at each stage in the process. Time (days) taken to develop tumour is shown, as is the site from which tumour cells were harvested. N1 114 is the mouse transplanted with tumour cells from RMP 114, where subsequent transplants are denoted N2 114 and N3 114. Wherever possible, tumour cells were harvested, prepared and stored for subsequent Western (W) or DNA analysis (Southern, S). *In vitro* culture of neoplastic thymocytes from mouse RMP 114 was unsuccessful. *In vitro* growth of tumour cells harvested from the transplanted mice (N1 114, N2 114 and N3 114) was successful. MLN; Mesenteric lymph node.

Table 6.4: Assessment of tumour dissemination in MFI *nu/nu* N1 mice

Mouse	thymus	spleen	mln	sln	kidney	liver	Other tissues	Latent period
N1 112	-	+	+	-	+	-	-	25
N1 113	-	-	+	-	-	-	Subcutaneous mass. Uterus and ovaries.	10
N1 114	-	+	+	-	-	+	Uterus, left ovary, Anterior abdominal mass	27
N1 115	-	+	+	-	+	+	Uterus and ovaries	14
N1 118	-	+	+	+	-	-	Subcutaneous mass. Uterus and ovaries	15
N1 119	n/a	n/a	n/a	n/a	n/a	n/a	n/a	n/a

Table 6.4 summarises the gross postmortem changes in the MFI *nu/nu* mouse cohort and depicts which organs were enlarged. - = no gross involvement of the tissue and + = gross enlargement (this includes cases where enlargement was only very mild). Latent period in days post inoculation is given. It also shows which other tissues were found to be enlarged by the tumour at post mortem. n/a = not applicable (N1-119 did not develop tumours). Mln =mesenteric lymph node. Sln =subcutaneous lymph node. For each mouse, N1 is the transplant from RMP.

Table 6.5: Assessment of tumour dissemination in MFI *nu/nu* N2 mice

Mouse	thymus	spleen	mln	sln	kidney	liver	Other tissues	Latent period
N2 112	-	-	++	SE	-	-	-	18
N2 113	-	SE	+	-	-	SE	Uterus	11
N2 114	-	-	++	-	-	-		16
N2 115		-	+	-	-	-	Uterus	18
N2 118	-	SE	SE	-	-	-	-	11

Table 6.5 summarises the gross postmortem changes in the MFI *nu/nu* N2 secondary transplants and depicts which organs were enlarged. – represents no enlargement, + = extent of gross enlargement of organs (+ < ++). SE = slightly enlarged. Latent period in days is also shown. It also shows other tissues that are enlarged by the tumour. mln=mesenteric lymph node. sln=subcutaneous lymph node. For each mouse, N2 is the transplant from N1.

Table 6.6: Tumour sites harvested from mice

Tumour #	Primary (RMP)	Recipient 1	Recipient 2	Recipient 3
112	Thymus	Mln	mln	n/a
113	Thymus	mln mln (western) uterus (Southern)	mln	n/a
114	Thymus	Mass in ant abdomen	mln Mass in ant abd (Western)	mln
115	Thymus	Mln	mln	n/a
118	Thymus	Mln	mln	n/a
119	Thymus	n/a	n/a	n/a

Table 6.6 shows the tumour sites harvested from primary (RMP), and recipient (N1, N2 and N3) mice. Cells taken from site listed are used for all procedures. Where cell yield was insufficient, tumour tissue was also taken from a separate site in order to complete procedures and this is specified in the table. Mln =mesenteric lymph node. ant abd = anterior abdomen. n/a = not applicable.

At each passage stage of the *in vivo* process, tumour tissue from each mouse was concurrently cultured *in vitro*. As can be seen from Table 6.7, although the majority of primary tumours successfully propagated *in vivo*, only one of six tumours established *in vitro*. It should be noted that *in vitro* culture conditions did not include addition of cytokines which might have increased the success rate. Success of *in vitro* establishment was increased when transplanted tumours were cultured *in vitro* with 1 out of 3 of the N1 tumours, 2 out of 3 of the N2 tumours and the single N3 tumour growing to late passage (passage 8-12). The transplantation history and success of *in vitro* culture for each tumour is summarized in Table 6.7.

