p53 and Responses to DNA Damage in Small Airway Epithelium

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Declaration

The written composition of this thesis is my own work, and the work described in this thesis was planned and performed by myself. Assistance from others and any collaborations are acknowledged at the relevant places within the text.

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May, 2001

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Abbreviations

8-oxo-dG	8-oxodeoxyguanosine
AAH	atypical adenomatous hyperplasia
ADP	adenosine diphosphate
ARDS	adult respiratory distress syndrome
A-T	ataxia telangiectasia
ATP	adenosine triphosphate
BaP	benzo[a]pyrene
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BrdU	5-bromo-2'-deoxyuridine
CBP	CREB-binding protein
CC10	Clara cell 10kD protein
CCSP	Clara cell secretory protein
Cdk	cyclin-dependent kinase
CFA	cryptogenic fibrosing alveolitis
CIP	Cdk-inhibitory protein
COPD	chronic obstructive pulmonary disease
Cu(I)	cuprous; univalent copper
CYP	cytochrome P450
DAB	diaminobenzidine
dNTP	deoxynucleoside triphosphate
DSBs	double strand breaks
EDTA	disodium ethylenediamine tetraacetate
Fe(II)	ferrous; bivalent iron
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
Flox	flank by loxP
GADD	growth arrest and DNA damage-inducible
GM-CSF	granulocyte-macrophage colony-stimulating factor
GST	glutathione-S-transferase
HD	homozygous deletion

IARC	International Agency for Research on Cancer
ICAM	intercellular adhesion molecule
IFN	interferon
IL	interleukin
IPF	idiopathic pulmonary fibrosis
LB	Luria broth
LOH	loss of heterozygousity
LoxP	locus of crossover of P1
LPS	lipopolysaccharide
LTR	long terminal repeat
MGMT	methylguanine-DNA methyltransferase
MIF	macrophage inhibitory factor
MMP	matrix metalloproteinase
MMTV	mouse mammary tumour virus
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MNU	N-methyl-N-nitrosourea
NER	nucleotide excision repair
NHEJ	non-homologous end joining
NNK	4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone
NSCLC	non-small cell lung carcinoma
O^6meG	O^6-methylguanine
PAGE	polyacrylamide gel electrophoresis
PAH	polycyclic aromatic hydrocarbons
PBS	phosphate buffered saline
PCAF	p300/CBP-associated factor
PCNA	proliferating cell nuclear antigen
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	phosphoinositol
ROS	reactive oxygen species
RT-PCR	reverse transcriptase polymerase chain reaction
SAP	shrimp alkaline phosphatase

SCID	severe combined immunodeficient
SCLC	small cell lung carcinoma
SDS	sodium dodecyl sulphate
SOD	superoxide dismutase
SP	surfactant protein
SSC	standard saline-citrate
SV40	simian virus 40
TBE	tris borate EDTA
TBST	tris buffered saline with 0.1% tween
TdT	teminal deoxynucleotidyl transferase
TE	tris EDTA
TEMED	N,N,N,N'-tetramethylethylenediamine
TGF	transforming growth factor
TNF	tumour necrosis factor
TTF	thyroid transcription factor
TUNEL	TdT-mediated dUTP nick end labelling
UV	ultraviolet
WAF	wild-type p53 activated factor
WHO	World Health Organisation
XP	xeroderma pigmentosum

Abstract

Acute lung injury is implicated in many respiratory diseases, including lung adenocarcinoma and emphysema. In this thesis, the hypothesis that the stress protein p53 is important in acute lung injury was investigated. p53 is a short-lived, latent transcription factor which is activated and stabilised in response to a wide range of cellular stresses, including DNA damage. In certain cell types, wild type p53 protein mediates a variety of DNA damage responses and transcriptionally modulates a battery of downstream genes involved in DNA repair, growth control, and apoptosis.

The effects of p53 were investigated in a mouse model of acute lung injury and in short-term primary cultures of isolated Clara cells. Gene targeted mice, germline deficient in p53, were exposed to γ-irradiation and compared to wild type controls. The in vivo response to DNA damage was characterised in terms of growth arrest, apoptosis, morphology, and gene expression. An acute stress response was observed in vivo, and localised to a subpopulation of the lung epithelium, the bronchiolar cells. p53 was stabilised in this population and was associated with transcriptional induction of Bax, but not other bcl-2 family members. p53 deficient mice did not display this rapid accumulation of Bax transcripts, as assessed by RNase Protection Assay. Within wild type and p53 null mice, γ-irradiation did not induce apoptosis in lung epithelial cells at any timepoints studied, as assessed by morphology, but did induce strand breaks that were detectable by TUNEL. Cell cycle activity, as assessed by BrdU incorporation, was infrequent in the lung at all timepoints, regardless of p53 status, and hence an effect of p53 on cell cycle progression was not detected in vivo.

The effects of p53-deficiency were additionally investigated in short-term primary cultures of murine bronchiolar Clara cells. Culturing of Clara cells allowed an assessment of the functional consequences of p53 deficiency in proliferating cells. Clara cells, isolated from gene-targeted p53-deficient mice, were compared to cells derived from wild type littermates. Baseline proliferation rates, as determined by BrdU incorporation, were similar irrespective of p53 status. p53 null cultures displayed abnormal morphology; specifically, a high incidence of multinucleation, which increased with time in culture. Multinucleated cells maintained expression of the Clara cell marker CC10, and were proficient in S phase DNA synthesis, as determined by BrdU incorporation. Nucleation defects in p53 -/- Clara cells associated with abnormalities in mitosis and cytokinesis, as documented by time-lapse videomicroscopy, and with increased centrosome number, determined by confocal microscopy. Defects in centrosome homeostasis, mitotic fidelity, and cytokinesis in p53-null Clara cells may reflect a novel role of p53 in preserving genomic integrity in lung epithelium.

Effects of p53-deficiency were also studied following exposure to DNA damage. A p53-dependent reduction in the BrdU index was observed in Clara cells following ionizing radiation. The reduction in BrdU index in wild type cells displayed serum-dependency, and occurred only in the absence of serum. Apoptosis was infrequent in both genotypes, as determined by time-lapse videomicroscopy. Taken together, these findings demonstrate that in murine primary Clara cell culture, growth arrest

but not apoptosis is a p53-mediated response to DNA damage, and that extracellular factors, such as serum, influence this response.

In addition, a transgenic model employing lung-specific Cre/lox technology was evaluated. The Cre/lox recombinase system evolved within bacteriophage P1 as a mechanism to maintain correct unit copy segregation of the prophage within host cells. This thesis reports application of this system to regulate gene expression in murine lung epithelial cells in vivo, with the eventual goal of generating improved mouse models of acute lung injury. Transgenic mice expressing Cre from the lung-specific promoter human SP-C were crossed to a Floxed DNA Ligase I line, and transgene stability and function assessed by PCR and Southern blot methodologies. The SP-C Cre transgene was demonstrated as stable, but of low copy-number. Excision of the floxed allele, as determined by PCR, was specific to the lung, and was not observed in other tissues. However, the level of excision was poor as assessed by Southern analysis of the excision event. Furthermore, Cre expression was undetectable by RT-PCR. Low expression levels of Cre may reflect the low copy-number of the SP-C/Cre transgene.

In summary, this thesis reports on the role of p53 in lung epithelial cells, and on the feasibility of using Cre/lox technologies to regulate gene expression in the lung epithelium of transgenic mice. Bax mRNA induction, but not apoptosis, is a DNA damage response of small airway epithelial cells. Transactivation of Bax was p53-dependent in irradiated lung, as determined by RNase protection assay, and did not occur in p53-/- mice. To further investigate the role of p53 in the lung, a method for

extracting, isolating, and culturing Clara cells, a progenitor cell of the lung, was established and incorporated into this analysis. Absence of p53 favours multinucleation and loss of cell cycle arrest in primary Clara cell culture, and highlight additional roles of p53 in cell division and growth control. In addition, this thesis explored the feasibility of using Cre/lox technologies to regulate gene expression in murine lung epithelium. SP-C/Cre mice were assessed in their ability to excise a Floxed DNA Ligase I cassette in the tissues of double transgenic mice. Cre-mediated excision was specific to lung epithelium, but infrequent.

1. Introduction

1.1. BIOLOGY OF SMALL AIRWAY EPITHELIUM

1.1.a. THE BRONCHIOLE

At least eight principal types of epithelial cells have been identified in the linings of the tracheobronchial conducting airways in mammals (reviewed in Harkema et al., 1991). Four of these cell types – mucous goblet cells, serous cells, small mucous granule cells, and basal cells – are restricted anatomically to the proximal airways and will be discussed no further. The remaining four cell types – the ciliated cell, the neuroendocrine cell, the brush cell, and the Clara cell – are often found in both the proximal and distal conducting airways of mammals, although with marked interspecies differences in distribution. The behaviour of these cell types is integral to our understanding of bronchiolar physiology, and their biology is summarised below (see Figure 1.1.i).

1.1.a.1. Ciliated cells

The ciliated cell engenders mucous flow towards the tracheobronchial escalator, a function generated by the numerous motile cilia that line the luminal surface, facilitating pulmonary clearance of particulates, dead cells and excess surfactant. The cilium is composed of a set of characteristic tubules the structures and arrangement of which vary over its length. Long microvilli are interspersed among the cilia along the apical surface of the cell. The numerous microvilli and cilia

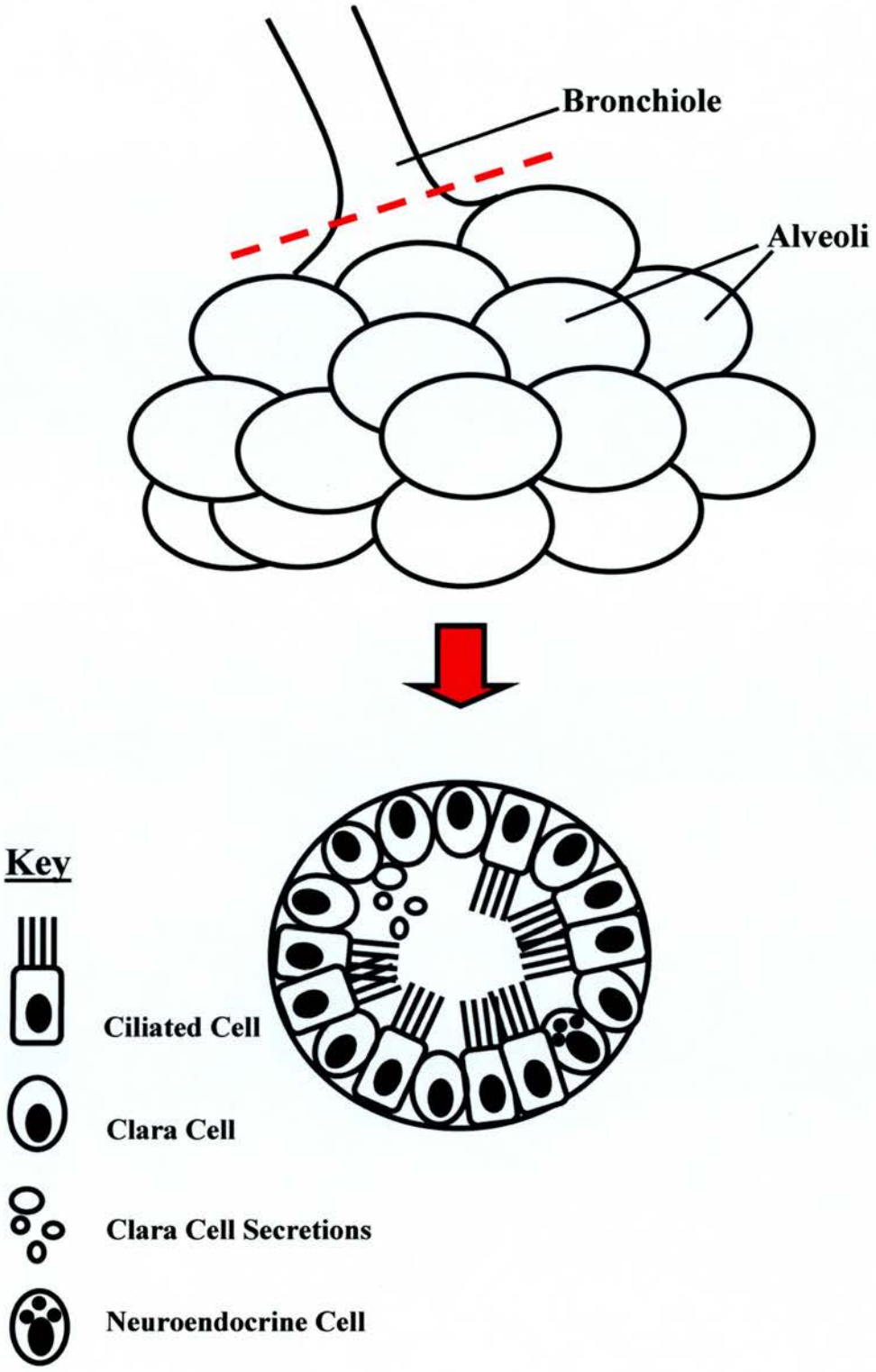


Figure 1.1.i. A model describing the cellular constituents of the bronchioli. The bronchioli consist of multiple cell types, including ciliated cells, Clara cells, and neuroendocrine cells. Ciliated cells engender mucous flow towards the bronchi, and thus facilitate pulmonary clearance. Clara cells secrete surfactant, which assist in alveolar integrity and pulmonary clearance. Clara cells additionally serve as progenitor cells of the bronchioles, and differentiate into ciliated cells as required. Neuroendocrine cells are occasionally observed in the bronchioli. It is suspected that neuroendocrine cells participate in remodelling of the conducting airways.

provide the cell with added luminal surface area that may additionally be important for ion exchange between the extracellular airway fluid and the intracellular cytoplasm (Harkema et al., 1991).

Ciliated cells are the prominent cell type in the bronchi and bronchioles, but are particularly susceptible to injury from inhaled irritants (Harkema et al., 1987; Nikula et al., 1988). Ciliated cells are terminally differentiated cells, and regeneration of denuded epithelium, a consequence of irritant exposure, is dependent on the recruitment of Clara cells (Plopper & Dungworth, 1987).

1.1.a.2. Neuroendocrine cells

Neuroendocrine cells are rare in the airway epithelium of most species (Harkema et al., 1991). When present, they usually lie in a basal position, but may have a cytoplasmic projection to the airway surface. These cells can occur individually or as clusters of cells, termed neuroepithelial bodies, and are thinly scattered at the laryngotracheal junction and at bifurcations of intrapulmonary bronchi (Terzakis et al., 1972; Sorokin et al., 1983). The presence of these cells increases from the main bronchi to the bronchioli, but neuroendocrine cells are infrequently found in terminal bronchioli (Tateishi, 1973).

Numbers of neuroendocrine cells are thought to be increased in chronic lung diseases such as bronchopulmonary dysplasia, cystic fibrosis, and asthma (Aguayo, 1993; Gosney et al., 1989; Johnson et al., 1993). Extended exposure to a variety of pollutants such as tobacco smoke (Sunday et al., 1988), nitrosamines (Linnoila,

1982), and napthalene (Peake et al., 2000) results in neuroepithelial body cell hyperplasia. Neuroepithelial body hyperplasia occurs principally at airway branch points and, by virtue of their association with proliferating Clara cells, a role in remodelling of the conducting airways has been suggested (Reynolds et al., 2000).

1.1.a.3. Brush cells

Brush cells have been identified in the conducting airways of a number of species and are present along the full length of the respiratory airway (Harkema et al., 1991). Their name is derived from the brush border of closely packed microvilli that coats their apical surface. They are present infrequently, making up fewer than 1% of the total cell population. However, their presence has not been demonstrated convincingly in humans (Harkema et al., 1991). The function of the brush cell is unknown, but roles in absorption of periciliary fluid, chemoreception, and ciliogenesis have been suggested (Jeffery, 1987). Lack of brush cells in human columnar epithelium dictates that their contribution to small airway disease is improbable.

1.1.a.4. Clara cells

Clara cells express Surfactant proteins SP-A, B, and D, which are secreted into the lumen and assist in alveolar integrity (reviewed in Plopper et al., 1997), and a secretory protein homologous to uteroglobin with similar antiinflammatory properties (Gupta et al., 1987; Gupta & Hook, 1988; Lopez de Haro et al., 1988; Singh et al., 1988; Johnston et al., 1999). This secreted protein, Clara cell 10kDa protein (CC10), is expressed specifically in Clara cells and is frequently used as a

cell marker (Boers et al., 1999; McBride et al., 2000). Clara cells additionally express Cytochrome P450 isoenzymes, the nature of which vary between species (Plopper et al., 1997; Harkema et al., 1991). The pattern of P450 isoenzyme expression accounts for Clara cell sensitivity to certain aromatic hydrocarbons, such as napthalene.

The observation that ciliated cells develop from Clara cells (Plopper & Dungworth, 1987; Evans et al., 1976; Brody et al., 1987; Hook et al., 1987) revived an interest in the Clara cell as a potential stem cell in the airways. The distal migration of Clara cells in the lungs of animals exposed to ozone, and the frequent 'bronchiolisation' of alveoli in lungs with chronic injury further suggest a role of the Clara cell in alveolar remodelling. However, it is noteworthy that Clara cells display marked interspecific heterogeneity in their distribution (Plopper et al., 1980a,b,c; Widdicombe & Pack, 1982). Significantly, in rodents Clara cells occupy tracheal, bronchial, and bronchiolar epithelium, whereas primates accommodate Clara cells in only the epithelium lining terminal and respiratory bronchioles (Harkema et al., 1991; Boers et al., 1999). It is suggested that adenocarcinoma of the lung, a tumour of the small airways, represents malignancy of the Clara cell (see section 1.2.c). Consequently, there is great interest in the effects of xenobiotics and other DNA-damaging agents on Clara cells.

Clara cell isolation and enrichment, a procedure involving dissociation of airway epithelia and separation of cell types by density and differential attachment, has been used to investigate key features of Clara cell physiology including xenobiotic

transformation and lectin binding patterns (Murphy et al., 1999; McBride et al., 2000). Short term primary Clara cell cultures from the mouse can be maintained for up to 5 days in serum-free medium, and represent a powerful tool for assessing the effects of gene deficiency in lung epithelial cells.

1.1.b. THE ALVEOLUS

The alveolus is the quintessential compartment of the lung and is the principal site of gas exchange (see Figure 1.1ii). Gas exchange is accomplished over the respiratory membrane, where epithelium is separated from endothelium by, in health, only a thin interstitium. Thickening of the alveolar interstitium is a common response to inhaled irritants and induces respiratory distress via loss of the surface area over which gas exchange can take place. Alveolar epithelium is composed of two cell types, named the Type I and Type II cell.

1.1.2.1. Type I cells

Type I cells are flattened, highly branched squamous cells, and are the principal cell type associated with gas exchange (reviewed in Harkema et al., 1991). Massive surface area to volume ratio of these thread-like cells enables efficient gas exchange between the capillary endothelium and the alveolar lumen. Type I cells are particularly susceptible to damage by inhaled pollutants, but are regenerated from cuboidal Type II cells that otherwise reside in the corner of the alveolus.

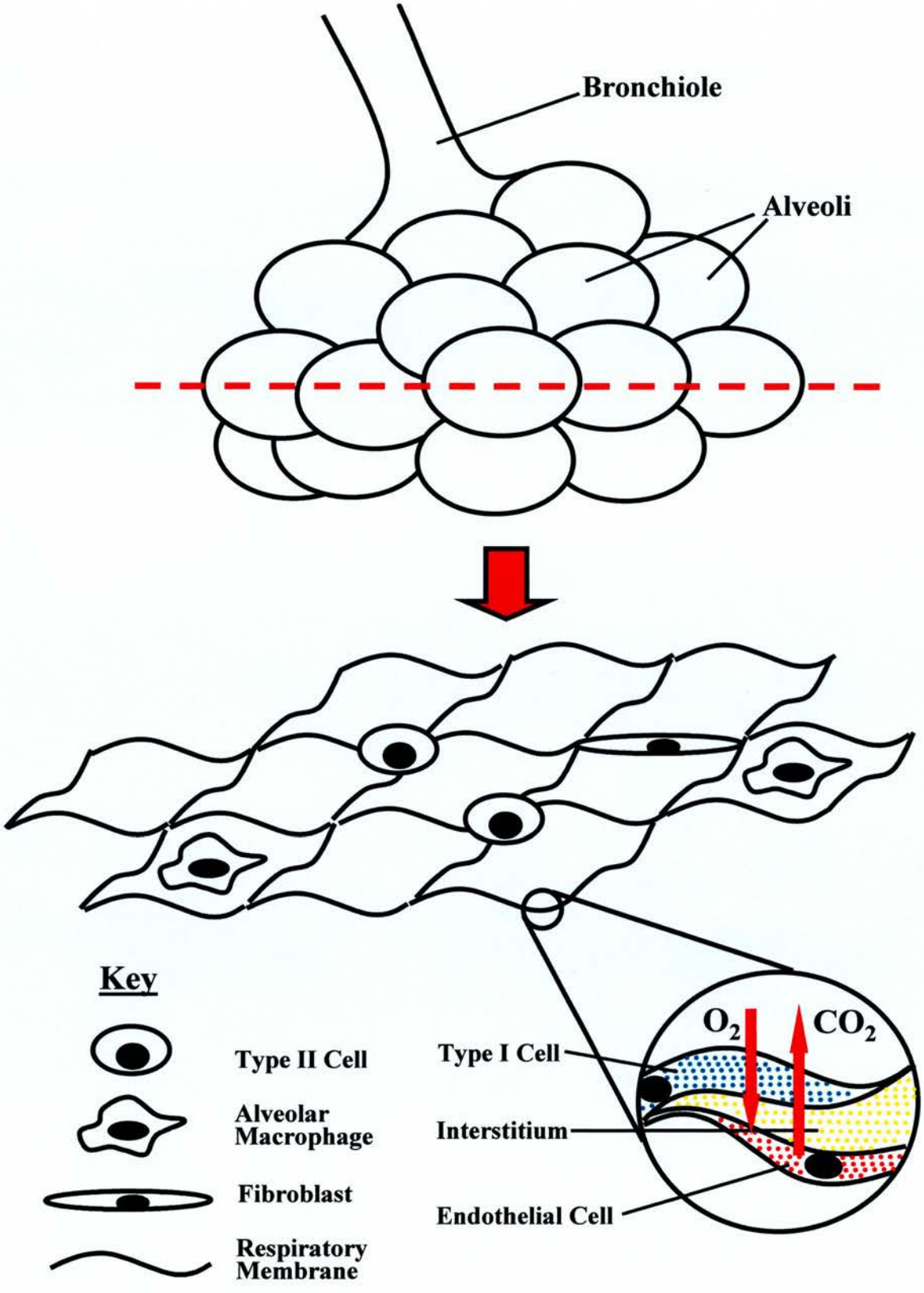


Figure 1.1.ii. A model describing the cellular constituents of the alveoli. The alveoli consist of multiple cell types, including Type and Type II epithelial cells, endothelial cells, fibroblasts and macrophages. Gas exchange is accomplished over the respiratory membrane, where Type I cells are juxtaposed to endothelial cells with only a thin interstitium in between. Type II cells assist in alveolar integrity via secretion of surfactant. Fibroblasts are occasionally observed in the alveoli, scattered throughout the interstitium. Alveolar macrophages reside in the alveolar lumen, where they assist in pulmonary clearance of irritants and infectious particles.

1.1.2.2. Type II cells

Type II cells are the progenitor species of the alveolus and differentiate into Type I cells as required (reviewed in Plopper et al., 1997). Type II cells comprise about 15% of the cells in the distal lung, but because of their cuboidal shape they account for only 10% of the alveolar surface. Type II cells additionally express surfactant proteins and P450 enzymes, and thus show some similarities with Clara cells. In addition, a role of type II cells in the transepithelial transport of sodium, a process that minimises alveolar fluid, has been reported (Harkema et al., 1991). To date, type II cells have been isolated from rabbit (Devereux & Fouts, 1981; Finkelstein et al., 1983), rat (Mason et al., 1977; Mason, 1982; Richards et al., 1987), hamster (Murphy et al., 1999), guinea pig (Sikpi et al., 1986), pig (Murphy et al., 1999), and human (Robinson et al., 1984; Devereux et al., 1986), but not mouse lungs which require ex vivo expansion from foetal explants (Glasser et al., 1991; Shannon, 1994). The major uses of freshly isolated type II cells are for studies of metabolism (Mason, 1978; Devereux, 1984; Devereux et al., 1986; Geppert & Elstein, 1983; Maniscalco et al., 1983) and injury response (Freeman et al., 1986; van Klaveren et al., 1997a,b; Planus et al., 1999; Piotrowski et al., 2000). The relative difficulty in culturing type II cells from adult mice has hindered genetic dissection of the injury response pathways specific to this cell type.

1.2. DISEASE OF THE SMALL AIRWAYS

The small airways are a frequent site of injury to inhaled irritants and infectious particles. In certain pathologies, such as the infectious pneumonias, the aetiological agent is known and its contribution to disease understood. In other pathologies, including ARDS, CFA, lung cancer, and emphysema, DNA damage has been proffered as a significant factor in the aetiology of the disease.

1.2.a. ARDS

Acute Respiratory Distress Syndrome (ARDS) first became recognised as a major clinical phenomenon during the Vietnam War (Ashbaugh et al., 1967). An unconventional way of killing people with conventional weapons, ARDS was primarily recognised as a common response to smoke inhalation and, later, to reactive oxygen species in general. ARDS is additionally a complication of sepsis.

ARDS is a common response to high dose ionizing radiation, hyperoxia, and chemotherapeutics such as bleomycin (reviewed in Canonico & Brigham, 1997). Insidious oxidative species generated by exposure to these agents activate an inappropriate vascular response, culminating in thickening of the alveolar interstitium and destruction of the respiratory membrane. Mutant mice deficient in antioxidant enzymes, such as Extracellular Superoxide Dismutase (Carlsson et al., 1995), are particularly susceptible to ARDS and display reduction in survival time and an earlier onset of oedema. ARDS is additionally a feature of sepsis, and can be induced by intratracheal or intravenous administration of lipopolysaccharide (LPS)

or tumour necrosis factor-α (TNF-α) (Murakami et al., 2000; Burdon et al., 2000). ICAM-1 induction in the pulmonary vasculature, which is TNF-α dependent, is considered a necessary event in the aetiology of the disease (Sato et al., 2000; Welty et al., 1997). In addition, Migration Inhibitory Factor (MIF), secreted by alveolar macrophages, has been identified as a critical determinant in the development of ARDS (Donnelly et al., 1997). MIF is observed in the alveolar airspaces of patients with ARDS and augments proinflammatory cytokine secretion and glucocorticoid responses in the small airways. The authors suggest that MIF may act as a mediator sustaining the pulmonary inflammatory responses in ARDS (Donnelly et al., 1997). The latter analysis highlights the importance of the alveolar macrophage in the aetiology of ARDS.

DNA damage has been observed in alveolar epithelium of ARDS in both humans and mice, and is associated with p53 induction (Guinee et al., 1996; O'Reilly et al., 1998; Okudela et al., 1999). Given the importance of p53 in DNA damage responses in other cell types, it is plausible that p53 may additionally contribute to the aetiology of ARDS. However, mice germline deficient in p53 are equally susceptible to the oedematous changes characteristic of ARDS (Santana et al., 1996; see 1.5.b). Thus, although p53 is induced in ARDS, this implies no causal role of p53 in disease aetiology. It is probable that p53 induction in the latter syndrome reflects genotoxic damage, a direct consequence of exposure to reactive oxygen species (see 1.3.a.2).

1.2.b. Cryptogenic Fibrosing Alveolitis

An alternative outcome to bleomycin and radiation-induced pneumonitis is the organisation condition Cryptogenic Fibrosing Alveolitis (CFA), also known as Idiopathic Pulmonary Fibrosis (IPF). CFA is a progressive illness recognisable histologically by thickening of the alveolar walls and collagen deposition, and immunochemically by inappropriate cytokine expression (Rube et al., 2000; Hong et al., 1999; Buttner et al., 1997). Animal models of CFA have been developed by exposure to ROS (Franko et al., 1991; Haston & Travis, 1997; Piguet et al., 1997), and by intratracheal instillation of the fluorescent hapten FITC (Roberts et al., 1995). In the latter analysis, fibrotic lesions developed principally at sites of hapten deposition, highlighting local effects of toxic particulates.

CFA is observed infrequently in immunodeficient SCID and nude mice (Roberts et al., 1995; Schrier et al., 1983; Szapiel et al., 1979), thus appears to be at least partially dependent on the presence of T and/or B lymphocytes. High levels of TNF-α and IFN-γ segregate with fibrosis in bleomycin-treated animals, and suggest a role for inappropriate cytokine responses in the induction of fibrosis (Helene et al., 1999). Furthermore, transgenic mice overexpressing the proinflammatory cytokines TGF-α, TNF-α, and PDGF display pulmonary fibrosis from an early age (Korfhagen et al., 1994; Miyazaki et al., 1995; Hoyle et al., 1999). The tissue remodelling characteristic of CFA is thus unlikely to represent a response to DNA damage, but rather inflammatory and fibrotic sequelae induced by inappropriate leukocyte activation. Importantly, CFA can be induced independently of p53 as it is observed following bleomycin and ionizing radiation-treatment in p53-null mice in a manner

indistinguishable from wild type littermates (Okudela et al., 1999; Santana et al., 1996).

1.2.c. Lung Cancer

Lung cancer is a major health problem worldwide due to its high frequency, especially in developed countries, and accounts for over a million deaths, annually (reviewed in Hecht, 1999). Cigarette smoking is responsible for 90% of lung carcinoma in men, and 75-80% in women, and there is epidemiological evidence for a dose-response relationship with the duration and amount of smoking. Due to the advanced stage of malignancy at which it is often first diagnosed, lung cancer in humans is often difficult to treat and exhibits an extremely high mortality rate (Aisner et al., 1996; Hecht, 1999). Consequently, there is increasing interest in the normal molecular mechanisms that prevent lung tumourigenesis, which allow for improved definitions of carcinogenesis, improved patient diagnosis, and offer novel sites of therapeutic intervention.

Lung cancer is the result of a sequential accumulation of multiple genetic alterations involving key genes involved in growth control and apoptosis. The malignant transformation of lung epithelial cells involves mutational/dysregulational activation of oncogenes, including Ki-ras, c-Myc, C-erb-B2, and mutational inactivation of tumour suppresor proteins, such as p53 and Rb (Table 1.2). Inactivation of tumour suppressor genes removes important regulatory constraints on the cell cycle, and is considered a necessary feature of lung tumorigenesis. Smoking induces a range of lung carcinomas in humans, and is associated with the development of squamous,

Table 1.2. – Molecular and Genetic Abnormalities in Lung Cancer

Gene	Abnormality	Frequency (%)		References
		SCLC	NSCLC	
Oncogenes				
Ki-ras	point mutation (codon 12)	0	30	Slebos & Rodenhuis, 1992; Mitsudomi et al., 1991; Rodenhuis et al., 1988
Bcl-2	overexpression	75	30-60	Stefanaki et al., 1998; Laudanski et al., 1999
c-erbB2	overexpression	<10	25-35	Salgia & Skarin, 1998; Volm et al., 1992; Kern et al., 1990
c-Myc	overexpression	10-40	10-50	Prins et al., 1993; Volm et al., 1992; Kiefer et al., 1987; Wong et al., 1986
Tumour Suppressor Genes				
p53	LOH + mutation	80-100	50-80	Sameshima et al., 1992; Takahashi et al., 1989
Rb	LOH + mutation	80-90	20-30	Mori et al., 1990; Harbour et al., 1988
p16	HD, LOH + mutation, methylation	<10	60	Okamoto et al., 1995; Kamb et al., 1994

HD, homozygous deletion; LOH, loss of heterozygousity; SCLC, small cell lung carcinoma; NSCLC, non-small cell lung carcinoma

neuroendocrine, and non-small cell lung carcinomas (Hecht, 1999; Aisner et al., 1996). The term adenocarcinoma is used to define a subsection of non-small cell lung carcinomas defined histologically by the presence of secretory vesicles, glandular differentiation, and/or mucin production (Travis et al., 1999). Lung adenocarcinomas can be experimentally induced in animals by treatment with tobacco carcinogens (reviewed in Hecht, 1999). Consequently, rodent lung adenocarcinoma is considered a model of human lung carcinogenesis. Spontaneous lung adenocarcinomas are observed in certain strains of mice, notably the A/J strain, and highlight the effect of genetic background on lung tumourigenesis (Wattenberg, 1999; Moody et al., 2000; Wardlaw et al., 2000). Spontaneous lung adenocarcinomas are additionally a feature of p53-mutant mice and humans, the significance of which is discussed in later chapters (see 1.5.b and 4.2).

Lung adenocarcinoma proceeds through a series of defined morphological stages, from atypical adenomatous hyperplasia (AAH), through carcinoma in situ (adenoma), to overt carcinoma (adenocarcinoma) (Kitamura et al 1995; 1996). Metastases of lung adenocarcinoma are often observed in brain, liver and gut (Aisner et al., 1996). Malignant transformation of lung epithelial cells involves mutation and/or dysregulation of key oncogenes and tumour suppressor genes. Mutations in Ki-ras, Rb and p53 are early events in lung adenocarcinoma, and may highlight critical steps in lung tumourigenesis (Rodenhuis et al., 1988; Harbour et al., 1988; Takahashi et al., 1989; Mori et al., 1990; Mitsudomi et al., 1991; Sameshima et al., 1992; Slebos & Rodenhuis, 1992). In this respect, the effects of combined p53 and Rb deficiency have been investigated in the lungs of transgenic mice. SV40 T

antigen inhibits both p53 and Rb signalling, and induces adenocarcinoma formation in transgenic mice when expressed from the human SP-C promoter (Wikenheiser et al., 1992). The human SP-C promoter directs ectopic gene expression in the distal portion of the developing lung, and in both Clara cells and type II cells of adult transgenic mice. It is suggested that lung adenocarcinoma of SP-C/SV40 T antigen mice represents malignancy of the Clara cell (Wikenheiser et al., 1992). Notwithstanding this, early-stage tumours of these mice frequently express Clara cell markers in vivo. However, it is noteworthy that in late-stage adenomas of SP-C/SV40 T antigen mice, expression of Clara cell markers is often lost. By contrast, type II cell markers are observed in late-stage adenomas and in cell lines established from SP-C/SV40 T antigen mice. Thus in both tumours and cell lines there is a blurring of the distinction between Clara and type II cells, a phenomenon which the authors suggest may reflect altered differentiation of the advancing neoplasms (Wikenheiser et al., 1992).

1.2.d. COPD and Emphysema

Several terms historically have been used to designate the group of conditions recognised by obstruction to airflow. Chronic obstructive pulmonary disease (COPD) is a clinical term denoting a group of diseases "characterised by persistent slowing of airflow during expiration" (Calverley & Pride, 1995). Thus, the term is a clinical rather than a pathological diagnosis, and embraces both chronic bronchitis and emphysema, which often develop together, as the underlying cause of disease. Emphysema is one pathological correlate of COPD and is an alternative outcome of life-long tobacco smoking. Defined pathologically as the distension of alveolar

airspaces and destruction of alveolar walls, emphysema is a progressive disease which induces respiratory distress via loss of the surface area/volume ratio over which gas exchange is accomplished. Two major patterns of emphysema - panacinar and centriacinar - are prevalent in the human species (Figure 1.2). Panacinar emphysema is an inherited recessive disorder in humans, and is primarily associated with germline disruption of the alpha-1-antitrypsin gene. Humans and mice displaying alpha-1-antitrypsin deficiency develop spontaneous panacinar emphysema from an early age, and are additionally prone to cirrhosis of the liver. Panacinar emphysema is additionally a feature of the spontaneous mutant mouse strains beige (Keil et al., 1996), tight-skin (Kielty et al., 1998), blotchy (Fisk & Kuhn, 1976), and pallid (Keil et al., 1996), each strain incorporating mutations in single distinct genetic loci. Consequently, panacinar emphysema is a polygenic disease associated with disturbances in extracellular matrix metabolism. Panacinar emphysema can be induced experimentally by intratracheal instillation of elastolytic enzymes, including papain, pancreatic elastase, neutrophil elastase, and proteinase 3 (Gross et al, 1965; Snider et al., 1986). Airspace enlargement is additionally a feature of transgenic mice overexpressing collagenase, interleukin-11, or PDGF-B specifically in lung epithelium (D'Armiento et al., 1992; Ray et al., 1997; Hoyle et al., 1999). Importantly, panacinar emphysema is not induced in experimental animals by non-elastolytic proteolytic enzymes, such as bacterial collagenase (Shapiro, 2000).

