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The role of p53 in mouse skin keratinocytes

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Thesis submitted to the University of Glasgow for the degree of
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Abbreviations

DNA	deoxyribonucleic acid
cDNA	complementary DNA
RNA	ribonucleic acid
mRNA	messenger RNA
A	adenine
C	cytosine
G	guanine
T	thymine
dNTP	deoxyribonucleoside
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dTTP	deoxythymidine triphosphate
bp	base pairs
kb	kilo base pairs
mm	millimeters
ml	milliliter
mM	millimolar
uM	micromolar
ug	microgram
W	watts
mA	milliamps
°C	degrees centigrade
EDTA	ethylenediamine tetra-acetic acid
EtBr	ethidium bromide
Tris	tris (hydroxymethyl) methylamine
SDS	soduim dodecyl sulphate
FCS	fetal calf serum
PBS	phosphate buffered formalin
PBF	phosphate buffered formalin
SLM	special liquid medium
KBM	keratinocyte basal medium
DMBA	7,12- dimethyl benzaanthracene
TPA	12-O tetradecanoylphorbol-13- acetate
PCR	polymerase chain reaction
H *ras*	harvey ras
PCNA	proliferating cell nuclear antigen

Lac Z	Beta galactosidase
Gy	Gray

Abstract.

The aim of this project was to investigate the cellular events mediated by the tumour suppressor gene p53. Initially we chose to look *in vitro* at the response of p53 wild type and knockout keratinocyte cells to transforming growth factor beta (TGFβ1) and ionising radiation, as p53 has been implicated in the cellular response to these agents. *In vitro* analysis showed that wild type p53 is not essential for mouse skin keratinocytes to execute a block in DNA synthesis in response to TGFβ1. Furthermore, we found that the presence of wild type p53 does not alter the survival of mouse skin keratinocyte cells after exposure of up to 10Gy of ionising radiation.

As the growing number of reports of p53 mediated cell type specific responses to ionising radiation became apparent, we decided to further our initial *in vitro* analysis of mouse skin keratinocytes and their response to ionising radiation by looking at specific keratinocyte populations in mouse epidermis after exposure to ionising radiation *in vivo*. We choose the relatively low dose of 4Gy for our *in vivo* experiments to avoid the complications of p53 independent cell death by necrosis at higher radiation doses.

In epidermis, distinct populations of keratinocytes where identified which responded to p53 stabilisation by entering the apoptotic pathway or executing a cell cycle block. Cells within the matrix population of the hair follicle underwent p53 mediated apoptosis after exposure to radiation.

The creation of p53 reporter mice allowed us to analyse the transcriptional activity of p53 induced by a number of agents in mouse epidermis. We found no evidence of p53 transcriptional activity in any population of keratinocytes in the skin after treatment with ionising radiation or tumour promoters.

As an extension to the original study we looked at chemically induced tumour development in mouse skin pre treated with radiation which contained fewer matrix cells. The number of carcinogenic lesions which developed was significantly more than in normal mouse skin untreated with radiation. This was the case for both p53 wild type and knockout mouse skin. This led us to re-examine the role of mouse skin stem cells in tumour formation.

Our results demonstrate that p53 induction and downstream events are regulated in not just a tissue specific manner but also a cell type specific manner within a tissue. These findings are of significant importance in the evaluation of sensitivity of individual cell types to DNA damaging agents, and are also relevant to the assessment of therapeutic treatment of tumours of varying origins.

Chapter 1. Introduction

1.1 The discovery of p53

p53 was originally discovered as a protein complexed to SV40 T antigen in SV40 transformed cells. Antisera to the SV40 T antigen was used to demonstrate that T antigen formed a protein complex with the p53 protein (Lane and Crawford, 1979). Shortly after the cloning of mouse p53 it was reported that cotransfection of p53 with activated *ras* oncogene caused the transformation of primary rat fibroblasts (Eliyahu *et al,* 1984). The p53 gene was also found to be able to act on its own to immortalise early pass rat Chondrocytes (Jenkins *et al,* 1984). The conclusion from these studies, was that uncontrolled expression of normal p53 was a major step in cellular transformation. The p53 gene, as a result of these initial discoveries, was classified as a cellular oncogene and was classed alongside *myc, myb,* and *fos.* This definition proved to be incorrect. The p53 constructs used in the early experiments contained mutations in the coding region of the p53 gene that were subsequently found to affect the biological properties of the p53 protein (Eliyahu *et al,* 1988; Hinds *et al,* 1989). When the initial experiments were repeated with genes coding for wild type *p53,* it was found that wild type p53 not only failed to transform the cells, but was able to suppress cellular transformation mediated by other oncogenes.

Reintroduction of wild type p53 into tumour cells was shown to result in the reversal of tumorigenicity, and often the transformed phenotype of the tumour cell (Wolf *et al,* 1984). p53, was next classified as a tumour suppressor gene. It was subsequently shown that reintroduction of p53 into a tumour or transformed cell led to growth arrest of the cells in G1 of the cell cycle. As a result of these experiments wild type p53 was termed a negative regulator of cell growth, and elimination of wild type p53 function was assumed to be required for unregulated cellular proliferation to occur. Deletions or mutations of the p53 gene were subsequently found to be very common in mouse and human tumours. (Hollstein *et al,* 1996).

Cellular proliferation is very tightly controlled. Cell growth occurs in a series of steps which includes a period of DNA synthesis (S) where the DNA replicates, a period of mitosis (M) in which the chromosomes are separated, and temporal gaps in between these two events termed G1 and G2. It is now firmly established that a family of protein kinases, called cyclin dependent kinases (cdks), controls the transitions between

successive phases of the cell cycle in eukaryotic cells (Pines *et al*, 1993). All the cdks are structurally related to each other and require associated cyclin proteins for activity. Cyclins are frequently classified as G1 cyclins or mitotic cyclins depending on when they function in the cell cycle. In vertebrates D type cyclins (D1, D2, D3) are considered to be key regulators of G1 progression (Sherr, 1993). Formation of active complexes between D type cyclins and cdk4 / cdk6 are thought to be needed for G1 progression. Cyclin E in association with cdk2 is required for G1/S transition (Knoblich *et al,* 1993) and cyclin A, in complex with cdk2 is essential for progression through S phase (Reviewed in Fisher, R. P. 1997). Both A and B type cyclins associate with cdc2 to promote entry into mitosis. (Knoblish *et al,* 1993).