It was anticipated that the tumours may exhibit increased aggressiveness with each *in vivo* transplant and that this may manifest as a decreased latent period for tumour development with each serial passage. In general this was what was observed in the passaging of the 6 RMP tumours, albeit this was not a strict rule. Latency to tumour development in the primary transgenic RMP animals was an average of 28.7 days (range 26-36) compared to the primary transplants which developed with an average latency of 18.2 days post inoculation (range 10-27). Secondary transplants were observed at an average of 14.8 days post inoculation (range 11-18). This was not unexpected since, in the primary transgenic animal, there has to be time for initiating tumour events to occur whereas the transplanted animals receive a transplant of preformed malignant cells. Nonetheless, two of the primary transplanted recipients (N1) had a latency period similar to that seen in the original RMP mouse ie RMP 112 had a latency of 26 days and the transplant animal (N1 112) had a latency of 25 days. Similarly RMP 114 and the transplant N1 114 both succumbed to tumour at 27 days. Furthermore, although the latent period for tumour development was always decreased in the secondary transplant animals (N2 mice) compared to the original RMP mice, the latent period in the N2 animals was not always less than in the primary transplants (N1). Two out of 5 of the secondary transplants (N2 113 and N2 115) took slightly longer than the primary transplant (N1) to develop tumours. It should be noted that this murine model is highly prone to tumour development and that not many spontaneously arising primary tumours occur as early as 28 days of age. A more significant decrease in tumour latency after transplant is seen in the MMLV-induced lymphoma model. Here primary tumours arise with a mean latency of 106 days (range 62-134), while transplanted recipients succumb to disease at 20 and 28 days. The longer latency of the primary tumours in this case clearly reflects the need to accumulate multiple

hits of insertional mutagenesis and possibly other genetic or epigenetic changes (Fan 1997).

6.2.2.2 Southern analysis shows that wild type p53 allele is retained with prolonged in vivo passage

It has been reported that the majority of primary tumours developing in RMP mice retain the wild type p53 allele after one round of *in vivo* transplantation (Blyth et al 2006) although *in vitro* establishment is associated with loss of the p53 allele. In order to investigate whether this is due to a difference between *in vitro* and *in vivo* conditions or whether it reflects the fate of the allele in later tumour progression, the status of the p53 allele was investigated during sequential *in vivo* transplantation of these tumours. To this end, Southern analysis was carried out on primary and transplanted tumours. The results demonstrate that the p53 allele is retained after prolonged *in vivo* passages as summarized in Figure 6.5.

Southern analysis was also undertaken on cell lines derived from both primary and transplanted tumours at early and late passages wherever possible. The results are shown in Figure 6.5 and Table 6.8. In most cases (except tumour 115) the wild type allele was lost early on during *in vitro* culture, suggesting that allelic loss occurs in response to *in vitro* pressures.

6.2.2.3 Tumours do not always express functional p53 as assessed by western blot for $p19^{ARF}$

The Southern results indicate the presence of a p53 allele with first, second and third *in vivo* passage of tumour cells. This analysis does not, however, exclude the possibility that the allele is mutated. Western blot analysis was carried out on the tumours to investigate whether or not there was expression of p53 protein. Western blot analysis revealed that the primary RMP tumours express the p53 protein which is in accord with the Southern results (Figure 6.6, Figure 6.7). In all cases the primary, secondary and tertiary tumour transplants (N1, N2 and N3 tumours) also expressed p53. In the case of N3 114 and N1 115 a larger protein was expressed alongside the p53 protein.

Table 6.7: Fate of *in vitro* and *in vivo* passaged cells explanted from RMP tumours

Tumour	RMP		N1		N2		N3	
	***In vivo* LP**	***In vitro***	***In vivo* LP**	***In vitro***	***In vivo* LP**	***In vitro***	***In vivo* LP**	***In vitro***
112	26	No	25	n/a	18	Yes		
113	27	No	10	No	11	n/a		
114	27	No	27	Yes	16	Yes	16	Yes
115	27	Yes	14	No	18	No		
118	29	No	15	n/a	11	n/a		
119	36	No	-	-	-	-		

Table 6.7 shows the success of *in vitro* passage for each tumour. No = *in vitro* growth was not successful. Yes = successful *in vitro* growth was achieved. n/a = not applicable and is where *in vitro* growth was not attempted due to insufficient tumour cell yield. RMP = primary tumour. N1, N2, N3 represent tumours after primary, secondary and tertiary *in vivo* transplantation. *In vivo* LP = *in vivo* latent period (days).