Centrilobular emphysema is the dominant form of the disease in humans, is principally associated with life-long tobacco smoking, but is an additional feature of occupational exposure to cadmium, asbestos, silica, and coal dust. Centrilobular

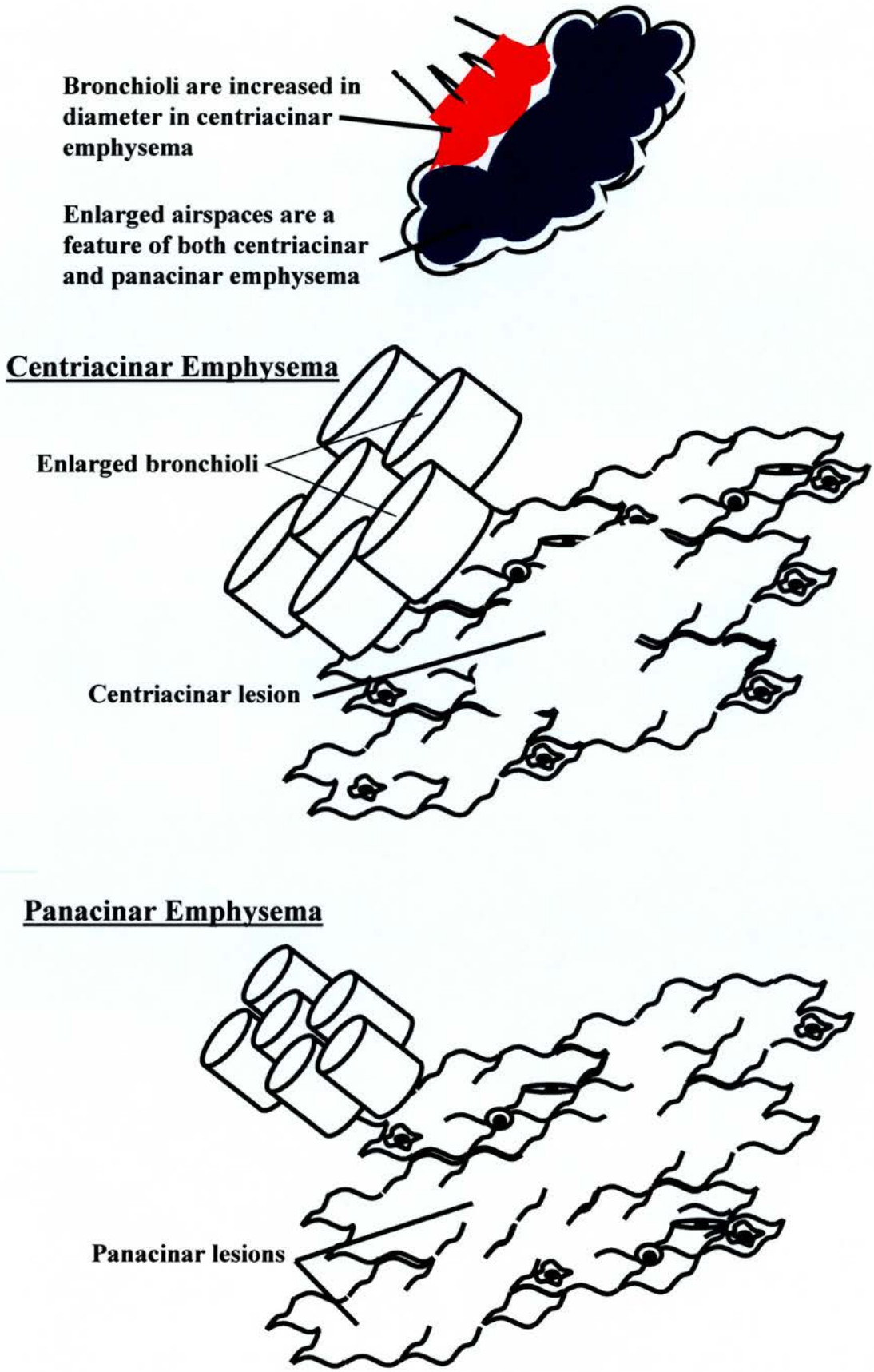


Figure 1.2. A model describing patterns of emphysema. Centriacinar emphysema is characterised by enlarged airspaces, and typically involves respiratory bronchioles. Panacinar emphysema involves the entire acinus, or more commonly entire lobules (panlobular), but is not localised to the centriacinus.

emphysema is characterised grossly by enlarged airspaces, generally measuring from 1 to 10mm in diameter, surrounded by normal lung tissue (Calverley and Pride, 1995). A typical centrilobular lesion involves respiratory bronchioles, which gives the lesion a centriacinar location (Figure 1.2). The mechanism by which tobacco smoke induces emphysema is complex, but with macrophage elastase identified as a candidate intermediate. Macrophage elastase (MMP-12) is expressed in human alveolar macrophages of cigarette smokers and in patients with emphysema, but not in normal lung tissue (Shapiro, 2000). Knockout mice deficient in MMP-12 mice have been generated and are resistant to the emphysemous changes typical of chronic cigarette smoke exposure (Hautakami et al., 1997). Surprisingly, MMP-12-/- mice also fail to recruit macrophages into their lungs in response to cigarette smoke (Hautakami et al., 1997; Shapiro, 2000). Based on these observations, Shapiro (2000) suggests a model of human emphysema by which cigarette smoke induces constitutive macrophages to produce MMP-12, which cleaves elastin, generating fragments chemotactic for monocytes. A positive feedback loop is generated which perpetuates macrophage accumulation and concomitant lung destruction. However, it should be emphasised that these observations do not account for the bronchiolar enlargement typical of human smoking-related emphysema. Indeed, centriacinar emphysema is infrequent in experimental animals. Consequently, animal models of emphysema are interesting, but limited. An alternative strategy is to understand precise features of injury responses in bronchiolar cells. Methods for culturing bronchiolar Clara cells from the mouse have been developed and have been used to discern the role of bronchiolar cells in xenobiotic metabolism. Primary Clara cell culture technology could additionally be exploited to determine injury responses of

bronchiolar cells. Understanding the genetic basis of bronchiolar cell injury responses in vitro may represent a method of addressing the molecular mechanisms underlying human centriacinar emphysema.

1.3. DNA DAMAGE AND THE LUNG

1.3.a. Tobacco Smoking and Lung Disease

The literature on the toxic effects of tobacco smoke is huge, hence the following discussion is brief. Tobacco smoking has proved a most effective method of delivering toxins to the lung. Extensive epidemiological data establish cigarette smoking as the major cause of lung cancer, and it is estimated that about 90% of male lung cancer deaths, and 75-80% of female lung cancer deaths in the United States each year are caused by smoking (reviewed in Hecht, 1999). The risk of lung cancer diminishes after smoking cessation, but not during the first five years, and the relative risk never returns to that of a non-smoker (Hecht, 1999). Chronic Obstructive Pulmonary Disease, the clinical manifestation of emphysema, is an additional smoking-related disease, and represents the 4th leading cause of death in the United States (WHO, 1999). Smoking-related emphysema is characterised biochemically by protease/antiprotease and oxidant/antioxidant imbalances, and is considered a consequence of chronic neutrophil and macrophage hyperactivity in the airways (Shapiro 1999; 2000). DNA damage of brochioalveolar cells is a feature of smokers (De Flora et al., 1996), and chronic DNA damage may contribute to the development of smoking-related emphysema.

Tobacco smoke contains a rich source of DNA-damaging agents, which can be mechanistically subdivided into carcinogens requiring metabolic activation, and reactive oxygen species.

1.3.a.1. Tobacco Carcinogens

There are 55 carcinogens in tobacco smoke that have been evaluated by the International Agency for Research on Cancer (IARC) and for which there is "sufficient evidence for carcinogenicity" (reviewed in Hecht, 1999). Of these, the polycyclic aromatic hydrocarbon (PAH) benzo[a]pyrene (BaP), and the nitrosamine 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (NNK) are the most extensively studied. BaP's ability to induce lung tumours upon local administration or inhalation is well documented (Hecht, 1999; Thyssen et al., 1981; Morse et al., 1995). It is however noteworthy that when administered systemically, it causes lung tumours in mice, but not in rats (Hecht, 1999; Culp et al., 1998). The tobacco-specific N-nitrosamine NNK is a potent lung carcinogen in rats, mice, and hamsters (Hecht, 1998). NNK is organospecific for the lung, and induces adenoma and adenocarcinoma formation independent of the route of administration (Hecht, 1999). Tobacco smoke is additionally a tumour promoter, the majority of this activity being dependent upon uncharacterised weakly acidic compounds (Hecht, 1999). Substantial levels of cocarcinogens, such as catechol, decane, pyrene, and benzo[e]pyrene are present in tobacco smoke. In addition, cigarette smoke contains high levels of acrolein, which is cytotoxic to the pulmonary cilia (Hecht, 1999).

Carcinogens in tobacco smoke are enzymatically transformed to a series of metabolites as the exposed organism attempts to convert them to forms that are more readily excreted. The initial steps are usually carried out by the cytochrome P450 (CYP) enzymes, which oxygenate the substrate. Other enzymes, such as lipoxygenases and cyclooxygenases, may additionally be involved. The oxygenated

intermediates formed in these initial reactions may undergo further transformation by glutathione-S-transferases (GST), uridine-5'-diphosphate-glucuronosyl-transferases, sulphatases, and other enzymes, a process known as detoxification (Spitz et al., 1999; Shields & Harris, 2000). Some of the metabolites produced by the P450s react with DNA to form covalent adducts, and thus elicit damage requiring excision and mismatch repair processes. Genes encoding carcinogen-metabolising enzymes, such as CYP1A1, N-acetyltransferase 2, and GSTM1 are polymorphic within the human population, and it is thought they may confer both resistance and susceptibility traits to smoking-related disease (Spitz et al., 1999; Shields & Harris, 2000).

1.3.a.1.i. O^6-methylguanine

Several studies have documented the presence of alkylated guanosine residues, particularly, 7-methyldeoxyguanosine in the lungs of smokers (Kato et al., 1993; Shields et al., 1990; Mustonen et al., 1993; Kato et al., 1995;Blomeke et al., 1996; Petruzzelli et al., 1996). While 7-methylguanine is not generally considered a miscoding adduct, other methyl adducts which do have miscoding properties, such as O^6-methylguanine (O^6meG), are formed at the same time, but at lower levels (Hecht, 1999). O^6meG is a mutagenic and cytotoxic DNA adduct that can be formed in vivo by such diverse agents as tobacco smoke, methylnitrosurea, and other methylating agents (Hecht, 1999). In vitro studies (Singer et al., 1989; Dosanjh et al., 1993; Snow et al., 1984), as well as in vivo mutagenesis assays (Bhanot & Ray, 1986; Loechler et al., 1984), have shown that O^6meG preferentially base pairs with dTMP instead of dCMP, thus giving rise to G to A transition mutations. The importance of these lesions has been demonstrated in vivo. Specifically, a strong correlation was

found between the persistence of O^6meG lesions and tumours in rodents (Goth & Rajewsky, 1974). Furthermore, the persistence of O^6meG lesions after treatment with 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone (a component of tobacco smoke) has been found to correlate with the activation of the Ki-ras oncogene in lung tumours in mice (Devereux et al., 1993).

The O^6meG lesion can be readily repaired in a saturable manner by the suicide enzyme methylguanine-DNA methyltransferase (MGMT) (Pegg, 1990). Repair occurs via the transfer of the methyl group to a cysteine residue in the MGMT protein, a process that is stoichiometric and autoinactivating (Margigson & O'Connor, 1990; Pegg, 1990). This type of repair is error free and noncytotoxic. However, in many human solid tumour cell lines and in some non-tumour tissues the ability to repair O^6mG is lacking due to a failure to activate MGMT (Zaidi & O'Connor, 1995; Pegg, 1990; Yarosh et al., 1983, Yarosh et al., 1984; Day et al., 1980). In this respect, it is noteworthy that MGMT induction following DNA damage is p53-dependent in tissues of wild type mice (Rafferty et al., 1996). The in vivo consequences of MGMT deficiency are best understood in the lethal response to chemotherapeutic agents. Knockout mice, germline deficient in MGMT, are 2- to 10-fold more sensitive to the lethal effects of the O^6-alkylating agents MNU, BCNU, and temozolamide than wild type mice (Glassner et al., 1999), but show similar sensitivities to cross-linking agents such as cyclophosphamide and mitomycin C (Shiraishi et al., 2000; Glassner et al., 1999). Consequently, MGMT is of critical importance in protecting against the toxic effects of alkylating agents, such as those used for cancer chemotherapy. Furthermore, transgenic mice overexpressing MGMT

have been generated, and are relatively resistant to NNK-induced lung tumourigenesis (Liu et al., 1999), documenting tumour suppressor properties of MGMT, and highlighting a potential protective role of MGMT in tobacco carcinogenesis. Based on these observations, Hecht (1999) and others (Liu et al., 1999) speculate that formation of O^6-methylguanine lesions is the principle mutagenic effect of the tobacco-specific nitrosamine NNK.

1.3.a.2. Reactive Oxygen Species

Tobacco smoke additionally contains high concentrations of reactive oxygen species (ROS), which are strongly implicated in diseases related to smoking, particularly cancer (Ames, 1983; Church & Pryor, 1985; Pryor, 1987). Free radicals from tobacco smoke severely deplete the pool of intracellular antioxidants in living cells, and induce oxidative damage in humans and experimental animals (Leanderson & Tagesson, 1993). Free radicals can attack DNA bases or deoxyribose residues to produce damaged bases or strand breaks (Dizdaroglu, 1993). Alternatively, ROS can oxidise lipid or protein molecules to generate intermediates that react with DNA to form adducts (Cheeseman, 1993). It is established that superoxide radical, lipid peroxides and hydrogen peroxide are not sufficiently reactive per se to attack DNA (Meneghini & Hoffman, 1980; Brawn & Fridovich, 1981; Lesko et al., 1980). It is instead proposed that relatively stable species, such as hydrogen peroxide and lipid peroxide, migrate from their generation site and react with nuclear Fe(II) or Cu(I) to produce hydroxyl radical and alkoxyl radicals in site-specific Fenton reactions (Mello-Fihlo & Meneghini, 1991) (Figure 1.3). Being generated close to DNA, these radicals may then induce DNA damage.

Figure 1.3 – The Fenton Reaction. Hydrogen peroxide (i) and alkylated hydroxides (ii) react with Fe(II) to generate hydroxyl radical and alkoxyl radical, respectively. It is assumed that iron ions are bound to DNA and therefore that the Fenton reaction generates hydroxyl/alkoxyl radicals *in situ*. Neocurpoine, a copper specific lipophilic chelator which blocks the Cu-mediated Fenton reaction, does not protect cells from H_2O_2, implying that iron, and not copper, is the metal mediating the damage in most cases (Mello-Fihlo & Meneghini, 1991).

i) $H_20_2 + Fe^{2+} \rightarrow OH^{\bullet} + OH^{-} + Fe^{3+}$

ii) $ROOH + Fe^{2+} \rightarrow RO^{\bullet} + OH^{-} + Fe^{3+}$

1.3.a.2.i. 8-oxo-dG

Free radicals induce a number of lesions in DNA, including base damage, sugar damage, strand breaks, cross-links, and the generation of abasic sites (Dizdaroglu, 1993). 8-oxo-dG is not necessarily the most abundant DNA product, nor is it the most mutagenic, but it has been the most extensively studied because it is the most easily measured. 8-oxo-dG levels are increased in the lungs of smokers, and can additionally be detected in the urine. 8-oxo-dG causes miscoding by DNA polymerase in vitro, and site-specific mutagenic experiments verify that 8-oxo-dG is mutagenic in mammalian cells. However knockout mice, germline deficient in the Ogg1 gene responsible for 8-oxo-dG repair, display increased 8-oxo-dG levels, but no discernible pathology (Klungland et al., 1999). Consequently, the biological significance of 8-oxodG damage is not understood.

1.3.a.2.ii. Double Strand Breaks

Double Strand Breaks (DSBs) induce both chromosomal abnormalities and gene mutations, and are considered integral to radiation carcinogenesis (Little, 2000). DSBs are an additional consequence of tobacco smoke exposure in vitro (Leanderson & Tagesson, 1993). In mammalian cells, DSBs are repaired by homologous and non-homologous mechanisms (reviewed Karran, 2000). Homologous recombination (HR) is a highly accurate process which can restore the precise DNA sequence at the double strand break. Homologous recombination requires extensive regions of homologous DNA and takes place by means of replication, using information on the undamaged sister chromatid or homologous chromosome. DSBs are initially processed by the Mre11/Rad50/NSB1 nuclease complex to produce a single-stranded region with 3'overhang (Karran, 2000). Rad52 has DNA double-strand end binding activity and protects DNA ends from exonucleases. Interactions between Rad52 and Rad51 are thought to promote polymerisation of Rad51 onto single-stranded DNA to form a nuceloprotein filament that searches for the homologous duplex DNA. The formation of joint molecules between homologous strand of damaged and undamaged DNA is followed by exchange of DNA strands, which allow for semi-conservative replication.

In contrast, non-homologous end-joining (NHEJ) is a process in which two broken strands are joined directly, often resulting in deletions or small insertions. Characteristically, NHEJ requires no homology with an undamaged DNA partner and no, or only a few base pairs of sequence homology between the two broken ends. The process of NHEJ involves the DNA-PK complex, the kinase activity being

dependent upon binding of Ku70 and Ku80 proteins to the broken DNA ends. Ku70/Ku80 heterodimers bind to DSBs in a sequence-independent manner and recruit the catalytic subunit (DNA-PKcs) to the DSB, facilitating interactions between DNA-PKcs and DNA and stimulating DNA-PKcs kinase activity. DNA-PKcs recruits and regulates the activities of other protein complexes involved in NHEJ, including the XRCC4/DNA ligase IV complex required for joining the filled-in DNA ends, and the RNA polymerase I complex, repression of which facilitates NHEJ (Kuhn et al., 1995; Labhart, 1995). Most DSBs are repaired by this illegitimate recombination process, which is error-prone, and thus likely accounts for many of the potentially mutagenic lesions induced by clastogenic agents. It is further noteworthy that Ku80 expression has been observed to suppress chromosomal rearrangements in undamaged murine cells (Difilippantonio et al., 2000), documenting a housekeeping function of DNA-PK in maintaining genome integrity.

1.3.2. Ionizing Radiation

Nuclear events in Hiroshima and Nagasaki testify to the carcinogenic potential of ionizing radiation. Long-term follow up studies of the atom bomb survivors from Hiroshima and Nagasaki reveal radiation to be a universal carcinogen, capable of inducing cancer in most tissues at all ages, including the foetus (Pierce et al., 1996; Little, 2000). X- and gamma-emissions induce the formation of hydroxyl radicals from water (radiolysis) which may subsequently attack DNA to produce DNA strand breaks (see 1.3.1.b). DNA double strand breaks allow for non-reciprocal recombination events and the formation of aberrant chromosomes, perhaps critical events in tumourigenesis (Duesberg et al., 2000). The major clinical effects of lung

irradiation are conventionally divided into two stages: radiation pneumonitis and radiation fibrosis. Pneumonitis can be regarded as the episode during which the specific effects of lung damage are expressed and fibrosis as the subsequent wound-healing phase. One reason for this opinion is provided by the chronology of changes following thoracic irradiation. Phillips and Margolis (1972) irradiated the thorax of mice in an atmosphere of 5% oxygen. Deaths occurred between 80 and 160 days later and were due to pneumonitis. The survivors did not die immediately afterward; this has held true in experiments by others also (Kurohara & Casarett, 1972). It is additionally noteworthy that mice that are irradiated while breathing 100% oxygen exhibit greater mortality at 7 months than mice breathing atmospheric air (Gross, 1994). These observations highlight the critical importance of oxic conditions in pathological sequelae, and indicate that pneumonitis is a dose-limiting consideration in thoracic radiotherapy (Gross, 1994). It has also been shown that inhibition of collagen synthesis does not greatly reduce mortality in irradiated rats (Gross, 1994), suggesting that fibrosis is not a factor in mortality due to lung irradiation. Consideration of the pathogenesis of radiation reactions in the lung therefore concentrates on the mechanisms of the acute episode of radiation pneumonitis and the events leading up to it, a process that is probably similar in humans and experimental animals.

1.4. BIOLOGY OF p53

1.4.a. Biology of p53

The p53 tumour suppressor protein is a short-lived, latent transcription factor which is activated and stabilised in response to a wide range of cellular stresses including DNA damage, mitotic spindle damage and activated oncogenes (for recent review see Ljungman, 2000). p53 was discovered more than 20 years ago by virtue of its association with SV40 T antigen (Lane and Crawford, 1979). Initial p53 expression studies demonstrated that many immortalised and primary human tumour cell lines expressed elevated levels of p53 relative to the amounts expressed in normal wild-type cells (Zakut-Houri et al, 1983). The notion that p53 possessed cellular transformation associated properties was supported by further experiments which demonstrated that elevated levels of p53 expression could co-operate with activated Ha-Ras and transform primary fibroblast cells in culture (Eliyahu et al, 1985). Since those early days, there has been a plethora of data supporting the alternative hypothesis that p53 is an inhibitor of cellular transformation, and that accumulation of wild-type p53 protein following genotoxic stress reflects an attempt to preserve genomic integrity. The extravagant significance of p53 in regulating the integrity of the genome is supported by several observations, including: 1) the majority of human cancers have lost wild-type p53 function (Hollstein et al., 1994); 2) humans and mice with p53 mutations develop tumours early in life (Purdie et al., 1994; Jacks et al., 1994); 3) p53 deficient embryos are susceptible to spontaneous exencephaly (Armstrong et al., 1995; Sah et al., 1995); 4) p53 deficient mice display increased radiation and chemical carcinogenesis (Lee et al., 1994; Harvey et al., 1993); 5)

damaged embryos are less frequently aborted in mice that lack p53 (Komarova et al., 1997). Elegant animal models have demonstrated that p53 dysfunction is commonly associated with either tumourigenesis or tumour progression in many cell types, and lesions affecting wild-type p53 gene expression are the most frequently observed molecular abnormalities in a wide spectrum of human malignancies (Hollstein et al., 1994). Accumulation of p53 protein is observed in certain cell types following DNA damage, and is associated with the induction of apoptosis and/or growth arrest (Midgley et al., 1995). It is hypothesised that it is this 'p53 response' that underlines the tumour suppressor properties of p53 relevant to human malignancy.

1.4.a.1. p53 Structure and Function

p53 is a 53kDa phosphoprotein that can be subdivided into an N-terminal transactivation domain, a proline-rich domain, the core DNA-binding domain, and the C-terminal oligomerization and regulatory domains (Figure 1.4.i). Central to the regulation of p53 is its interaction with the MDM2 oncoprotein, which blocks p53 transactivation function by binding to a region of the transactivation domain (reviewed in Meek, 1999). MDM2 targets p53 for ubiqitin-mediated proteolysis and thus mediates rapid turover of the p53 protein. In addition to this role in regulating p53, the MDM2 gene is itself stimulated by p53-dependent transactivation. Consequently, MDM2 participates in an autoregulatory feedback loop which keeps p53 under tight control under normal conditions of cell maintenance. These findings suggest that MDM2 is pivotal to the regulation of p53 protein. MDM2 is frequently overexpressed in human lung adenocarcinoma, and in one study was observed in over 30% of cases. It is possible that MDM2 overexpression represents an

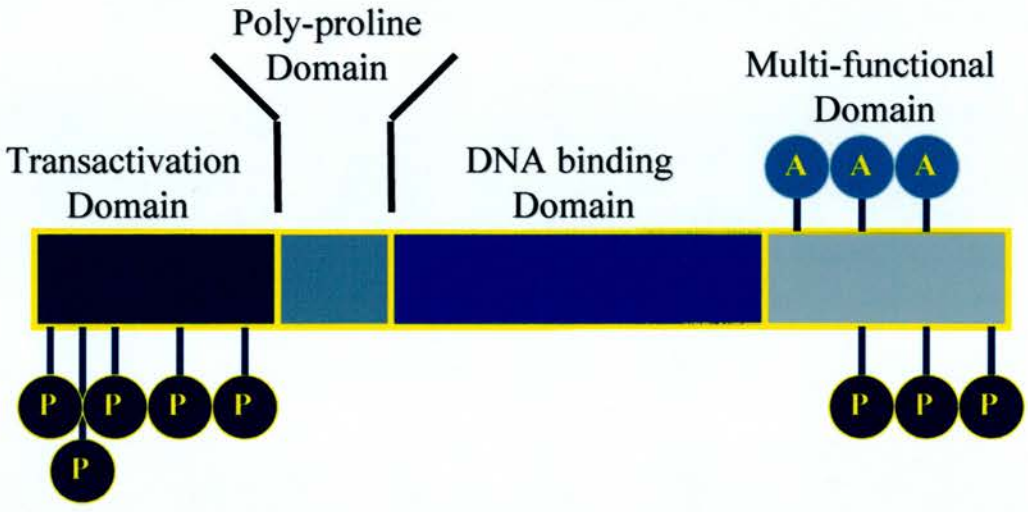


Figure 1.4.i – A model describing the structure and function of p53. The N- terminal, or transactivation domain of p53 binds various proteins associated with the basal transcriptional machinery including TBP (TATA-binding protein) (Horikoshi *et al,* 1995), $TAF_{II}40$ and $TAF_{II}60$ (TATA-associated factors; Thut *et al,* 1995) and p300/CBP (Gu and Roeder, 1997 and Lill *et al,* 1997). The core domain encompasses the DNA binding domain, additionally the site recognised by SV40 T antigen, and is a mutational hotspot of the p53 locus. The C- terminal domain of p53 contains both the regions necessary for protein tetramerisation and for non-sequence specific DNA binding. Monomer units of p53 protein form homodimer complexes through direct interaction between their respective tetramerisation domains (amino acids 320-360), and functional p53 tetramers subsequently form as a result of p53 homodimerisation. The non-sequence specific DNA binding region within the C- terminal domain of p53 (amino acids 363-393) confers ability to bind to both damaged DNA and denatured DNA templates, and may represent a mechanism by which p53 detects DNA damage within the cell (Levine *et al,* 1997). This latter sub-domain additionally enables p53 to re-anneal both complementary RNA and single stranded DNA (Wu *et al,* 1995), and may indicate a role in transcription-coupled repair.

epigenetic mechanism of ablating p53 function. However, it is noteworthy that oncogenic properties of MDM2 distinct from its regulation of p53 have been observed in certain cell types, such as mammary epithelium (Lundgren et al., 1997).

1.4.a.2. p53 Activation

The p53 response is triggered by many different stresses involving both DNA-damaging and non-DNA damaging agents (Figure 1.4.ii). These include ribonucleotide synthesis inhibitors, thymidine dinucleotides, media depletion, hypoxia, antioxidants, DNA strand breaks, bulky DNA lesions, DNA topoisomerase inhibitors, blockage of RNA polymerase II, heat shock, cold shock, viral infection, pRb deregulation, and oncogene expression. The emerging picture from cellular and molecular studies is that multiple, distinct sensors and pathways exist that activate p53 in response to stress.

The mechanisms by which p53 is activated are best understood in response to the DNA damaging agents UV and ionizing radiation. Both UV and ionizing radiation induce a rapid induction of p53 in certain mammalian cell types. UV induces bulky adducts in DNA that, if formed in the transcribed strand of an active gene, will impede the elongation of RNA polymerases. Blockage of RNA polymerase is additionally accomplished by cisplatin and nitrogen mustard, and may represent a common trigger for p53 accumulation following exposure to bulky adduct-forming agents. Ionizing radiation induces a number of different types of DNA lesions, of which strand breaks are considered the most relevant to p53 stabilisation. Notwithstanding this, microinjection of DNA with free ends into mammalian cells is

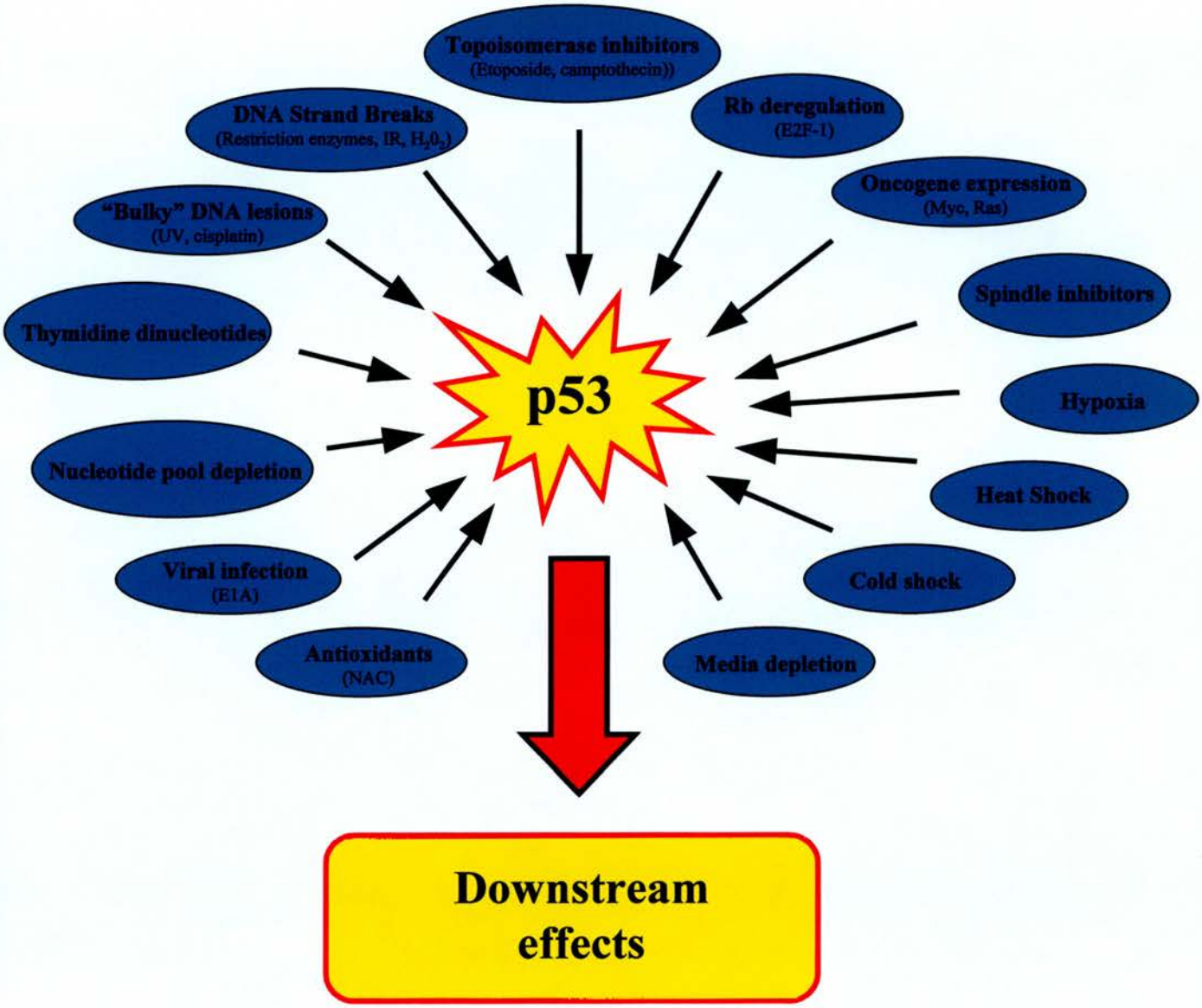


Figure 1.4.ii. The p53 response is triggered by many different stresses involving both DNA-damaging and non-damaging agents. These include ribonucleotide synthesis inhibitors (Linke et al., 1996), thymidine dinucleotides (Eller et al., 1997), media depletion (Zhan et al., 1993), hypoxia (Graeber et al., 1994), antioxidants (Plaumann et al., 1996; Liu et al., 1998), DNA strand breaks (Nelson & Kastan, 1994; Midgley et al., 1995), bulky DNA lesions (Maltzmann & Czyzyk, 1984; Fritsche et al., 1993), DNA topoisomerase inhibitors (Fritsche et al., 1993; Tishler et al., 1993), heat shock (Graeber et al., 1994; Matsumoto et al., 1994), cold shock (Ohnishi et al., 1998), viral infection (de Stanchina et al., 1998; Debbas & White, 1993), pRb deregulation (Bates et al., 1998; Hsieh et al., 1999), and oncogene expression (Zindy et al., 1998; Palmero et al., 1998).

sufficient to activate p53. The p53 phosphorylation events characteristic of radiation-injury are thought to be mediated by members of the PI-3 kinase family.

Activation of p53 in response to DNA damage is complex, requiring multisite phosphorylation and acetylation at key residues of the p53 protein (Figure 1.4.iii; reviewed in Meek, 1999). N-terminal phosphorylation permits recruitment of key transcription factors including p300/CBP and PCAF, which in turn acetylate residues at the C-terminus of p53 leading to stimulation of the site-specific DNA binding function. Phosphorylation of human p53 occurs in the N-terminus domain at serines 15, 20, 33, and 37 in response to ionising and UV radiation, and DNA-damaging drugs (Meek, 1999; Shieh et al., 1997; Banin et al., 1998; Canman et al., 1998; Shieh et al., 1999). The serine 15 phosphorylation site is juxtaposed to the MDM2 binding site, and phosphorylation of p53 at serines 15 and 37 can block its interaction with MDM2 in vitro (Pise-Masison et al., 1998; Shieh et al., 1997). The consequence of disrupting the p53-MDM2 complex is the release of p53 from the inhibition of transactivation and the prevention of further proteasome-mediated p53 degradation. An attractive quality of this model is that it explains how p53 could be both induced and activated simultaneously.

In response to ionizing radiation, the N-terminus of p53 is phosphorylated by, at a minimum, three different DNA damage-responsive protein kinase activities, each of which belongs to the PI-3 kinase family. These are the DNA activated protein kinase (DNA-PK), the ATM kinase, the product of the ATM gene mutated in the genetic disorder ataxia telangiectasia (A-T), and the ATM-Rad3-related kinase (ATR).

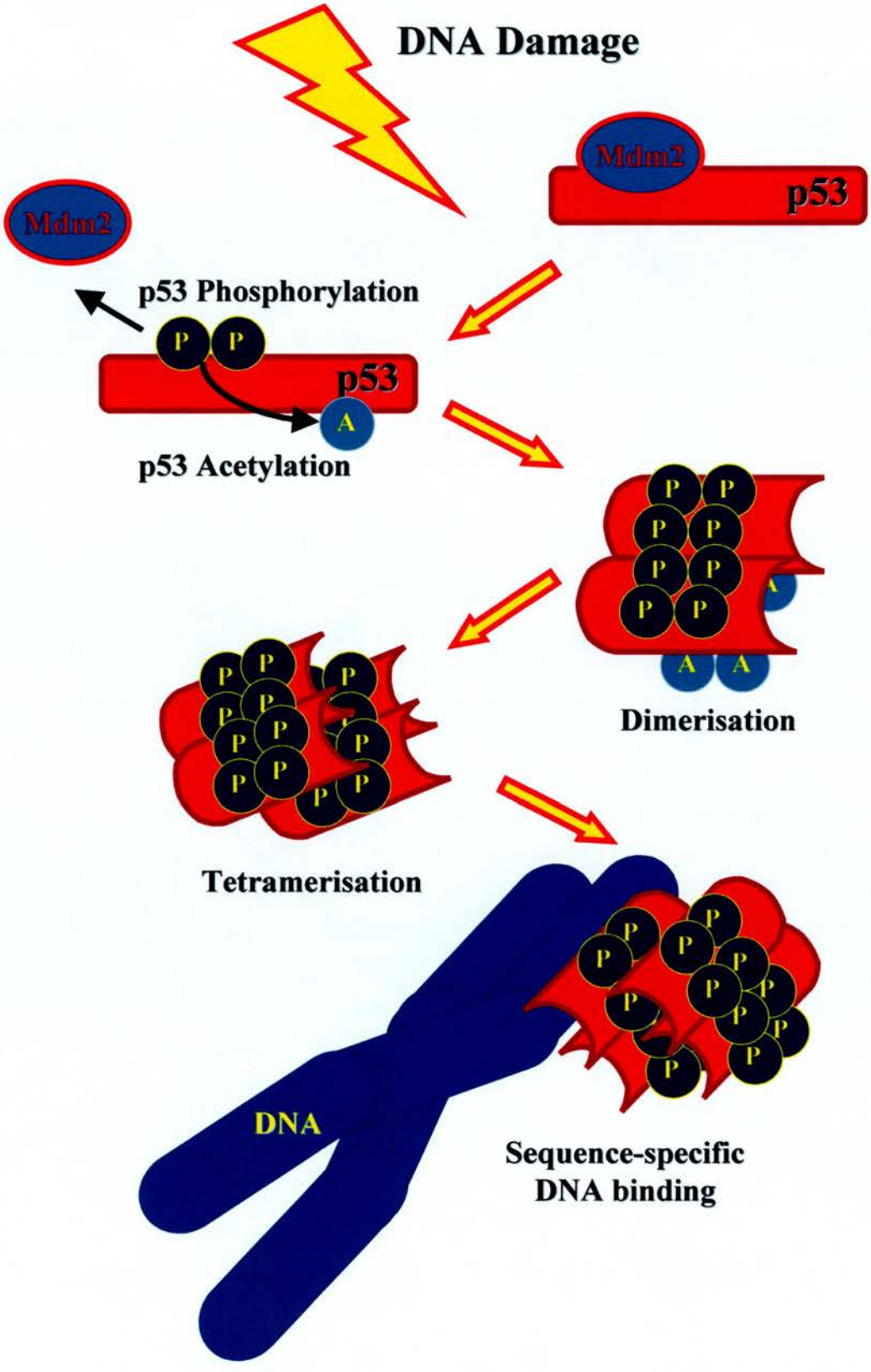


Figure 1.4.iii - A model describing DNA damage-induced p53 activation. Phosphorylation of the N-terminal transactivation domain occurs in response to DNA damage, displacing Mdm2 and facilitating recruitment of key transcription factors, which in turn acetylate the C-terminus, inducing tetramerisation and stimulating site-specific DNA binding.