The G1/S and S phase substrates of cdk/cyclin complexes fall into two classes. They are either transcriptional regulators involved in controlling the expression of S phase genes, or they are components of the DNA synthesis machinery itself. The most intensely studied G1/S substrate is the Retinoblastoma gene product (pRb). Early in the cell cycle, pRb sequesters transcription factors, of the E2F/DP family, which are involved in regulating genes required for S phase progression. During progression through G1 pRb becomes phosphorylated by cyclin dependent kinases, which causes the release and activation of E2F/DP transcription factors (Hinds and Weinberg, 1994).

p53 knockout mice were created by the introduction of a null mutation into the p53 gene by homologous recombination in murine embryonic stem cells (Donehower *et al*, 1992; Clarke *et al.* 1993; Lowe, *et al* 1993a.; Tsukuda *et al* 1993). As p53 had been implicated in cell cycle control it was thought it may be an essential component in the regulation of development. However mice homozygous for the p53 null allele developed normally but were prone to spontaneous development of neoplasms at an early age. Mice heterozygote for the inactivated p53 allele also showed an increased incidence of spontaneous malignancies compared to p53 wild type mice. In the vast majority of tumours in heterozygote animals, the wild type p53 allele was deleted in tumours (Donehower *et al,* 1992). Initial analysis of the p53 null mice did not support the hypothesis that p53 is important in the cell cycle regulation of normal cells, as the mice developed normally with no defects. Although p53 is mainly regarded as the guardian of

the genome, recent results suggest a role for p53 in normal development of the neural tube and ocular structures. (Sah, *et al* 1995 & Armstrong, *et al* 1995)

It was finally concluded that p53 is dispensable for the growth and development of normal cells, but is required to prevent the occurrence of malignant genetic alterations. Consistent with the role of p53 as guardian of the genome, fibroblasts from p53 deficient mice show chromosomal abnormalities that become apparent at early passage. Aneuploidy and evidence of chromosomal instability has also been shown in to exist in tumours from p53 null mice. Embryonic fibroblasts derived from p53 null mice possess (1) shorter doubling time (2) increased ability to grow under conditions of low cell density, (3) a lack of senescence even at high passage, compared to equivalent p53 wild type cells.(Harvey *et al,* 1993).

The p53 protein is now widely described as a negative regulator of cell growth whose inactivation is a major contribution for the development of malignancy.

The amino terminus of p53 has the ability to modulate the transcription of other genes by interaction with transcription factors such as the TATA box binding protein, a component of the general transcription factor TFIIH as well as several TATA box binding protein associated factors (Horikoshi *et al,* 1995). Wild type p53 can bind to TBP and act as transcriptional repressor for a number of target genes, including *PCNA* (Proliferating Cell Nuclear Antigen), *c-fos* ,*c-jun, IL-6* and *bcl2* (Horikoshi *et al,* 1995).

p53 is present predominantly as tetramers, with dimers and monomers accounting for less than 5% of the total p53 molecules in a cell. This observation is important in the evaluation of the interaction of p53 with TBP. Given the low fraction of monomeric p53 in cells and that wild type p53 is present in low amounts of about 100 molecules per cell, p53 mediated transcriptional repression by binding to TBP is most likely an effect of grossly overexpressed p53.

The central core of the p53 gene contains the sequence specific DNA binding domain (Barogonetti *et al,* 1993; Halazonetis and Kandil, 1993; Pavletich *et al,* 1993; Wang *et al,*1993). Intrinsic to the function of p53 is its ability to regulate transcription through

sequence specific DNA binding. P53 tetramers bind specifically to a p53 responsive element that contains repeats of the consensus binding sequence Pu-Pu-Pu-C-A/T-T/A-G-Py-Py-Py where Pu and Py are purine and pyrimidine nucleotides respectively. (El-Deiry *et al*,1992) The binding of wild type p53 to its cognate response elements regulates transcription of nearby promoters.

The carboxyl terminus of p53 is capable of binding non-specifically to different forms of damaged DNA and re annealing complementary strands of RNA and DNA (Wang *et al*, 1993; Balkalkin *et al*, 1994; Bayle *et al*; Lee *et al*, 1995; Reed *et al*, 1995). The C-terminus of the p53 gene can be subdivided into three regions. The linker region which spans around 300 residues connects the DNA binding domain to the tetramerisation domain. This is followed by a small region which is rich in basic residues.

1.2 p53 and phosphorylation.

P53 protein is extensively post-translationally modified, mostly by phosphorylation. The major phosphorylation sites in p53 have been mapped within two distinct domains at the N- and C- termini of the protein. Most of the phosphorylation sites are clustered in the transactivation domain, and in a region containing PEST sequences which are thought to mediate rapid protein turnover. (Samad *et al*, 1986).

Several protein kinases are known to modify p53 at its N terminus. One of these is the double stranded DNA activated protein kinase (DNA-PK) (Lees-Miller *et al*, 1990). This nuclear kinase is potently activated by linear double stranded DNA. Activation of DNA-PK by DNA breaks is of special interest as a p53 kinase since p53 itself is stabilised in response to DNA damage. Phosphorylation of p53 by DNA-PK may play a major role in the response of p53 to DNA damaging agents.

Casein kinase 1 (CK1) phosphorylates p53 at three N terminal sites (Milne *et al*, 1992). It is interesting to note that a DNA repair defective mutation in the yeast *S. cerevisiae* lies in a gene encoding a protein kinase with a high similarity to CK1 (Hoekstra *et al*, 1991). This represents another potential link between DNA damage and p53 phosphorylation.

Li Oncogene 1995

A third protein kinase, Mitogen Activated Protein (MAP) kinase phosphorylates p53 at its N-terminus (Milne *et al,* 1994). MAP kinase encompasses a family of related protein kinases which are thought to play a role in mediating the effect of various mitogens and differentiation factors acting through protein tyrosine kinase receptors. MAP kinase is activated following exposure of cells to ultraviolet radiation, (Devary *et al,* 1992). It is possible that regulation of gene transcription following UV damage is mediated through the MAP kinase pathway. Phosphorylation of p53 by MAP kinase may therefore be important in the response of p53 to DNA damage, or changes in the cellular environment which occur as a result of mitogenic or differentiating signals.

Protein kinase C is thought to play an important role in the phosphorylation of p53, particularly at the C terminus. The site of PKC phosphorylation is believed to lie in the region of the protein which is important for oligomerisation. At the cellular level, stimulation by phorbol esters, which are potent activators of PKC leads to increased phosphorylation of p53. This may be direct or indirectly through the activation of a PKC activated kinase such as MAP kinase (Baudier, *et al* 1992).

Relatively little is known about the enzymes which catalyse the dephosphorylation of p53 *in vivo.* However protein phosphatase 2A has been shown to dephosphorylate several phosphorylated sites on p53 *in vitro* (Scheidtmann, *et al* 1991).