Figure 6.5: Southern blot analysis of p53 status in RMP transplanted tumours

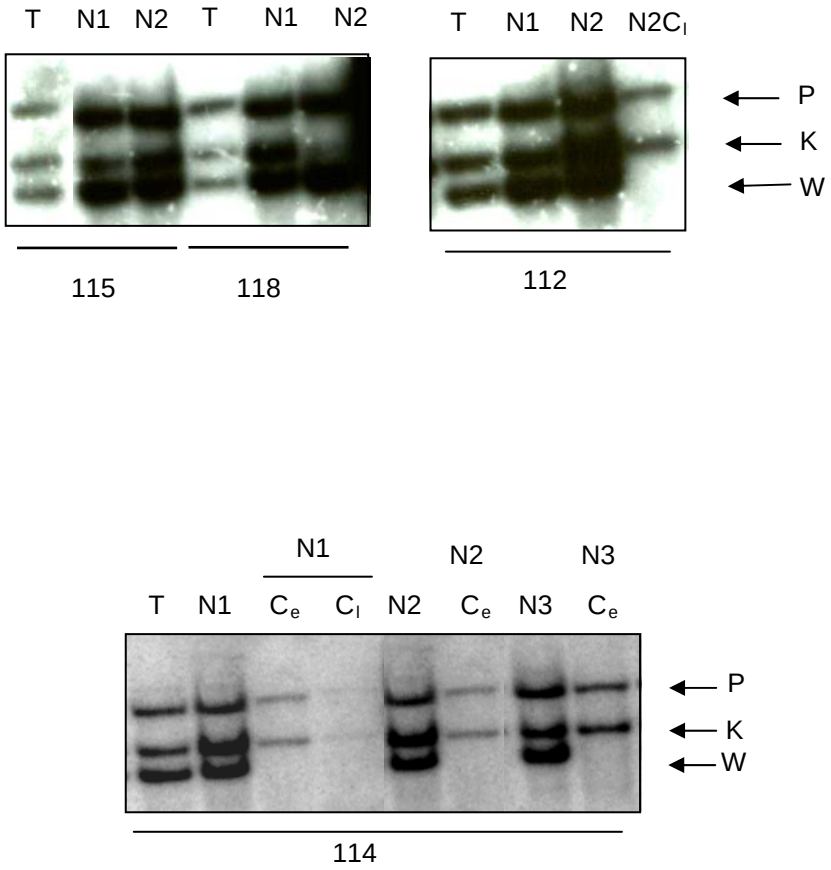


Figure 6.5: Southern blot analysis results for p53 status of tumours #112, 114, 115 and #118 after *in vivo* and *in vitro* passage. RMP is the primary tumour; N1, N2 and N3 represent the first, second and third *nu/nu* transplantations. C_e and C_l refer to early and late passage cell lines derived from these tumours. Pseudogene (P), knock-out (K) and wild type (W) p53 alleles are indicated.

Table 6.8: Western and Southern results for RMP tumours

Tumour ID	p53 Southern	p53 Western	p19 Western
Tumours			
Primary			
RMP 112	+/-	+	-
RMP113	+/-	+	-
RMP 114	+/-	i/c	-
RMP 115	+/-	+	-
RMP 118	+/-	+	-
Primary transplants			
N1 112	+/-	+	i/c
N1 113	+/-	+	-
N1 114	+/-	+	+
N1 115	+/-	Faint *	-
N1 118	+/-	+	-
Secondary transplants			
N2 112	+/-	+faint	++
N2 113	+/-	+	-
N2 114	+/-	+	+
N2 115	+/-	+	-
N2 118	+/-	+	-
Tertiary transplants			
N3 114	+/-	+ *	+
Cell lines			
Primary tumour			
RMP 115 P1	+/-	+	-
RMP 115 P12	+/-	+	+
Primary transplant			
N1 114 P1	-/-	-	+
N1 114 P10	-/-	-	+
Secondary transplant			
N2 112 P2	-/-	-	+
N2 114 P2	-/-	-	+
N2 114 P11	-/-	-	+
Tertiary transplant			
N3 114 P2	-/-	-	+
N3 114 P10	-/-	-	+

Table 6.8: this summarises p53 status by Southern blot and also p53 and p19 expression by Western blot analysis for RMP tumours. +/-= p53 heterozygote; -/- = p53 null. n/a= not applicable. Results are shown for primary tumours and also for tumours derived from primary (1˚), secondary (2˚) and tertiary (3˚) *in vivo* transplants as well as for cell lines derived from these tumours where P reflects passage number. i/c = inconclusive. * Denotes larger p53 band (see text).