DNA-PK phosphorylates human p53 at serines 15 and 37 in vitro and is dependent for activity of interaction with double-stranded DNA structures containing nicks or gaps (Anderson, 1994; Jackson & Jeggo, 1995). ATM and ATR also phosphorylate p53 at serine residues 15 and 37, and are additionally associated with hypersensitivity to DNA-damaging agents (Meek, 1999). p53 induction displays delayed kinetics in irradiated cells isolated from A-T individuals (Enoch & Norbury, 1995), but not SCID mice (deficient in DNA-PK), insinuating ATM as the dominant p53-activation factor in the ionizing radiation response in vivo.

1.4.b. DNA Damage Responses of p53

In response to DNA damage, p53 affects transcriptional regulation of gene expression and inhibits tumour cell growth by either inducing G1 growth arrest or apoptosis (Figure 1.4.iv). Some 100 genes are thought to be transactivated by p53. In addition, p53 reduces transcription of other genes, a mechanism at least partially accomplished by histone deacetylation. The great majority of p53 mutations observed in human tumours are found in the DNA-binding domain of the protein, abolishing transactivation function of p53. Consequently, transactivation functions of p53 are considered integral to its role in tumour suppression.

The transactivation capacity of p53 in vivo was initially demonstrated by the use of transgenic mice expressing a lacZ reporter gene in a p53-dependent manner (Gottlieb et al., 1997; Komarova et al., 1997). Following γ-irradiation, sites of maximal reporter expression colocalised with sites of maximal p53 stabilisation in most tissues examined of adult mice. However, it is noteworthy that a p53

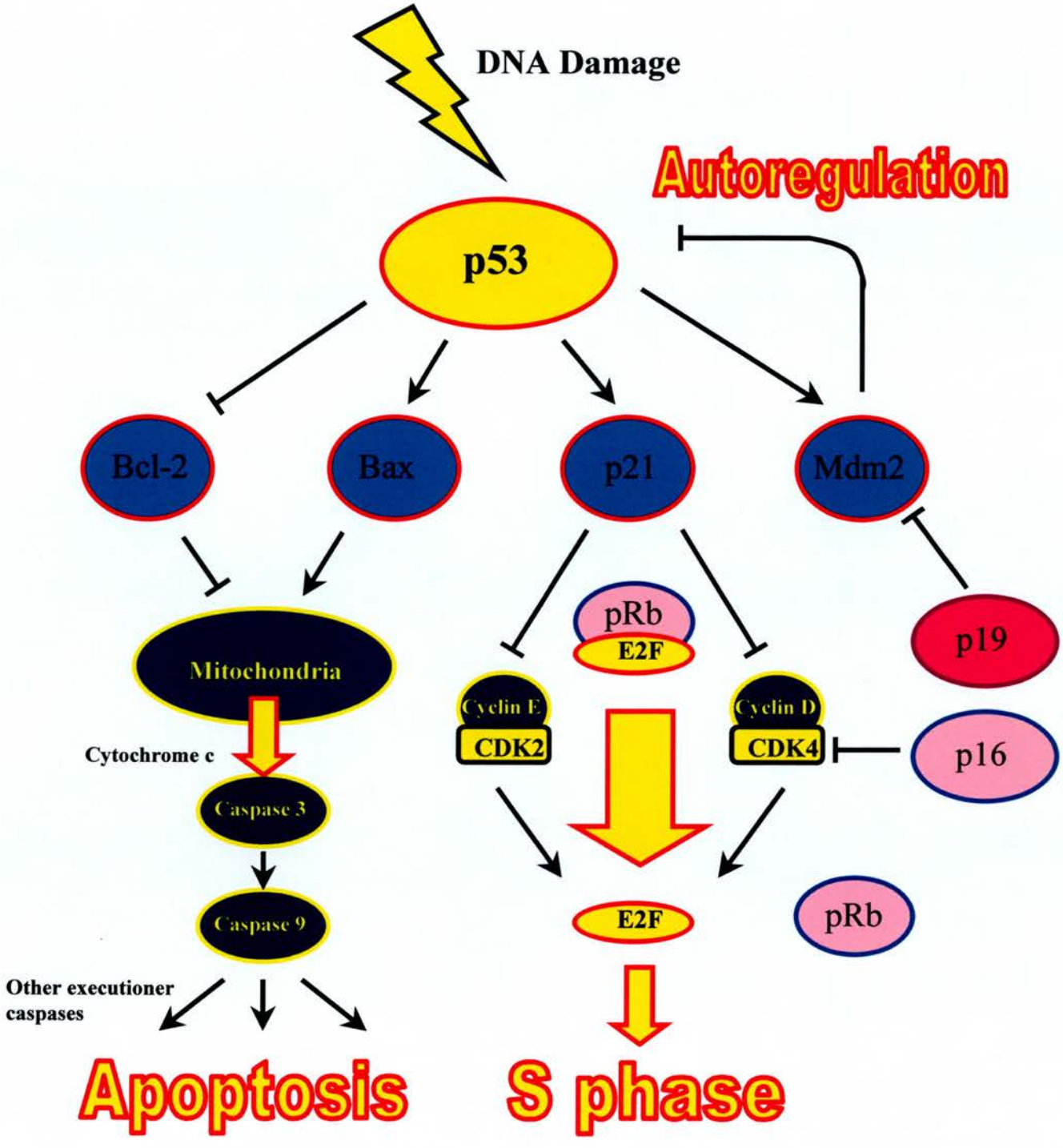


Figure 1.4.iv. A model describing transactivation functions of p53. p53 upregulates expression of Bax, p21, and Mdm2 and represses Bcl-2 expression thereby impacting on both the cell cycle and steps that initiate the caspase cascade. Autoregulation of p53 is accomplished by induction of Mdm2, a p53 target gene. Regulation of Mdm2 is additionally dependent on the p19 gene product.

stabilisation/transactivation infidelity was observed in certain elements of the spleen; specifically, although both red and white compartments of the spleen exhibited high levels of p53 stabilisation in response to DNA damage, reporter gene expression was only detected in the red pulp. In other cell types, such as the hepatocyte (Bellamy et al., 1997b), p53 transactivation, but not stabilisation, is a feature of radiation-injury. Consequently, the relationship between p53 stabilisation and transactivation would appear cell-type specific, and may vary between different cell lineages.

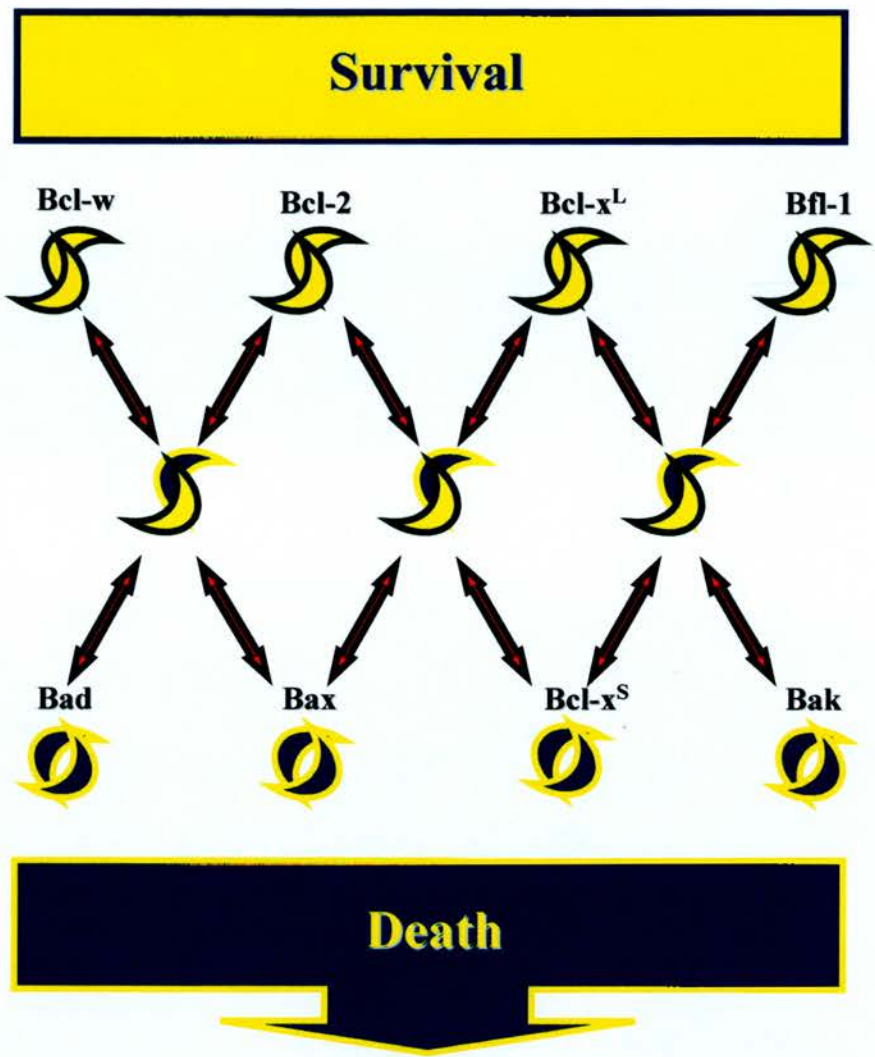
1.4.b.1. Apoptosis

Apoptosis is an evolutionary conserved, morphologically defined form of cell death characterised by chromatin condensation and collapse of the nucleus. p53 status is critical for the induction of a rapid apoptotic response in many mammalian cell types in response to DNA damage (reviewed in Bellamy, 1997c) and, by removing the damaged cell, is considered a major cellular strategy in the prevention of tumourigenesis. Consequently, although not proven, it has been suggested that the attenuated apoptotic responses exhibited by p53 deficient cells contribute to an overall increase in the probability of their malignant conversion.

p53-dependent apoptosis can be triggered by a variety of stimuli including DNA damage, oncogene activation, and growth factor withdrawal. In vitro studies demonstrate that p53 can induce apoptosis through both transactivation dependent and independent mechanisms. Indeed, cells cultured in the presence of both actinomycin D and cycloheximide (inhibitors of RNA and protein synthesis respectively) display p53-dependent apoptosis following DNA damage (Caelles et

al., 1994) or c-myc overexpression (Wagner et al., 1994). It is additionally noteworthy that a C-terminal deficient, and consequently transactivation deficient, p53 molecule retains the ability to induce HeLa cell apoptosis in response to inappropriate oncogene expression (Haupt et al., 1995). The mechanisms underlying p53-dependent apoptosis in the absence of downstream gene transactivation remain to be elucidated, however direct interaction between p53's C-terminal domain and both XPB and XPD helicases appear to be essential in certain cellular contexts (Wang et al., 1996). Furthermore, an ability of activated p53 to mediate the translocation of the Fas receptor from Golgi appartus to the cell surface has been observed in certain cell types, such as vascular smooth muscle cells (Bennett et al., 1998). Activated p53 additionally stimulates interaction between FADD and the Fas receptor, thus transiently sensitising cells to Fas-induced apoptosis (Bennett et al., 1998).

p53 additionally induces apoptosis via transcriptional modulation of downstream target genes. Of these, the family of Bcl-2-related proteins constitutes the biologically most relevant class of apoptosis-regulatory gene products known to be modulated by p53 (reviewed in Zamzani et al, 1998). Bcl-2 related proteins may either promote cell survival (Bcl-2, Bcl-x^L, Bcl-w, Bfl-1) or encourage cell demise (Bax, Bcl-x^S, Bad, Bak)(Cory, 1995; Kroemer, 1997; Reed, 1994; 1997; Thompson, 1995; Yang and Korsmeyer, 1996). The consensus is that the relative amount of death agonists and antagonists from the Bcl-2 family constitute a regulatory rheostat whose function is determined, at least in part, by selective protein-protein interactions between family members (Figure 1.4.v). Notwithstanding this,



Key

Death Antagonist Homodimers

Agonist/Antagonist Heterodimers

Death Agonist Homodimers

Figure 1.4.v. A model describing protein-protein interactions of bcl-2 family members. The death/life rheostat is mediated, at least in part, by competitive dimerisation between selective pairs of death agonists and antagonists (Sedlak et al., 1995). Since each of the members of the Bcl-2 family is expressed in a cell-type-, differentiation-, and activation stage-specific fashion, a network of pairs of interacting proteins influences cell fate.

transgene-enforced overexpression of Bcl-2 and Bcl-x^L confer apoptosis resistance to a number of different tissues including lymphocytes (McDonnell et al., 1989; Sentman et al., 1991; Strasser et al., 1991), neurons (Martinou et al., 1994), hepatocytes (Lacronique et al., 1996), keratinocytes (Pena et al., 1997) and mammary epithelium (Jager et al., 1997). By contrast, ectopic expression of propapoptotic derivatives, such as Bax and Bcl-x^S, sensitises cells to apoptosis induction following genotoxic insult (Pena et al., 1997; Brady et al., 1996) and glucocorticoid stimulation (Brady et al., 1996). Knock-out studies have revealed that Bcl-2 deficiency is deleterious for certain cell types, including lymphocytes (Nakayama et al., 1994), melanocytes (Yamamura et al., 1996), intestinal epithelial cells (Kamada et al., 1995), and some classes of neurons (Michaelidis et al., 1996), whereas deficiency of Bcl-x causes intrauterine death accompanied by massive loss of postmitotic immature neurons (Motoyama et al., 1995). Importantly, Bax -/- Bcl-x-/- double knockout mice do not mainfest the increased cell death of immature neurons observed in Bcl-x -/- mice (Schindler et al., 1997). Similarly, the thymic hypoplasia characteristic of Bcl-2-/- mice is largely absent in mice also deficient in Bax (Knudson & Korsmeyer, 1997), highlighting the importance of an equilibrium between death agonist and antagonist proteins in the regulation of cell survival.

Bcl-2 and its homologues insert into intracelluar membranes, including those of the mitochondria, and is suggested that regulation of apoptosis is dependent on protein-protein interactions within the outer and inner mitochondrial membranes (Zamzami et al., 1998)(see Figure 1.4.vi). Notwithstanding this, Bcl-2 overexpression prevents disruption of mitochondrial membrane potential ($\Delta\psi_m$), cytosolic displacement of

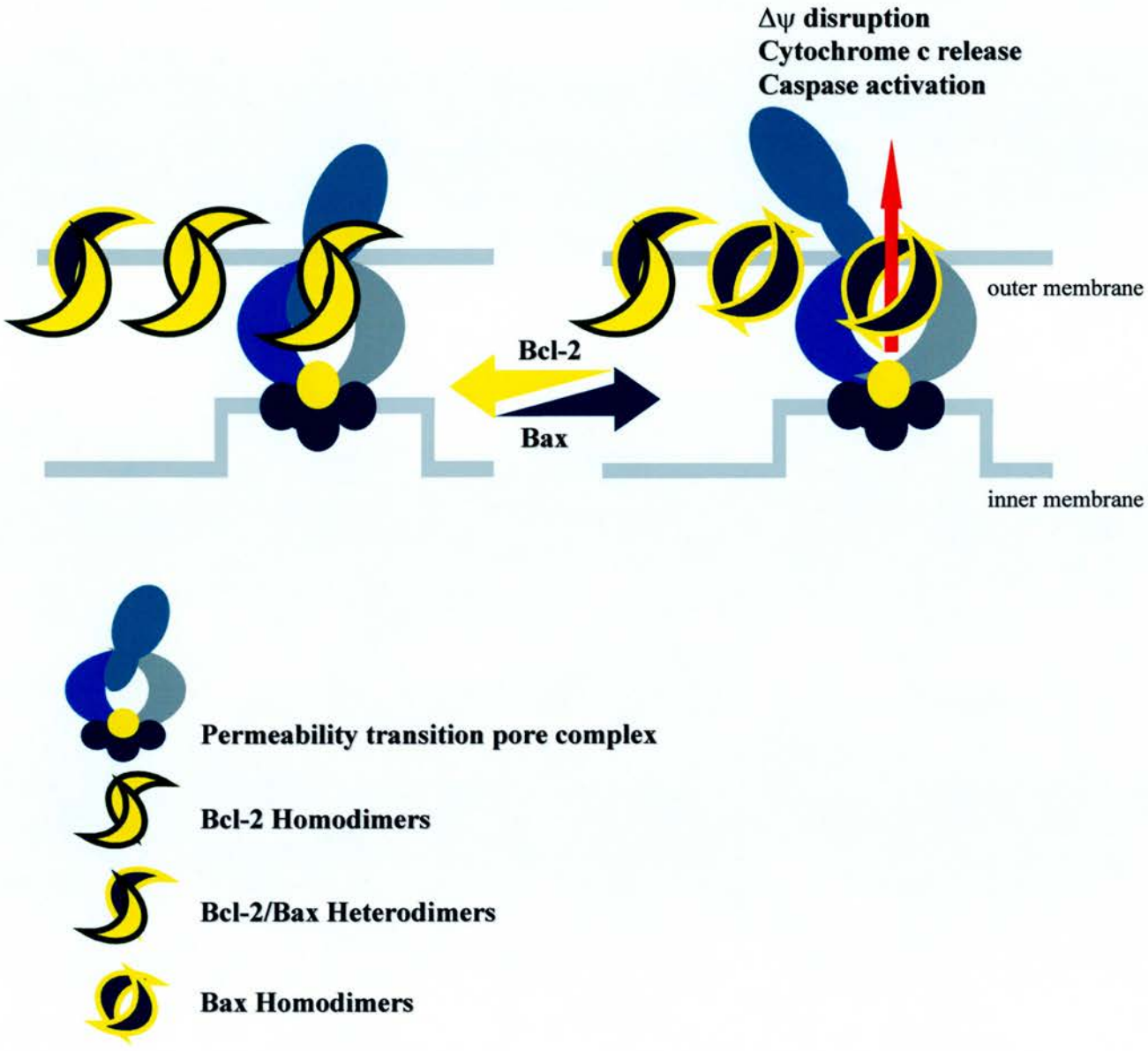


Figure 1.4.vi – A model describing regulation of apoptosis by bcl-2 family members. The mitochondrial permeability transition (PT) pore is a multiprotein complex formed at the contact site between the mitochondrial inner and outer membranes, exactly the same localisation at which Bax, Bcl-2 and Bcl-x^L are particularly abundant. Experiments involving the purified PT pore complex indicate that Bax, Bcl-2, and Bcl-x^L exert at least part of their apoptosis-regulatory function by facilitating (Bax) or inhibiting (Bcl-2, Bcl-x^L) pore opening.

cytochrome c, and caspase activation typical of apoptosis (Kluck et al., 1997). Furthermore, Bax, Bcl-2 and, Bcl-x^L are particulalrly abundant at the contact site between the mitochondrial inner and outer membranes, additionally the site of the permeability transition (PT) pore complex from which $\Delta\psi_m$ and mitochondrial efflux are regulated. Data obtained with isolated mitochondria indicate that Bax protein can induce both $\Delta\psi_m$ dissipation and cytochrome c release. This finding has been confirmed in mammalian cells and yeast cells transfected with Bax (Manon et al., 1997; Rosse et al., 1998; Xiang et al., 1996), suggesting that Bax acts to destabilise mitochondrial membrane function and thus induce apoptosis via its interaction with the PT complex.

In vivo, the p53-dependent apoptotic response is best understood following ionizing radiation insult, which induces DNA strand breaks. In mice, there is consensus that p53-dependent apoptosis is observed in thymocytes, splenocytes, and intestinal enterocytes from approximately 4 hours following irradiation, and that this associates with the induction of Bax. By contrast, intestinal cells and thymocytes of p53-deficient mice do not display this rapid wave of apoptosis following ionizing radaition injury. Instead, these cell types from p53-deficient mice exhibit apoptosis at the later time points of 24-48 hours post-irradiation (Clarke et al., 1994; 1993). It has been suggested that the p53-dependent wave of apoptosis reflects Bax upregulation in intestinal cells and thymocytes of irradiated mice. However, Bax deficient mice do not display the apoptotic defects characteristic of p53-null mice. In this respect, it is noteworthy that p53 can inhibit the expression of Bcl-2 in cultured cells, thus it is conceivable that p53 influences the expression of both target genes to

promote apoptosis in vivo. Notwithstanding this, Bax and Bcl-2 deficient mice display thymic hyperplasia and intestinal exfoliation, respectively, insinuating their involvement in normal tissue dynamics. The genetic basis of the p53-independent apoptotic pathway is not understood, but in considering their prominent role in life/death decisions there is reason to suspect Bcl-2 family involvement.

1.4.b.2. Growth Arrest and Repair

p53 induces G1 and G2 growth arrests in mammalian cells, phenomena dependent on the ability of p53 to transcriptionally activate the expression of downstream loci. Initiation of p53 dependent cell cycle arrest appears to create a cellular environment in which damaged DNA may be repaired with greater efficiency. The $p21^{WAF1/CIP1}$ gene product (p21) is induced by p53 in a range of cell types, and is a critical determinant of DNA-damage induced growth arrest (Brugarolos et al., 1995; El-Deiry et al., 1994; Waga et al., 1994; Li et al., 1994). p21 inhibits cyclin-dependent kinase activity, thus inducing dephosphorylation of Rb and inhibition of the E2F transcription factor complex (reviewed in Nevins, 1998). Consequently, p21, although not a transcription factor, regulates a distinct network of genes involved in cell-cycle progression, including several E2F regulated genes (Chang et al., 2000). p21 has also been demonstrated to interact with proliferating cell nuclear antigen (PCNA), a protein that facilitates DNA synthesis (Flores-Rozas et al, 1994; Waga et al., 1994). PCNA is also involved in nucleotide excision repair (NER), but interaction with p21 has been demonstrated to block DNA replication selectively without affecting PCNA dependent NER (Li et al., 1994). The relationship between p53, p21, and mediation of the G1-S cell cycle checkpoint is not exclusive, however,

as fibroblasts derived from mice deficient in p21 are still partially able to arrest at G1-S following DNA damage.

p21 induction is observed in murine lung epithelial cells following hyperoxia and ionizing radiation insults, and in a mouse model of acute immune complex alveolitis (McGrath, 1998; Macleod et al., 1995; Kuwano et al., 1997). In irradiated lung, p21 induction is p53-dependent, as determined by Northern analysis, and does not occur in the lungs of p53-deficient mice (Macleod et al., 1995). It is hypothesised that p21 induction may reflect cell cycle changes in damaged lung.

Gadd45 is a growth arrest and DNA damage-inducible gene implicated in the p53-dependent G1 and G2 growth arrests. Gadd45 directly interacts with PCNA in response to DNA damage in such a manner as to enhance DNA repair whilst inhibiting DNA replication (Hall et al., 1995). Gadd45 deficient mice exhibit several of the phenotypes of p53-deficient mice, including genomic instability, increased radiation carcinogeneis, and a low frequency of exencephaly. Consequently, it is probable that that gadd45 is a critical component of the p53 pathway in most cell types. However, gadd45 induction is p53-independent in the lungs of irradiated mice, as determined by Northern analysis, and occurs equally in p53 deficient mice. The mechanisms of regulating gadd45 expression in the lung following DNA damage are not understood.

In addition to inducing cell cycle arrest, which facilitates DNA repair, p53 has been directly implicated in the repair of damaged DNA. p53 has 3' to 5' exonuclease

activity and the ability to recognise and bind damaged DNA template. Furthermore, the C-terminal domain of p53 is able to interact with the XPB and XPD helicase components of the TFIIH repair complex (Wang et al., 1996). The in vivo significance of these interactions has yet to be established, however the in vivo consequences regarding p53 status and mutation rate are presented and discussed below.

1.4.c. p53 and Mutation Frequency

Abrogation of wild type p53 function is predicted to lead to an increase in the number of cells bearing DNA damage as a consequence of altered apoptotic and DNA repair profiles. Experiments designed to test these predictions suggest that p53 dependent differences may only become apparent following exogenous damage. Two different groups have used a transgene target to monitor spontaneous mutation frequency, but in both cases no p53 dependent differences were observed (Sands et al., 1995; Nishino et al., 1995). By contrast, experiments using either short pre-B cell cultures (Griffiths et al., 1997) or a transfected fibroblast line (Yang et al., 1995) have provided data in support of a p53-dependent decrease in the number of mutation bearing cells following DNA damage. In vivo experiments utilising the endogenous locus Dlb-1 as a marker of mutation have demonstrated a p53 dependent decrease in mutation frequency in the murine small intestine (Clarke et al., 1997), although this was only observed at high levels of DNA damage. A criticism of these studies is that by concentrating on a relatively small target locus, they do not address the possibility of clastogenic damage, such as that induced by ionizing radiation. However, taken together, these different experiments do support a role of p53 in preventing the

acquisition of mutations, but it is clear that it is not the sole mechanism, particularly at low levels of DNA damage.

1.5. p53 AND LUNG DISEASE

1.5.a. p53 and Lung Cancer

Lung cancer is the leading cause of cancer death in the Western world (WHO, 1999). Lung cancer incidence is sporadic within the population with tobacco smoking identified as a major aetiological agent (WHO, 1999). Tobacco smoke is DNA damaging, and induces DNA strand breaks and point mutations in mammalian cells (Poli et al., 1999; Hecht, 1999). Progressive accumulation of mutations in specific oncogenes and tumour suppressor genes compromises genomic integrity and is considered a hallmark of tumourigenesis. It has been suggested that specific mutations induced by carcinogens in tobacco smoke may explain the natural history of lung malignancy (Tuveson & Jacks, 1999).

A hotspot for point mutations and deletions in lung cancer is the gene encoding the stress protein p53 (Takahashi et al., 1989; Bennett et al., 1999). p53 is frequently mutated in human malignancy and its presence is considered rate-limiting in tumour progression. In certain cell types, wild type p53 protein affords an invaluable DNA damage response and transcriptionally modulates a battery of downstream genes involved in growth control, DNA repair, and apoptosis following genotoxic insult (reviewed in El-Deiry, 1998). Growth arrest facilitates DNA repair, and is instrumental in maintaining genomic integrity. Apoptosis is a morphologically defined form of cell death and, by removing the damaged cell, is considered a major cellular strategy in the prevention of tumourigenesis. Consequently, in certain cell types, absence of p53 permits propagation of mutant cells following genotoxic insult

(Griffiths et al., 1997; Clarke et al., 1997). Point mutations of the p53 locus observed in human lung malignancy frequently ablate DNA binding and hence abolish transactivation ability of the p53 protein (Bennett et al., 1999). Tumour suppressor properties of p53 in the lung thus abide within its ability to transactivate downstream genes. In lung adenocarcinoma, where progression of the disease is histologically defined, mutation of p53 occurs early in tumour formation, possibly at a preneoplastic stage (Kitamura et al., 1995; Kitamura et al., 1996). Presumably, loss of p53 confers a selective advantage in preneoplastic lesions of the lung and is mutated accordingly. However, the mechanism by which p53 loss contributes to lung carcinogenesis is not understood.

Knockout mice, germline deficient in p53, display a complex phenotype of genomic instability, increased radiation carcinogenesis, and a low frequency of exencephaly (Fukasawa et al., 1997; Donehower et al., 1992; Harvey et al., 1993; Purdie et al., 1994; Jacks et al., 1994; Armstrong et al., 1995; Sah et al., 1995). p53 deficient mice additionally display spontaneous lung adenocarcinoma, albeit infrequently. It is probable that lung adenocarcinoma represents malignancy of the Clara cell, a progenitor species of the small airways (Massaro et al., 1994). Indeed, targeted expression of SV40 T antigen in small airway epithelium robustly induces adenocarcinoma in transgenic mice, and tumours of these mice frequently express Clara cell markers in vivo (Wikenheiser et al., 1992; Wikenheiser & Whitsett, 1997). p53 influences the response to DNA damage in certain cell types, and loss of p53 has many effects including decreased apoptosis, cell cycle checkpoint disturbances, reduced DNA repair, and abnormalities in cytokinesis (Ko & Prives, 1996). It is

possible that tumour suppressor properties of p53 in the small airways reflect its role in the pluripotent Clara cell.

1.5.b. p53 and Acute Lung Injury

Effects of p53 in metaplastic lung have been partially elucidated in the context of DNA damage, and include induction of the cell cycle-dependent kinase inhibitor p21 (Macleod et al., 1995), and the repair enzyme O-6-methylguanine DNA methyltransferase (MGMT) (Rafferty et al., 1996) (see 1.4.b). Induction of the death-related gene Bax is a feature of acute lung injury (Guinee et al., 1997), and may represent an additional p53 response of lung epithelium. In certain cell types, notably those of the gut, thymus, and spleen, DNA damage-induced Bax induction is p53-dependent and is associated with apoptosis (Kitada et al, 1996). Bax induction is a feature of acute lung injury, and localises to bronchioalveolar cells (Guinee et al., 1997), although its association with apoptosis is unclear, as discussed below.

p53 stabilisation is observed in human small airway epithelium in the inflammatory condition Adult Respiratory Distress Syndrome (ARDS), and in mice following hyperoxia and bleomycin exposure (Guinee et al., 1996; O'Reilly et al., 1998; Okudela et al., 1999). ARDS is a complication of smoke inhalation, acute exposure to radiation, and sepsis and pathological features of the disease include small airway cell apoptosis and oedema. High-dose radiation, bleomycin, and hyperoxia treatments model ARDS in experimental animals. Mice treated with these toxins display many features of ARDS, including vascular permeability changes and apoptosis of small airway cells (Mantell et al., 1999). Hyperoxia, bleomycin, and

ionizing radiation increase the amount of reactive oxygen species (ROS) in the cell. ROS are both genotoxic, causing DNA strand breaks, and proinflammatory, inducing cytokine release. Notwithstanding this, the lungs of ROS-treated mice display p53 stabilisation, TNF-alpha induction, and apoptosis of airway cells (O'Reilly et al., 1998; Okudela et al., 1999; Ortiz et al., 1998). Endothelial cell apoptosis is Acidic Sphingomyelinase (ASMase)-dependent in irradiated lung, and does not occur in ASMase-deficient mice (Santana et al., 1996). ASMase mediates TNF-alpha signalling in endothelial cells, and ASMase mice are deficient in TNF-alpha, but not p53, responses to radiation injury. Apoptosis of airway epithelial cells is an additional feature of acute lung injury, as determined by TdT-mediated dUTP end-labeling (TUNEL) of DNA strand breaks, an in situ method for detecting apoptosis (Kazzaz et al., 1996; Bardales et al., 1996; Guinee et al., 1996). TUNEL indices increase in airway cells following ROS-exposure, and are enhanced in p53-deficient mice (Okudela et al., 1999). It is thus conceivable that p53 influences the response to injury via a role in apoptosis and, indeed, such a role has been hypothesised in the airways (Guinee et al., 1996). However, other reports suggest that the pathogenic response to ROS is p53-independent, and instead correlates with the aforementioned TNF-alpha induction (Santana et al., 1996; Ortiz et al., 1998). Discrepancies in the literature may reflect the current concern over the accuracy of TUNEL in determining apoptosis. It is documented that TUNEL staining does not discriminate between necrosis and apoptosis in certain cell types (Grasl-Kraupp et al., 1995). DNA fragmentation is additionally a feature of ROS-exposure (Dizdaroglu, 1993), and it is possible that ROS generated by hyperoxia, bleomycin, and ozone induce strand breaks detectable by TUNEL. Consequently, TUNEL is a useful marker, but

not a surrogate method of determining apoptosis, which still requires the demonstration of distinct morphological features. The relationship between p53, the bcl-2 family, and epithelial cell apoptosis is thus currently unresolved in the context of acute lung injury, and requires clarification.

In this analysis, the author sought to identify functions of p53 in small airway epithelial cells. p53 stabilisation has been observed in the small airways previously, but its significance is unclear. To determine the role of p53 in bronchioalveolar cells, the author exploited gene targeted mice, germline deficient in p53, to establish unequivocally the functions of p53 over a 72-hour time course in response to ionizing radiation. Apoptosis was evaluated by two methods - morphology and in situ end-labelling (TUNEL), and regulation of Bcl-2 family transcription was assessed by sensitive ribonuclease protection assays of whole-lung extracts as there is controversy on this subject. This thesis additionally explored the hypothesis that Clara cells, as a major progenitor species of the small airways, are relevant to neoplasia of the lung and centriacinar emphysema, and that phenotypic effects of p53 deficiency would predominate in this cell type. A method for extracting, isolating, and culturing Clara cells from the mouse has been described previously (Masek & Richards, 1990) and was incorporated into this analysis to facilitate a greater understanding of the role of p53 in small airway epithelial cells.

1.6. TRANSGENIC MOUSE MODELS OF LUNG INJURY

Knowledge of biochemical and physicochemical interactions helps in understanding injury mechanisms and highlights new avenues of prophylaxis and treatment. Transgenic technology offers a way of examining the genetic determinants of acute lung injury, and has been used to illustrate the in vivo importance of key genes in damage response pathways. Control elements of genes expressed in a lung-selective manner have been identified, and have been used to express transgenes in subsets of respiratory epithelial cells in the developing and mature lung. Distinct temporal and spatial patterns of expression are observed with such constructs, depending on the promoter element used and the species from which it was cloned (Table 1.6.i). Of these, the SP-C promoter has been most extensively studied. Cis-acting sequences from human Surfactant Protein-C gene confer pulmonary-specific gene expression in transgenic mice. Unlike the endogenous murine SP-C promoter, which drives expression specifically in type II alveolar cells of adult mice, transcriptional elements from the human SP-C gene direct expression in the primordial respiratory epithelium of mid-gestation embryos, and in both Clara cells and type II cells of adult transgenic mice.

Existing mouse models of lung injury exploit lung-epithelium specific promoters to address the functional consequences of gene overexpression in vivo. By the use of such models, significant progress has been made in understanding mechanisms by which cytokines and antioxidant enzymes influence remodeling processes in the lung (Table 1.6.ii). These studies additionally highlight the potential importance of lung

Table 1.6.i – Lung epithelium-specific promoter elements

Gene	Species	5' flanking region	Cell specificity
SP-A	Mouse	1.4kb	bronchiolar/alveolar type II (Bruno et al., 1995)
SP-B	Mouse	0.218 to 2.1kb	bronchiolar/alveolar (Bohinski et al., 1994; Bruno et al., 1995)
SP-C	Human	3.7kb	bronchiolar/alveolar type II (Glasser et al., 1991; Wert et al., 1993)
SP-C	Mouse	4.4kb	type II (Wert et al., 1993)
CC10	Rat	2.4kb	nonciliated bronchiolar (Hagen et al., 1990; Stripp et al., 1992)
CCSP	Rabbit	1.4kb	uterus/nonciliated bronchiolar (Hagen et al., 1990)
TTF-1	Mouse	1.4kb	bronchiolar/alveolar type II (Bohinski et al., 1994; Bruno et al., 1995)

SP, surfactant protein; CC10, Clara cell 10kDa protein; CCSP, Clara cell secretory protein (homologue of CC10); TTF, thyroid transcription factor.