1.3 P53 induction, apoptosis, cell cycle arrest, and the development of cancer.

Genomic integrity is continuously under threat from environmental factors. p53 is thought to be involved in maintaining stability of the genome. In cell culture systems, exogenous wild type p53 expression has been shown to be capable of eliciting cessation of growth, increased apoptosis, or increased cellular differentiation.

The disruption of the p53 gene in a high percentage of tumours highlights the importance of disabling p53 dependent pathways in order to achieve cellular transformation. Abnormalities in p53 function are thought to contribute to the development of transformed phenotypes in two ways: 1)Loss of p53 function in cells where p53 mediates cell cycle arrest after DNA damage is most likely to result in the

replication of a damaged DNA template and the development of heritable genetic changes in daughter cells (Kastan *et al,* 1991) ; 2) Loss of p53 mediated cell death leads to survival of cells in inappropriate physiological conditions (Kinzler *et al,* 1996). The latter situation is particularly detrimental since the cell escapes death despite DNA damage. These two factors are thought to work together to contribute to the genetic alterations associated with cancer development as a result of DNA damage. P53 has also been implicated in the development of cancer by its proposed involvement in growth factor signalling and angiogenesis.

1.4 p53 stabilisation after DNA damage.

After DNA damage, p53 protein levels rise in cells (Kuerbitz *et al,* 1992). The amount and kinetics of p53 induction vary greatly depending upon the genotoxic agent. UV light produces an increase in p53 levels in cultured cells almost immediately. The extent of the increase is over ten fold and is maintained for 30 hours post UV exposure before falling back to levels observed in control cells. In contrast, in response to ionising radiation p53 levels increase by three fold in the space of an hour and return to background levels within three hours. 3-Aminobenzidine can inhibit poly-ADP ribose polymerase and hence prevent DNA repair. In a series of experiments, Lu *et al* (1993) showed that the p53 response lasts until 3- Aminobenzidine is removed and that DNA repair commences upon withdrawal of the drug. These observations suggest that the presence of DNA damage prolongs the p53 response.

DNA strand breaks are an adequate signal for p53 stabilisation in response to DNA damage (Hartwell *et al,* 1989). The nature of the break that induces p53 stabilisation was discovered using microinjection techniques. Defined plasmids of DNA were used in microinjection studies to show that double stranded ends of linear DNA longer than 27 base pairs, circular DNA with large gaps and single stranded circular DNA all possessed signalling ability. Dilution experiments led to the conclusion that as little as one double strand break per cell can initiate the p53 pathway. (Huang *et al* 1996)

Cells derived from Ataxia telangiectasia (AT) patients have helped to unravel the signalling pathway between DNA damage and p53. AT cells are hypersensitive to DNA damaging agents, such as irradiation and restriction enzyme digestion which induces

double strand DNA breaks (Thacker *et al,* 1994) Furthermore the ability of AT cells to induce cell cycle arrest following DNA damage is deficient, indicating defects in cell cycle checkpoint control (Meyn *et al,* 1995).

The signal for apoptotic induction may involve cytoplasmic events as well as DNA damage. A number of cellular events following the exposure of cells to non - ionising radiation are independent of the nucleus and radiation induced DNA damage. For example the response to ultraviolet light depends on the activation of *Src* like kinases in the cytoplasm, and can occur in enucleated cells (Devary *et al,*1992). Consistent with this, high levels of membrane tyrosine phosphorylation are observed seconds after exposure of mammalian lymphocytes to γ radiation. The rapidity of this response makes it unlikely that the initiating signal involves the nucleus. It is also interesting to note that inhibition of the membrane phosphorylation reactions blocks radiation induced apoptosis. This is again consistent with the initiation of apoptosis occurring outside the nucleus.

Exposure of cells to hypoxic conditions results in increased p53 protein levels and can lead to p53 dependent apoptosis (Graeber *et al,* 1996). It is widely believed that this induction of p53 occurs in the absence of DNA strand breaks. It has been recently shown that depletion of ribonucleotide triphosphates (rNTP) using inhibitors of purine and pyrimidine biosynthesis, is sufficient to induce p53 and p53 - dependent G1 arrest (Linke, et al 1996). It may be the case that an inadequate supply of rNTPs results in changes in the RNA macromolecule population, analogous to the way that dNTP inhibitors lead to DNA strand beaks. This suggests a common link in p53 signalling which is dependent on changes in nucleic acid macromolecules.

1.5 p53 induction and cell cycle arrest

Most cells delay transit through the cell cycle in response to DNA damage. This is thought to allow the repair of damaged DNA before replication and chromosome segregation. Cell cycle delay in mammalian cells in response to DNA damage has been observed in G1, S and G2 phases of the cell cycle (Maity *et al,* 1994). Early studies showed exposure of mammalian cells to ionising radiation prolongs both S and G2 phases (Leeper *et al,* 1972), the delay in S phase lasting only a few hours, while the G2

delay is much longer. A delay in G1 was not observed in early studies with ionising or UV radiation. This was probably due to the fact that cultured cells are very commonly deficient in this pathway (Domon *et al,* 1968).

Studies with primary cultures were the first to reveal the existence of a G1 block (Little, 1986). Subsequently other cell lines have been shown to block in G1 after DNA damage (Kastan *et al,* 1991). Using a subtractive hybridisation technique, a gene highly induced by wild type, but not mutant p53 was discovered . The gene was named *WAF1* for wild type p53 activated fragment. (El-Diery *et al,* 1993). Simultaneously Harper *el al* (1993) identified a gene that they called *CIP1* whose product was found to bind to cyclin complexes and inhibits the function of cyclin dependent kinases. The two labs soon realised they had characterised the same gene by different methods. *WAF1/CIP1* proved to be identical to a p21 protein found complexed with cyclin and cyclin dependent kinases in normal cells. Yet another group had independently discovered the same protein, denoted it Sdi1 and described the protein as being involved in growth arrest accompanying cellular senescence (Noda *et al,* 1994).

$p21^{waf}$ mRNA levels, as well as protein levels, increase following exposure of cells to ionising radiation in a p53 dependent manner (Xiong *et al,* 1993). $p21^{waf}$ can inhibit the growth of human tumour lines as well as normal diploid fibroblasts when it is introduced via transfection. Cells which lack functional p53 do not show an increase in $p21^{waf}$ levels after radiation, nor do these cells arrest at G1/S following irradiation (Dulic *et al,* 1994). $p21^{waf}$ knockout mice show no abnormal development. However fibroblasts from these animals are impaired in their ability to undergo G1 arrest following DNA damage. (Brugarolas *et al,* 1995).