Figure 6.6: Western blot analysis of RMP tumour series

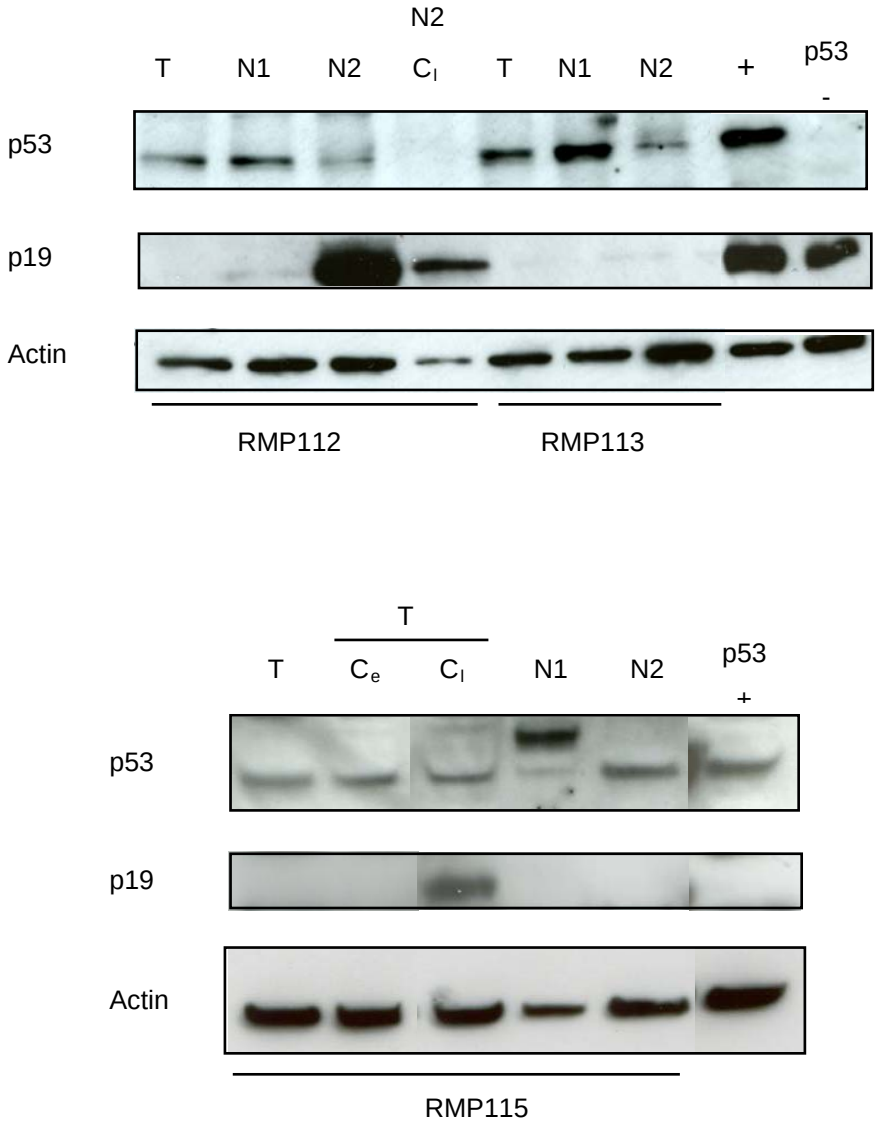


Figure 6.6: Protein expression by Western blot analysis for tumours 112, 113 and 115. Levels of p53 (top), and $p19^{ARF}$ (middle) are shown. Actin (bottom) is included as a loading control. Results are shown for the primary tumour (RMP), primary (N1) and secondary (N2) tumour transplants and also for *in vitro* culture.

Figure 6.7: Western analysis of RMP tumour series 114

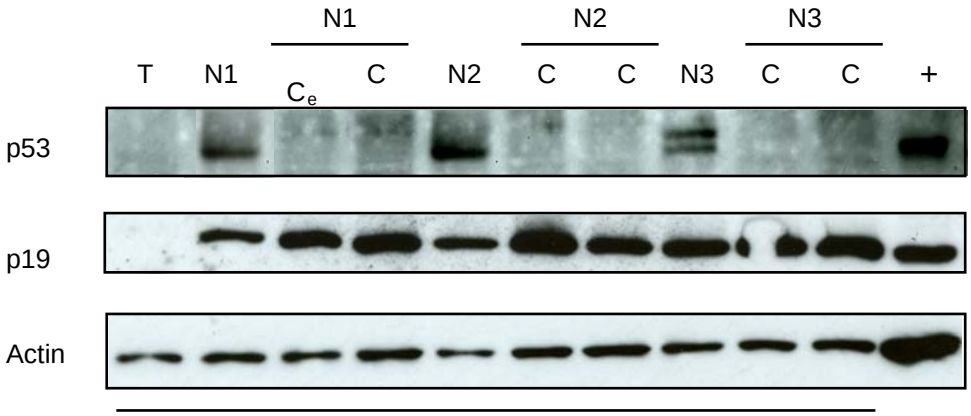


Figure 6.7: Protein expression by Western blot analysis for tumour series 114. Levels of p53 (top), and $p19^{ARF}$ (middle) are shown. Actin (bottom) is included as a loading control. Results are shown for the primary tumour (RMP), primary (N1), secondary (N2) and tertiary (N3) tumour transplants and also for *in vitro* culture (C: cell line e: early, l: late passage).

The primary transplant for tumour 112 (N1 112) expressed p53, analysis of $p19^{ARF}$ expression for this tumour showed only a very faint band suggesting that the p53 protein remained functional in most of the transplanted tumour cells. It is interesting that for tumours 112 and 113 there was lower p53 expression in the second tumour transplant (N2) compared to the first transplant (N1) suggesting that p53 expression may have decreased with *in vivo* passage. The results show a very strong $p19^{ARF}$ band expressed by the secondary transplant 112 (N2 112) alongside weak expression of p53. Surprisingly there was less $p19^{ARF}$ expressed with *in vitro* culture of N2 112 despite the lack of p53 expression. However, the faint actin loading control band (N2 112 late passage) casts doubt on the significance of this observation. The primary transplant for tumour 115 (N1 115) expressed p53 (faint band) which was presumed to be functional based on the lack of concurrent $p19^{ARF}$ expression, however it also expressed a protein larger than p53 the identity of which is unknown but is suspected to be a p53 mutant.