Table 1.6.ii – Transgenic mouse models of lung injury

Gene of Interest	Promoter	Phenotype
SV40 T antigen	SP-C	Tumourigenesis and immortalization of epithelial cells (Wikenheiser et al., 1992, 1993)
	CCSP	Tumourigenesis (Demayo et al., 1991)
TGF-α	SP-C	Lung fibrosis and altered morphogenesis (Korfhagen et al., 1994)
TGF-β	SP-C	Altered morphogenesis (Zhou et al., 1996)
TNF-α	SP-C	Lung inflammation (Miyazaki et al., 1995)
FGF receptor (mutant)	SP-C	Altered morphogenesis (Peters et al., 1994)
GM-CSF	SP-C	Type II cell hyperplasia (Huffman Reed et al., 1997)
PDGF	SP-C	Lung inflammation, fibrosis and emphysema (Hoyle et al., 1999)
IL-6	CC10	Airway inflammation (DiCosmo et al., 1994); Protection from hyperoxic acute lung injury (Ward et al., 2000)
IL-11	CC10	Bronchial remodeling and airway inflammation (Tang et al., 1996); Protection from hyperoxic acute lung injury (Waxman et al., 1998)
SOD	SP-C	Protection from hyperoxic acute lung injury (Folz et al., 1999)

SV40, simian virus 40; TGF, transforming growth factor; CFTR, cystic fibrosis transmembrane conductance regulator; TNF, tumour necrosis factor; FGF, fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; PDGF, platelet-derived growth factor; IL, interleukin; SOD, superoxide dismutase; SP-C, human surfactant protein C; CC10, rat Clara cell 10kDa protein; CCSP, rabbit Clara cell secretory protein.

epithelial cells in disease processes. A caveat of existing lung-transgenic models is that they are restricted to overexpression studies, and do not address the significance of gene deficiency in lung epithelial cells in vivo.

One method for addressing the significance of gene deficiency in vivo is conditional gene targeting. Conditional gene targeting is an invaluable method for overcoming the high incidence of embryonic lethality observed in constitutive knockouts, and for discerning the in vivo significance of gene deficiency in specific tissues. Two methods of conditional gene targeting, the Cre-lox and Flp-frt systems, are in existing use in mammals. Of these, the Cre-lox system has been used with the most frequent success. The Cre/lox recombinase system evolved within bacteriophage P1 as a mechanism to maintain correct unit copy segregation of the prophage within host cells. Bacteriophage P1 encodes the 38-kDa Cre recombinase that catalyses site-specific DNA recombination between 34-base pair repeats termed loci of recombination or 'lox' sites (Sauer & Henderson, 1988). Cre recombinase, expressed ectopically in mammalian cells, induces either deletion or inversion of the sequences flanked by the lox sites dependent upon lox site orientation (Figure 1.6). The Cre-lox system can function in a highly efficient manner in directing tissue-specific, site-specific, and heritable chromosomal DNA recombination events and has been used to generate tissue-specific knock-outs, inducible knock-outs, and site-directed chromosomal translocations in transgenic mice in vivo (Table 1.6.iii). The ability to generate temporally and/or spatially restricted gene alterations largely resolves two of the main problems associated with conventional gene 'knock-outs', namely embryonic lethality and secondary effects of the targeting event, such as

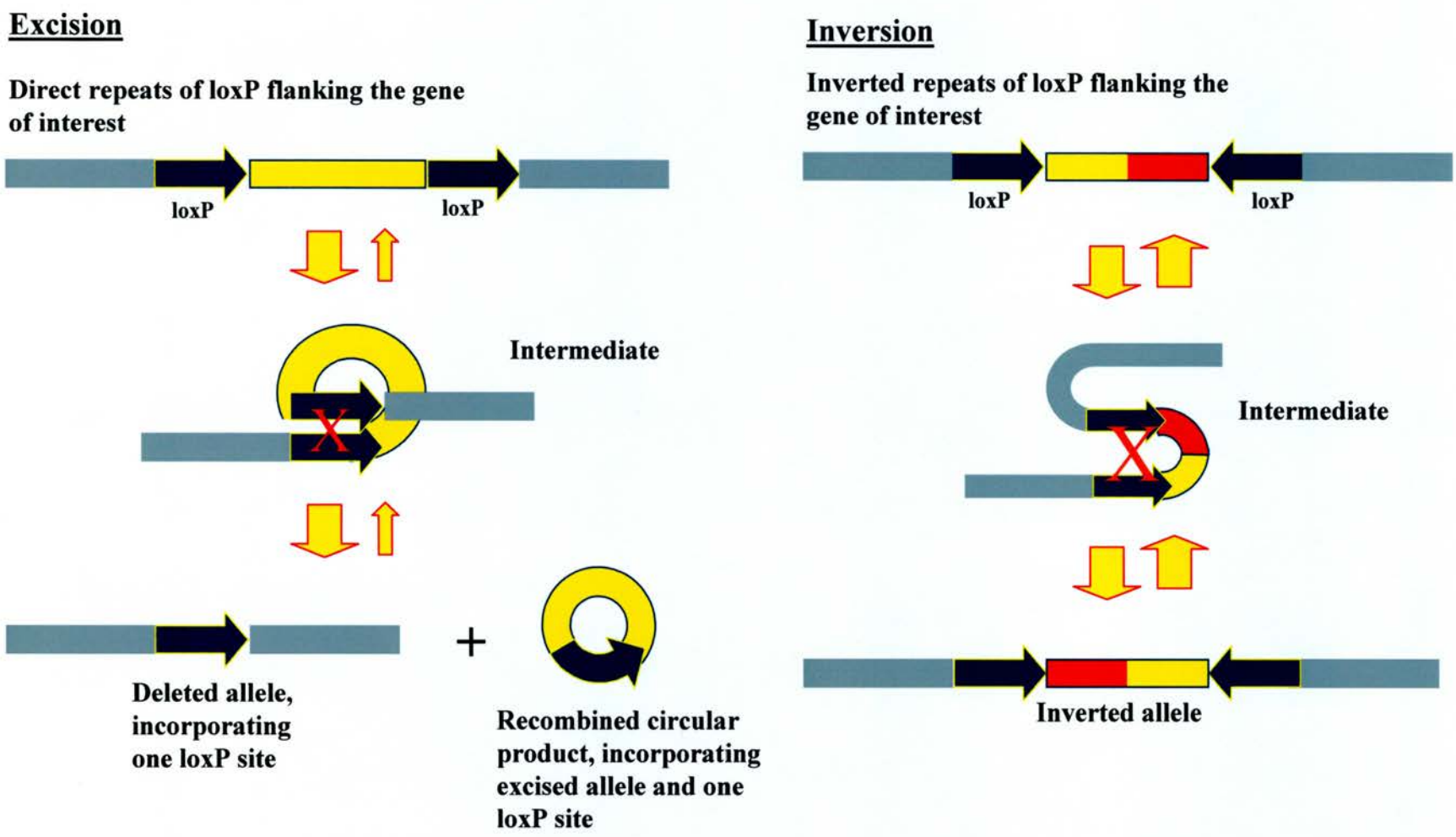


Figure 1.6. A model describing the Cre-lox system of site-directed recombination. Recombination of loxP sites is mediated by Cre recombinase (denoted by yellow arrows). Excision of the floxed allele is energetically favourable over the reverse reaction. Inversion is reversible in the continued presence of Cre.

Table 1.6.iii – Use of Cre-lox technology in the mouse

Tissue-specific Cre	
Adipose tissue (aP2-Cre)	**Barlow et al., 1997**
Mammary gland (BLG-Cre)	**Selbert et al., 1998**
Skin (Keratin-5-Cre)	**Tarutani et al., 1997**
Cardiac muscle (α-MHC-Cre)	**Agah et al., 1997**
Kidney (AQP2-Cre)	**Nelson et al., 1998**
Pancreas (Rat Insulin-Cre)	**Ray et al., 1998**
Endothelium (Tie-1-Cre)	**Gustafsson et al., 2001**
Cerebellum (GFAP-Cre)	**Marino et al., 2000**
Smooth Muscle (SM-MHC-Cre)	**Regan et al., 2000**
T cell (lck-Cre)	**Gu et al., 1994**
CNS (Synapsin 1-Cre)	**Zhu et al., 2001**
Macrophages / Granulocytes (LysM-Cre)	**Clausen et al., 1999**
Inducible-Cre	
Tetracycline-inducible Cre	**St-Onge et al., 1996**
Interferon α/β-inducible Cre	**Kuhn et al., 1995**
4-hydroxytamoxifen-inducible Cre	**Feil et al., 1996**
Chromosomal Translocations	
Modelling the t(8;21) reciprocal translocation found in human acute myeloid leukaemia	exploits Nestin-driven Cre to overcome lethal effects in early embryogenesis **Buchholz et al., 2000**
Modelling the t(9;11) reciprocal translocation found in human leukaemia	exploits ubiquitously expressed Cre (deleter mice, Schwenk et al., 1995) **Collins et al., 2000**

developmental compensation.

Recently, in this department, a SP-C/Cre transgene has been developed in which the 3.7kb fragment of the human SP-C promoter described previously (Glasser et al., 1991; Wert et al., 1993; see Table 1.6.i) is used to express Cre recombinase specifically in small airway epithelial cells of transgenic mice (S.O'Dea, ongoing work). A single founder mouse genotypically positive for Cre was generated by blastocyst injection of the SP-C/Cre construct, and was crossed onto a Floxed mouse line (Floxed DNA Ligase I) to facilitate analysis of Cre-mediated recombination in vivo. As an additional project of this thesis, the author sought to determine the feasibility of this approach in attaining conditional gene targeting in the lung, with the eventual goal of generating improved mouse models of lung injury.

1.7. AIMS

The aims of this thesis are the following:

1) To describe the effects of DNA damage in small airway epithelial cells in terms of growth arrest, apoptosis, and gene expression

2) To discern the role of p53 in small airway epithelial cells in vivo, and in primary Clara cell culture

3) To determine the feasibility of Cre-lox conditional gene targeting in small airway epithelium

2. MATERIALS AND METHODS

2.1. IN VIVO STUDIES

2.1.a. DNA-damaging agents

2.1.a.1. Irradiation

Mice were 5 Gray γ-irradiated by a Cs-137 source at 0.33 Gy/minute. Unirradiated controls were otherwise transported and handled identically. At 2, 4, 6, 8, 24, 48 and 72 hours following irradiation, mice were killed and tissues harvested. The left lung was transferred to a vial of formalin for fixation and paraffin embedding. The right lung was transferred to a cryotube and snap frozen in liquid nitrogen for subsequent RNA and protein extraction.

2.1.a.2. Methylating Agents

Mice were injected intraperitoneally with 50mg/kg MNNG (N-Methyl N-Nitro N-Nitrosoguanidine, Sigma). At 6 and 24 hours post-injury, mice were killed and tissues harvested for histology and protein analysis, as described previously (see section 2.1.a.1).

2.1.a.3. Alkylating Agents

Mice were injected intraperitoneally with 10mg/kg Nitrogen Mustard (Mechloroethamine Hydrochloride, Sigma). At 6 and 24 hours post-injury, mice were killed and tissues harvested for histology and protein analysis, as described previously (see section 2.1.a.1).

2.1.b. Gene-targeted mice

Generation of the homozygous p53-deficient mice used in these experiments has been described previously. Briefly, murine E14 embryonic stem cells (derived from strain 129/Ola) bearing a targeted deletion of exons 2 through 6 of the p53 gene were injected into blastocysts to generate germline chimaeras, which were bred to homozygousity. The targeting construct consisted of a fragment from which intron 1 of the p53 gene, ligated to a pgk-neo cassette and a 5kb fragment that incorporated exons 7-11 of the p53 gene. The neo cassette contained numerous STOP codons in all three reading frames to prevent downstream transcription of the truncated gene.

p53 genotypes were established by a standard operating procedure, specifically, a PCR strategy of DNA prepared from tail biopsies of appropriate lines (progeny of Het x Het crosses). Alleles were selectively amplified by using a primer for intron 7 (common to both genotypes) and primers specific for exon 6 (wild type) and the neo construct.

Mice used in the experiments described here were outbred on a mixed background, segregating for 129, Ola, and Balb/c. For each experiment the mice were age and sex-matched, and were littermates if possible. p53 genotype was rechecked on tail samples after killing. The mice were housed in plastic cages in a room with a 12 hour day-night cycle and controlled temperature and humidity. They were given a standard diet and water ad libitum.

2.1.c. Histology

Histological analysis of wild type and p53-/- mouse lungs was accomplished on 3μm haematoxylin and eosin sections by the author and a pathologist (DJH).

2.1.d. Evaluation of apoptosis

2.1.d.1. Morphology

The morphology of apoptosis was easily recognised by light microscopy on 3μm haematoxylin and eosin stained paraffin sections.

2.1.d.2. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling (TUNEL)

Endonucleolysis is considered a key biochemical event of apoptosis. DNA strand breaks were detected on paraffin sections by the enzymatic in situ end labelling method TUNEL. Terminal deoxynucleotidyl transferase catalysed the polymerisation of nucleotides to free 3'-OH DNA ends in a template-independent manner. Two TUNEL kits, In Situ Cell Death Detection Kit (Roche) and ApopTag (Oncor) were used in accordance with the manufacturer's instructions. The former kit exploited fluorescein as a label of dUTPs, whilst the latter used digoxigenin. Incorporated dUTPs were detected on sections by anti-fluorescein (Roche) and anti-digoxigenin (Oncor) antibodies, respectively. DNase I-treated sections served as a positive control. Negative controls omitted TdT amplification step.

Fluorescein-labelled DNA strand breaks were undetectable by immunofluorescence, hence peroxidase conjugated anti-fluorescein antibodies were necessary to visualise

TUNEL reaction. Positively stained nuclei were visualised by Liquid DAB Chromogen with DAB enhancer (Biogenex), used according to the manufacturer's instructions.

2.1.e. Western analysis

Frozen tissue samples were homogenised and lysed in the presence of a cocktail of protease inhibitors. The lysate was vortexed briefly and centrifuged at 10,000g for 2 minutes at 4°C to pellet cell debris. The supernatant was transferred to a fresh Eppendorf tube and stored at –20°C for up to 2 months. Protein concentrations of extracts were determined using a derivation of the Bradford method (BioRad Protein Assay, BioRad) in accordance with the manufacturer's instructions. Comparisons of Absorbance at 595nm relative to a standard curve of Bovine Serum Albumin facilitated determination of protein concentration.

SDS-polyacrylamide gel electrophoresis was accomplished on precast gels in a XCell II™ tank (Novex) in accordance with manufacturer's instructions. An appropriate amount of protein was diluted 1:1 with loading buffer, boiled for 1 minute, and loaded onto 4-10% Tris-HCl precast gels (Novex). Electrophoresis was accomplished in NuPAGE™ running buffer (Novex) at a constant voltage of 100V per gel for 2 hours, or until dye front reached the bottom of the gel. Protein was transferred onto Hybond™ECL™ nitrocellulose membranes (Amersham) via electrophoresis at a constant current of 250mA per gel for 90 minutes. Gel sandwich apparatus (BioRad) and transfer tank (BioRad) were used in accordance with

manufacturer's instructions. Transfer buffer was made up fresh and chilled to 4°C prior to use.

Protein blots were transferred to 50ml centrifuge tubes. All subsequent steps were accompished on a roller mixer. Blots were washed three times in TBST (5 minutes each wash), and stained with Ponceau solution to assess equal loading and quality of transfer. Blots were destained of Ponceau solution, washed three times in TBST (5 minutes each wash) and blocked in 10% Marvel (Nestlé, UK) in TBST for 1 hour. Blots were then subjected to immunolabelling.

2.1.e.1. p53 protein

Blots were incubated with CM5 rabbit polyclonal antiserum (Novocastra), diluted 1:100 in 10% Marvel/TBST, overnight at 4°C. Blots were washed in TBST (3x5 minute wash steps) and incubated with peroxidase-conjugated donkey-anti rabbit IgG (Santa Cruz), diluted 1:2,000, for 30 minutes at room temperature. Visualisation was accomplished by chemiluminescent labeling. This stepwise protocol involved incubation with ECL™ (Amersham), exposure to ECL™ film (Amersham), and development of films (Hyperprocessor, Amersham), all carried out in accordance with manufacturer's instructions. Developed films were digitally scanned (DeskScan II, Hewlett Packard) and bands quantified with densitometry software (Aida, Microsoft).

CM5 immunopositivity was not observed in whole lung extracts at a load of 40μg protein per well. 150μg protein per well was required to unequivocally demonstrate

immunopositivity in whole lung extracts. 20μg irradiated spleen extract served as a positive control. Negative controls omitted primary antibody, and were accomplished in parallel.

2.1.e.2. $p21^{WAF1/CIP1}$ and bcl-2 family proteins

The presence of $p21^{WAF1/CIP1}$ and Bax proteins in whole lung extracts was determined by immunoblotting with goat polyclonal antibodies M-19 and P-19 respectively (Santa Cruz). Primary antibodies were incubated for 1 hour at room temperature at dilutions of 1:200 in 10% Marvel/TBST.

Blots were washed in TBST (3x5 minute wash steps) and incubated with peroxidase-conjugated donkey-anti goat IgG (Santa Cruz), diluted 1:5,000, for 30 minutes at room temperature. Visualisation and densitometry were accomplished by methods described previously (see 2.1.e.1). bcl-x long and short isoforms were demonstrated by immunoblotting with rabbit polyclonal antibody M-125, diluted 1:500 in 10% Marvel/TBST, and incubated for 1 hour at room temperature. Subsequent steps were identical to those used in p53 immunoblotting (see 2.1.e.1).

$p21^{WAF1/CIP1}$, Bax, and $bcl\text{-}x^{L/S}$ proteins are of similar size, hence different blots were required for each analysis. 40μg whole lung extract was loaded per well. Negative controls omitted primary antibody, and were accomplished in parallel.

2.1.f. Immunohistochemistry

2.1.f.1. p53 protein

CM5 rabbit polyclonal antiserum (Novocastra), the antibody used in this analysis, recognises seven distinct epitopes throughout the p53 protein. CM5 antibody has been used previously to demonstrate p53 stabilisation in formalin-fixed paraffin embedded murine tissues. Tissues derived from p53-null mice served as a negative control, irradiated spleen from wild type mice served as a positive control.

CM5 immunohistochemistry was performed on formalin-fixed low temperature paraffin sections on polylysine coated slides after microwave pretreatement (10 minutes, 700 Watts) in 10mM citrate buffer (pH 7.6). Positive-nuclei were visualised by an avidin-biotin peroxidase labelling procedure.

Slides were rehydrated, exposed to 1.5% hydrogen peroxide/methanol for 15 minutes, washed in TBST, and incubated for 10 minutes in 20% swine serum/TBST. Endogenous biotin was blocked using a kit (Vector laboratories), according to the manufacturer's instructions. Paraffin sections were incubated with primary antibody, diluted 1:500 in 20% swine serum/TBST, in a humid chamber overnight at 4°C. Negative controls included irradiated spleen derived from p53-null mice, and wild type irradiated spleen of which primary antibody was omitted. After washing in TBST, biotinylated-swine anti-rabbit F(ab')2 (Dako) at 1:400 dilution was applied for 30 minutes. Avidin-biotin horseradish peroxidase complex (Dako) was used as the final labeling step and the chromogen was Liquid DAB (Diaminobenzidine) with DAB enhancer (Biogenex), each applied according to the manufacturer's

instructions. Slides were counterstained with haematoxylin, dehydrated, mounted in pertex, and coverslipped.

2.1.f.2. bcl-2 family proteins

bcl-x long and short isoforms were immunohistochemically localised on paraffin sections with M-125 rabbit polyclonal antiserum (Santa Cruz). Microwave pretreatment in citrate buffer (10 minutes, 700W) produced superior immunostaining to that accomplished with trypsin pretreatment or no pretreatment. Rehydrated paraffin sections were blocked in 20% swine serum/TBST and incubated with primary antibody overnight at 4°C (diluted 1:400 in 20% swine serum/TBST). After washing in TBST, biotinylated swine anti-rabbit F(ab')2 (Dako) at 1/400 dilution was applied for 30 minutes.

Avidin-biotin alkaline phosphatase complex (Dako) was used as the final labeling agent and the chromogen was Vector Red. Advantages of Vector Red as a chromogen include its ability to fluoresce under UV light, allowing enhanced visualisation by immunofluorescence. Immunofluorescence was viewed under UV illumination from a mercury lamp using a dichroic filter appropriate for Vector Red (green) fluorescence.

Bax immunohistochemistry involved identical pretreatment and labeling steps as bcl-x, with the exception that blocking and antibody incubation steps were accomplished in 20% donkey serum/TBST. P-19 anti-Bax goat polyclonal antiserum, diluted 1:100, was incubated overnight at 4°C. Biotinylated donkey ant-goat IgG, diluted

1:500, was incubated for 30 minutes at room temperature. Negative controls omitted primary antibody.

2.1.f.3. $p21^{WAF1/CIP1}$ protein

M-19 goat polyclonal antiserum was used to detect $p21^{WAF1/CIP1}$ on paraffin sections. Trypsin and microwave pretreatments followed by avidin-biotin amplification steps were insufficient to label $p21^{WAF1/CIP1}$ on paraffin sections. Hence superior amplification with biotinylated tyramide (NEN Life Sciences) was necessary to assess protein distribution. Microwave pretreatment in citrate buffer (10 mins, 700W) improved immunodetection with the latter system.

Slides were rehydrated, exposed to 1.5% hydrogen peroxide/dH_2O for 15 minutes, washed in TBST, and incubated for 10 minutes in 20% swine serum/TBST. Endogenous biotin was blocked in accordance with manufacturer's instructions (TSA^{TM}, NEN Life Sciences). M-19 goat polyclonal IgG, diluted 1:100, was incubated overnight at 4°C, washed in PBS, and incubated with biotinylated donkey anti-rabbit secondary antibody, diluted 1:500, for 30 minutes. Sections were washed, and biotinylated derivatives amplified with a Tyramide Signal Amplification^{TM} Kit (TSA^{TM}, NEN Life Sciences) in accordance with manufacturer's instructions. TSA^{TM} exploited Horseradish Peroxidase activity to catalyse the deposition of biotin-labeled tyramide onto tissue sections, resulting in the deposition of numerous biotin labels. Biotin was labeled with avidin-biotin peroxidase complex (Dako), and visualised with DAB. Added labels were deposited proximal to the enzyme site, resulting in minimal loss of resolution.

2.1.f.4. 5-Bromo-2'-deoxyuridine (BrdU)

24 hours prior to killing, mice were injected intraperitoneally with BrdU. Tissues were harvested, formalin-fixed, and paraffin-embedded. Intestinal epithelium is constitutively proliferating, and served as a positive control of BrdU incorporation. Primary antibody was omitted in negative controls.

Slides were rehydrated, exposed to 1.5% H_2O_2 in distilled water for 10 minutes, rinsed in PBS, and incubated in 5M HCl for 1 hour, followed by three 5-minute washes in PBS to restore neutrality. Cells were incubated in 20% rat serum/TBST for 10 minutes, and then incubated with purified monoclonal rat anti-BrdU IgG-direct peroxidase conjugate (Roche) diluted 1:10 in 20% rat serum/TBST for 1 hour. Negative control slides omitted the primary antibody. Positively stained nuclei were visualised by Liquid DAB Chromogen with DAB enhancer (Biogenex), used according to the manufacturer's instructions. Slides were counterstained with haematoxylin, mounted, and coverslipped.

2.1.g. Flow cytometric evaluation of cell proliferation

Fresh lung tissue was collected, disaggregated and nuclei stained by propidium iodide as described by Vindelov (1985). This method produces clean, single nuclei essentially devoid of residual cytoplasm. Ten thousand nuclei from each sample were analysed for DNA content by a Coulter EPICS CS flow cytometer, measuring fluorescence emitted by each nucleus at 610nm (red fluorescence) in 488nm Argon laser light. The gate was set to red fluorescence and the results of each analysis were viewed as a histogram of recorded red fluorescent events. The total red fluorescence

emitted by each nucleus is proportional to the amount of bound propidium iodide. Propidium iodide binds stoichiometrically to DNA hence the integral red fluorescence recorded by the flow cytometer for each nucleus is proportional to DNA content. The histograms of red fluorescence are therefore histograms of nuclear DNA content.

2.1.h. Ribonuclease protection assays

Total RNA was extracted from frozen tissues using TriGene (Sigma) according to the manufacturer's instructions. Quantitative determination of RNA yield was accomplished by spectrophotometry in a 70µl cuvette (GeneQuant, Pharmacia Biotech). Quality of RNA was assessed by visualisation of Ethidium Bromide-stained extracts electrophoresed on a formaldehyde gel. Streaking of ribosomal RNA bands was considered indicative of degradation.

Generation of radiolabelled antisense riboprobes was accomplished with the use of RiboQuant™ MultiProbe mAPO-2 Template Set (Pharmingen) and In Vitro Transcription (Pharmingen) kits, in accordance with manufacturer's instructions. Riboprobe templates are transcribed from pPMG plasmids containing the gene fragment of interest, in this case, members of the bcl-2 family and housekeeping genes L32 and GAPDH. Transcription from SP6 promoters produce sense riboprobes, whilst polymerisation from T7 promoters generates antisense probes. Templates were transcribed with T7 polymerase in the presence of an excess of ^{32}P-dUTP (32030X, ICN) for 1 hour at 37°C, in accordance with manufacturer's instructions. Subsequent incubation with DNase I removed plasmid templates, and

thus allowed purification of riboprobes. Riboprobes were purified by stepwise phenol:chloroform extraction and ammonium acetate/absolute ethanol precipitation, in accordance with manufacturer's instructions. The RNA pellet was air dried and dissolved in 50µl hybridisation buffer (Pharmingen). Counts/min of dissolved riboprobe were analysed in a scintillation counter (LKB Wallac). The acceptable minimum was $3x10^5$ counts/min.

20µg RNA extracts in 1.5 ml eppendorf tubes were vacuum-dried and redissolved in 8µl hybridisation buffer (Pharmingen). 2µl probe (diluted between $2.3\text{-}4.3x10^5$ counts/min) was added to each sample. Samples were covered with nuclease-free mineral oil, centrifuged briefly, and removed to a heat block at 90°C. Heat block was immediately reduced to 56°C and maintained at this temperature overnight to allow hybridisation.

The following day, eppendorfs were removed to a 30°C water bath for 15 minutes prior to RNase treatment. A mixture of RNase A and T1 in an appropriate buffer (Pharmingen) was incubated with each sample for 45 minutes at 30°C to digest single-stranded RNA. Double-stranded RNA, procured from overnight hybridisation of total RNA with antisense probes, was purified and resolved by polyacrylamide gel electrophoresis, in accordance with manufacturer's instructions. Protected probes were quantified with Phosphorimager apparatus (Fuji), and expressed relative to housekeeping gene L32.

2.2. IN VITRO STUDIES

2.2.a. Primary Clara cell culture

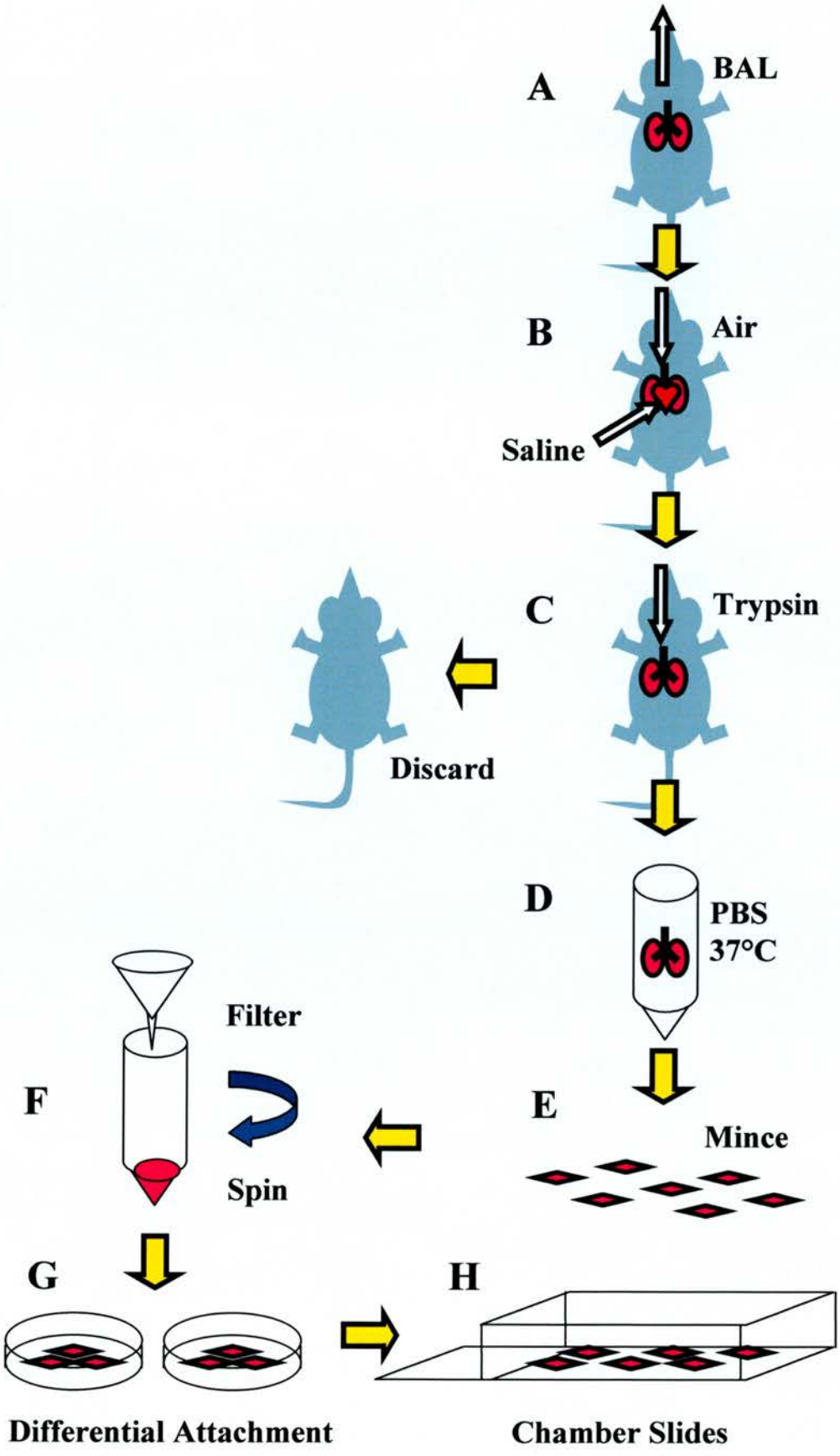
The perfusion apparatus was sterilised and cleaned before and after use by running through 70% absolute ethanol/tissue culture grade water followed by tissue culture grade water. All reagents and equipment in contact with isolates were cleaned to tissue culture grade standard and sterilised. Perfusions were carried out under still air conditions and once Clara cells were isolated all subsequent handling was performed in a sterile laminar flow tissue culture hood (Class II) using sterile technique. The Clara cell isolation and culture method is summarized in Figure 2.2.

2.2.a.1. Dissection and lavage of lung

Young adult mice were given a lethal intraperitoneal injection of 0.2 ml Sagatal. The ventral surface skin was removed and the midline incised to allow entry into the peritoneal cavity. The gastrointestinal tract was displaced to the right and the major dorsal blood vessels severed, thus achieving exsanguination. The thyroid gland was excised allowing entry to the trachea. The trachea was separated from the underlying oesophagus and cannulated (1mm Luer cannula, CAN1004, Scientific Lab Supplies).

The diaphragm was opened via an incision at the caudal-most aspect of the xiphisternum. The rib cage was removed followed by a portion of thymus, facilitating ease of access to the myocardium. Care was taken not to puncture the lungs. A haemostat was attached to the apex of the myocardium and rotated 180 degrees counter-clockwise, facilitating entry into the right ventricle. The right ventricle was incised perpendicular to the right coronary artery, and a cannula

Figure 2.2. Clara cell isolation method and culture method. A) The 1st catheter is inserted into the trachea and used to extract BAL fluid. B) The 2nd catheter is inserted into the right ventricle of the heart and used to lavage the pulmonary circulation with ice-cold saline. C) Trypsin is administered to the lungs *in situ* via the trachea (1st catheter). D) Intact lungs are harvested and removed to a 37°C water bath to facilitate digestion. E) Lungs are minced and filtered through a 100μm mesh. Low centrifuge spins (30xG) removes blood cells and selects for bronchiolar clumps. F) Macrophages and fibroblasts are removed by differential attachment to charged plastic. G) Isolated bronchiolar cell clumps are washed in PBS, resuspended in pre-warmed medium (37°C) and seeded to fibronectin-coated slides.


A
BAL
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Air
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D
PBS
37°C
E
Mince
F
Filter
Spin
G
H
Differential Attachment
Chamber Slides

(1.7mm Luer cannula, CAN1008, Scientific Lab Supplies) inserted through the semi-lunar valve into the pulmonary artery. Sterile saline (0.15M NaCl) was gravity fed through the cannula, causing expansion of the left atrium. An incision was made in the left atrium to allow fluid exit. Lungs were artificially ventilated with a 1.0ml syringe of air. After 5 ventilations, the lungs were largely devoid of blood, and appeared white. Intracardial cannula was removed and discarded.

2.2.a.2. Digestion and removal of lung

Ice-cold trypsin was intratracheally instilled into the lungs in situ via the remaining cannula. Myocardium was removed and phrenic nerve, oesophagus, posterior vena cava, and strands of mediastinum severed, allowing detachment of the lungs from the peritoneal cavity. Trachea was severed rostral to the insertion point of the cannula and the lungs and trachea, with syringe and cannula attached, removed to a vial of PBS preheated to 37°C. The plunged syringe, necessary to minimise leakage of trypsin throughout the dissection, was at this point discarded. Trypsin-instilled lungs were incubated for 10 minutes at 37°C.

2.2.a.3. Cell purification by centrifugation

Trypsinised lungs were removed to a sterile plastic tissue culture dish and minced to ¼ mm diameter portions with sterile scissors. Foetal Bovine Serum (2mls/lung) was added to shredded tissue to inhibit further trypsin activity. Shredded tissue was transferred to a 50ml centrifuge tube, diluted 1:1 with DNase I solution, and hand-shaken for 4 minutes. Treatment with DNase I reduced viscousity. Clumps of tissue were removed by passing preparation through a 100μm mesh gauze. Low centrifuge

spins (30g) in ice-cold PBS removed remaining blood cells and selected for bronchiolar cell clumps. Pellets, devoid of erythrocytes and thus white, were resuspended in 10mls medium (5% FBS) and plated onto a sterile plastic tissue culture dish at 37°C, 5%CO2/air for 90 minutes, to allow differential attachment.

2.2.a.4. Cell purification by differential attachment

At this point in the procedure, the isolate consists predominantly of bronchiolar cell clumps, with typical 'bunch of grapes' morphology, but also macrophages and fibroblasts. The latter two cell types have an increased capacity to adhere to charged plastic than epithelial cell clumps. Serum was added to the medium to promote the adhesion of non-parenchymal cells.

Isolates were incubated at 37°C, 5%CO2/air in 5% FBS medium for 90 minutes to allow non-parenchymal cells to attach. Supernatants were collected into 50ml tubes and centrifuged at 100g for 10 minutes. Pellets were washed in PBS and resuspended in 1ml serum-free medium. A 10µl aliquot was taken for an approximate cell count (see 2.c.1). Suspensions were diluted accordingly and seeded to fibronectin-coated chamber slides. Cells were maintained in serum-free conditions in 5% CO2/air at 37°C unless otherwise stated. Fresh medium was introduced every 24 hours.

2.2.a.5. Plating of freshly isolated Clara cells

It is a standard practice to evaluate the yield of isolated cells using a haemocytometer. However, the latter apparatus is designed primarily for quantifying single cell suspensions and not clumps of adherent cells. Instead, aggregates of Clara cell isolates with typical 'bunch of grapes' morphology were counted using the haemocytometer, and diluted to approximately of $1x10^4$ clumps/ml in serum-free medium. 400µl Clara cell suspension was seeded to each well of Lab Tek II 8-well chamber slides. 3ml Clara cell suspension was seeded to each chamber flask.

2.2.a.6. Cell substratum

Clara cells were cultured on fibronectin-coated glass. Clara cells do not attach readily to charged plastic hence non-charged glass chamber slides and flasks were used throughout. Glass flasks were adapted for use with the Leica videomicroscope via the addition of a glass platform, sandwiched between the chamber flask and videomicroscope moveable platform.

Glass chamber slides (Lab-Tek™ II, Life Technologies) and flasks (Flaskette, Life Technologies) were coated overnight with fibronectin (Sigma), diluted 10µg/ml in PBS, at 4°C. 30 minutes prior to plating, slides and flasks were washed in PBS and allowed to air-dry in a Class II tissue culture hood.

2.2.a.7. Culture medium

Medium formulation is a major determinant of both culture survival and maintenance of in vivo phenotype. Serum-free medium is desirable because serum is undefined and promotes growth of non-parenchymal cells. The medium used throughout the isolation and culture procedure was a 1:1 mixture of Hams F12 (GIBCO) and M-199 medium (GIBCO) with supplements. Supplements included the amino acid glutamine, the antibiotics penicllin and streptomycin, the non-haem iron-transport protein transferrin, the strerol hydrocortisione, the retinol derivative retinyl acetate, and insulin and epidermal growth factors. This medium composition is a derivation of the formula used previously by Masek & Richards (1990) and is identical to that used by McBride and others (2000) in the primary culture of murine Clara cells.