As an inhibitor of cyclin dependent kinase complexes, $p21^{waf}$ may contribute to DNA damage induced G1 arrest by preventing the phosphorylation of the Retinoblastoma gene product (pRb). $p21^{waf}$ can also arrest cell cycle progression by direct inhibition of DNA replication, independent of interactions with cyclin cdk complexes (Luo *et al,* 1995). $p21^{waf}$ is found in complexes in normal cells. These complexes consist of a cdk, and the essential DNA replication factor, PCNA, which is a 36Kd protein capable of

interacting with DNA polymerase α. When separately overexpressed in cells the Cdk binding domain and the PCNA binding domain of $p21^{waf}$ both prevent DNA replication. This suggests a dual role for $p21^{waf}$ as a cell cycle inhibitor *in vivo* (Lou *et al,* 1995). The binding of $p21^{waf}$ to PCNA does not affect the DNA repair activity of PCNA but is thought to alter the availability of PCNA for DNA replication.

In summary, p53 can control cell cycle progression at the G1/S boundary, at least in part through the transcriptional activation of $p21^{waf}$. $p21^{waf}$ appears to be the most pleiotropic mediator of p53 dependent cell cycle arrest However other genes transcriptionally activated by p53 may play a role in DNA damage arrest.

Cyclin G was identified in a differential screen experiment as a gene whose expression is induced by wild type p53 (Okamoto and Beach, 1994). Little is known about the function of cyclin G. It may be involved in DNA repair by complexing with a cdk responsible for phosphorylating and activating repair proteins. Cyclin G is unlikely to be involved directly in cell cycle arrest as overexpression does not result in a cell cycle block.

Another good candidate for a p53 response gene involved in DNA damage induced growth arrest is the proto-oncogene *mdm-2* (Barak *et al,* 1993). *mdm2* is amplified in 40% of human sarcomas (Oliner *et al,* 1992). The gene encodes a protein that has the ability to complex with p53 and inhibit its transcriptional activity. *mdm2* is transcriptionally activated by wild type p53 in response to UV irradiation. It is thought that an auto regulatory feedback loop exists between *mdm2* and p53 Overexpression of *mdm2* inhibits p53 dependent G1 arrest in response to irradiation (Barak *et al,* 1993; Perry *et al,* 1993). Recently it was been shown that *mdm2* promotes the degradation of p53. This is though to be an important mechanism in ensuring that the p53 signal is quickly terminated. (Haupt, *et al* 1997)

GADD45 (Growth Arrest and DNA damage) (Kastan *et al,* 1992), like *mdm2* and $p21^{waf}$ is induced when cells are exposed to DNA damaging agents leading to G1 arrest. This damage induced gene also contains a p53 binding site and has been shown to

be induced by p53 (Lu *et al,* 1993). Recent evidence suggests that GADD45 can interact with PCNA and block DNA replication co-ordinately enhancing nucleotide excision repair of damaged DNA. (Smith *et al,* 1994)

The insulin-like growth factor binding protein 3 gene has also been identified as a p53 target gene that is induced in cells after DNA damage (Buckbinder *et al,* 1995). IGF-BP3 protein has the ability to inhibit the signalling of insulin- like growth factor receptors and so plays an antimitogenic role in the cell. This represents another possible way by which p53 can suppress growth.

Several other candidate response genes have also been identified, including transforming growth factor α (Shin *et al,* 1995), thrombospondin (Dameron *et al,* 1994), fas/APO1 (Owen- Schaub *et al,* 1995), PCNA (Shivakumer *et al,* 1995), and the epidermal growth factor receptor gene (Deb *et al,* 1994). However these genes have not yet met as many criteria as those described previously and so it is not clear if they are true p53 target genes.

p53 also has the ability to repress transcription of cellular and viral genes with promoters that lack p53 binding sites. Transcriptional repression may represent another mechanism that p53 uses to elicit cell cycle arrest.

1.6 P53 and cell cycle arrest at G2.

G2 delay after DNA damage presumably prevents problems in chromosome condensation and segregation. The possibility that G2 delay may be critical in preventing irreversible fixation of DNA damage is suggested by the correlation between delay in G2 and increased radioresistance in cells transfected with various oncogenes. However in fission yeast radiosensitivity and G2 delay share common pathways but have separable phenotypes.

Bouffler, *et al* (1995) looked at chromosomal abnormalities in p53 null and wild type mice. They found that the frequency of spontaneous aberrations in bone marrow cells was greatly elevated in p53 null and heterozygote mice compared to wild type mice. They did not observe any increased induction of stable aberrations after γ radiation,

however they found that lack of p53 resulted in increased radiation induced hyperploidy. No effect of p53 status was observed on sister chromatid exchange or G2 chromatid damage, however they did observe a p53 mediated effect on post irradiation entry into mitosis. This led them to suggest a G2/M checkpoint role for p53 in mouse bone marrow cells after irradiation.

Stewart *et al* (1995) also found evidence of p53 involvement at G2/M. Using REF52 cells transfected with temperature sensitive p53 ^{val135} they showed a conditional block at G1/S and G2/M when wild type p53 expression was induced. These results are comparable to a study by Martinez *et al* who showed a modest peak of G2/M cells when wild type p53 expression was induced (Lu *et al,* 1994).

Many studies have failed to observe any evidence of p53 induced G2/M block. The G2/M transition is the shortest part of the cell cycle, so the number of cells at this point in the cycle is small at any given time (Baserga, 1985). This may provide some explanation as to why some have failed to recognise the block. Kulez-Martin *et al* recently found a p53 splice variant ($p53^{as}$) that is preferentially expressed at G2 (Lu *et al* , 1994). Stewart *et al* used a p53 clone capable of producing $p53^{as}$ in their transfection studies. However much of the work on p53 induced cell cycle arrest made use of p53 cDNA clones that are not capable of making the G2 $p53^{as}$ variant. This may be another explanation as to why there are few reports of p53 mediated arrest at G2. As yet very few G2 specific p53 transcriptional target genes have been identified, Cyclin G perhaps being the most attractive possibility.

1.7 Cell cycle arrest in S phase.

Cell cycle arrest in S phase after DNA damage, presumably acts to prevent the propagation of genetic lesions during DNA synthesis. The p53 induced cell cycle inhibitor $p21^{waf}$ can inhibit cyclin A-cdk2 which is active at the G1/S cell cycle boundary. It can also inhibit cyclin A-CDC2, which is active in S and early G2 phase (Dulic *et al,* 1994). This led to the hypothesis that P53 may be involved in establishing S phase arrest by switching on the transcription of $p21^{waf}$. However, S phase delay after

radiation is observed in virally transformed cells in which p53 is non functional. $p21^{waf}$ may be involved in the establishment of S phase arrest, but it is probably induced by a pathway independent of p53.