The western results for the primary tumour (RMP 114) are difficult to interpret (Figure 6.7). Although the blot does not demonstrate obvious p53 expression (there is possibly a very faint band), the lack of concurrent $p19^{ARF}$ expression is unexpected. The tertiary transplant for this tumour (N3 114) expresses a protein that is larger than p53 (presumed to be a mutant protein) as well as expressing p53. The concurrent expression of $p19^{ARF}$ suggests, however, that the p53 protein is non functional.

Assuming, therefore, that expression of p53 in the absence of $p19^{ARF}$ represents the presence of functional p53 protein and that concurrent expression of p53 and $p19^{ARF}$ represents the presence of non functional p53 protein, the western blot results suggest that all the primary tumours are expressing functional p53 (RMP 114 results are difficult to analyse). Three of the primary tumour transplants (113, 115, 118) appear to retain functional p53 expression throughout *in vivo* culture. One tumour (112) retains functional p53 expression with the first *in vivo* transplantation, however it then expresses non functional p53 with the second *in vivo* passage (N2 112). Tumour 114, on the other hand, appears to express non functional protein with *in vivo* transplantation despite retention of the p53 wild type allele (as assessed by Southern blot analysis). The results are summarized in Table 6.8 and Table 6.9.

Table 6.9: Tumours expressing apparently functional p53

	Tumours expressing functional p53	Tumours with non functional p53
Primary tumours	RMP 112, RMP 113, RMP 114?, RMP 115, RMP 118	None
Primary transplant	N1 112, N1 113, N1 115, N1 118	N1 114
Secondary transplant	N2 113, N2 115, N2 118	N2 112, N2 114
Tertiary transplant		N3 114

Table 6.9 shows the tumours that express functional p53 as determined by lack of concurrent $p19^{ARF}$ expression by western blot analysis. RMP represents the primary tumour. N1, N2, N3 represent primary, secondary and tertiary transplants respectively.

6.2.2.4 Functional p53 is lost with in vitro passage

Of the 6 primary RMP tumours investigated in this study only one was successfully grown in culture. By contrast, the results suggested that it was easier to culture tumour cells once they had been passaged in *nu/nu* mice and the success rate of *in vitro* culture improved after each subsequent *in vivo* transplant. Tumours demonstrated loss of the wild type allele with early *in vitro* passage (with one exception). Consistent with this observation, western blot analysis revealed absence of p53 expression and concurrent expression of $p19^{ARF}$. Tumour RMP 115 was unusual in two ways. Firstly, it was the only primary tumour to establish successfully in culture and secondly the wild type allele was retained with prolonged *in vitro* passage. Western blot analysis of p53 and $p19^{ARF}$ expression suggested, however, that the p53 protein, whilst apparently functional at early passage was no longer functional, presumably mutant, at late passage.

As discussed before, the p53 allele was retained with prolonged *in vivo* passage of tumour 114 in association with expression of non-functional p53 protein. This would imply that the allele had mutated and one would have expected this to be a sufficiently advantageous change to be able to support subsequent *in vitro* growth. Surprisingly, however, complete loss of the p53 allele was seen to occur with *in vitro* culture which would lead one to surmise that this mutation alone may have been less potent in cell survival than the null allele.

6.3 DISCUSSION

In this Chapter the role of p53 in restricting the growth of transplanted tumours was explored. Two mouse model systems were investigated, a MMLV-induced group and a surrogate oncogene- induced group, both of which display lymphoid tumours which retain a wild type p53 allele, although both subsequently lose this gene after *in vitro* explant (Baxter et al 1996; Blyth et al 2006). *In vitro* passaging was compared with passaging tumours *in vivo* using an immunocompromised transplant model and the status of the p53 pathway in these concurrent systems was compared.

MMLV infection of mice heterozygous for the p53 tumour suppressor gene has previously been carried out using breeding stock of a mixed genetic strain (C57Bl/6, CBA/CA, NIH, SV129). Mice of this strain were seen to develop tumours, predominantly lymphoma, at between 99 and 267 days (Baxter et al 1996). In the current study an inbred C57Bl/6 strain was used and mice developed lymphoma at between 62 and 134 days. It has been shown previously that altering the strain of p53 null animals can alter the tumour spectrum observed (Harvey et al 1993a) and it is probable that this is also responsible for the alteration in latency period. Furthermore, the C57Bl/6 background strain can itself predispose mice to spontaneous lymphoma development, albeit after a prolonged latency (Stutman 1975). Differences between viral supernatant preparations used in this study and in the one reported by Baxter et al (Baxter et al 1996) may also have contributed to the different observed latent periods. The results of this chapter are consistent with previous reports showing that MMLV-infected mice generally do not exhibit loss of heterozygosity of p53 and retain the wild type p53 allele in an apparently functional form (Baxter et al 1996). The retention or loss of p53 with i*n vivo* passage of MMLV induced tumours has not previously been investigated. The results here demonstrate that there is retention of the p53 heterozygote status (Southern blot analysis) with *in vivo* passage of MMLV-infected tumours. Western results to assess expression and functionality of p53 were unfortunately not available due to insufficient tumour tissue.