Medium composition:

1:1 Hams F12 (GIBCO) and M199 (GIBCO)
2mM L-Glutamine (GIBCO)
100U/ml Penicillin (GIBCO)
100μg/ml Streptomycin (GIBCO)
1X Insulin/Transferrin/Selenium (ITS-X, GIBCO)
10ng/ml Hydrocortisone (Sigma)
0.1ng/ml Retinyl Acetate (Sigma)
10ng/ml Epidermal Growth Factor (rhEGF, Sigma)

2.2.b. Morphology

Clara cell cultures from wild type and p53-/- mice were stained with haematoxylin/methyl green and assessed for morphological abnormalities by light microscopy.

2.2.c. Immunocytochemistry

2.2.c.1. Clara cell 10kDa protein (CC10)

Cultures plated onto Lab Tek II Glass chamber slides were fixed overnight at 4°C in 80% ethanol/PBS. Slides were incubated with CC10 rabbit polyclonal IgG (courtesy of S. O'Dea), diluted 1:100 in 20% swine serum/PBS for 1 hour. Slides were washed in PBS and incubated with biotinylated swine anti-rabbit IgG, diluted 1:500 in 20% swine serum/PBS. Avidin-biotin alkaline phosphatase complex (Dako) was the final labeling agent and the chromogen was Vector Blue (Vector Laboratories). Nuclei were Feulgen counterstained, mounted, and coverslipped. Negative controls omitted primary antibody.

2.2.c.2. 5-Bromo-2'-deoxyuridine (BrdU)

Cultures plated onto Lab Tek II Glass chamber slides were exposed to medium containing 40μM BrdU for 24 hours, after which they were fixed overnight at 4°C in 80% ethanol/PBS. Cells that had incorporated BrdU into DNA were labelled by direct peroxidase immunocytochemistry, as described previously (section 1.f.4). Slides were counterstained with haematoxylin and light green, mounted, and coverslipped.

Evaluation of BrdU positivity in primary Clara cell culture was accomplished by AxioHOME microscopy. 500 Clara cells (sufficient to achieve a stable running mean) were counted from at least seven randomly selected fields and the results expressed as a percentage (BrdU labelling index).

2.2.c.3. Pericentrin

Cultures plated onto Lab Tek II chamber slides were fixed in ice-cold methanol for 10 minutes, air-dried, washed in PBS (3x5mins), and blocked in 3% Bovine Serum Albumin in PBS. Slides were incubated with rabbit anti-pericentrin IgG (BAbCO), diluted 1:100 in 3% BSA/PBS, overnight at 4°C. Slides were washed in PBS (3x5mins) and incubated with FITC-conjugated swine anti-rabbit secondary antibody (Dako), diluted 1:20 in 3% BSA/PBS, for 1 hour at room temperature. Nuclei were counterstained with propidium iodide, mounted, and coverslipped. Staining was visualised by Confocal Microscopy.

Evaluation of centrosome number was accomplished by Confocal microscopy. 300 Clara cells (sufficient to achieve a stable running mean) were counted from five high power randomly selected fields and the results expressed as a percentage.

2.2.d. Irradiation

After 48 hours in culture, Clara cells were 5 Gray γ-irradiated by a Cs-137 source at 33 Gy/minute. Unirradiated controls were otherwise transported and handled identically.

2.2.e. Time-lapse videomicroscopy

Cultures plated onto glass chamber flasks were subjected to time-lapse videomicroscopy for a period of 24 hours. Cultures were gassed with 5%CO_2/Air prior to their insertion onto the videomicroscope moveable platform (Leica). Cultures and microscope apparatus were maintained at 37°C throughout the analysis. Digital images were taken from 5 fields per flask every 15 minutes with the videomicroscope (Leica) and associated software (QUIPS, Leica). Images pooled from the resultant succession of TIFF files (Microsoft) were converted to GIF files (Microsoft) for video analysis (GIF animator, Microsoft). Videos were analysed blind for abnormal mitosis and cytokinesis events.

2.3. CHARACTERISATION OF SP-C/Cre TRANSGENIC MICE

2.3.a. Subcloning

2.3.a.1. Agarose Gel Electrophoresis of DNA

The agarose gel concentration used was dependent on the size of the DNA molecules to be resolved. TBE gels were prepared in accordance with Standard Operating Procedures. Samples to be run were mixed with loading buffer and loaded directly onto the gel. Molecular weight markers (1 kb Ladder, Life Technologies) were diluted in loading buffer and used where appropriate. Electrophoresis was accomplished in 1X TBE at 50-100V.

2.3.a.2. Restriction Digest Analysis of Plasmid DNA

A suitable amount of DNA was mixed with 0. 1 volumes of 10X reaction buffer (supplied by the manufacturer) and 0.1 volumes of 10X Bovine Serum Albumin as required (manufacturer's instructions). 0.1 volumes of restriction endonuclease were added to the reaction mix and any remaining volume was made up to 50µl with ddH20. The digests were incubated for 1~24hrs at the temperature recommended by the manufacturer (New England Biolabs). Digested fragments were visualised on agarose gels.

2.3.a.3. Extraction of DNA Fragments from Agarose Gels

The QIAEX H Gel Extraction Kit (QIAGEN) was used for the purification of DNA fragments from agarose gels. DNA was digested with appropriate restriction endonucleases and electrophoresed on an agarose-TBE gel containing ethidium

bromide. The bands were visualised with UV light (Herolab, gel documentation equipment) and excised from the gel with a scalpel. The gel slice was weighed and appropriate volumes of solubilisation buffer and DNA-binding resin were added to the sample according to the manufacturers instructions. Following solubilisation and adsorption of the DNA to the resin, the sample was centrifuged at 10,000g for 30 seconds. The resin pellet was washed three times and air-dried for 15 minutes. To elute the DNA, the pellet was resuspended in 20µl of water. DNA samples extracted from gels in this manner were stored at -20°C.

2.3.a.4. DNA Ligation

Purified DNA fragments encoding SP-C/Cre were ligated into bluescript plasmid to facilitate the manufacture of probes for Southern analysis. pBS was linearised, and subsequently dephosphorylated with Shrimp Alkaline Phosphatase (SAP, United States Biochemical). SAP removed 5' terminal phosphate groups from linearised DNA and thus prevented vector recircularisation in ligation reactions. SAP reaction was accomplished in the presence of 0.1 volumes of 10X SAP reaction buffer for 30 minutes at 37°C. 1 unit SAP was used per reaction.

It is crucial to ensure that all SAP activity is removed before a treated DNA sample is used in a ligation reaction otherwise other reaction components could also be dephosphorylated preventing efficient ligation. This was achieved by incubation of SAP reaction mixtures at 65°C for 10 minutes.

Equal ratios of insert and SAP-treated vector were mixed together for the ligation reaction. The total volume of reaction mix was 20 µl. This was then added directly into a tube of dehydrated Ready-To-Go^{TM} T4 DNA ligase (Pharmacia Biotech). The ligation reaction was incubated at room temperature for 5 minutes, mixed by gentle pipetting and then transferred to a 16°C water bath for 30 minutes. After this time the ligation is complete. T4 DNA ligase was inactivated by heating to 70°C for 10 minutes. The sample was then used to transform bacteria.

2.3.a.5. Transformation of Bacteria With Plasmid DNA

A 5µl aliquot of Epicurian Coli XL-2 Blue ultracompetent cells (5 x 10^9 cfu/ug pUC18 DNA, Stratagene) was removed from storage at -70°C and thawed on ice. Plasmid DNA was added directly onto the cells and then mixed by pipeting. The cells and the DNA were incubated on ice for 30 minutes, heat shocked at 42°C for 45 seconds and returned to ice for a further 2 minutes. 80µl Luria Broth (LB) was added to the tube, which was then incubated at 37°C for 1 hour in an orbital incubator at 225rpm. The transformed bacteria were plated onto LB-agar plates containing appropriate antibiotics that selected for transformants.

2.3.a.6. Preparation of Plasmid DNA

Plasmid DNA was purified from transformed E. coli with QIAGEN Miniprep and Maxiprep kits, the kit used determined by the yield required. Both procedures are similar, and were accomplished in accordance with the manufacturer's instructions. Briefly, bacterial pellets are resuspended in Tris-HCl/EDTA and RNase A is added to this solution to avoid RNA contamination in later nucleic acid purification steps.

Bacterial lysis is accomplished by exposing suspensions to a mixture of NaOH and SDS. The lysis reaction is subsequently neutralised by addition of potassium acetate. This latter step produces a protein precipitate, subsequently removed by centrifugation. Supernatants were transferred to fresh eppendorfs, and the DNA purified by stepwise phenol:chloroform extraction, isopropanol precipitation, partial rehydration in 70% ethanol, and dissolved in TE pH 8.0. Plasmid DNA was stored at –20°C.

This "miniprep" method is a modification of the procedures described in Sambrook *et al.* (Sambrook *et al,* 1989). A single bacterial clone was picked from a LB-agar plate using a sterile p200 pipette tip. The clone was transferred to a Falcon 2059 tube containing 5 ml of LB supplemented with appropriate antibiotics and incubated in an orbital incubator (225rpm) at 37C overnight. The following day, bacterial pellets were harvested by centrifugation.

Large-scale isolation of plasmid DNA was carried out using a Plasmid Maxi Kit (QIAGEN) according to the manufacturer's instructions. A flask containing 100ml of LB, supplemented with the appropriate antibiotics, was inoculated with bacteria from suitable miniprep cultures. The bacteria were grown for 12- 16 hours in an orbital incubator, harvested by centrifugation (4,000rpm for 10 minutes at 4°C, Sorvall GSA rotor, Centrikon H-40IB), resuspended and lysed just as for the miniprep method. Protein precipitate was removed by centrifugation at 11,500rpm for 30 minutes at 4°C (Sorvall GSA rotor, Centrikon H-401B). The need for an organic extraction was avoided by use of QIAGEN-tips with a DNA-binding resin.

Supernatants were added directly to QIAGEN-tips. QIAGEN-tip columns with bound DNA were washed twice prior to elution of DNA. DNA was precipitated, rehydrated, and dissolved in TE, in accordance with manufacturer's instructions.

2.3.b. Southern Blotting

2.3.b.1. Preparation of DNA

10 μg of genomic DNA was digested with an appropriate restriction enzyme. Following digestion, the entire sample was electrophoresed on a 0.8% gel at low voltage (40V) until dye front had reached the distal most aspect of the gel. Fragments of interest were predicted to be larger than 15kb, hence transfer of the DNA to the nylon membrane was improved by partially depurination ('acid nick') of the DNA prior to transfer. Depurination was achieved by soaking the gel for 10 minutes 0.2M HCI. The gel was then rinsed in deionised water and the DNA was transferred immediately.

2.3.b.2. DNA Transfer

DNA was transferred from the gel onto a positively charged nylon membrane, Zeta-Probe® GT, Bio-Rad) according to the manufacturers instructions. 0.4M sodium hydroxide was used as the transfer buffer. Blotting was accomplished overnight. The membrane was subsequently rinsed in 2XSSC, air-dried, and baked *in vacuo* at 80°C for 30 minutes. Membranes were stored between sheets of Whatman 3MM paper at room temperature until required.

2.3.b.3. Prehybridisation

Hybridisation buffer was preheated to 68°C to ensure complete all components were in solution. 10mls buffer was transferred to a hybridisation tube (Hybaid) containing the baked nylon membrane to allow the membrane to equilibrate. Membrane and buffer were incubated at 68°C for 1 hour in a hybridisation oven (Hybaid).

2.3.b.4. Generation of Radiolabelled Probes

50ng of appropriate DNA was radiolabelled to a high specificity with ^{32}P-dCTP. Labelling was accomplished with Ready-To-Go T4 DNA polymerase beads, in accordance with manufacturer's instructions. Labelled probe was separated from unincorporated nucleotides using a Sephadex G50 column (Pharmacia). 20µl carrier salmon sperm DNA (10mg/ml) was mixed with radiolabelled material prior to addition to column. Unincorporated nucleotides were eluted by addition of 400µl TE buffer. The addition of a further 400µl TE eluted the purified probe. Quality of radiolabelling was assessed by comparison of the counts/minute of both elution products. Labelled probe was only used if it had incorporated >50% radionucleotides.

2.3.b.5. Hybridisation

Following prehybridisation, radiolabelled probe was added to the hybridisation tube. The membrane was incubated with the radiolabelled probe in hybridisation buffer overnight at 68°C. Subsequently, the membrane was washed in solutions of increasing stringency, removed from the hybridisation tube, sealed in a plastic bag, and exposed to Hyperfilm (Amersham) in an autoradiography cassette. Cassettes

were incubated at –70°C for 2-5 days. Autoradiography films were subsequently developed (Hyperprocessor, Amersham).

2.3.c. PCR Strategies

2.3.c.1. Genomic PCR Strategies

PCR strategies were accomplished using RedTaq™ DNA polymerase (Sigma) and accompanying reaction buffer. Magnesium chloride (11mM) and potassium chloride (500mM) were included in RedTaq™ buffer at 10X stock concentrations. Pharmacia Biotech supplied all deoxyribonucleoside-triphosphates (dNTPs). Oligonucleotides were synthesised by Cruachem. PCR reactions were accomplished in a thermocycler (Hybaid), PCR products were analysed on agarose gels.

Conditions in which PCR was accomplished are summarised in Table 2.3:

2.3.c.2. RT-PCR Strategies

An appropriate amount of RNA was diluted in a total volume of 11µl with nuclease-free water and 1µl of oligo dT primer (500ug/ml, Cruachem). The sample was heated to 70°C for 10 minutes then transferred onto ice. After 1 minute on ice, a further 4µl of 5X First Strand Buffer, 2µl of 0.1M Dithiothreitol and 2µl of dNTP mix (10mM) was added. The samples were mixed and heated to 42°C for 2 minutes. 1ul (200 Units) of Superscript™ II RNase H-Reverse Transcriptase was added to each tube. Reactions were allowed to proceed at 42°C for a further 50 minutes, by

TABLE 2.3 – PCR Protocols

PCR Primers

Cre 660	CRE360FOR	AAACGTTGATGCCGGTGAACG
	CRE3'REV	CTAATCGCCATCTTCCTGCAGG
β-actin	BA15	CTCCGGCATGTGCAAAG
	BA4	CGTAGATGGGCACAGTG
LigI genotyping	262W	AGCCTACCCTCTGGTAGATTGTCG
	VO727	GTCAGATTCAGAACCAACAAAG
	V2014	AGTGTTCCCATGGCACAAGTGGCTGGAAGC
LigI deleted	N2469	CCTGTGAGGGCCTGATGGTGAAGACCTTGG
	V2014	AGTGTTCCCATGGCACAAGTGGCTGGAAGC

Thermocycler Protocols

	Melting	*Annealing*	*Extension*	*Cycles*
Cre660	94°C, 45 sec	55°C, 1 min	72°C, 1 min	22
β-actin	94°C, 45 sec	55°C, 1 min	72°C, 1 min	22
LigI genotyping	94°C, 40 sec	65°C, 1 min	72°C, 1 min	35
LigI deletion	95°C, 45 sec	70°C, 1 min	72°C, 1 min	35

which time cDNA synthesis is complete. Reverse transcriptase enzyme was heat inactivated by a 15 minute incubation at 70°C.

PCR of mouse β-actin and Cre recombinase cDNAs was accomplished in conditions summarised in Table 2.3:

3. Results

3.1. The Role of p53 in the Lung

3.1.a. DNA Damage Response of Small Airway Epithelial Cells

3.1.a.1. Induction of p53 in bronchioalveolar cells

In an initial experiment, Balb/c mice were treated with a range of DNA-damaging agents to determine their ability to elicit p53 stabilisation in lung epithelial cells. Mice were treated with the methylating agent MNNG, which induces O^6-methylguanine lesions and other types of base damage, the crosslinking agent nitrogen mustard, which induces bulky adduct formation requiring nucleotide excision repair, and ionizing radiation, which induces DNA strand breaks. Lungs were harvested at 6 hours post-insult, processed as described in materials and methods (section 2.1), and subjected to SDS-PAGE for p53 protein analysis. The lungs of Balb/c mice displayed a rapid 3-fold increase in p53 protein by 6 hours following MNNG, N2M, and IR treatment, as determined by SDS-PAGE of total protein extracts (150μg protein/well)(Figure 3.1.i). The level of p53 stabilisation in damaged lungs was similar between the different treatments, but was low relative to irradiated spleen, where only 20μg of protein was loaded (Figure 3.1.i).

To more accurately monitor induction of p53 in DNA-damaged lung, a timecourse of p53 stabilisation following 5 Gray γ-irradiation was assessed in Balb/c mice by CM5 immunohistochemistry. p53 protein was detected in the nuclei of both ciliated cells

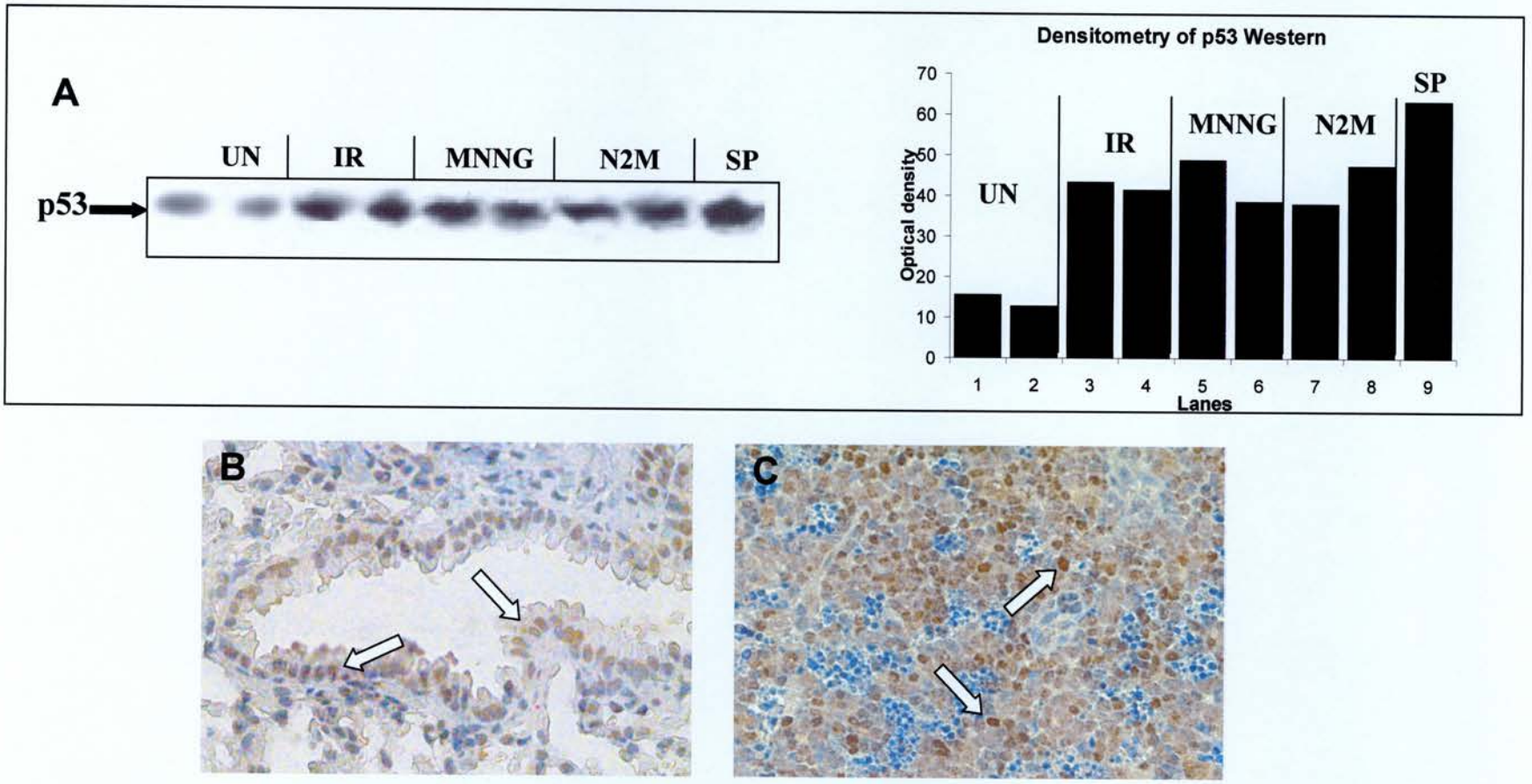


Figure 3.1.i Induction of p53 in airway epithelial cells

A) Western blot analysis of p53 expression in whole-lung protein extracts prepared from untreated (UN), γ-irradiated (IR), methylating agent (MNNG), and alkylating agent (N2M) treated mice. A rapid increase in p53 protein was observed in mouse lungs by 6 hours following exposure to DNA-damaging agents, as determined by densitometry (optical density=integrated optical density/number of pixels of thresholded image, MetaMorph version 4.5, Universal Imaging Inc.) Protein extracts from irradiated spleen (SP) served as a positive control. Mean optical density (n=2) of total lung protein extracts were 14 (UN), 42 (IR), 43 (MNNG) and 40.5 (N2M).

B-C) p53-Immunoperoxidase staining of paraformaldehyde-fixed tissue sections. Immunopositive nuclei (arrows) were observed in bronchiolar cells (B) and splenocytes (C) of irradiated mice (4 hours post-insult).

and non-ciliated cells of the bronchioles (Figure 3.1.i). Increased staining of p53 was observed by immunohistochemistry from the earliest timepoint of 2 hours and was maintained until the latest timepoint of 48 hours. p53 protein was additionally observed in irradiated cells of the spleen, but not the liver, as reported previously (Midgley et al., 1995).

3.1.a.2. Bax and Bcl-x, but not $p21^{WAF1/CIP1}$ proteins are expressed in bronchiolar epithelial cells

To determine additional effects of DNA damage in small airway epithelium, expression analysis of the candidate p53 stress-response genes Bax, Bcl-x, and p21 was accomplished in irradiated and unirradiated mouse lungs. In the mouse, three splice isoforms of Bax have as yet been reported (Krajewski et al., 1994). The most common transcript encodes a 21kDa protein that topographically resembles the Bcl-2 protein by virtue of the presence of a transmembrane domain. Expression of the Bax 21kDa isoform is associated with apoptosis in certain cell types. The other reported variants of Bax include 24kDa and 5kDa isoforms, both lacking the transmembrane domain. Their function is unknown, but is distinct from apoptosis (Krajewski et al., 1994). P-19, the antibody used in this analysis, recognised a region conserved between the three isoforms of Bax.

The only Bax isoform detected in murine lung was the 21kDa protein. Bax was detected in the lungs of untreated mice, and did not display induction following irradiation, as demonstrated by SDS-PAGE. (Figure 3.1.ii). Bax protein localised to

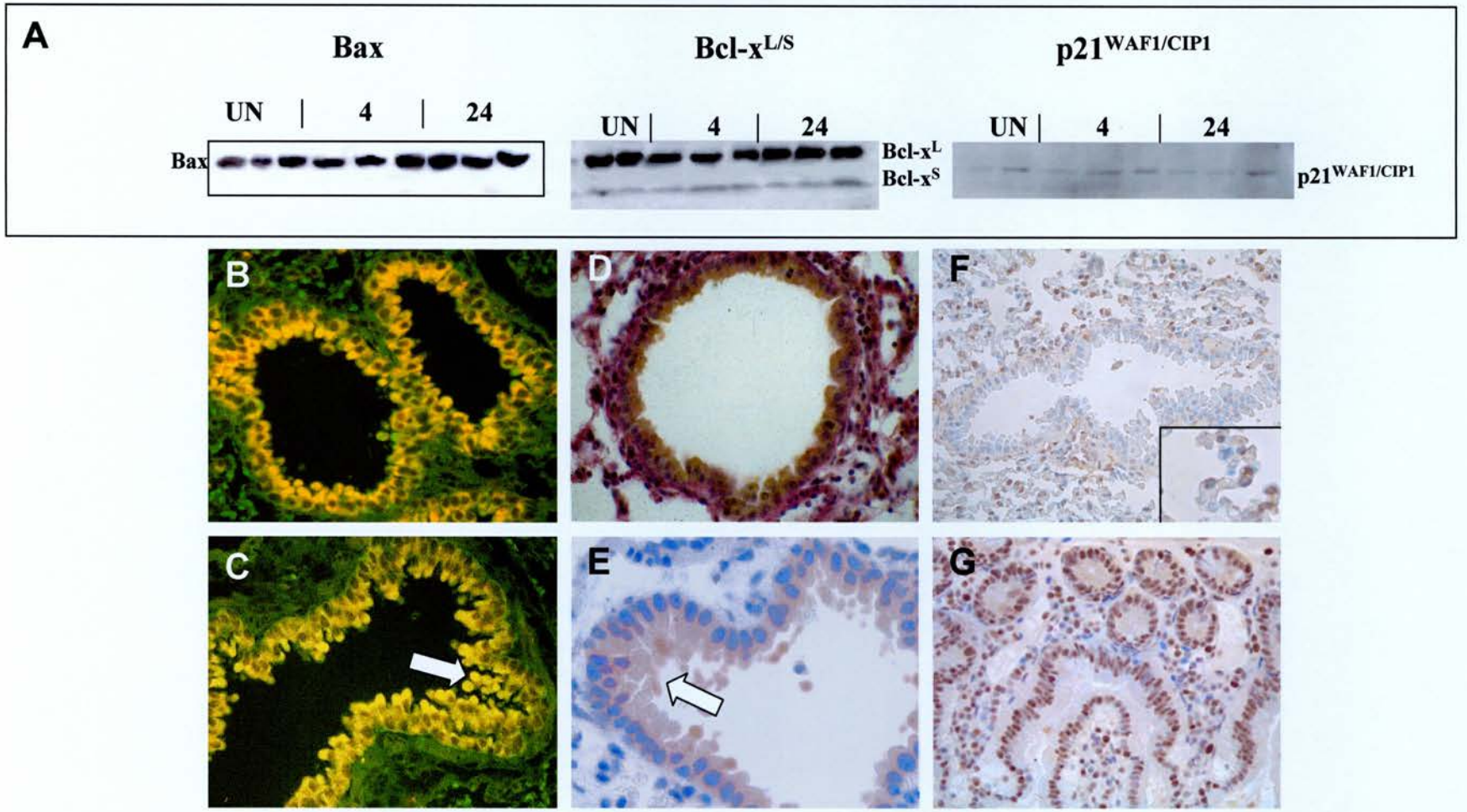


Figure 3.1.ii Expression of Bax, Bcl-x, and p21 in airway epithelial cells

A) Western blot analysis of Bax, Bcl-$x^{L/S}$ and $p21^{WAF1/CIP1}$ expression in whole-lung protein extracts prepared from untreated (UN) and γ-irradiated mice (4 and 24 hours post-irradiation). Mild induction of Bax, but not Bcl-$x^{L/S}$ and $p21^{WAF1/CIP1}$ proteins, was observed in irradiated mouse lungs. B-E) Bronchiolar cells immunohistochemically labelled with Bax (yellow) and Bcl-$x^{L/S}$ (brown) antibodies in untreated (B,D) and irradiated (C,E) mouse lungs. Labelling was cytoplasmic in columnar epithelium and was additionally observed in Clara cell secretions of irradiated mouse lungs (arrows, C,E). $p21^{WAF1/CIP1}$ expression was observed in alveolar cells of irradiated mouse lungs (F, inset), but expression was weak relative to mouse intestinal villi (G). $p21^{WAF1/CIP1}$ expression was not observed in bronchiolar epithelium at all time points.

bronchiolar epithelium, additionally a site of p53 stabilisation, and was observed within Clara cell secretions (Figure 3.1.ii).

Two major splice isoforms of the Bcl-x locus exist. M-125, the antibody used in this analysis, recognises an amino terminus epitope conserved between the long and short isoforms. Both protein isoforms were constitutively expressed in murine lung and did not increase following DNA damage, as demonstrated by SDS-PAGE (Figure 3c). Immunohistochemical analysis with M-125 revealed constitutive Bcl-$x^{L/S}$ expression in columnar epithelium (Figure 3.1.ii). Bcl-$x^{L/S}$ expression was additionally observed in Clara cell secretions (Figure 3.1.ii).

The CDK inhibitor $p21^{WAF1/CIP1}$ has been implicated in DNA damage-induced G1 growth arrest (Brugarolos et al., 1995; El-Deiry et al., 1994; Waga et al., 1994; Li et al., 1994). Transcriptional upregulation of $p21^{WAF1/CIP1}$ in the lung in response to ionizing radiation has been documented previously (Macleod et al., 1995). In this analysis, regulation of p21 $^{WAF1/CIP1}$ was assessed at the protein level. p21 $^{WAF1/CIP1}$ expression levels were similar in the lungs of unirradiated and irradiated Balb/c mice, as determined by SDS-PAGE analysis of total protein extracts (Figure 3.1.ii). Immunohistochemical analysis revealed that $p21^{WAF1/CIP1}$ expression was localised to alveolar epithelial cells of the small airways, and was not observed in bronchiolar epithelium (Figure 3.1.ii). Staining was nuclear in irradiated alveolar cells, but was weak in comparison to intestinal epithelial cells, where a role of p21 $^{WAF1/CIP1}$ in the induction and maintenance of terminal differentiation has been documented previously (Doglioni et al., 1996; Sasaki et al., 1996).

3.1.a.3. Radiation induces DNA strand breaks, but not apoptosis in lung epithelium

To more accurately determine the effects of DNA damage in small airway epithelium, analysis of gene expression in murine lungs was supplemented with morphometric analysis of proliferation and apoptosis. Cell cycle activity was assessed in the lung by bromodeoxyuridine (BrdU) incorporation. Intestine harvested from BrdU-treated animals served as a comparison. Epithelial cells of the intestinal crypts incorporated BrdU (Figure 3.1.iii), consistent with documented cell proliferation in the gut (Clarke et al., 1994). By contrast, lung epithelial cells did not incorporate BrdU prior to and following irradiation, and thus were unlikely to be proliferating. Vindelov flow cytometry analysis confirmed these findings and demonstrated that > 95% of lung tissue was G0/G1 growth arrested prior to irradiation exposure (Figure 3.1.iii).

Two methods, TUNEL and morphology, were compared in their ability to detect apoptosis on paraffin sections of damaged lung. TUNEL is a method for detecting DNA strand breaks in situ. DNA strand breaks are not exclusively a feature of apoptotic cells, but also occur in damaged and necrotic cells (Grasl-Kraupp et al., 1995). TUNEL staining was observed in small airway epithelial cells following radiation insult, but the staining was titratable and required a high concentration of TdT in the TUNEL reaction (Figure 3.1.iv). Apoptotic splenocytes, by contrast, remained TUNEL positive in reduced TdT concentrations. TUNEL was thus a poor surrogate method for the detection of apoptosis in lung epithelium. The incidence of apoptosis was more accurately determined by morphology on haematoxylin and eosin sections. Apoptosis was infrequent in lung epithelium at all timepoints,

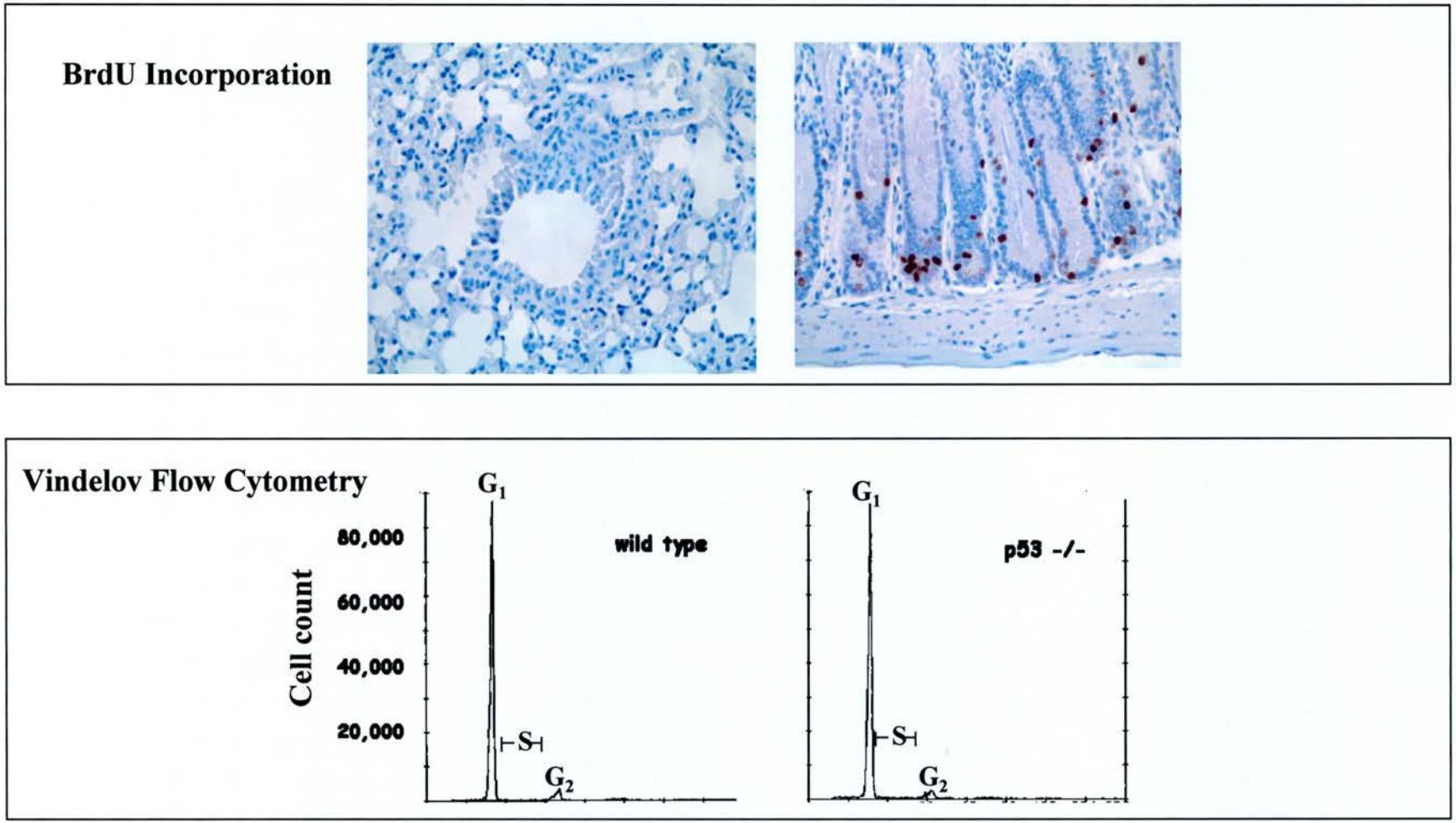


Figure 3.1.iii Cell cycle analysis of wild type and p53-/- mouse lungs

BrdU incorporation assay (upper panel) demonstrating proliferation in intestinal epithelial cells (brown), but not lung epithelial cells. Vindelov flow cytometry profiles (lower panel) of wild type and p53-/- mouse lungs were similar regardless of genotype, and confirmed lack of S phase activity in lung epithelium.