1.8 P53 - a direct role after DNA damage?

As well as transcriptional activation and/or repression, a direct involvement of p53 in the regulation of cellular DNA synthesis should be considered as a possible method of p53 mediated growth regulation. Lane *et al* showed that p53 associates with viral replication complexes in Herpes virus infected cells (Lane, et al 1979). It has also been shown that addition of wild type p53 *in vitro* to DNA replication assays inhibits SV40 DNA replication. Expression of p53 in SV40 transformed monkey cells inhibits *in vivo* replication of plasmids containing the SV40 origin of replication (Mercer *et al* 1990). Mutant p53 is not capable of reproducing these functions. The inhibitory function of wild type p53 in SV40 replication is thought to be due to the binding of p53 to the SV40 large T antigen, resulting in the inhibition of the large T intrinsic DNA helicase activity (Wang, *et al* 1989).

It should be noted that p53 levels in these experiments were grossly elevated and far higher than normal physiological levels. However it is interesting to note that lytic infection with SV40 leads to stabilisation of p53. It may be the case that the abnormally high levels of p53 in infected cells directly mediates inhibition of viral DNA replication.

p53 can bind to replication protein A (RPA). RPA is a multisubunit complex which contains three polypeptides of molecular weight 70,000, 34,000, and 13,000. The binding of RPA to single stranded DNA is believed to be an initial step in DNA replication and is thought to be required for the unravelling of cellular origins of replication and for DNA excision repair (Dutta *et al*, 1993; He *et al,* 1993). Interaction of p53 with RPA inhibits the ability of RPA to bind to single stranded DNA *in vitro.* RPA also binds to mutant p53 proteins which are unable to induce G1 arrest. However the binding of mutant p53 also abolishes the binding of RPA to single stranded DNA. Wild type and mutant p53 may exert different functions when bound to RPA.

1.9 P53, DNA damage, and apoptosis.

Apoptosis, or programmed cell death, is the process by which a cell actively commits suicide under a tightly controlled cascade of events. Apoptosis is recognisable microscopically by characteristic condensed basophilic cytoplasm, darkly staining karyorrhectic nuclei, and the presence of apoptotic cell bodies, which are usually phagocytosed by macrophages or by neighbouring cells. DNA from cells undergoing apoptosis is cleaved into fragments of roughly 200 base pairs. However, there are a number of reports that cell death can occur without DNA fragmentation (Shuze-Osthoff *et al*, 1994).

The signals that trigger apoptosis, occurring either naturally during development or as a response to insults imposed on the cell include the presence of DNA damage, growth factor or nutrient withdrawal, disruption of cell matrix interactions, altered expression of potent cellular oncogenes such as *myc*, and viral infection.

In mammalian cells, many of the gene products that activate or suppress apoptosis have been identified. p53 is known to be responsible for directing apoptosis in some systems (Clarke *et al,* 1993; Lotem and Sachs, 1993; Lowe *et al,* 1993). In tumorigenesis of the brain choroid plexus epithelium for example, the absence of p53 correlates with aggressive tumour growth and a decrease in apoptosis, suggesting that p53 dependent apoptosis normally acts as a check to tumour growth and progression in the tissue.

p53 independent apoptosis has also been observed. The induction of apoptosis in mouse thymocytes treated with glucocorticoid and calcium, and proliferating lymphoid cells treated with genotoxic agents is p53 independent. It is important to note that p53 knockout mouse embryos do not exhibit any gross abnormal cell death patterns in development, although some neural abnormalities have recently been discovered (Sah, *et al* 1995; Armstrong *et al* 1995). This suggests that apoptosis in normal development, in the absence of any abnormal stress occurs largely through p53 independent pathways.

1.10 p53, and effectors of apoptosis

The most common genetic alteration in human B cell lymphoma is a chromosome translocation that juxtaposes the bcl-2 gene with the immunoglobulin heavy chain gene (Tsujimoto, *et al* 1985). The high incidence of p53 loss and accompanying high levels of bcl-*2* in human tumours prompted exploration of the effects of p53 on *bcl-2* gene expression. p53 protein was found to be capable of transcriptional repression of the *bcl-2* gene (Miyashita *et al*, 1994). *bcl-2* was subsequently characterised as a cell survival gene. BCL-2 protein is an intracellular membrane protein that resides in the outer mitochondrial membrane, nuclear envelope and parts of the endoplasmic reticulum. The protein has been shown to block cell death induced by numerous stimuli including growth factor deprivation, Ca^{2+} ionophores, reactive oxygen species, some viruses, heat shock, and irradiation (Reviewed in White 1997). BCL-2 levels have been shown to correlate *in vitro* with sensitivity to apoptosis. In clinical studies, high levels of BCL-2 protein or alterations which lead to deregulation of the protein production are associated with poor responses to therapy.

Transgenic mice which harbour an expression construct in which the *bcl-2* gene is under the transcriptional control of the IG heavy chain enhancer show accumulation of B lymphocyte cells as a result of prolonged cell survival. The mice also develop high grade malignant lymphomas (McDonnel *et al,* 1989). *bcl-2* knock out mice show stunted growth and post natal mortality. Initially, haematopoietic development is normal but soon complete apoptotic involution of the primary lymphoid organs takes place. The animals also develop polycystic kidneys and renal failure. Due to the lack of development of pigment in the second hair cycle the mice turn grey at about six weeks old (Kamada, *et al* 1995).

BCL-2 is one of a growing family of related proteins that have been conserved throughout multicellular evolution. This family includes Ced-9 of *C.elegans* (Spector *et al,* 1992), the mammalian proteins BCL-Xs and BCL-Xl (Biose *et al,* 1993), MCL-1 (Kozopaz *et al,* 1993*),* A1(Lin *et al, 1988),* BAX (Oltvai *et al 1993),* and viral proteins which include P30 (baculovirus) (Clem *et al,* 1991), BHRF1 (Epstein Barr Virus)

(Pearson *et al,* 1987), VG16 (Herpesvirus) (Albrecht *et al,* 1987), LMW5 (African Swine Virus) (Neilan *et al,* 1993), and p19E1B (Adenovirus) (White *et al,* 1992).

Members of this growing family can be divided into two groups. BCL-2, BCL-XL, BHRF1, p19EIB, MCL1and CED-9 all have the ability to inhibit programmed cell death. However BAX , and BCL-Xs antagonise the anti apoptotic activity of BCL-2. p53 can transactivate *bax* (Selvakumaran *et al,* 1994) which contrasts with its ability to repress transcription of *bcl2.*

It has been proposed that when BCL-2 is in excess, BCL-2 homodimers predominate and cells are protected and when BAX is in excess, BAX homodimers predominate and cells are susceptible to apoptosis (Oltvali *et al,* 1993). BCL-2 and EIB 19K proteins can block apoptosis that is both p53 dependent and independent, suggesting that both function downstream of p53 and inhibit multiple pathways.