When tumours from the $Runx2^{tg/-}Myc^{tg/-}p53^{+/-}$ (RMP) mouse cohort were investigated it was observed that the primary tumours retained the wild type p53 allele and that this was functional in every case. Many of the tumours also retained functional p53 with two consecutive *in vivo* transplantations. By contrast, however, although the wild type allele was retained for three consecutive *in vivo* transplants of RMP114, it appeared to be non-

functional, presumably due to mutation. As mentioned before, it is plausible that retention of a functional p53 allele is influenced by other genetic events that are likely to be specific to the individual tumour.

I observed loss of functional p53 during *in vitro* culture of all the tumours, including those tumours in which the allele was presumed to be mutant as assessed by $p19^{ARF}$ expression *in vivo*. Pressure to lose the allele during culture of the tumour would not be expected, as the presence of a mutant isoform would be anticipated to give tumour cells a growth advantage (Eliyahu et al 1984; Jenkins et al 1984). One possible explanation is that the *in vivo* tumour is expressing p53 with reduced function rather than expressing non functional p53 and that further inactivation/complete loss of p53 is necessary for successful *in vitro* growth. This is plausible since it is known that a high proportion of p53 mutations lead to synthesis of a stable full length mutant protein that may display partial, rather than complete, loss of DNA binding activity resulting in loss of some, rather than all, wild type functions (Soussi and Lozano 2005).

Another factor that should be borne in mind is that RMP mice carry only a single functional copy of p53. Other studies have suggested that p53 may be haploinsufficient for tumour suppression, which means that the reduced expression arising from a single copy is insufficient to suppress tumorigenic signals (Cook and McCaw 2000). Moreover, the degree of haploinsifficiency for p53 may vary according to tissue and tumour context (French et al 2001). Ability to suppress $p19^{ARF}$ expression may not therefore be a completely reliable guide to the functionality of the p53 allele in the tumour cell. Unfortunately, there was no time available to explore this hypothesis further by sequencing the apparently intact p53 alleles in the transplanted tumours that expressed $p19^{ARF}$.

In conclusion, the results show that loss of heterozygosity of the p53 allele and, consequently, absolute loss of protein expression, is a consistent features of *in vitro* culture. This contrasts with the *in vivo* situation where loss of heterozygosity does not occur and the p53 allele generally appears to remain functional despite prolonged *in vivo* culture of tumour cells. A reduction in the function of the p53 protein that is expressed is sometimes seen however, suggesting that this may be advantageous, although not essential, for tumour progression in late stage lymphomagenesis.

CHAPTER 7
GENERAL DISCUSSION

This study was stimulated by observations on the interaction of Moloney murine leukaemia virus (MMLV) and the p53 pathway. Thymic lymphomas arise from cells at a broad range of stages of T cell differentiation in both MMLV-infected and p53 null mice, yet interestingly synergy between the two is relatively weak (Baxter et al 1996)**.** Baxter *et al* proposed that this may be due to functional similarities between MMLV and p53 loss and it has been suggested that MMLV may have some anti-apoptotic function that spares the need to lose p53. Alternatively, it is possible that the virus may neutralise the effects of p53, either directly or via activation of target genes. I was interested therefore to investigate the role of p53 during MMLV infection and associated leukaemogenesis.

Firstly, in Chapter 3 the effect of MMLV on the growth of primary murine embryonic fibroblasts (MEFs) was investigated both in the presence and absence of functional p53. Many retroviruses demonstrate a correlation between pathogenicity and their ability to produce cytopathic effects, notably apoptosis (Weller and Temin 1981) (Bonzon and Fan 1999) (Meyaard et al 1994) (Rojko et al 1996). For instance, Mink cell focus-forming murine leukaemia viruses have been shown to trigger endoplasmic stress, induced by accumulation of high levels of a viral envelope precursor protein, which leads to apoptosis in host cells (Yoshimura et al 2000) (Yoshimura et al 2001) (Nanua and Yoshimura 2004b) (Yoshimura and Luo 2007) (Zhao and Yoshimura 2007). It has also been shown that the cytopathic effect of murine leukaemia virus strains is dependent on p53 in some instances and independent of p53 in others (Unnikrishnan et al 1999) (Nanua and Yoshimura 2004a). Similarly, evidence exists that retroviral integration induces DNA damage signals by both p53 dependent and independent routes (Skalka and Katz 2005).