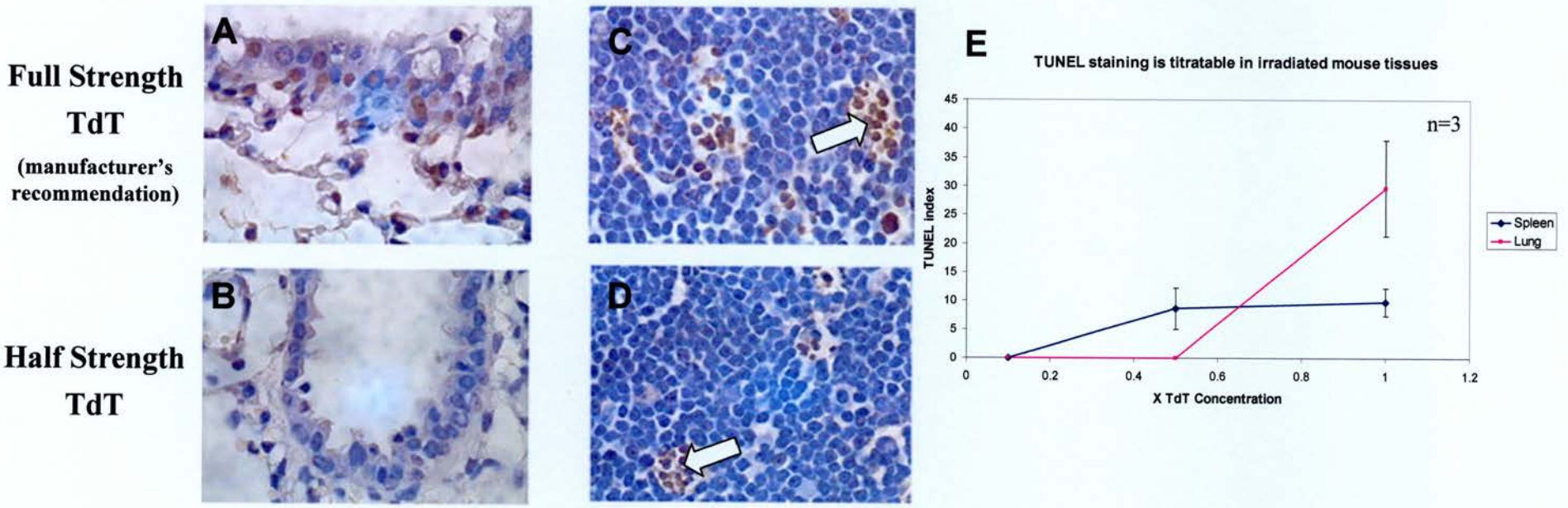


Figure 3.1.iv TUNEL analysis of irradiated lung and spleen

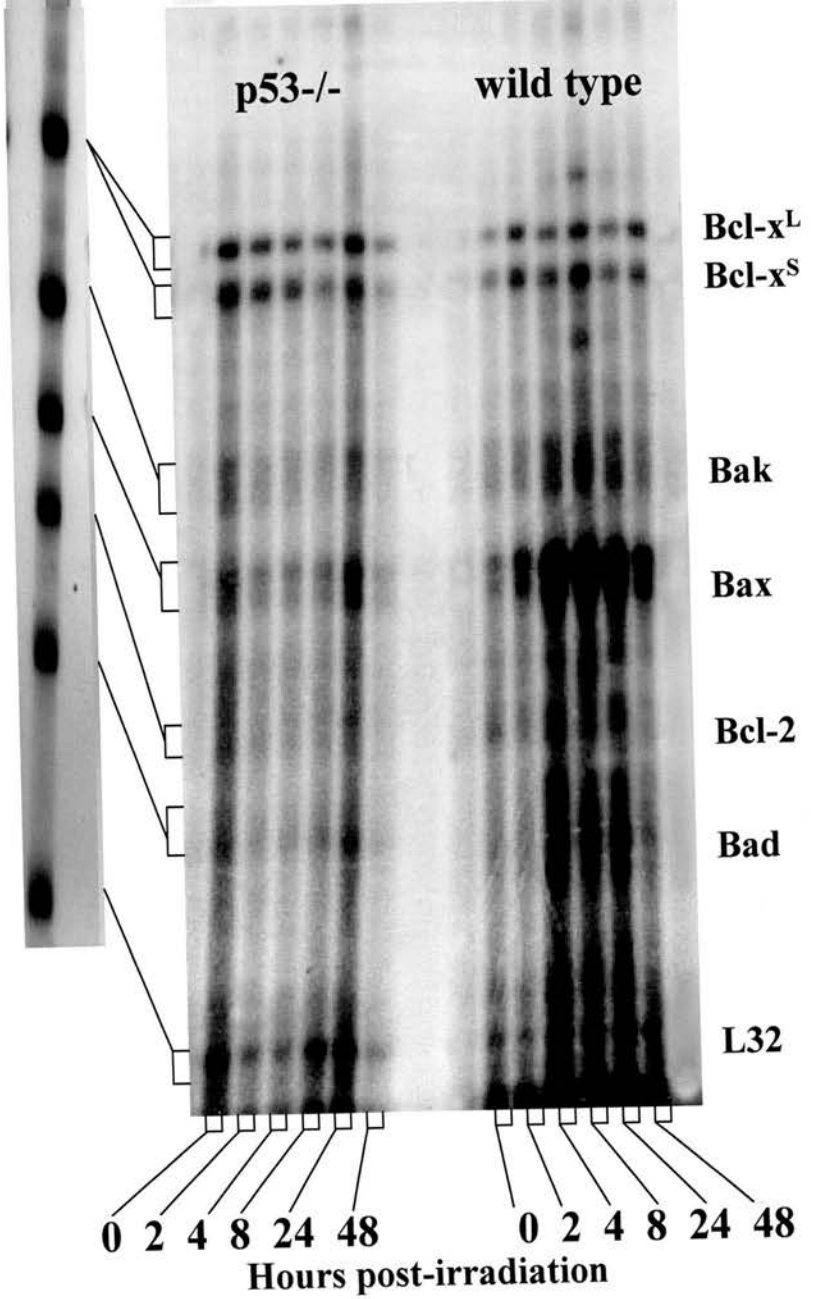
TUNEL staining (brown) was observed in small airway epithelial cells (A) and splenocytes (C) of mice at 4 hours post-γ-irradiation. Staining was titratable in irradiated lung (B), but not spleen (D), by reducing the terminal deoxynucleotidyl transferase (TdT) concentration by 50% (E). Cells with morphological features of apoptosis were additionally observed in irradiated spleen (arrows, C, D), but not lung epithelium. TUNEL index scores represent the mean (n=3) ± standard deviation at 1X, 0.5X, and 0.1X TdT concentration.

regardless of genotype. Apoptosis was observed in lymphoid cells upon irradiation as reported previously (Malcolmson et al., 1997). Significantly, lymphoid cell apoptosis was absent in lungs of p53-deficient animals. Apoptosis was observed in a few scattered endothelial components of irradiated lung and mediastinum. Typically, counts per field of view were less than 1 per cent.

3.1.b. Trans-modulatory effects of p53 in irradiated lung

To more accurately determine transactivation functions of p53, Ribonuclease Protection Assays were used to determine the abundance of bcl-2 family transcripts over a 48 hour timecourse following irradiation. The lungs of gene-targeted mice, germline deficient in p53, were harvested at 0, 2, 4, 8, 24, and 48 hours following irradiation and compared to those of wild-type controls. In the lungs of untreated animals, bcl-2 family mRNA profiles were similar regardless of genotype. Induction of the death-related gene Bax was observed in response to DNA damage (Figure 3.1.v-vi). Wild type animals displayed a twofold increase in Bax mRNA from 4 hours post-injury, and Bax expression levels remained significantly greater than that of untreated animals at the later timepoint of 8 hours. The lungs of p53 -/- littermates did not display this rapid induction of Bax mRNA, and over the 48 hours following irradiation, Bax transcripts were maintained at levels similar to those observed in unirradiated controls.

A



B

	Wild Type			p53 -/-		
	Bcl-x^L	Bcl-x^S	Bax	Bcl-x^L	Bcl-x^S	Bax
0	-	-	-	-	-	-
2	0.76 ± 0.05	0.23 ± 0.02	2.32 ± 0.93	1.00 ± 0.06	1.19 ± 0.33	1.18 ± 0.20
4	0.75 ± 0.12	0.34 ± 0.13	2.16± 0.75	0.80± 0.44	1.22 ± 0.85	0.84± 0.17
8	0.49 ± 0.02	0.24 ± 0.04	1.84± 0.61	0.88± 0.28	1.40 ± 0.50	0.87 ± 0.07
24	1.18± 0.63	0.49 ± 0.17	2.03 ± 0.67	0.95 ± 0.41	1.66 ± 0.55	1.27 ± 0.86
48	0.76 ± 0.10	0.34± 0.04	1.08 ± 0.57	0.73± 0.05	1.83 ± 0.40	1.25 ± 0.26

Figure 3.1.v RNase Protection Assay of Bcl-2 family expression

A) Representative RPA displaying detectable levels of Bcl-xL, Bcl-xS, and Bax mRNA transcripts over a 48 hour timecourse. Undigested probes (left) served as size markers. B) Table summarising data obtained from RPA densitometry analysis of wild type and p53-deficient mouse lungs (see Materials and Methods). mRNA scores (e.g. Bax/L32) are expressed relative to unirradiated controls. Data represents the mean (n=3) ± standard deviation (for complete data set, see appendix).

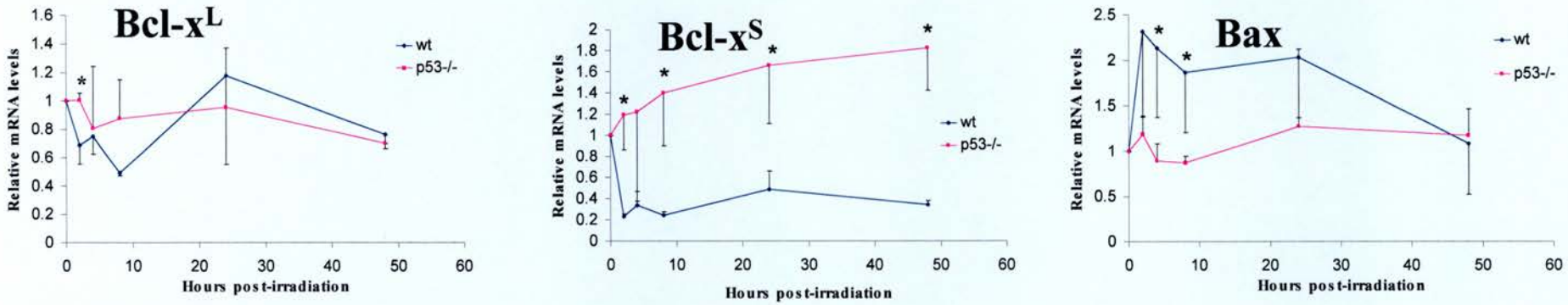


Figure 3.1.vi RNase Protection Assay of Bcl-2 family expression

Graphs summarising the data presented in Figure 3.1.v. Asterisks (*) acknowledge statistically significant differences in Bcl-2 family member mRNA abundance relative to L32 mRNA, as determined by a one-tailed *t* test (P<0.05). For complete data set, see appendix.

A decrease in abundance of the Bcl-x short isoform mRNA was observed in irradiated lungs of wild type mice (Figure 3.1.v-vi). From 2 hours following irradiation, Bcl-x^S transcripts were approximately one third of that of unirradiated controls, and these reduced expression levels were maintained until the latest timepoint considered of 48 hours. p53-/- mice did not display this reduction in Bcl-x^S transcripts following radiation, but instead showed a steady induction of Bcl-x^S mRNA over this 48 hour timecourse when compared with unirradiated controls. Bcl-x^S mRNA expression levels in p53-/- mice were at their maximal (80% increase relative to unirradiated controls) at the latest timepoint considered of 48 hours following irradiation.

The other family members, Bcl-w, Bfl-1, Bak, and Bcl-2 did not show altered regulation upon DNA damage. Constitutive expression of Bcl-x^L transcripts was observed at all timepoints, which was variable between individual mice but did not vary significantly between genotypes (Figure 3.1.v-vi).

3.1.c. Effects of p53-deficiency in Primary Clara cell culture

The effects of p53-deficiency were additionally investigated in short-term primary cultures of bronchiolar Clara cells. Clara cells, isolated from gene-targeted p53-deficient mice, were compared to cells derived from wild type littermates. The isolation and culture method used represents an adaptation of the method described by Richards. Briefly, mice were killed, lungs lavaged and intratracheally perfused in situ with trypsin, harvested, and incubated at 37°C in PBS for 10 mins. Subsequently, lungs were minced to 1/4 mm diameter portions and passed through a 100micron mesh. Low centrifuge spins (30G) removed blood cells and selected for bronchiolar cell clumps. Macrophages and fibroblasts were removed by differential attachment to charged plastic. Isolated bronchiolar cell clumps were washed in PBS, and seeded to fibronectin-coated chamber slides. Clara cells, identified by histochemical markers, were maintained in serum-free conditions unless otherwise stated.

3.1.c.1. Morphological abnormalities in p53-deficient Clara cells

Prior to isolation for culture from adult lung, virtually all bronchiolar cells are in the G0 phase. They are thus effectively synchronised before receiving the proliferative stimulus of isolation and primary culture. In culture, both wild type and p53 null Clara cells displayed a rise in BrdU positivity from near zero at plating to a peak at 48-72 hours in culture (Figure 3.1.vii). BrdU indices were similar between wild type

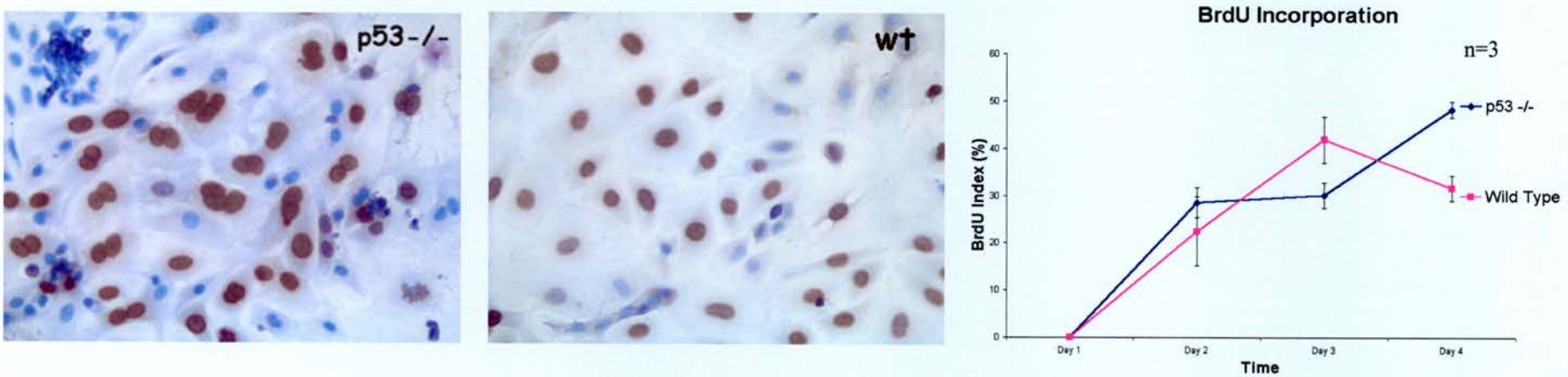


Figure 3.1.vii BrdU incorporation in primary cultured Clara cells

Both wild type and p53-/- Clara cells displayed similar rates of proliferation over the first 3 days in culture, as determined by counts of BrdU incorporation (counts of 200 cells were sufficient to obtain a running mean). In p53-/- Clara cell cultures, multiple BrdU positive nuclei were frequently observed in a single cell. Data represents the mean (n=3) ± standard deviation.

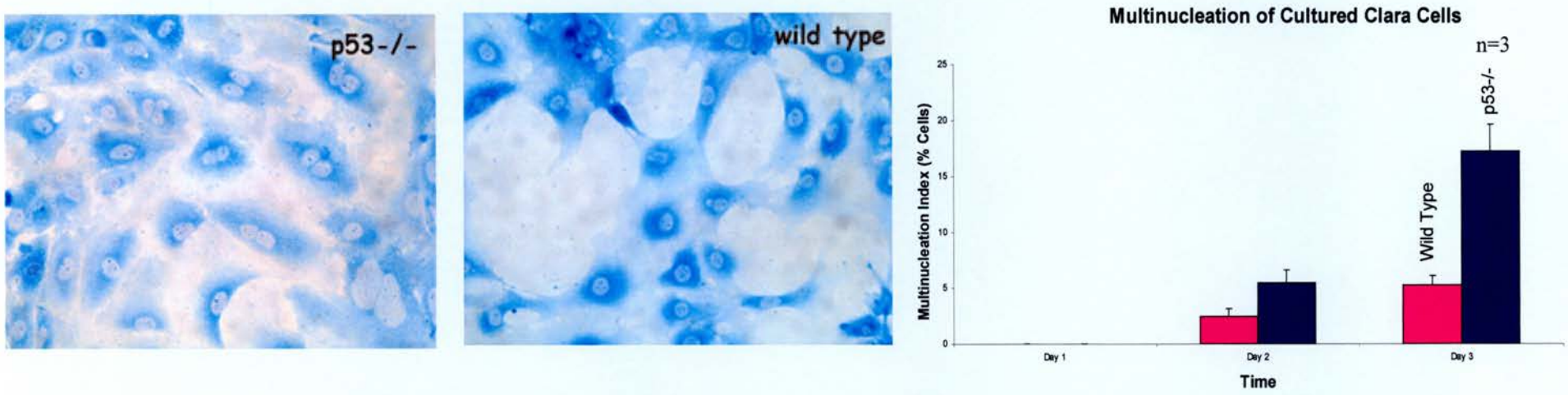


Figure 3.1.viii Morphological abnormalities in p53-/- Clara cells

Primary cultured Clara cells immunohistochemically labelled with anti-CC10 IgG (blue). Two or more nuclei were frequently observed in p53-/- cultures. The number of abnormal cells increased as a feature of time in culture (multinucleation index = no. of multinulceated cells / total no. of cells, expressed as a precentage). Counts of 250 cells were sufficient to achieve a running mean. Data represents the mean (n=3) ± standard deviation.

and p53 null cells at all timepoints. Thus, wild type and p53 null Clara cells display similar kinetics of entry from G0 to S phase.

From 48 hours in culture, a proportion of p53-/- Clara cells displayed morphological abnormalities. Specifically, two or more nuclei were frequently observed in p53-/- cultures. Counts of nuclei/cell (nucleation index) in primary cultures revealed that multinucleation increased over time in cultures of both genotypes, but was significantly enhanced in the p53 null cells (Figure 3.1.viii). Multinucleated cells maintained expression of Clara-cell marker genes and were often BrdU positive (Figure 3.1.viii). However, multinucleation was not associated with DNA synthesis, which was similar irrespective of p53 status.

It was hypothesised that multinucleation represents a failure to undergo cytokinesis in cultured Clara cells. Time-lapse videomicroscopy was employed to assess cytokinesis abnormalities. p53 null cells displayed an array of mitosis and cytokinesis abnormalities, including multiple spindle formation (Figure 3.1.ix, for video, see appendix). By contrast, M phase abnormalities were not observed in wild type cells. Cell death was infrequently observed in time-lapse videos of both genotypes. Thus, p53 has a role in mitotic fidelity and cytokinesis in cultured Clara cells.

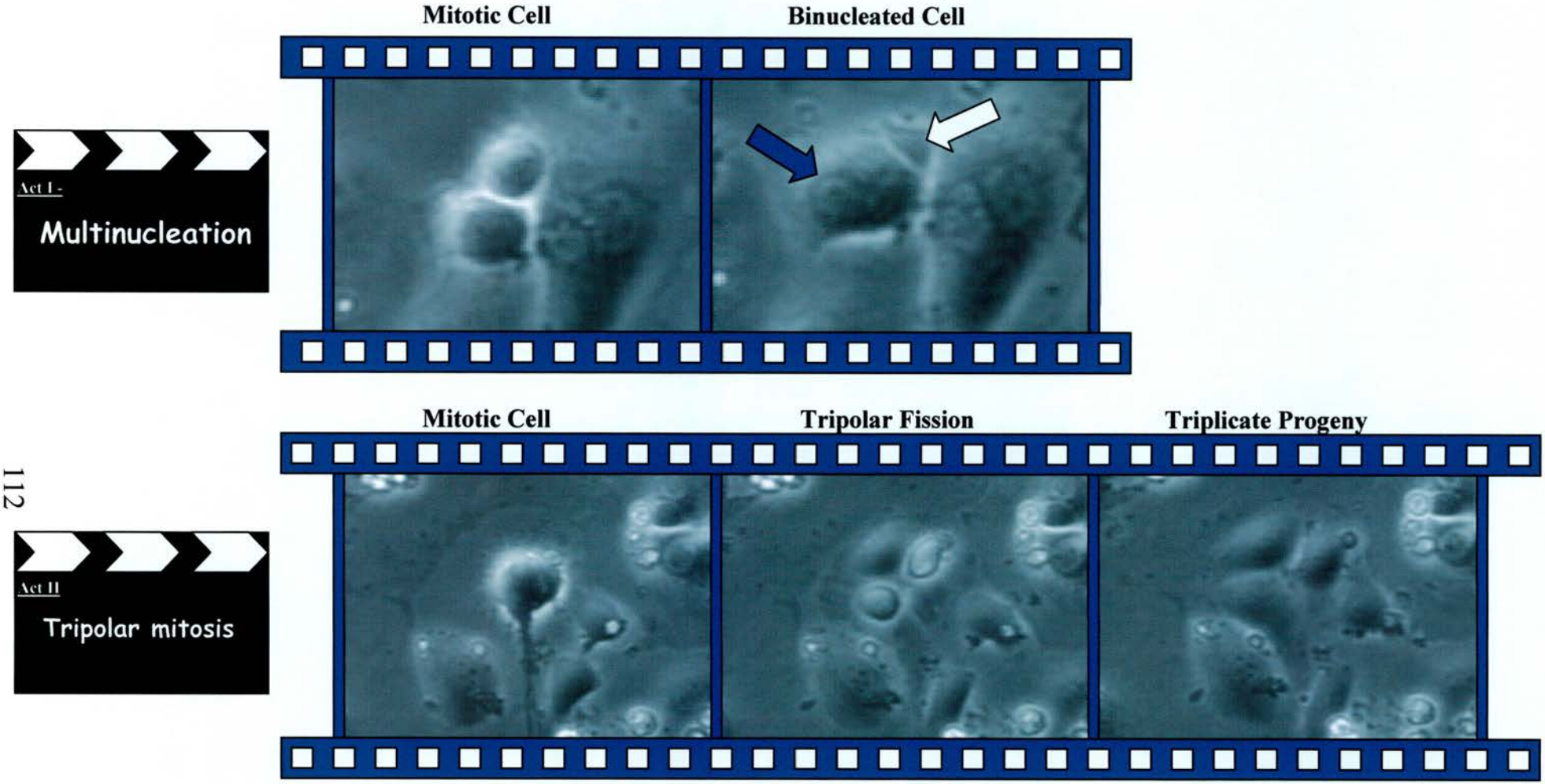


Figure 3.1.ix Multinucleation in p53-/- Clara cells

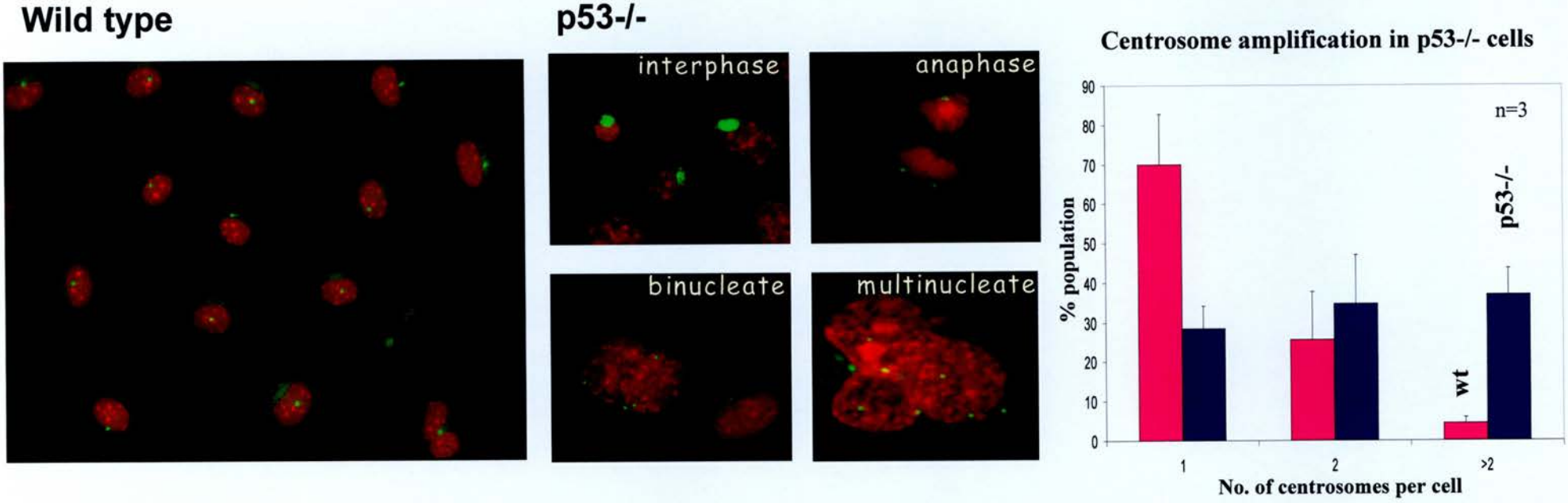
Time-lapse videomicrographs of p53-/- Clara cells demonstrating failure to segregate nuclei mitotically. Upper panel demonstrates the formation of a multinucleated cell (blue arrow) and a cytoplast (white arrow) from a single M phase cell. Lower panel demonstrates the formation of a 3 nuclei from a single M phase cell. Time-lapse videos of p53-/- Clara cells are additionally available on CD-ROM (see appendix).

3.1.c.2. Centrosome amplification in p53-deficient Clara cells

The observed role of p53 in mitosis and cytokinesis may reflect a role in regulation of centrosome number. Centrosomes are essential for the formation of bipolar spindles and the balanced segregation of chromosomes. Defects in shape, size, and number of centrosomes are frequently observed in malignant tumours and are associated with gross genome instability (Pihan et al., 1998; Lingle et al., 1998). A role of p53 in maintaining centrosome number has been documented previously (Fukasawa et al., 1996; Fukasawa et al., 1997).

Centrosome abnormalities were assessed by Confocal Microscopy in Clara cell primary culture. 72 hour-cultured cells were fixed in methanol and immunofluorescently stained for pericentrin, a protein specific to the centrosome. Nuclei were counterstained with propidium iodide. Wild type Clara cells contained predominantly one or two centrosomes juxtaposed to the nucleus (Figure 3.1.x). By contrast, > 40% of p53 null cells contained more than two centrosomes (Figure 3.1.x). The presence of multiple centrosomes was observed in interphase, mitotic, and multinucleated cells (Figure 3.1.x).

Thus, p53 regulates centrosome number, mitotic fidelity, and cytokinesis in primary cultured Clara cells. This role of p53 is independent of DNA damage and may represent a novel, constitutive role of p53 in maintaining genome integrity.



Pericentrin staining of wild type and p53-/- Clara cells

Figure 3.1.x - Centrosome amplification in p53-/- Clara cells

Confocal micrographs of wild type and p53 null Clara cell cultures immunoflourescently labelled with ant-pericentrin IgG (green) and counterstained with propidium iodide (red). More than two centrosomes (green) were frequently observed in p53 null, but not wild type cells. Data represents the mean (n=3) ± standard deviation.

3.1.c.3. Growth arrest abnormalities in p53-deficient Clara cells

In response to DNA damage, cells undergo growth arrest and repair, or apoptosis. In many cell types, these DNA damage responses are regulated by p53. Wild type and p53 null Clara cell cultures were gamma-irradiated and their response to injury documented over 24 hours. BrdU incorporation, a measure of entry into S phase, decreased substantially in wild type cells following irradiation (Figure 3.1.xi). By contrast, p53 null cells maintained BrdU indices similar to those of unirradiated controls. The observed growth arrest in irradiated Clara cells is thus p53-dependent.

Apoptosis was not observed in irradiated wild type and p53 -/- Clara cells, as monitored by time-lapse videomicroscopy. Apoptosis was not observed in 96 frames displaying 150-200 cells over a 24 hour period (1 frame every 15 minutes), as determined by morphology. Consequently, the incidence of apoptosis in irradiated wild type and p53-/- primary Clara cell cultures over the 24 hour period post-injury is less than 0.5%.

Response to genotoxic injury can be influenced by extracellular factors including hormones, cytokines, and unspecified serum factors (Bellamy et al., 1997a). The addition of serum to the medium was sufficient to abolish DNA-damage mediated growth arrest in wild type Clara cells, which maintained BrdU indices similar to those of unirradiated controls (Figure 3.1.xi). In addition, p53-/- cultures, but not wild type cells, displayed a higher rate of proliferation in the presence of serum. The

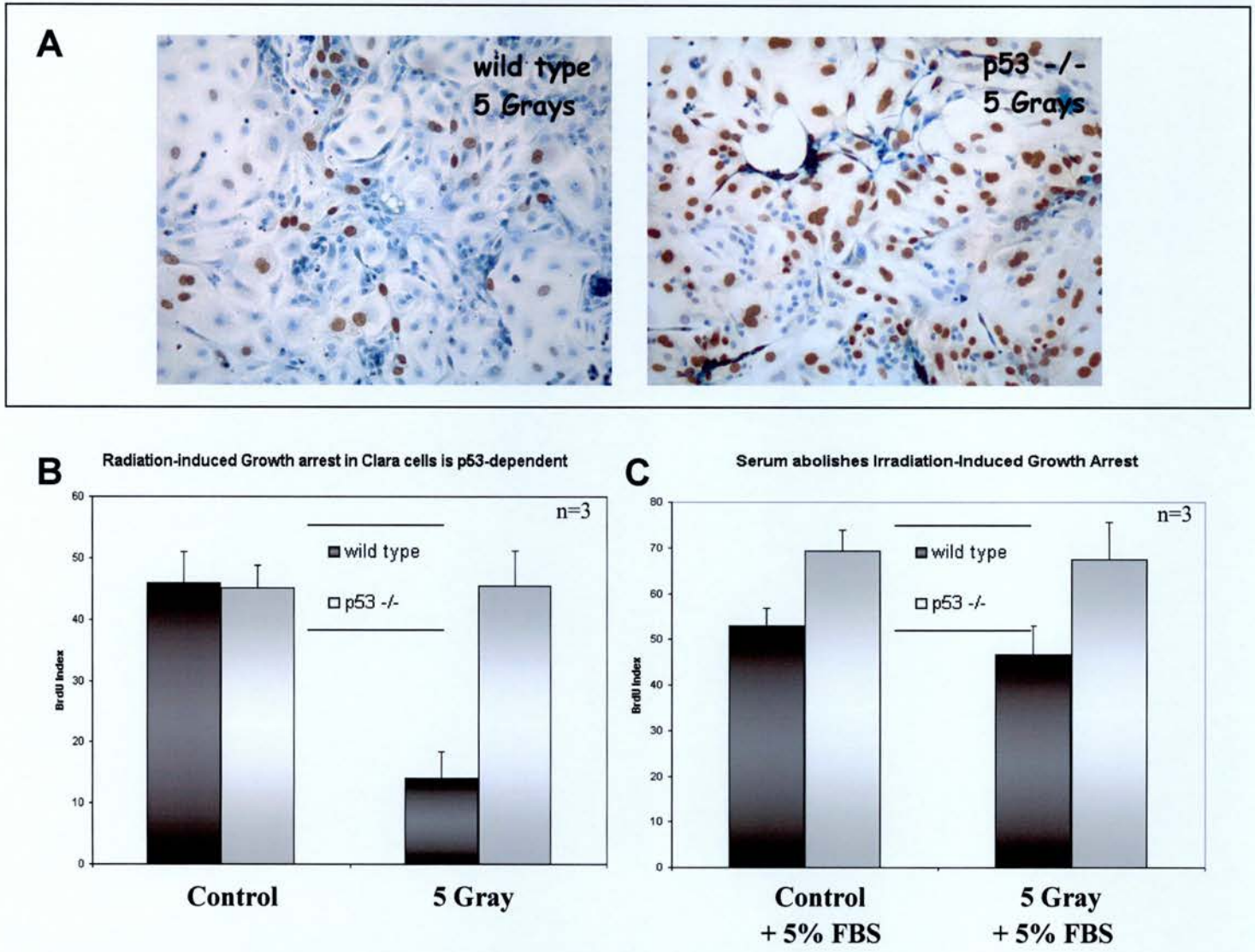


Figure 3.1.xi Growth arrest abnormalities in p53-/- Clara cells

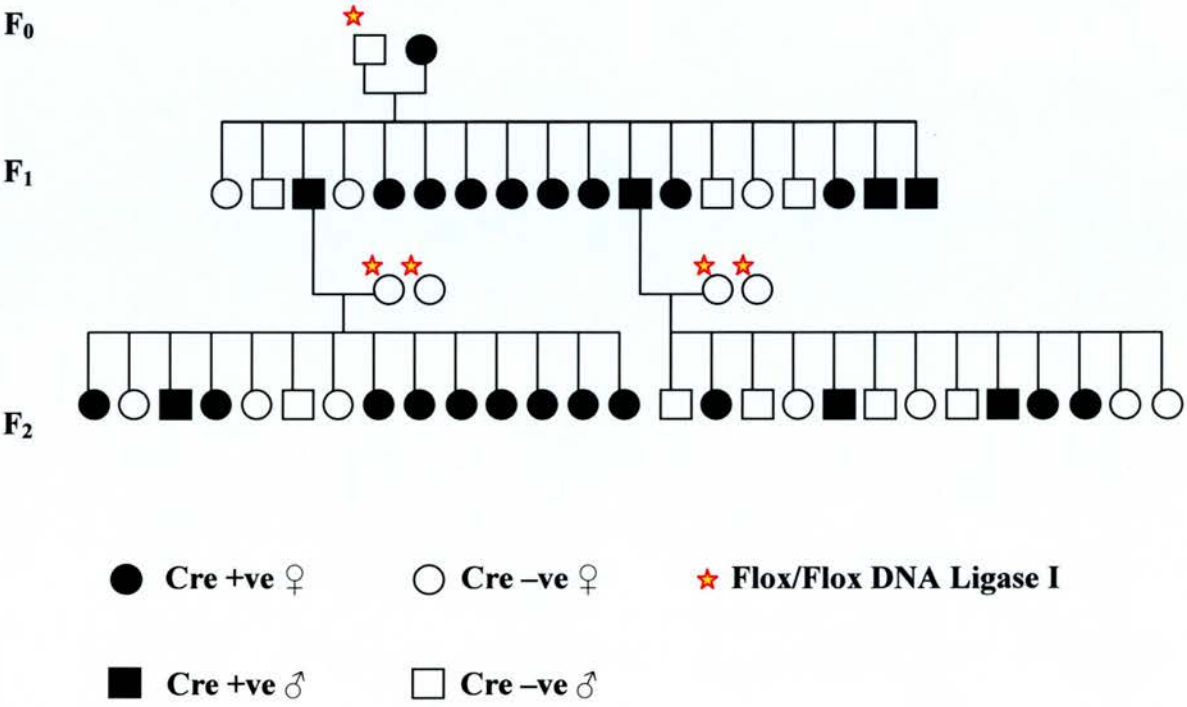
A) 24 hour-BrdU incorporation assays demonstrating growth arrest abnormalities in irradiated p53-/- Clara cells. B) The growth arrest response to DNA damage is entirely p53-dependent in Clara cells cultured in serum-free conditions. C) Addition of 5% foetal bovine serum to the culture medium abolishes the p53-dependent DNA damage response. B,C) p53-/- cultures additionally display a higher rate of proliferation in the presence of serum, highlighting synergistic effects of p53 and extracellular factors in Clara cell cycle regulation. Data represents the mean (n=3) ± standard deviation.

BrdU index of p53-/- Clara cells was 50% greater when cultured in the presence of 5% foetal bovine serum (70% BrdU incorporation with FBS versus 45% in serum-free conditions), regardless of radiation injury. The latter observation highlights synergistic effects of p53 and extracellular factors in Clara cell cycle regulation.

3.2. CHARACTERISATION OF SP-C/Cre TRANSGENIC MICE

3.2.a. Overview

It was the intention of this project to determine the feasibility of tissue specific Cre-lox conditional gene targeting in lung epithelial cells of transgenic mice. In collaboration with Dr. Shirley O'Dea, 3.7kb of the human SP-C promoter was used to express bacteriophage P1 Cre recombinase and thus promote Cre-mediated recombination specifically in the small airway epithelial cells of transgenic mice. Floxed DNA Ligase I mice (FloxligI) were employed to determine the function of SP-C/Cre in vivo (see section 1.6).



In brief, SP-C/Cre plasmid was NotI/NdeI digested and the resulting 5.1 kb fragment microinjected into blastocysts and introduced into pseudopregnant recipients to produce transgenic mice expressing Cre specifically in small airway epithelium. A single founder mouse was generated which was crossed onto a FloxligI/FloxligI line to produce F_1 mice heterozygous for FloxligI and transgenic for Cre. Three of these mice were further crossed onto a FloxligI/FloxligI line to generate transgenic animals hemizygous for Cre, and both heterozygous and homozygous for FloxligI. Transgene analysis of F_2 animals was restricted to FloxligI heterozygotes.

SP-C/Cre was characterised in vivo via analysis of transgene stability and function. Transgene trans-generation stability was determined by a Southern blot strategy, whereby resolution of fragments reflected integration sites and concatemerisation of SP-C/Cre. To facilitate this analysis, an internal probe that labeled a sequence specific for the human SP-C promoter was required. The human SP-C promoter has only been partially sequenced, hence restriction mapping and subcloning of the SP-C/Cre vector was necessary to generate an internal probe suitable for assessing transgene stability.