It was presumed that the prime function of p53 would be to control the relative levels of BAX and other cell death/ survival proteins by transactivation and or repression of the gene promoters. However p53 mediated apoptosis has been reported in cells treated with the protein synthesis inhibitor cycloheximide. Caelles *et al* showed that GHFT1 cells expressing a temperature sensitive p53 mutant could undergo p53 mediated apoptotic cell death in response to UV damage, which was independent of protein transcription or translation (Caelles *et al,* 1994).

Two recent papers reported contradictory results with a p53 mutant that abrogates the transcriptional activating function of p53. Based on the results from transient transfection assays it was concluded that p53 mediated apoptosis does not require transcription function in HeLa cells (Yonish-Rouach *et al,* 1994; Haupt *et al,* 1995). In contrast, the White laboratory demonstrated that BRK cells stably expressing E1A plus temperature sensitive p53 ^{val135} underwent apoptosis after temperature shift to 32^{o}C, whereas cells expressing the same mutant used in the HeLa cell experiments did not (Sabbatini *et al,* 1995).

Cisplatin treatment of Epstein-Barr virus immortalised human B lymphocyte cell lines results in p53 mediated apoptosis which occurs in cells at the G1/S cell cycle boundary. Transfection studies showed a correlation between p53 transcriptional activation and the induction of apoptosis. Mutant alleles that were incapable of transactivation caused apoptosis to occur in the G2/M after cisplatin treatment. The results of this study suggest that DNA damage can induce apoptosis from G1/S when p53 is transactivation competent, and from G2/M when it is transactivation defective in certain cell types. (Allday *et al,* 1995).

Ludwig *et al* analysed the transcriptional activity of a number of p53 mutants, and showed that not all p53 responsive cell promoters are equally sensitive to different p53 mutants. They identified two mutants that whilst retaining the ability to induce cell cycle arrest through transactivation of $p21^{waf1}$ have defective ability to transactivate *bax* and *IGF-BP3*. The amino acid substitutions present in these mutants are in the DNA binding domain. Transcriptional activity of both correlated with their ability to bind DNA containing the p53 binding site present in each of the promoters tested. (Ludwig, *et al* 1996) It seems likely that modifications which effect DNA binding capacity of p53 such as phosphorylation may also play a role in the transcriptional targets of p53 in a given cell.

The generation of transgenic mice, in which a *Lac Z* transgene is driven by a p53 response element allowed the analysis of the transcriptional activity of p53 protein *in vivo* (McCallum *et al* 1996). In the spleen of irradiated p53 lac Z reporter mice, p53 was induced in all the cells of the tissue. Although apoptosis was observed in both the red and white pulp of the tissue, LacZ expression was only apparent in the red pulp. These results suggest that not all p53 dependent apoptosis is the result of transcriptional activation by p53. These results are similar to results published recently (Gottlieb, *et al,* 1997)

1.11 Apoptosis or cell cycle arrest *in vitro*?

Irradiation induced cell cycle arrest and apoptosis appears to be cell type specific. Mouse embryo fibroblasts derived from homozygous and heterozygous wild type p53 mice respond to DNA damage with growth arrest as do cell lines that contain wild type

p53. Fibroblasts from p53 knockout mice fail to arrest. It seems that in normal fibroblasts p53 functions to produce growth arrest in response to DNA damage. However thymocytes from homozygous and heterozygous wild type p53 undergo apoptosis in response to radiation and thymocytes from p53 knockout animals do not. The evidence to date suggests different cell types respond to DNA damage and p53 accumulation with either cell cycle arrest or apoptosis (Kastan *et al,* 1992, Clarke *et al,* 1993; Lowe *et al,* 1993; Slichenmyer *et al,* 1993).

When p53 dependent apoptosis was first observed some reports suggested that small amounts of DNA damage resulted in cell cycle arrest while significant amounts of DNA damage resulted in apoptosis. It was later proposed that p53 induction by DNA damage results in cell cycle arrest in some physiologic settings and apoptosis in other settings, that is at a given low amount of damage, some cells are programmed to die and some programmed to arrest. However this is not to imply that a cell cannot be overwhelmed with a massive DNA insult which results in cell death.

Kastan *et al* looked at the decision fork between cell cycle arrest and apoptosis *in vitro* in the non malignant cell line Baf-3, an interleukin 3 dependent (IL3) murine lymphoid lineage cell line. Exposure of this cell line to ionising radiation results in apoptosis in the absence of IL3. However when IL3 is present no apoptosis occurs. (Canman *et al, 1*995). It was shown that in the presence of IL3 the cells arrested in both G1 and G2/M phases of the cell cycle. The G1 arrest observed was dependent on p53 function. In contrast, in the absence of IL3 at the time of radiation the cells did not arrest in G1 and entered the apoptotic pathway rapidly. Irradiation and p53 induction caused an increase in $p21^{waf}$ levels in the absence of IL3, however, the levels were significantly less than levels found in irradiated cells with IL3. $p21^{waf}$ mediated inhibition of cyclin dependent kinases is thought to be dependent on absolute levels of $p21^{waf}$ in the cell. This may explain why, despite an increase in $p21^{waf}$ levels in the cells subjected to irradiation and growth factor withdrawal, the cells fail to arrest. No changes in BCL- 2, BAX or BCL-X were observed that may have accounted for the modulation of G1 arrest versus apoptosis. This suggests that modulation of the level of these proteins is not responsible for dictating cellular outcome after p53 induction by radiation. However alterations in

the activity, rather than levels of these proteins may play a role in the determination of cell fate after radiation.(Kastan *et al,* 1995). The results of Kastan are comparable to recent results from the Vogelstein lab which demonstrated that loss of $p21^{waf1}$ causes a switch in cell fate after DNA damage from cellular growth arrest to apoptosis.