Our results did not show any cytopathic effect when MEFs were infected with MMLV *in vitro*. Nor did the virus appear to induce p53 expression in these cells. It is not, however, inconceivable that MMLV may produce a cytopathic effect or induce p53 at a higher multiplicity of infection. The results did demonstrate an additive effect on cellular growth properties when MMLV was used to infect p53 null MEFs. The explanation for this may be consistent with the prominent role murine leukaemia viruses play in oncogenesis and

reflect the activity of these viruses as insertional mutagens whereby they activate target genes by promoter or enhancer insertion (Jonkers and Berns 1996). Selection of target genes by retroviral insertional mutagenesis can be rapid (Stewart et al 2007). It is possible that high throughput screening of retroviral integration sites in MMLV infected MEFs may illustrate specific gene sets that are selected *in vitro* to complement p53 loss. It might also demonstrate that some of the target genes are not represented in MMLV infected p53 null MEFs, indicating a possible functional redundancy. A correlation of these genes could then be made with those recently published in a study from the Berns laboratory identifying target genes in a high throughput screen of MMLV infected p53-/- lymphomas (Uren et al 2008).

The remaining chapters of this thesis aimed to investigate the role of p53 in MMLV and oncogene-induced T cell transformation. In Chapter 4 I confirmed the presence of a preleukaemic phase of increased apoptosis occurring *in vivo* in MMLV infected mouse thymus. Comparison with the results of Fan *et al* (Bonzon and Fan 1999) suggested that timing of the wave of increased apoptosis may vary between mouse strains. The mechanism of this apoptotic burst is not clear. It is not known whether it reflects a direct viral effect due to local replication and high concentration of the virus or whether the viral effect is more indirect and simply compromises cell function making it more susceptible to other influences leading to apoptosis. The results in this study were not conclusive although there was some suggestion that loss of p53 may interfere with MMLV-induced preleukaemic apoptosis and that p53 may, therefore, play a role in this apoptotic burst which is considered to be a significant stage in eventual tumour development. This would further imply that the synergy between MMLV and p53 loss does not arise at this early preleukaemic phase of tumorigenesis.

In Chapter 5, $Runx2^{tg/-}Myc^{tg/-}p53^{+/-}$ (RMP) mice were used as a genetic surrogate for the MMLV oncogenic programme to look at later stages of T cell lymphomagenesis. It was shown that metastasis of the rapid onset lymphomas in these mice involved no detectable loss of the p53 wild type allele. It was interesting however, that there were some tumours that demonstrated induced $p19^{ARF}$expression suggesting pressure on the p53 pathway or functional loss in at least a subset of tumour cells. Whether this pressure was greater during metastasis to non-lymphoid cells was unclear as a limitation of this study was that RMP mice show relatively restricted involvement of non-lymphoid tissues. It would be interesting to re-examine this issue in other models with a greater propensity to metastatic

spread. It is possible, of course, that functional p53 is limiting metastatic spread in these mice. In humans, loss of p53 function has been found to correspond clinically with increased metastasis. One such example is with breast cancer where accumulation of mutated p53 protein is associated with a poorer prognosis and has a significant predictive role for relapse, distant metastasis and death (Silvestrini et al 1996) (Chen et al 2002). Another example is with colorectal tumours where p53 mutations have been found to play a role in the establishment of metastatic lesions (Kastrinakis et al 1995). The role of p53 in preventing tumour growth and metastasis is complex and not restricted to its anti-proliferative and apoptotic functions. p53 is able, for instance, to exert some control on cell motility and, in this way, is able to modulate cell migration, tumour cell invasiveness and metastasis (Moskovits et al 2006) (Roger et al 2006). p53 also has an inhibitory effect on angiogenesis which impinges on tumour growth and metastasis which are dependent on neovascularisation (Dameron et al 1994) (Van Meir et al 1994) (Teodoro et al 2006) (Abbas et al 2007) (Futamura et al 2007).

The role of p53 in tumour progression was further explored in Chapter 6. The p53 allele was retained with *in vivo* transplantation of both MMLV-induced tumours and RMP tumours. This is in contrast to other viral models in which loss of the p53 pathway is critical for disease progression, as exemplified by Friend leukaemia virus (Lee et al 2003; Prasher et al). It has also been reported that Friend erythroleukaemias developing in p53 null animals are biologically more aggressive than those arising on a wild type background implying that the timing of p53 loss/mutation has a significant impact on disease progression (Prasher et al). Abelson murine leukaemia virus (Shore et al 2002; Thome et al 1997) and Human T lymphotropic virus type 1 (HTLV-1) (Pise-Masison and Brady 2005) are also examples in which inhibition of p53 is significant in disease pathogenesis.