3.2.b. SP-C/Cre Transgene Stability

3.2.b.1. Probe generation

From restriction digest analysis, a novel PstI site 1kb upstream of a diagnostic NcoI site was detected in the human SP-C promoter (Figure 3.2.i). A 1.3kb PstI fragment (containing the diagnostic NcoI site) was gel-purified and ligated into bluescript plasmid to facilitate bulk generation of probe (Figure 3.2.ii). Ligations were transformed into ultracompetent E.coli, plated on X-gal Ampicillin LB plates, and white colonies picked and cultured overnight in the presence of antibiotics. Minipreparations of plasmid DNA were purified from aliquots of cultures. Minipreparations were PstI digested, and resolved by agarose gel electrophoresis to determine success in ligation and subsequent culture steps. A successful transformant was cultured overnight in a 100ml flask of medium with antibiotics. Plasmid DNA was purified to generate 3.3ug yield of subcloned plasmid.

Subcloned plasmid was PstI digested and the 1.3kb fragment gel-purified (Figure 3.2.iii). The latter fragment was further digested with BstxI to generate 600bp and 700bp fragments. The 700bp fragment did not contain the NcoI site, and hence encoded the sequence upstream of this restriction site. The 700bp fragment was gel-extracted and purified for use as a probe template in Southern analyses of transgene stability.

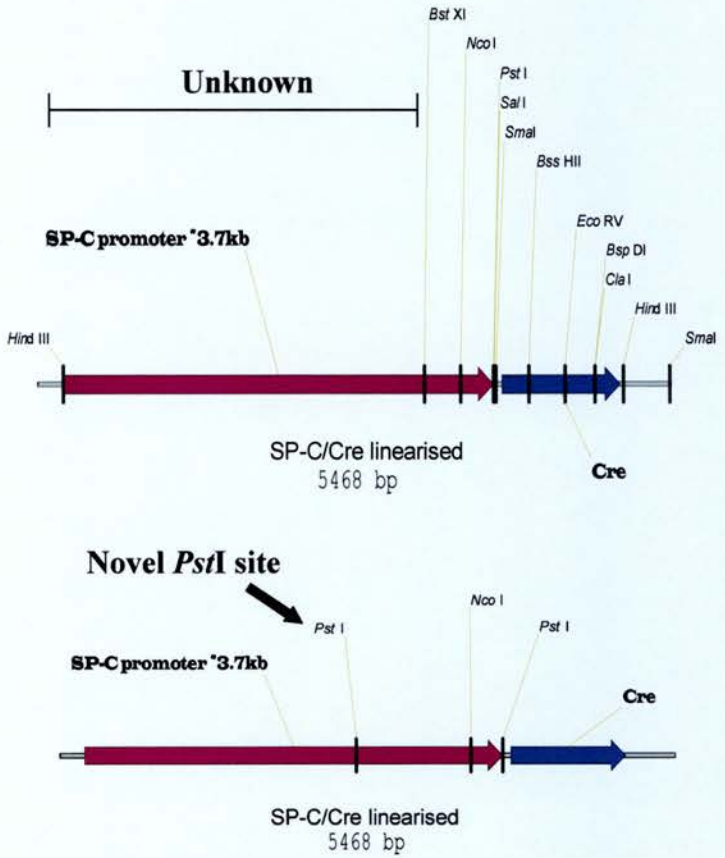
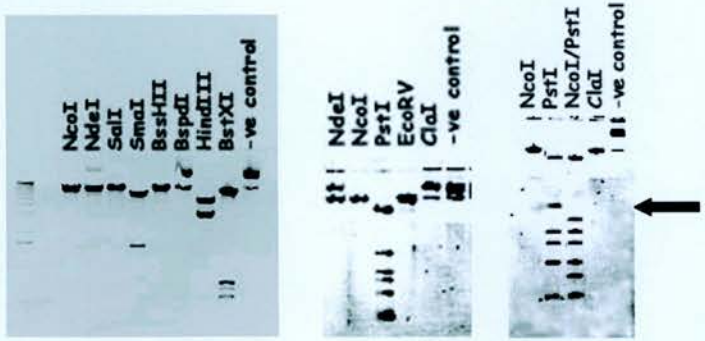


Figure 3.2.i - Manipulation of the Human SP-C Promoter

Restriction mapping of the Human SP-C Promoter. A 1.3kb *PstI* fragment (arrowed) incorporated a *NcoI* site, and thus a 3' sequence of the SP-C promoter.

Restriction Map of the human SP-C promoter



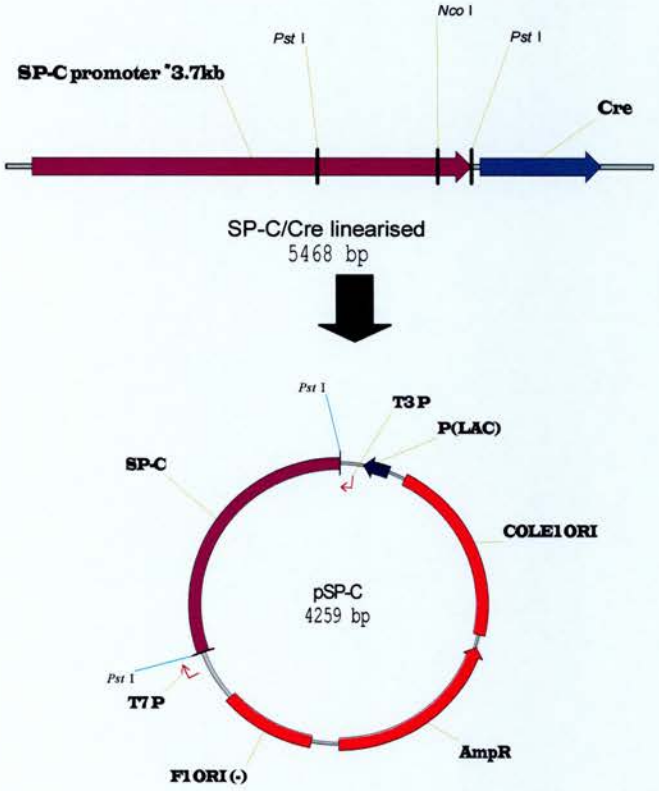
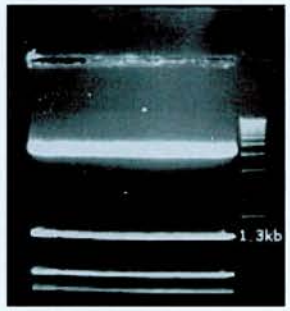


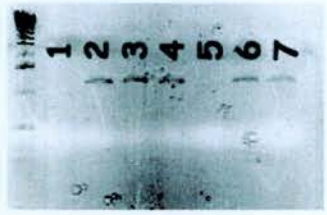
Figure 3.2.ii - Manipulation of the Human SP-C Promoter

Subcloning of the Human SP-C Promoter. The 1.3kb *PstI* fragment was gel-extracted and ligated into *PstI* digested, SAP-treated bluescript plasmid. Plasmid minipreps of white colonies demonstrated success in 5 of 7 ligations.

Gel Extraction
of 1.3kb *PstI* fragment



Gel Diagnostic
of *lacZ* mutated clones



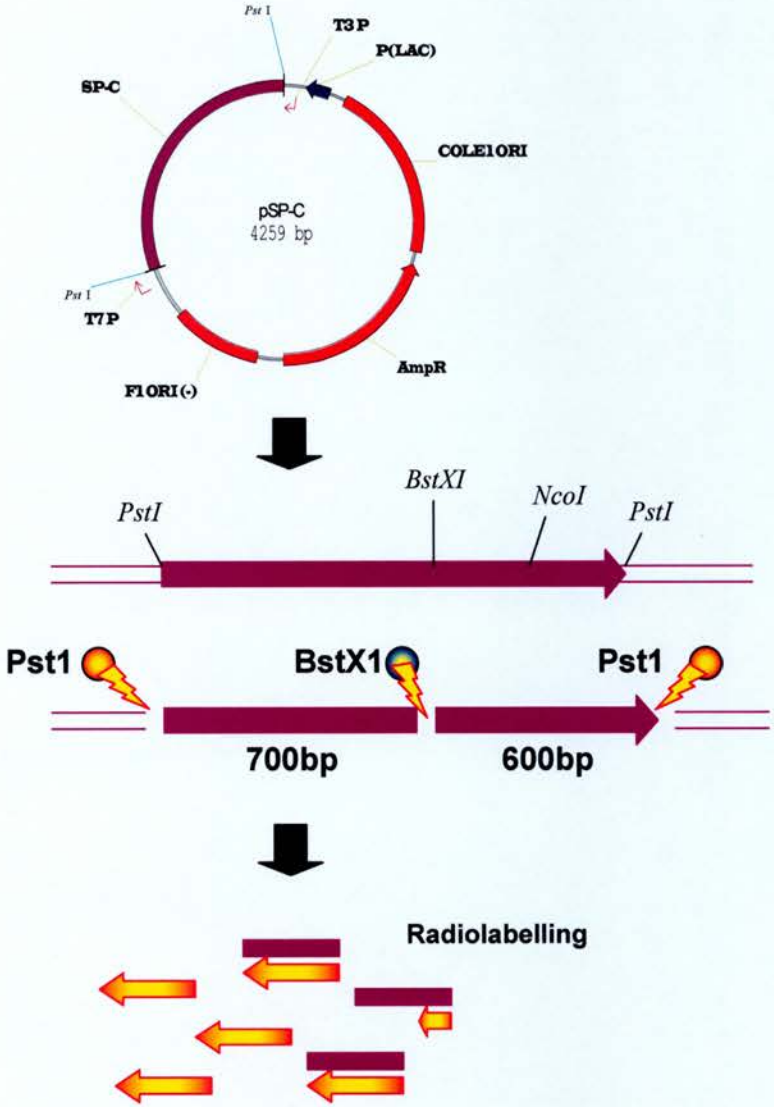
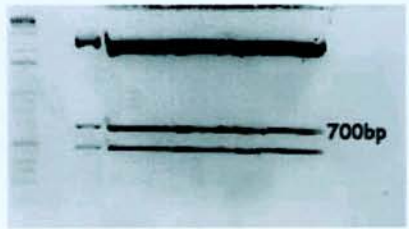


Figure 3.2.iii - Manipulation of the Human SP-C Promoter

Generation of SP-C Probe
The 700bp *PstI/BstXI* fragment was gel-purified and served as a probe template. Probes were radiolabelled as required.

Gel Extraction
of 700bp *PstI/BstX1* fragment



3.2.b.2. SP-C/Cre recombinase transgene is germline stable

Transgene stability was assessed on Southern blots of tail-biopsied DNA. Two genomic digests, NcoI and XbaI, were employed. Both NcoI and XbaI digest SP-C downstream of the BstxI site, and thus downstream of the 700bp probe (Figure 3.2.iv).

NcoI digested DNA of F_0, F_1, F_2 mice was electrophoresed on a 0.7 % agarose gel, blotted onto a nylon membrane, and incubated overnight with radiolabeled SP-C probe. Autoradiographs of Southern blot displayed two major bands. The predominant band resolved at 5.5kb, the approximate size of the construct, and represented multiple copies of the transgene concatemer (Figure 3.2.iv). A larger band resolving at 7 kb signified a sole integration of the SP-C/Cre transgene into the mouse genome.

Transgene stability was verified by probing of XbaI digested DNA. SP-C contains two XbaI sites, and Xba I digestion removes a 428 bp sequence incorporating the NcoI site. XbaI digested DNA of F_0, F_1, F_2 mice was electrophored on a 0.7 % agarose gel, blotted onto a nylon membrane, and incubated overnight with radiolabeled SP-C probe. Xba I Southern autoradiographs displayed two distinct bands (Figure 3.2.iv). The predominant band resolved at 5kb and signified multiple copies of the transgene concatemer. A single consistent integration band was observed at (>5kb). SP-C/Cre transgene was thus germline stable over three generations.

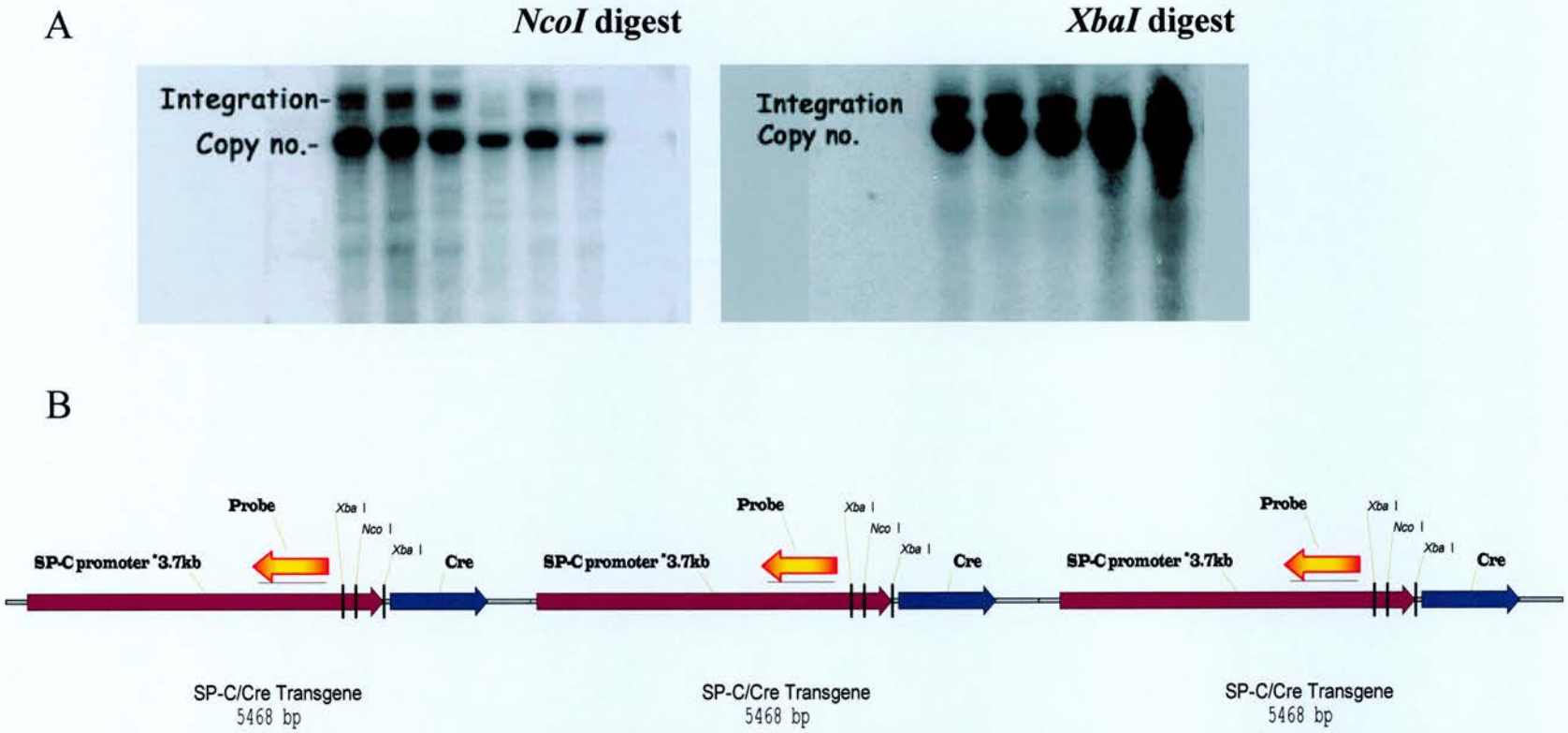


Figure 3.2.iv - Southern analysis of SP-C/Cre transgene stability

Integration analysis of transgene concatemer. A) NcoI and XbaI digested DNA was electrophoresed, blotted, and incubated with radiolabelled SP-C probe. Stable patterns of transgene integration were observed in founder mouse progeny. B) Model of SP-C/Cre concatemer based on Southern data.

Transgene copy number was quantified via densitometry of the concatemer band relative to the integration band. In both NcoI and XbaI digests, concatemer band was threefold more abundant than the integration band. Transgene copy number was thus low, and contained only three complete copies of the SP-C/Cre construct (Figure 3.2.iv).

3.2.c. SP-C/Cre Transgene Function

3.2.c.1. Cre mediated recombination is specific to airway epithelium, but infrequent

Excision of the floxed allele was assessed by two methods - PCR and Southern blotting. PCR amplification of the deleted allele in DNA extracted from a range of tissues demonstrated Cre-mediated excision in the lung, but not other tissues (Figure 3.2.v, for PCR primers, see Materials and Methods). Cre-mediated excision was thus specific to lung. Southern blot analysis allowed quantification of the excision event. A radiolabelled intronic ligase I probe encoding a 1.2kb sequence conserved by floxed, wild-type, and deleted alleles (generated by EcoRI/HindIII fragment of exons 21-22, courtesy of D.Melton), demonstrated equal densities of floxed and wild-type alleles in brain, kidney, liver, and lung (Figure 3.2.vi). The deleted allele was not detected in F_1 and F_2 lung extracts, including those demonstrating excision by PCR. Excision of the floxed allele was therefore not demonstrated by Southern analysis.

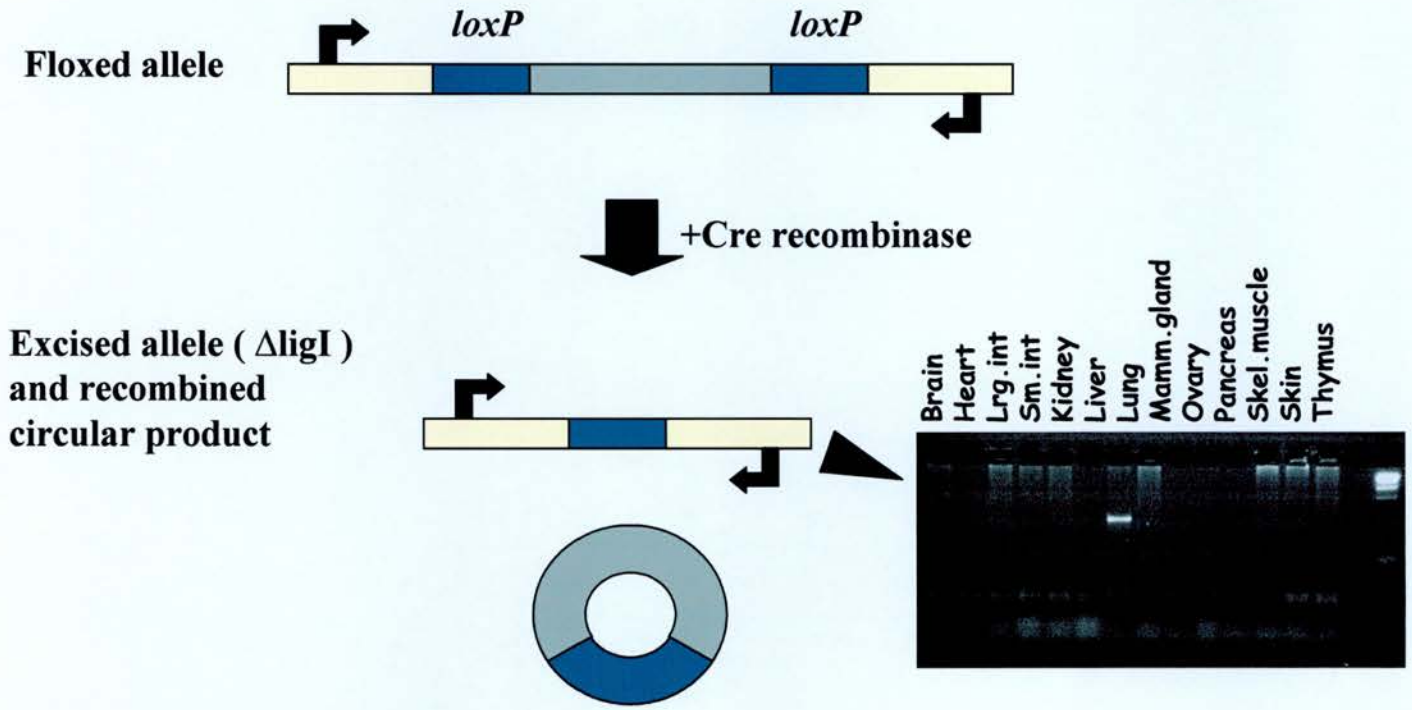


Figure 3.2.v - PCR Analysis of SP-C/Cre-mediated Excision

Cre-mediated excision was determined by a PCR-based strategy. This strategy is outlined in the schematic diagram above, where arrows denote primer recognition sites. Excision was lung-specific, as determined by PCR analysis of a range of mouse tissues.

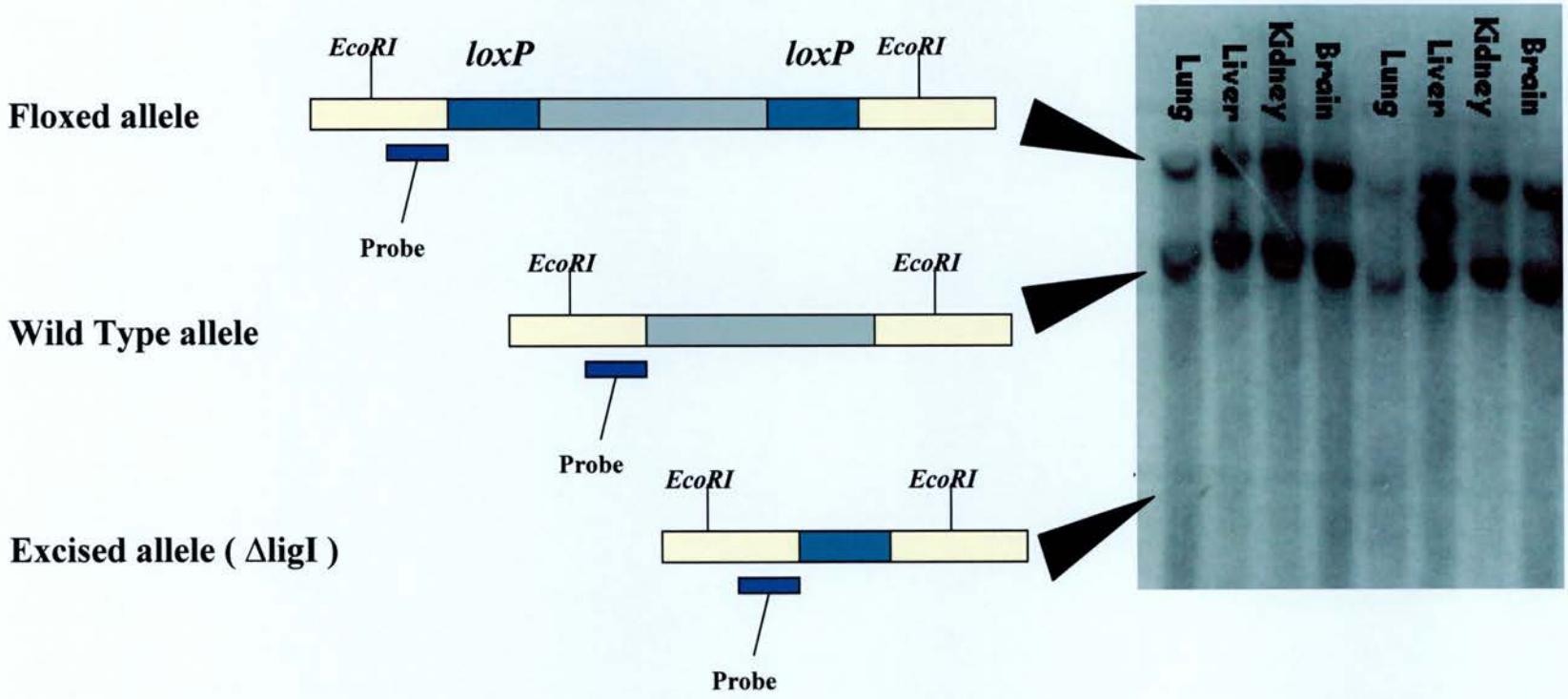


Figure 3.2.vi - Southern Analysis of SP-C/Cre-mediated Excision

DNA was *EcoRI* digested, electrophoresed, blotted, and incubated with a pan-*DNA ligase I* probe which recognised floxed, excised, and wild type alleles. Floxed and wild type DNA ligase I alleles were observed in tissues of transgenic mice. The *ΔligI* allele was not detected in lung or other mouse tissues by Southern blot.

3.2.c.2. Cre expression is not detected in airway epithelium

It was hypothesised that the low level of Cre-mediated recombination in murine lung, detectable only by PCR amplification, may reflect failure to transcribe Cre. RT-PCR of Cre and β-actin was performed on total RNA extracted from frozen lung tissue (Figure 3.2.vii). β-actin expression was observed in all lung extracts, demonstrating success of the reverse transcription technique. By contrast, Cre cDNAs were not detected in extracts of reverse transcribed lung total RNA. It is thus unlikely that SP-C/Cre transgene was transcriptionally active in adult mouse lung.

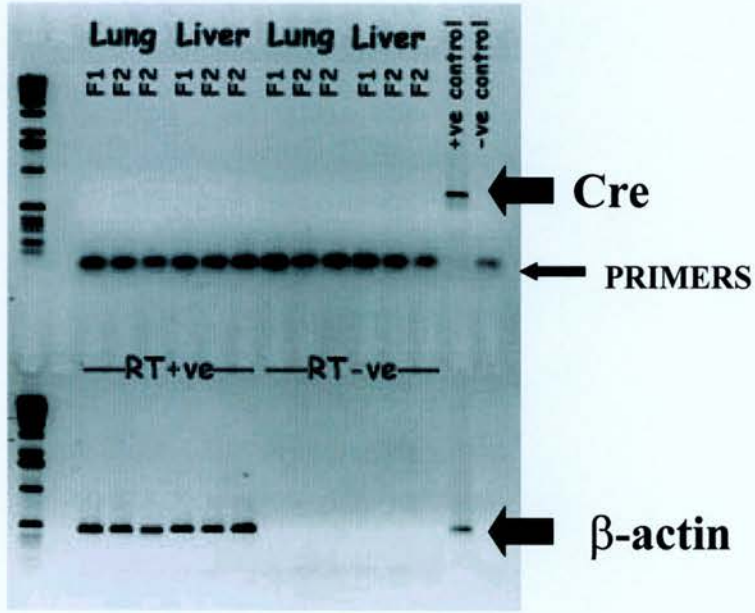


Figure 3.2.vii - RT-PCR of SP-C/Cre Mouse Lungs

β-actin expression was detected in all lung and liver extracts. Cre expression was not detected in extracts of reverse transcribed RNA. RNA isolated from Δ ligI primary hepatocyte cultures (courtesy of D.Rannie) served as a positive control. Negative controls omitted the reverse transcription reaction (RT-ve) and substituted dH2O for cDNA (-ve) in the subsequent PCR amplification step.

4. Discussion

4.1. The Role of p53 in Small Airway Epithelial Cells

Response to DNA damage can be understood in terms of apoptosis, growth arrest, and expression of genes related to both phenomena. In other cell types, tumour suppressor p53 is a critical determinant of the response to DNA damage. In this analysis, the author sought to identify functions of p53 in small airway epithelium. p53 stabilisation has been observed in the small airways previously, but its significance is unclear. Because Clara cells are a major progenitor species of the small airways, and are considered relevant to neoplasia of the lung and centriacinar emphysema (Massaro et al., 1994), it was hypothesised that phenotypic effects of p53 deficiency would predominate in this cell type. To discern the role of p53 in the Clara cell, a method for extracting, isolating, and culturing Clara cells from the mouse was developed and incorporated into this analysis.

An acute stress response of p53 is documented in mouse small airway epithelium in vivo, and in isolated Clara cells in vitro. In vivo, p53 stabilisation is observed in lung epithelium following 5 Gray γ-irradiation. Immediate induction of the death-related gene Bax, but not other bcl-2 transcripts, was p53-dependent following genotoxic damage. Bax and Bcl-x proteins localise to the columnar epithelium, additionally the site of p53 and p21 induction. DNA strand breaks, but not apoptosis were observed in small airway epithelium following irradiation. Clara cells, isolated from wild type and p53-deficient mice, were 5 Gray γ-irradiated from the same

radiation source and monitored for behavioural changes by Time-Lapse Videomicroscopy, and cell cycle changes by BrdU incorporation. Apoptosis was not observed in videos of either genotype. DNA-damage induced growth arrest, not demonstrable in vivo due to the low turnover of small airway epithelium, was observed in primary cultured Clara cells and was p53-dependent.

The author reports that Bax induction, but not apoptosis, is a DNA damage response of small airway epithelium of mice. Transactivation of Bax was p53-dependent in irradiated lung, as determined by RNase protection, with a twofold increase in Bax mRNA occurring in the lungs of wild type mice from 4-8 hours following irradiation. The observed p53 response is reminiscent of the in vivo radiation response of the murine small intestine, whereby a wave of apoptosis is observed in intestinal epithelial cells of wild type, but not p53-/- mice, within 4 hours following irradiation (Clarke et al., 1994). Bax induction is a feature of irradiated enterocytes and associates with apoptosis, as determined by morphological criteria (Kitada et al., 1996). TUNEL positive cells were observed in airway epithelium following DNA damage, but did not display the morphological features associated with apoptosis. Furthermore, TUNEL reactions were easily titratable in irradiated cells of wild type lung, but not the spleen where TUNEL positive cells additionally displayed the hallmarks of apoptosis including nuclear condensation and collapse of the nucleus. Consequently, it is unlikely that TUNEL staining in lung epithelium reflects the oligonucleosomal DNA fragmentation characteristic of lymphoid cell apoptosis. It is probable that the TUNEL reaction additionally recognises DNA strand breaks induced by ionizing radiation. In this respect, it is noteworthy that similar

observations have been reported previously in lung epithelium following insidious oxidative damage induced by bleomycin and hyperoxia (Okudela et al., 1999; Barrazone et al., 1997).

Transrepression of Bcl-x^S was additionally observed in irradiated lungs, and was p53-dependent, as determined by RNase Protection Assay. Bcl-x^S is a potent promoter of apoptosis when overexpressed in cultured cells, but can be inhibited by co-expression of either Bcl-2 or the Bcl-x long isoform (Tao et al., 1998; Lindenboim et al., 2000). A physiological role of Bcl-x^S in promoting apoptosis has been difficult to assess experimentally due to the native pre-mRNA being shared by both the long and short isoforms. However, induction of endogenous Bcl-x^S has been achieved through the control of Bcl-x pre-mRNA splicing by antisense oligonucleotides (Taylor et al., 1999), and correlates with the induction of apoptosis in certain cell lines (e.g. MCF7 cells, Simões-Wüst et al., 2000). p53-null mice do not display the rapid transrepression of Bcl-x^S observed in wild type lungs, as determined by measuring relative mRNA abundance, but rather display a modest (>50%) increase in levels of Bcl-x^S transcripts. It is unclear if this reflects a direct role of p53 in alternative splicing. An alternative possibility is that Bcl-x^S induction in p53-/- animals represents a compensatory function of an as yet undescribed DNA damage/oxidative injury response pathway in airway epithelial cells which serves to regulate the Bcl-2 family rheostat in the absence of p53. In this respect, it is noteworthy that both Bax and Bcl-x proteins colocalise to the same subset of lung epithelial cells of the mouse – the bronchiolar epithelium – consistent with overlapping functions in vivo. The Bcl-x long isoform, renowned for its death-

suppressor activity in other mouse tissues (Chao & Korsmeyer, 1997; Xu et al., 1999; Pena et al., 1998; Parsadanian et al., 1998), was constitutively expressed in small airway epithelium, as determined by SDS-PAGE analysis, and may represent one mechanism by which irradiated lung epithelial cells maintain resistance to apoptosis despite induction of pro-apoptotic Bcl-2 family members. Recently, induction of Bax and Bcl-x^L mRNAs have been reported in mouse lungs following exposure to 95% oxygen, and the authors argue that Bax and Bcl-x^L induction is a feature of ROS-damaged lungs (O'Reilly et al., 2000). The authors used an identical RNase Protection Assay kit to the one used in this analysis (mAPO-2, Pharmingen), and report induction of Bax and Bcl-x^L mRNAs in hyperoxic lungs from the earliest time point considered of 48 hours. By contrast, the results of this thesis, whereby little change in Bcl-x^L mRNA is observed in mouse lungs following γ-radiation, argue against Bcl-x^L induction as a general feature of oxidative injury, and instead invoke that there are qualitative differences between radiation- and hyperoxia-induced lung injuries. The underlying mechanisms by which these two oxidative injuries may differ have yet to be resolved, but the trans-regulatory effects reported here and elsewhere (O'Reilly et al., 2000) highlight the suitability of RNase Protection Assays in detecting changes in gene expression in mouse models of acute lung injury, and could be used to further address subtle differences between varying types of inhalational/oxidative injury (e.g. NO_2, SO_2, tobacco smoke).

To investigate the effects of p53 in proliferating airway cells, a method for isolating and culturing Clara cells, a progenitor cell of the lung, was incorporated into this research. A major finding of this analysis was that primary cultured Clara cells,

deficient in p53, displayed spontaneous multinucleation and the formation of bilobed nuclei, and that these nucleation abnormalities correlated with disturbances in centrosome homeostasis. Centrosomes ensure spindle polarity and thus correct chromosome segregation during mitosis (Hyman, 1996), and their assembly is tightly controlled with the cell cycle (Figure 4.1; for recent review, see Tarapore & Fukasawa, 2000). Because each daughter cell receives only one centrosome after mitotic division, the centrosome must duplicate during the mitotic cycle of the daughter cell. In considering the similarities between wild type and p53-/- Clara cell baseline proliferation rate, it would appear that in p53-/- cultures, centrosomes undergo multiple cycles of duplication in a single cell cycle. Similar observations have been reported in p53-/- murine embryonic fibroblasts (Fukasawa et al., 1996), whereby multiple copies of centrosomes are generated from early in G1. The adverse consequence of multiple centrosomes is most prominently seen in mitosis as multipolar spindles, a feature of p53-/- Clara cell cultures as documented by time-lapse videomicroscopy (for video, see appendix). Such events are likely to impair chromosomal segregation and induce karyotypic instability and mitosis by failure to form bipolar mitotic spindles (Figure 4.2). However, it is noteworthy that an abnormal centrosome number does not necessarily induce mitotic abnormalities, and that multiple copies of centrosomes may instead co-migrate to the poles, establishing bipolar spindles and normal chromosome segregation – a phenomenon known as pseudobipolarity (Tarapore & Fukasawa, 2000; see Figure 3.1.x, anaphase). Consequently, only a proportion of p53-deficient cells with an abnormal centrosome number would be expected to display an abnormal chromosome content.

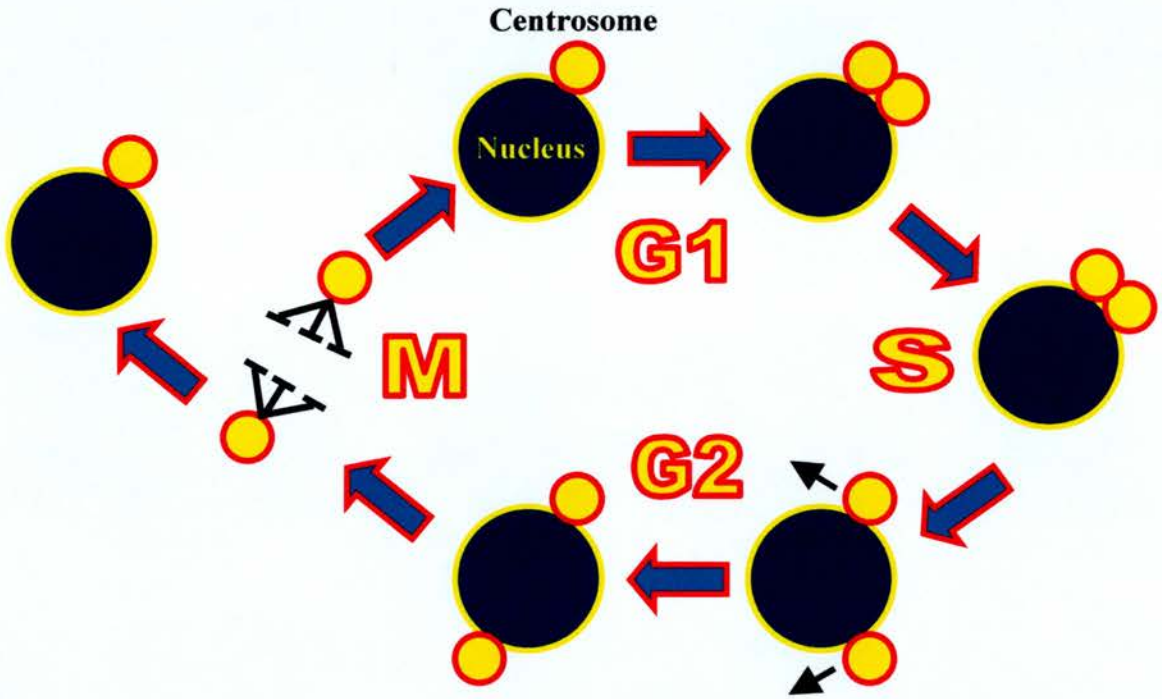


Figure 4.1 - The Centrosome Duplication Cycle. Centrosome duplication occurs once during each cell cycle, and is tightly coordinated with DNA replication. In wild type cells, centrosome duplication begins in late G1 and is completed in G2. Loss of centrosome duplication cycle regulation or uncoupling of the centrosome duplication cycle from the DNA replication cycle leads to abnormal amplification of centrosomes, which in turn profoundly affects mitotic fidelity.