As a result of these studies it was suggested that cellular outcome after irradiation of cells with wild type p53 was modulated by factors that determine the differentiation programme for individual cell types. This cellular specificity may be required since it is probably not desirable for epithelial derived cells to die upon exposure to DNA damage since these cells encounter frequent genomic insults in their normal physiological environment. However it is probable that epithelial stem cells responsible for repopulation of the epithelial tissue enter the apoptotic pathway after DNA damage to prevent the propagation of mutated cells

The proto-oncogene product c-Myc, which is transcriptionally repressed by p53, induces cell cycle progression and, in the absence of survival factors, apoptosis. Two models have been proposed to explain the induction of apoptosis by c-Myc following serum deprivation. Cell death may be due to a conflict of cellular proliferation signals from c-Myc and cytostatic effects of low serum from low serum levels. Alternatively proliferation and apoptosis may be naturally tightly coupled and for cellular proliferation to occur, the cell may need a signal to trigger proliferation as well as a separate signal to suppress apoptosis (Reviewed in Harrington, 1994). It is interesting to note that c-Myc has been implicated in the abrogation of p53 induced cell cycle arrest through modulation of $p21^{waf}$. c-Myc interferes with the inhibitory actions of $p21^{waf\ 1}$on cdk/cyclin complexes by inducing a heat labile inhibitor of $p21^{waf1}$. (Hermeking *et al* 1995)

1.12 p53 apoptosis or cell cycle arrest *in vivo*

The accumulation of p53 in response to DNA damage *in vitro* is now well documented to induce cell cycle arrest and apoptosis. However p53 induction *in vivo* seems to be more complex. Ultraviolet irradiation of human skin leads to in induction of p53 in a large proportion, but not all cells of the dermis and epidermis. (Hall, *et al* 1995). In the

mouse intestinal epithelium, radiation induced apoptosis, cell cycle arrest and p53 induction are temporally and spatially co-ordinated. (Merritt *et al*, 1994 Clarke *et al*, 1994). By looking at p53 induction *in vivo* in the tissues of irradiated mice Midgely *et al* found that some tissues show p53 induction after radiation whilst others, e.g. liver for show no response. They also showed that p53 induction does not always lead to p53 dependent apoptosis. In the spleen and thymus induction of p53 is associated with apoptosis, however in bone osteocytes p53 induction does not lead to apoptosis. These results are comparable with those of Hall *et al* and Merritt *et al*, all of which suggest that the induction of p53 and the p53 response are tightly regulated in a tissue specific manner.

The recently created *p53-Lac Z* reporter mice highlighted further the complex relationship between p53 induction and apoptosis *in vivo* MaCallum *et al* (1996) observed cell populations that induce p53 protein and undergo apoptosis after exposure to radiation such as thymus, spleen, bone marrow, choroid plexus, and intestinal crypts, and cell populations that induce p53 but do not enter apoptosis i.e. salivary gland, myocardium, adrenal, lung parenchyma and kidney as well as cell populations that do not induce p53 protein or apoptosis. This classification becomes even more complex within tissues. For example, in the salivary gland the ductal cells induce p53 while the acinar cells do not. The mechanism of this selectiveness is still unclear. However cell cycle status appears not to play a role in whether a cell induces p53 since proliferating and quiescent cell populations both induce p53 protein. The results generated from the p53 reporter mice do not rule out p53 mediated transcriptional repression

1.13 P53 and transforming growth factor beta induced cell cycle arrest

It is widely accepted that the development of malignant tumours involves aberrations in the regulation of the response to positive and negative growth regulators (Kinzler *et al*, 1996). TGFβ1 is a potent growth inhibitor of epithelial cells (Masui *et al*, 1986). Keratinocytes produce and secrete TGF β1 and express cell surface receptors for this growth factor. The biological actions of TGFβ1 include the inhibition of DNA synthesis and cell proliferation as well as the stimulation of keratinocyte differentiation (Sporn & Roberts, 1985; Goustin *et al* ,1986).

In vitro TGFβ1 can potently inhibit the growth of a variety of primary cells by inducing growth arrest at the G1/S boundary of the cell cycle. Many types of carcinomas, including squamous carcinomas of various origin are often refractory to growth inhibition by TGFβ1. In some cases the growth and tumorigenicity of tumour cells can be enhanced by TGF β1. This provides the tumour cells with selective growth advantage over normal cells (Hsu *et al,* 1994).

Malignant cancers are the result of complex and lengthy genetic changes (Kinzler *et al,* 1996). They progress through a series of benign precancerous stages to malignant, metastatic stages. Due to the step wise nature of malignant cancer development it seems logical that there may be a progressive loss of growth control by TGF β1 in cancer development. Each step on the way to loss of growth regulation may be attributable to a different genetic or epigenetic alteration that accumulate during malignant tumour progression.

The mouse skin carcinogenesis model is an ideal system to investigate the regulation of TGF β1 response in tumour development due to its stepwise progression (Brown *et al,* 1995). Initiation of mouse skin with DMBA results in the induction of a specific point mutation at codon 61 of the H *ras* gene. After initiation tumour promotion results in the formation of benign papillomas. Further genetic changes including the loss of p53 function and overexpression of mutant H *ras* cause a proportion of the benign lesions to progress to malignant carcinomas.

Haddow *et al* (1992) showed that activation of *ras* by DMBA treatment of mouse skin does not alter the epithelial cells response to the growth inhibitory action of TGFβ1. Several other studies looked at the effect of activated ras in TGFβ 1 signalling. Filmus *et al,* (1993) showed that rat intestinal cells expressing high levels of H *ras* oncogene lose sensitivity to the growth inhibitory actions of TGFβ1. Furthermore the loss of sensitivity was related to the degree of H *ras* expression. Burns *et al* showed that mutant H *ras* was able to transform primary thyroid epithelial cells, and that this transformation was associated with loss of responsiveness to TGF β1 and greatly increased nuclear levels of

p53. Mulder *et al* (1992) also showed ras is involved at some point in the TGFβ1 signalling pathway by showing that rat intestinal cells treated with TGBβ1 show activated ras within 10 minutes of treatment. In general, oncogenic ras induces some degree of resistance to the growth inhibitory effects of TGF β1, however the effect seems to be cell type and dose specific

H ras has also been implicated in changes in tumour cell invasiveness in response to TGF*β*1. It has been shown that TGFβ1 and mutant *H ras* collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumours. In ras transformed cells TGFβ1 treatment results in increased invasiveness which is dependant on epithelial stromal interactions. Normal epithelial cells do not show any change in invasiveness (Oft *et al* 1996). These observations highlight the diverse effects of TGFβ1 on cell growth and regulation as well as the importance of the H *ras* protooncogene in maintaining tight control of this pathway.