In vitro culture, by contrast, selected strongly for wild-type allele loss. Fundamental differences between the *in vitro* and *in vivo* systems presumably account for different pressures to lose the p53 allele although significance cannot be attributed to any single factor. One major difference is the high oxygen tension present in the *in vitro* growth environment, leading to oxidative stress of cells. This factor limits the growth of any primary cell type including mouse fibroblasts which enter a senescent state that can be relieved by plating cells in a reduced oxygen tension that more closely resembles the physiological state (Halliwell 2007). Cells grown *in vitro* also lack interactions with

neighbouring cells, stroma and extracellular matrix and lack exposure to extrinsic factors such as cytokines that would normally influence their growth and differentiation (Birgersdotter et al 2005). Perhaps *in vitro* growth would have been more successful and more akin to the *in vivo* situation if I had used cell substrates that were more supportive of T cell development such as the OP9-delta notch systems (de Pooter and Zuniga-Pflucker 2007). Supplementing the growth media with cytokines such as IL-7 may also have been beneficial (Wang et al 2006).

An interesting finding from the results of the RMP cohort was the emergence of a number of p53 mutants *in vivo* (114 and 115 tumour series). It is possible that these mutants still retained some functional capacity since they were lost, along with the associated wild type allele, with subsequent *in vitro* growth. If time had permitted it would have been interesting to sequence these p53 mutants which were selected on the $p53^{+/-}$ background to see whether these conform to the known spectrum of p53 mutations that are selected on a wild-type background.

Stewart *et al* (Stewart et al 2007) observed that MMLV infection accelerated tumour onset and further increased the metastatic spread of Runx2/Myc tumours to extra-thymic lymphoid tissues. They reported that genes controlling G1 progression were common targets for retroviral insertional mutagenesis, notably the *D cyclins*, *Jdp2* and *Pim-1*, which phosphorylate p21 (among other substrates). As p21 is a major down-stream mediator of p53-mediated growth arrest, this offers a potential functional link between p53 loss and MMLV. The implication is that a G1 restriction point represents the rate-limiting step in Runx2/Myc tumour growth and that this may be overcome in a similar manner either by MMLV activation of its target genes or by direct loss of p53. The impact in both cases is limited to a slight acceleration of tumour onset and hence might be sufficient to account for the observed weak synergy between MMLV and p53 and the apparent reduction in the number of insertional hits by MMLV required for p53 null lymphomas. The hypothesis that increased cell proliferation rather than loss of apoptosis is the key to tumour growth has been suggested before by Hsu *et al* who observed accelerated lymphoma development in mice with both inactivated p53 and overexpression of c-*myc* (Hsu et al 1995).

Since completing my experiments, Uren *et al* recently used high throughput screening of MMLV-infected mice in order to identify genes that collaborate with loss of both p53 and

$p19^{ARF}$ in lymphomagenesis (Uren et al 2008). Sites of insertional mutagenesis were compared between p53 null, $p19^{ARF-/-}$ and wild type mice. Well-established oncogenes such as *Myc* and *Pim 1* were recognized in this study as frequent common insertion sites (CIS). *Jdp2*, which has previously been identified in a number of MMLV-induced tumours, was also noted here (Hwang et al ; Rasmussen et al 2005; Stewart et al 2007) However there was no significant difference in the occurrence of these CISs between the 3 genotypes suggesting that the importance of these oncogenes in tumour progression is not influenced by p53 status.

The study was then able to identify distinct gene sets that were specific for $p19^{ARF-/-}$ or $p53^{-/-}$ mice. 47 CISs were recognized to occur exclusively in the p53 null genotype whilst 55 CISs were exclusive to the $p19^{ARF-/-}$ group. 11 CISs were common to both the $p53^{-/-}$ and the $p19^{ARF-/-}$ groups. These results are consistent with the knowledge that, although $p19^{ARF-/-}$ activates p53, it also has p53–independent tumour suppression functions and emphasise the fact that the two tumour suppressor genes do not simply operate by overlapping pathways (Kelly-Spratt et al 2004; Weber et al 2000).

In conclusion, the results of this thesis show that, although loss of p53 is not obligatory for the development of MMLV-induced lymphoma, the two are weakly collaborative. The findings further support the hypothesis that this weak synergy is due to an apparent functional overlap between MMLV and p53 loss which is due, in large part, to MMLV target genes, such as the Runx2/Myc combination, which are able to suppress p53 pathways *in vivo*.

Glossary

MEFs	Murine/mouse embryonic fibroblasts
LOH	Loss of heterozygosity
MMLV	Moloney murine leukemia virus
CDK	Cyclin dependent kinases
wt	Wild type
+/-	Heterozygote
-/-	Null
+/+	Wild type
KO	Knock out
#	Number
P	Passage
nu/nu	nude

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