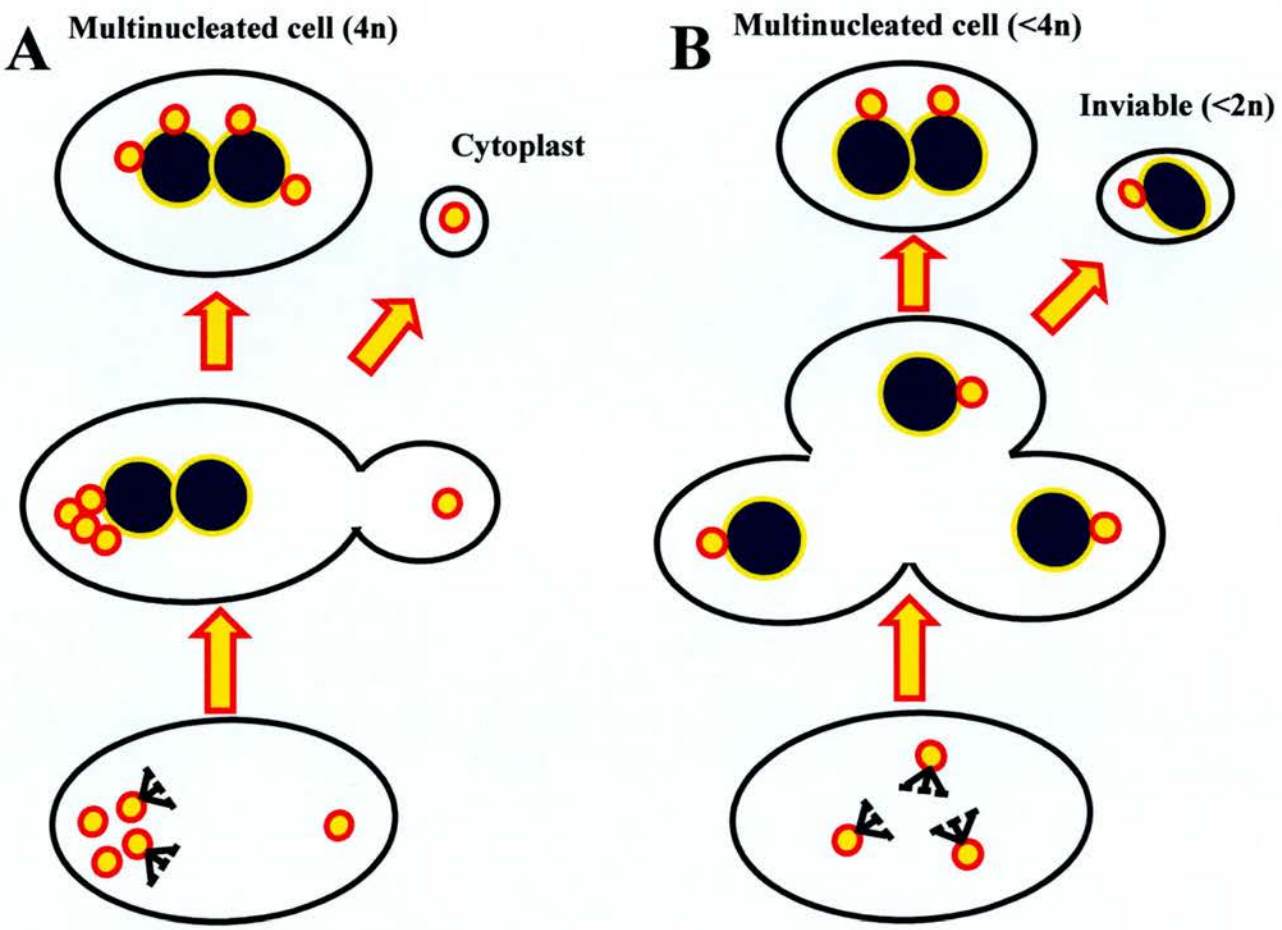


Figure 4.2 - Two possible mechanisms by which multinucleation of p53 -/- Clara cells is achieved based on Time-Lapse Videomicroscopy and centrosome data. Fig. a) depicts multinucleation without loss of chromsomes. Fig. b) illustrates the formation of multinucleated cells with abnormal chromosome content (aneuploid).

Effects of DNA damage were additionally characterised in primary cultures of Clara cells. The author documents that growth arrest, but not apoptosis, is a Clara cell response to ionizing radiation, and is p53-dependent. However, the Clara cell radiation response was additionally dependent on extracellular factors - specifically, the addition of serum to the medium was sufficient to abolish the p53-induced growth arrest. Effects of extracellular factors on the p53 response have likewise been observed in primary cultured murine hepatocytes (Bellamy et al., 1997a) and human lung cancer cell lines (Kannan et al., 2000), and highlight modulatory effects the tissue microenvironment may impose upon the p53 response in vivo. It should be emphasised that it is currently unclear to what degree cytokines and other growth factors expressed in injured lung (including Oncostatin M, Interleukin-8, Interleukin-1β, TNF-α, TGF-β_1) augment epithelial wound repair and/or the p53 response, especially in the context of the centriacinar damage typical of human emphysema. Suffice to say, interspecies differences in design of the airways, in the number and nature (e.g. P450 isoenzyme expression) of the various epithelial cell types has rendered centriacinar emphysema relatively resistant to modelling in experimental animals. In the latter respect, it is possible that the primary culture method described in this analysis could be used to distinguish the relative importance of growth factors and cytokines in influencing Clara cell behaviour from a genetic standpoint, and thus provide one method of determining the mechanisms underlying the bronchiolar remodelling typical of human emphysema.

4.2. p53 and Cancer of the Small Airways

p53 is frequently mutated in lung adenocarcinoma (Bennett et al., 1999), but the mechanism by which p53 deficiency contributes to malignancy is not defined. Various studies suggest that adenocarcinomas of the peripheral lung, like many other cancers, develop by multiple steps from atypical adenomatous hyperplasia through early in situ carcinoma to overt carcinoma (Kitamura et al., 1995; Kitamura et al., 1996; Anami et al., 1998). Mutation of p53 is an early event in the tumourigenesis of lung adenocarcinoma, and is often observed in hyperplastic lesions. In one study, overexpression of p53 protein, a reflection of missense mutations of the p53 DNA-binding domain locus, correlated with the Ki-67 labeling index and thus proliferation of hyperplastic cells (Hayashi et al., 1997). It is thus conceivable that p53 deficiency confers a proliferative advantage in hyperplastic lesions. In this analysis, the author reports that p53 regulates a growth arrest response in proliferating airway cells. The growth arrest response was characterised in primary cultured Clara cells in response to DNA damage, and was further dependent on extracellular factors, such as serum. However, p53 status did not alter baseline proliferation rates, which were similar between genotypes. Thus, loss of p53 per se does not confer a proliferative advantage in mitotically dividing airway cells. It is possible that the hyperplastic microenvironment is a p53-activating environment, and that the growth arrest response is a formidable selective pressure that a tumour must overcome, for example, in response to insidious oxidative species, such as those released by inflammatory cells, or from exposure to hypoxia, an in vivo consequence of disorganised neovascularisation of the tumour mass (Lakin & Jackson, 1999; Brown, 2000). However, it is additionally possible that p53 suppresses tumour formation by

mechanisms distinct from growth arrest. In the latter respect, a role of p53 in maintaining mitotic fidelity was observed in primary cultured Clara cells. Specifically, nucleation defects were apparent in p53-deficient cells, and correlated with centrosome amplification and resultant spindle abnormalities. Chromosomal instability is a feature of advancing lung tumourigenesis and the observed defects in centrosome homeostasis may underline one mechanism by which this is achieved. Notwithstanding this, centrosome amplification has been reported in a variety of human malignancies, including primary lung tumours and lung tumour metastatses (Pihan et al., 1998). It is currently unclear which of the observed functions of p53 predominates as the major tumour suppressor mechanism of the small airways. An attractive, alternative possibility is that the properties reported herein reflect the central role of p53 in maintaining genomic integrity via multiple mechanisms, and highlight the multi-functional nature of tumour suppressor p53.

In summary, effects of p53 deficiency were characterised in small airway epithelium in vivo, and in short-term primary cultures of isolated Clara cells. p53 stabilisation was a feature of DNA-damaged lung epithelium, and was associated with transactivation of the death-related gene Bax and trans-repression of Bcl-x^S, as determined by RNase Protection Assay. Radiation did not induce apoptosis in lung epithelial cells over a 72 hour time course, but did induce strand breaks that were detectable by TUNEL. Cell cycle activity was infrequent in lung epithelial cells at all timepoints, regardless of p53 status, thus an effect of p53 on cell cycle progression was not demonstrable in vivo. To investigate the effects of p53 in proliferating airway cells, a method for extracting, isolating, and culturing Clara

cells, a progenitor cell of the lung, was incorporated into this analysis. Proliferating Clara cells displayed DNA damage-induced growth arrest, which was p53-dependent, but not apoptosis. Defects in mitosis and cytokinesis were an additional feature of p53-deficient cells, irrespective of radiation injury, and were associated with disturbances in centrosome homeostasis. The author suggests that tumour suppressor properties of p53 in lung epithelium reflect its central tenet in preserving genomic integrity via multiple mechanisms, of which some are reported here. The author additionally wishes to highlight the importance of murine Clara cell primary culture as a powerful method for determining the functional significance of gene deficiency in airway epithelium.

4.3. Conditional Gene Targeting in Small Airway Epithelium

In addition, this thesis examined the feasibility of using a SP-C/Cre transgene to achieve conditional gene targeting in lung epithelial cells in vivo. Transgenic mice engineered to express Cre from the lung-specific promoter human SP-C were crossed to a Floxed DNA Ligase I line, and transgene stability and function assessed by PCR and Southern blot methodologies. Excision of the floxed allele was infrequent in the lungs of transgenic mice, and was not observed by a Southern analysis strategy that quantified the relative abundance of floxed, wild type, and excised DNA Ligase I alleles. RT-PCR strategies were further employed to address the question of Cre expression in SP-C/Cre transgenic mice. Cre expression was not detected in the lungs of SP-C/Cre transgenic mice, consistent with a failure to transcribe Cre in vivo. Failure to transcribe Cre may reflect 1) the low copy number of the SP-C/Cre transgene concatemer, 2) position effects relating to the site of transgene integration, or 3) methylational inactivation of the SP-C/Cre transgene established during the generation the C57/BL6 founder line. These three possibilities are not necessarily mutually exclusive, and all have the potential to induce heritable transcriptional silencing of the SP-C/Cre transgene.

Ligases are necessary for phosphodiester bond formation – the final step in repair of strand breaks. Strand breaks may be introduced directly by a DNA-damaging agent, such as ionizing radiation, or as a consequence of DNA repair proteins recognising and excising DNA damage. DNA-joining events are additionally required to link together the Okazaki fragments generated during lagging strand DNA synthesis. In prokaryotes, DNA-joining requirements are fulfilled by a single species of DNA

ligase. Consequently, DNA ligase mutants exhibit a pleiotropic phenotype that includes conditional lethality, hyperrecombination, increased sensitivity to DNA-damaging agents, and an increased mutation frequency (Lehman, 1974). By contrast, eukaryotes contain multiple species of DNA ligase with distinct, non-overlapping roles. DNA Ligase I represents the majority of the DNA ligase activity in proliferating cells, which may reflect a direct role of this gene product in DNA replication. Notwithstanding this, a specific requirement for DNA Ligase I to reconstitute lagging strand DNA synthesis has been demonstrated in vitro. Purified DNA Ligase I was necessary for repair of Okazaki fragments in reconstituting complete SV40 DNA replication with purified replication factors (Waga et al., 1994). Ligase I activity was demonstrated by biochemical criteria - specifically, ^{32}P-oligo(dT)/poly(dA) ligation in cell-free extracts. DNA Ligase III did not substitute for DNA Ligase I in repair of Okazaki fragments in this assay, highlighting substrate specificity of the ligation reaction. Failure to repair Okazaki fragments efficiently is considered integral to the haematopoietic failure and consequent embryonic lethality of DNA Ligase I-deficient mice (Bentley et al., 1996).

Direct evidence of a role of DNA Ligase I in DNA repair is from the study of a human, a DNA Ligase I compond heterozygote, who has become known to the scientific world as patient 46BR (reviewed in Friedberg et al., 1995). The individual from whom these cells were derived was a girl who manifested a complex clinical syndrome of dwarfism, the absence of secondary sexual characteristics, dilated venous capillaries, and severe photosensitivity. The patient also had severe immunodeficiency, characterised by a deficiency in IgA and IgG, and a failure of her

lymphocytes to respond to mitogenic stimuli in vitro. The patient suffered recurrent infections and died at the age of 19, apparently as a result of chronic lymphoma and an acute respiratory infection. No definitive diagnosis of the patient's disease was made; hence, this clinical picture has no formal designation. It is estimated that patient 46BR possessed less than 10% ligase I activity (Friedberg et al., 1995). A cell line derived from this patient demonstrated defective joining of Okazaki fragments during semiconservative DNA synthesis. In addition, the 46BR cell line was found to be markedly sensitive to killing by a variety of DNA-damaging agents, including UV radiation, γ radiation, and a number of alkylating agents. More recently, Comet assay analysis of 46BR cells, compared to two Bloom's syndrome cell lines, revealed a specific role of DNA Ligase I in repair of UV-induced damage, but not γ-irradiation induced injury (Nocentini, 1995). 46BR cells exhibited a marked DNA re-ligation defect after ultraviolet radiation damage. These observations highlight a role of DNA Ligase I in Nucleotide Excision Repair.

It should be emphasised that the Floxed DNA Ligase I cassette utilised in this analysis was used primarily as an investigative tool, a marker of SP-C/Cre-mediated recombination in the lungs of transgenic mice, and has been used previously to characterise BLG-Cre-mediated excision in mouse mammary epithelium (Selbert et al, 1998). Regardless, it is tempting to speculate as to the phenotype of the Floxed DNA Ligase I mice if the SP-C/Cre transgene had provided adequate levels of excision. For example, Cre-mediated deletion of DNA Ligase I in lung epithelial cells may render mice sensitive to killing by agents damaging to 46BR cells. Alternatively, deletion of Floxed DNA ligase I from a SP-C/Cre recombinase may

induce developmental abnormalities/perinatal lethality due to dysmorphogenesis of the developing lung buds, a feasible consequence of defective Okazaki fragment repair in embryonic tissues. In considering effects of acute lung injury relevant to this thesis, it is additionally noteworthy that mice conditionally deficient in Bcl-x using the Cre-lox system have recently been reported (Wagner et al., 2000). Conditional deletion of the Bcl-x gene was demonstrated in erythroid cells using a MMTV-LTR driven Cre, and highlight the effects of Bcl-x deficiency on cell proliferation, maturation, and survival in this cell type. Both the Bcl-x long and short isoforms are expressed in murine lung epithelial cells (see sections 3.1.a, 3.1.b), however their functions are unclear. It is possible that Bcl-x regulates life/death decisions in the developing lung comparable to what is observed during erythropoiesis. It is additionally possible that Bcl-x modulates the radiation response in adult lung epithelial cells, favouring survival over apoptosis and counteracting the effects of proapoptotic Bcl-2 family genes, such as Bax (see section 3.1.b). Discerning the role of Bcl-x in mouse lung epithelial cells would be a project particularly amenable to investigation using SP-C/Cre mice.

In conclusion, the SP-C/Cre mouse line reported in this thesis is unlikely to be useful in generating improved mouse models of acute lung injury. In theory, conditional ablation of a floxed gene of interest would be used to infer the significance of a gene/genetic pathway in toxic responses of the airways. The excised DNA Ligase I allele, when observed, was detected only by PCR and correspondingly is likely to reflect the sensitivity of PCR methodologies in detecting infrequent excision events in the lungs of SP-C/Cre transgenic mice. However, it is possible that the mouse line

reported herein may be of use in determining the effects of gene deficiency in other contexts e.g. malignancy, whereby loss of heterozygousity in but a tiny fraction of cells may nevertheless induce phenotypic change and pathological consequence in an in vivo environment. In this respect, it is noteworthy that Floxed Rb mice have been engineered and characterised (Marino et al., 2000), and present themselve as a promising line of future study, especially if crossed onto a p53 null background (by analogy with SP-C/SV40 T antigen mice, Wikenheiser et al., 1992; see Marino et al., 2000). With regards to developing a tool that dissects the roles of candidate genes in the context of acute lung injury, the current SP-C/Cre mouse line is clearly inadequate; however, the possibility remains that with the creation of more founder mice, a few may display higher i.e. detectable levels of Cre expression and the capacity for Cre-mediated recombination when bred onto a floxed background. As an improvement on the current SP-C/Cre strategy, whereby transgene expression analysis is accomplished post-mortem, the author suggests coinjecting the SP-C/Cre construct with an easily identifiable transgene, such as luciferase (as illustrated in Figure 4.3). This strategy has been used to successfully identify expression levels of the pituitary-specific POM-C/Cre transgene non-invasively in live mice, facilitating screening of founder mice prior to the adoption of lengthy breeding protocols (Berns group, NKI, Amsterdam).

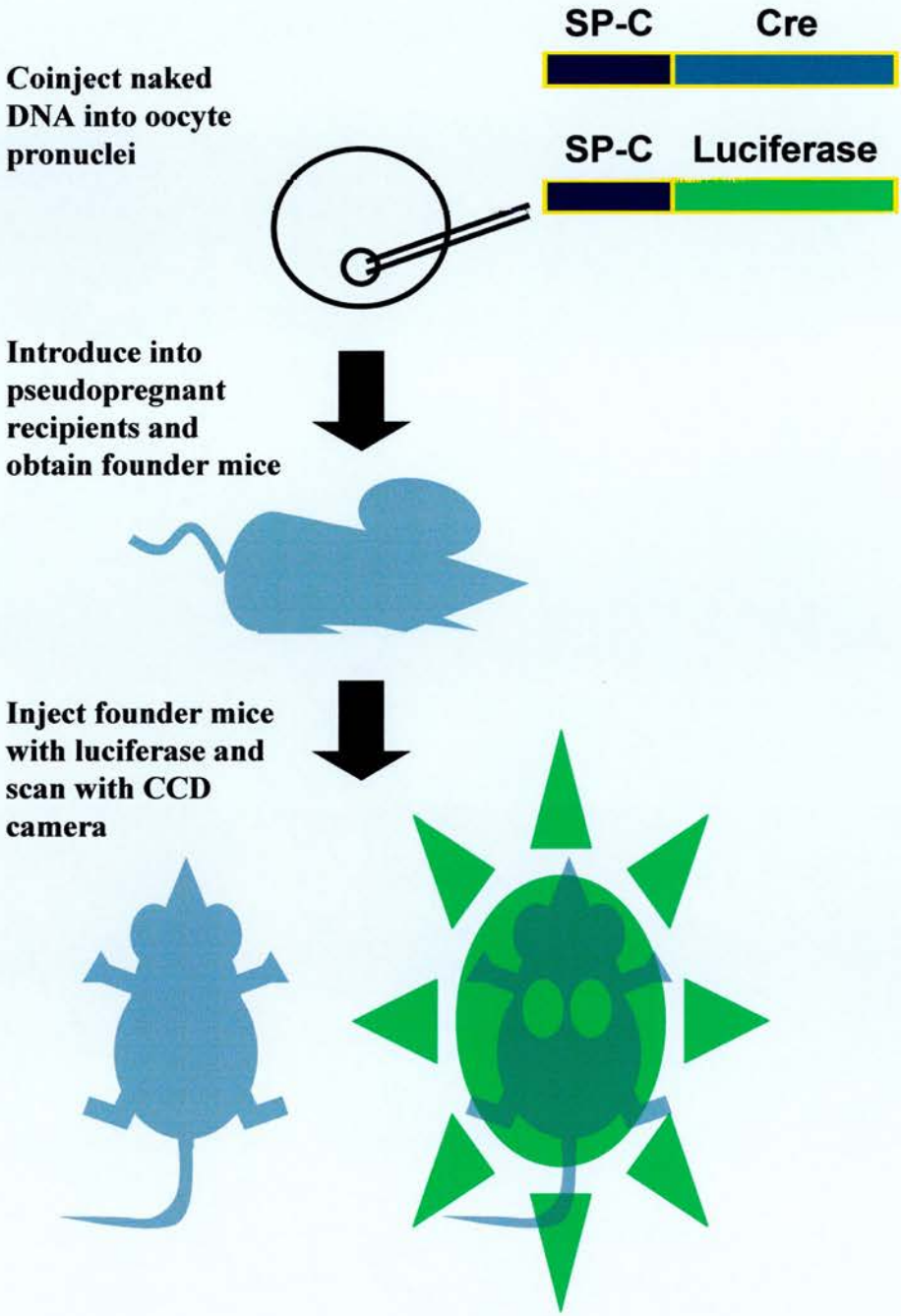


Figure 4.3. A noninvasive method of detecting Cre expression in vivo. Cre and luciferase transgenes intergate at a single site when coinjected into oocyte pronuclei. Founder mice expressing luciferase are detected by luciferin injection immediately prior to image analysis with a charged couple device (CCD) camera. Methodology based on that used to characterise the pituitary-specific POM-C/Cre transgene (Lyons et al., in press).

5. Appendix

5.1. Time-lapse videomicroscopy of p53-/- Clara cells

Isolated p53 null Clara cells were seeded to chamber flasks and subjected to time-lapse videomicroscopy for a period of 24 hours (from 24 to 48 hours in culture). p53-/- Clara cells displayed an array of mitosis and cytokinesis abnormalities, including multiple spindle formation and multinucleation.

Videos of p53-/- cells are available on CD-ROM and are attached to the inner leaf of this thesis. Videos are stored in a Flash (swf) format, and can be accessed with Flash, QuickTime, Netscape, or Internet Explorer.

5.2. Bcl-2 family Ribonuclease Protection Assays

Complete data sets for Bax, Bcl-x^L, and Bcl-x^S mRNA expression, as determined by RNase Protection Assay relative to housekeeping L32 mRNA, are presented in table format over the following 3 pages.

Bax mRNA Expression Profile Analysis

	wt			p53-/-				
Hours post-irradiation	Relative mRNA	Mean	STDEV	Relative mRNA	Mean	STDEV	t test	P value
2	3.39	2.323333333	0.928134329	1.15	1.183333333	0.202072594	0.087850088	0.912149912
	1.88			1.4				
	1.7			1				
4	3.02	2.156666667	0.749688824	0.98	0.843333333	0.165025251	0.036607822	0.963392178
	1.78			0.66				
	1.67			0.89				
8	2.53	1.836666667	0.605750224	0.93	0.87	0.065574385	0.046533886	0.953466114
	1.57			0.88				
	1.41			0.8				
24	2.81	2.036666667	0.670397892	0.77	1.266666667	0.860251901	0.213019342	0.786980658
	1.62			2.26				
	1.68			0.77				
48	1.73	1.08	0.56559703	1.23	1.246666667	0.25540817	0.341568937	0.658431063
	0.81			1.51				
	0.7			1				

Bcl-xL mRNA Expression Profile Analysis

	wt			p53-/-				
Hours post-irradiation	Relative mRNA	Mean	STDEV	Relative mRNA	Mean	STDEV	t test	P value
2	0.81	0.763333333	0.05033223	1.03	0.996666667	0.057735027	0.018874776	0.981125224
	0.77			0.93				
	0.71			1.03				
4	0.62	0.746666667	0.120554275	1.05	0.803333333	0.435928129	0.425720978	0.574279022
	0.76			0.3				
	0.86			1.06				
8	0.47	0.49	0.02	1.11	0.876666667	0.277368588	0.076360101	0.923639899
	0.51			0.57				
	0.49			0.95				
24	0.79	1.176666667	0.626923706	0.71	0.953333333	0.412835722	0.353664345	0.646335655
	0.84			1.43				
	1.9			0.72				
48	0.72	0.76	0.096436508	0.7	0.73	0.051961524	0.187228379	0.812771621
	0.69			0.7				
	0.87			0.79				

Bcl-xS mRNA Expression Profile Analysis

	wt			p53-/-				
Hours post-irradiation	Relative mRNA	Mean	STDEV	Relative mRNA	Mean	STDEV	t test	P value
2	0.25	0.233333333	0.02081666	0.96	1.193333333	0.32929217	0.018278786	0.981721214
	0.21			1.05				
	0.24			1.57				
4	0.2	0.34	0.127671453	1.26	1.223333333	0.845596436	0.115291815	0.884708185
	0.45			0.36				
	0.37			2.05				
8	0.2	0.24	0.036055513	1.64	1.4	0.495681349	0.028605859	0.971394141
	0.25			0.83				
	0.27			1.73				
24	0.3	0.49	0.168226038	1.07	1.66	0.550545184	0.020314515	0.979685485
	0.55			2.16				
	0.62			1.75				
48	0.31	0.343333333	0.04163332	1.51	1.83	0.401123422	0.009469356	0.990530644
	0.33			1.7				
	0.39			2.28				

5.3. Solutions

40% Acrylamide/Bisacrylamide
380g Acrylamide (Kodak 5521)
20g N,N-Methylene-bisacrylamide (Kodak 8383)
Dissolve in approx. 800ml of deionised water and adjust to a final volume of 1L with deionised water (store at 4°C).

10x Agarose gel loading dye
1.5g Ficoll (Sigma F-2637)
0.02g Bromophenol blue (Sigma B-0126)
0.02g xylene cyanole FF (Kodak T-1579)
dH_2O to 10ml (store at -20°C).

Alkaline lysis solution (NaOH/SDS)
20ml 1 N NaOH
10ml 10% SDS
dH_2O to 100ml (make fresh)

9.5M NH_4OAc (ammonium acetate)
73.23g NH_4OAc
dH_2O to 100ml

8.0M NH_4OAc:
61.69g NH_4OAc
dH_2O to 100ml

15% Ammonium persulfate (APS)
1.5g APS
dH_2O to 10ml (store at 4°C).

Ampicillin (Amp)
0.5g Ampicillin (Sigma A-9518)
dH_2O to 100ml (Add to media for final conc. 100 μg/ml)

10X denaturing buffer
2ml 1 M Tris-HCl, pH 9.5
20μl 0.5 M EDTA, pH 8.0
1ml 100 mM spermidine
dH_2O to 10ml (aliquot and store at -20°C)

DNase-free RNase A
200mg RNase A (Sigma R-5500)
3.3μl 3 M NaOAc, pH 4.5
dH_2O to 10ml
boil for 10 minutes (aliquot and store at -20°C).

0.5 M EDTA, pH 8.0 (disodium ethylenediamine tetraacetate)
186.1g Na_2EDTA
Dissolve in approx. 400ml dH_2O, adjust pH to 8.0 with 10 N NaOH, and adjust to 1 litre final volume with distilled water.

100 mM EDTA
20ml 0.5 M EDTA
80ml dH_2O

5mg/ml ethidium bromide (EtBr)
500mg EtBr (Sigma E-8751)
dH_2O to 100ml

1 M HEPES, pH 7.5
23.83g HEPES (Sigma H-3375)
dH_2O to 100ml
adjust pH to 7.5 with potassium hydroxide (KOH) (store at 4°C).

1M KCl (potassium chloride)
7.5g KCl
dH_2O to 100ml

Labelling Mix
7.5µM each of dGTP, dCTP, dTTP

LB Medium
10g Bacto-Tryptone (Difco 0123-01-1)
5g Bacto-yeast extract (Difco 0127-05-3)
10g NaCl
dH_2O to 1 L
adjust the pH to 7.0 and then autoclave to sterilize

LB plates
10g Bacto-Tryptone (Difco 0123-01-1)
5g Bacto-yeast extract (Difco 0127-05-3)
10g NaCl
15g Bacto-agar (Difco 0140-01)
dH_2O to 1 L
autoclave to sterilize, cool to 55°C, add antibiotic if desired, and pour into sterile petri dishes (approx. 20ml/plate).

10x Ligation buffer
5ml 1 M Tris-HCl, pH 7.6
1ml 1 M $MgCl_2$
1ml 1 M DTT
1ml 100 mM ATP
2.5mg BSA

dH_2O to 10ml (store in 25ml aliquots at -20°C)

Lysis/Solution P2
200mM NaOH
1% SDS
Stored at room temperature

1 M $MgCl_2$ (magnesium chloride)
20.33g $MgCl_2$-$6H_2O$
dH_2O to 100ml

1 M $MgSO_4$ (magnesium sulfate)
12.04g $MgSO_4$
dH_2O to 100ml (autoclave)

Methocarn
4 volumes methanol
2 volumes chloroform
1 volumes glacial acetic acid

1 M MOPS
20.93g MOPS (Sigma M-1254)
Dissolve in 80ml dH_2O, adjust pH to 7.5 with 1 N NaOH, and bring volume to 100ml.

10X MOPS buffer
400ml 1 M MOPS, pH 7.5
170ml 3 M NaCl
100ml 1 M MgCl2
330ml dH_2O

20 mM dNTP stocks:
80µl 100 mM dNTP
40µl 50:1 TE buffer
280µl dH_2O

Neutralisation/Solution P3
3.0M potassium acetate, pH 5.5
Stored at room temperature

PBS
120mM NaCl
2.7mM KCl
10mM phosphate buffer salts

Pre-Hybridisation solution

6x	SSC
5x	Denhardt's reagent
17mM	SDS

10X PCR buffer

5ml	1 M KCl
1ml	1 M Tris-HCl, pH 8.5
150µl	1 M $MgCl_2$
dH_2O to 10ml	

PCR Deoxynucleotide Preparation

250µl	100 mM dATP
250µl	100 mM dCTP
250µl	100 mM dGTP
250µl	100 mM dTTP
11.5ml	dH_2O

Aliquot this into 25 tubes with 500µl in each tube.

Phenol, TE-saturated

Add an equal volume of 10 mM Tris-HCl, pH 7.5-8.0, 1 mM Na_2EDTA to ultrapure phenol, mix well, allow phases to separate, remove and discard upper (aqueous) phase. Repeat until the pH of the aqueous phase is between 7.5-8.0 (store at 4° C).

Phenol/chloroform/isoamyl alcohol (25:25:1)

100ml	TE-saturated phenol
100ml	chloroform
4ml	isoamyl alcohol

Buffer QBT (Equilibration buffer)

750mM	NaCl
50mM	MOPS, pH 7.0
15%	isopropanol
0.15%	Triton® X-100

Buffer QC (wash buffer)

1.0M	NaCl
50mM	MOPS, pH 7.0
15%	isopropanol

Buffer QF (Elution buffer)

1.25M	NaCl
50mM	Tris, pH 8.5
15%	isopropanol

Resuspension/Solution P1

50mM	Tris-HCl, pH 8.0
10mM	EDTA
100µg/ml	RNase A

Stored at 4°C after addition of RNase A

RIPA Buffer

50mM	NaCl
1%	NP-40
12mM	deoxycholate
3mM	SDS
50mM	Tris-HCl pH 7.5

Sequencing Gel Solution (for RNase Protection Assay)

250g	Urea
75ml	19:1, 40% acrylamide solution
50ml	10xTBE
175ml	dH_2O

For each gel 60ml of the above solution was mixed with 60µl of TEMED and 60µl of fresh 25% ammonium persulphate (in dH_2O) and immediately poured.

SOC Media

2% w/v	Tryptone
0.5% w/v	Yeast Extract
0.05%	NaCl

Autoclave and add the following filter sterilised reagents.1% (v/v) 1M $MgCl_2$, 1% (v/v) 1M $MgS0_4$ and 0.1% (v/v) 2M glucose solution.

2M NaOAc (sodium acetate)

27.22g	$NaOAc\text{-}3H_2O$

dH_2O to 100ml

3M NaOAc, pH 4.5

408.24g	$NaOAc\text{-}3H_2O$

Dissolve in approx. 800ml dH_2O, adjust pH to 4.5 with glacial acetic acid and bring to a final volume of 1 L with dH_2O.

3M NaCl (sodium chloride)

17.53g	NaCl

dH_2O to 100ml

10N NaOH (sodium hydroxide)

40g	NaOH

dH_2O to 100ml.

1N NaOH

10ml	10 N NaOH

dH_2O to 100ml

10% SDS (sodium dodecyl sulfate)
10g SDS (Fisher S529-3)
dH_2O to 100ml

20X SSC (standard saline-citrate)
17.53g NaCl
8.82g sodium citrate
Dissolve in approx. 80ml dH_2O, adjust pH to 7.0 with HCl and bring final volume to 100ml.

1X SSC (standard saline-citrate)
5ml 20X SSC
95ml dH_2O

T4 DNA polymerase reaction buffer
50mM NaCl
10mM Tris-HCl
10mM $MgCl_2$
1mM DTT
pH 7.9 at 25°C

20X TAE buffer
96.9g Tris base
32.8g $NaOAc\text{-}3H_2O$
14.9g Na_2EDTA
Dissolve in approx. 700ml of deionised water, adjust the pH to 8.3 with glacial acetic acid, and bring to 1 L with dH_2O.

10X TBE
216g Tris base
110g boric acid
16.6g EDTA
Add water to 2 litres.

5x TBS
200g NaCl
5g KCl
75g Tris-HCl
make up to 4L with dH_2O, pH to 7.4 with HCl
make up to 5L

5x TBST
200g NaCl
5g KCl
75g Tris-HCl
make up to 4L with dH_2O, pH to 7.4 with HCl
add 12.5ml Tween-20
make up to 5L

TEMED (N,N,N',N'-tetramethylethylenediamine)
Severn 20-3000-01, store protected from light at 15°C.

TE (10:1) buffer
10ml 1 M Tris-HCl, pH 7.6
2ml 0.5 M EDTA
dH_2O to 1 L

1M Tris-HCl, pH 7.6, 8.0, 8.5, 9.0, 9.5
121.1g Tris base
dH_2O to 800ml
Adjust pH with concentrated HCl and then add dH_2O to 1 L.

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7.1.a. Jason - I know you didn't touch her. And, I know you didn't do anything with the other one either. No, I don't want any water, thank you. Give up the BAC-cloning strategy, instead adopt a FUC-cloning strategy. If you ever wish to pack it in, move to Cuba, and do genetic fingerprints of cigars, my beach hut awaits you. I must additionally remind you that you are contractually obliged to star in my Kung Fu / Techno / Horror production, due out in the Summer of 2001 (verbal agreement, Black Bo's, 6^{th}-7^{th} pint)...so don't go trying to leave the country on me now.

7.1.b. Dom - Raised in the swamps, and nurtured in the ways of the wild, the Mastery of this Man in the field of Southern Blotting was at one time legendary. It is said of this man that he was fuelled by a unique combination of philosophy, spirituality, and toxins from the night previous. He has now turned to the Dark Side of the Force, and can speak CC++ fluently, but has yet maintained his culinary values. I fear we have not heard the last of this one...

7.1.c. Jon - You are a danger to yourself and to others in the lab, and the sheer concept of giving you a PhD is ludicrous. You are nevertheless the solid backbone of our Teviot Campaign and, as resident postdoc, should be lending your academic weight to the finer matters of science, such as the possibility of an espresso machine in the office. Hedonism and pursuit of pleasure have never been so singularly personified, and one fears for the influence this one may have on any future nubile PhD students. Our theses will stand as Testaments to the Great God Dionysus, as monuments to those halcyon days at the latter part of the 20^{th} Century.

7.2. To Eleanor, Jan, Tracey, and Liz 'the Legs' Lovejoy...

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7.5. To my fellow scientists…

so much to say, so little time. No, seriously, I've got several hours to get this bastard in or they're gonna fine me £450. But that's bureacracy for you. There's a cockerel crowing (or whatever it is that cockerels do) suggesting that its almost Dawn. Either that or we've got a very confused cockerel on our hands…Difficult to say…Do cockerels crow?…Surely, its crows that crow…

Clearly I've been awake too long. Having slept a wink in 72 hours. But that's another story. **Shirley,** cheers for all the help through the years, for your ability to smile and tolerate my tantrums. We shared the pain of your Cre mouse. I wish you and Connor a happy life together…
Sharon, fashion guru and winner of the Most Irish Girl In Scotland award, your ability to eat banana sandwiches for breakfast, lunch and dinner astounds me. You are a vision with your doorstopper bread, a slab of cheese, and the omnipotent Cart-O-Death, and I wish you well in whatever you decide to do…
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All your stationery still looks
neat
I can't take it
when you
cry

But Natters,
Bat…
…ters
Ain't it time
we
said
goodbye…

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