Haddow *et al* (1992)also investigated TGFβ1 response in papilloma cell lines derived from tumours at various stages of the mouse skin tumorigenesis model. They reported that loss of response to TGFβ1 occurred at a stage in the system close to where p53 function is lost. More direct support for the role of p53 in TGFβ1 signalling came from results generated by Gerwin *et al,* 1992 and Reiss *et al,* 1993 who reported that the sensitivity of epithelial cell lines to TGFβ1 was reduced when vectors expressing mutant p53 were introduced. Transfection of mutant p53 into a pancreatic epithelium cell line with wild type p53 was also shown to cause resistance to the growth inhibitory effects of TGFβ1. The same construct also caused resistance to TGFβ1 in murine keratinocytes. However transfection of the same $p53^{143}$ mutation into a lung carcinoma cell line resulted in no significant reduction in response to TGFβ1

Blaydes *et al* (1995) analysed human thyroid cancer cell lines containing wild type or mutant p53 and a rat thyroid cell line with mutant H *ras* and high levels of wild type p53 but loss of p53 mediated cell cycle control. They found that loss of responsiveness to TGFβ1 was found in all the lines bearing mutant or non functional p53. They also

showed that introduction of a dominant negative mutant p53 into TGFβ1 responsive cell lines did not reduce responsiveness.

Recent discoveries have highlighted some of the ways that mutant p53 may be able to affect TGFβ1 signalling. As previously discussed in cells that sustain DNA damage p53 expression leads to G1 arrest or apoptosis. The increased levels of p53 transactivates $p21^{waf1}$, which inhibits cyclin dependent kinase activity. This in turn inhibits the phosphorylation of pRb preventing cell cycle progression. In cells that are rendered resistant to TGFβ1 by mutant p53, wild type p53 function may be abrogated causing the prevention of transcriptional activation of $p21^{waf1}$ gene, thus allowing unregulated phosphorylation of pRb and unregulated cell cycle progression. This circuitry suggests that TGFβ1 may suppress growth by inducing $p21^{waf1}$ via p53. It seems likely that the expression of $p21^{waf1}$ may represent an important mediator of TGFβ1 activity irrespective of p53 status.

Malliri *et al,* (1996) highlighted the importance of $p21^{waf1}$ in TGFβ1 signalling. In an study of cell lines derived from squamous cell carcinomas they showed that TGFβ1 activates numerous cell cycle inhibitory pathways connected with $p21.^{waf1}$ The same study also reported that expression of the cell cycle inhibitor $p15^{ink4b}$ was unnecessary for TGFβ1 mediated growth arrest in the cells analysed.

Two other mechanisms for TGFβ1 inhibition of pRb phosphorylation have been described, both involving members of cyclin-cdk complexes, one of which implicates p53 in TGFβ1 signalling. In Mink lung epithelial cells which express wild type p53 and are highly responsive to TGFβ1 mediated growth arrest, the synthesis and activity of cdk4 is downregulated by TGFβ1 treatment. This indirectly inhibits the activity of cyclin E-cdk2 complexes. Mutant p53 confers resistance to TGFβ1 in these cells by interfering with the down regulation of cdk4 in response to TGFβ1, furthermore wild type p53 is capable of repressing translation of cdk4 (Ewen *et al* 1996*).* Furthermore, HaCaT cells which express mutant p53 do not down regulate cdk4 expression after exposure to TGFβ1. These findings suggests that regulation of cdk4 translation by p53 may be involved in the control of G1 progression. In response to TGFβ1 wild type p53

may prevent cdk4 translation, and ultimately result in the accumulation of hypophosphorylated pRb, causing G1 arrest. Mutant p53, by complexing to its wild type counterpart may act to confer resistance to cdk4 translational repression, and hence to TGFB1 mediated growth arrest. Additionally, in Mink lung epithelial cells TGFβ1 causes an inhibition of cdk2 kinase activity. This inhibition involves the cdk inhibitor $p27^{kip1}$ which is induced by TGFβ1. $p27^{kip1}$ binds to and inactivates cyclin E- cdk2 complexes. No link has been established between p53 and $p27^{kip1}$. It should be noted however, that changes in cyclin cdk4, D1, and pRb, and $p27^{kip1}$ were found to occur occurs late in the TGFβ1 response and was not needed in some cell types at all after exposure to TGFβ1 by Malliri, *et al* 1996.

It seems likely, in view of the experimental evidence, that as in the case of H *ras*, p53 is involved in TGFβ1 signalling in a cell type and dose specific manner. However care should be exercised in the interpretation of *in vitro* experiments utilising well established cultured cell lines. Cell lines are usually devoid of normal p53 function or some downstream pathway, and are often, more genetically complex than their *in vivo* counterparts, making interpretation of signalling pathways difficult.

It is probable that other cellular oncogenes and tumour suppressor genes in addition to p53 and H ras, co-operate to induce an increased level of resistance to TGFβ1. The MAD-related family of proteins are essential components in the signalling pathways of serine threonine kinase receptors for the TGFβ1 superfamily. It has been shown that the *madr22* gene is a tumour suppressor gene located on human chromosome 18q near *dpc4*, yet another potential component in TGFβ1 signalling. Mutations in madr2 and/or loss of chromosome 18q are thought to function to disrupt TGFβ1 signalling in human colorectal cancer (Frank, *et al* 1997). The collaborative effect of H *ras* mutation, loss of p53 and alterations on MAD related genes may well vary from cell type to cell type. The availability of a range of knockout animals and skin targeted *H ras* transgenic animals provide an ideal model with which to study the factors that co operate to induce total resistance to the growth inhibitory effects of TGFβ1 in mouse skin keratinocytes.

1.14 P53 in mouse skin tumorigenesis

Three major concepts underlie the theory of skin tumorigenesis. The first is the current understanding of how populations of cells, such as the epidermis, reproduce. The second is the current concept of multistage carcinogenesis which involves distinct steps of initiation, promotion, and conversion. The last relates to the role of the hair follicle and the interfollicular epidermis in skin cancer formation.

The earliest event in skin carcinogenesis, initiation, is mutational and requires carcinogen treatment. It has been shown *in vitro* that initiated keratinocytes have an altered response to differentiation signals, which is thought to provide a selective growth advantage. At the molecular level, mutation of the H *ras* proto-oncogene has been shown to be an initiating event in skin tumours, particularly in tumours produced by *9,10*-dimethylbenzathracene (DMBA) (Quintanilla *et al,* 1986).

Exogenous tumour promotion causes the selective clonal expansion of initiated cells, giving rise to numerous benign papillomas. A single tumour is thought to arise from the clonal expansion of one initiated cell. However models of polyclonality have been proposed (Winton *et al,* 1989).

Potent tumour promoters of the phorbol ester class such as *12-0-* tetradecanoylphorbol-*B-* acetate (TPA) activate protein kinase C (PKC) (Nishizuka *et al,* 1986) This enzyme activity accelerates epidermal terminal differentiation (Yuspa *et al,* 1982). Initiated cells resist the induction of terminal differentiation by activators of PKC and, this confers a selective growth advantage in these cells in response to promotion. Common genetic changes during skin tumour promotion include trisomy of chromosomes 6 and 7 (Aldaz *et al,* 1989). Trisomy of chromosome 7 involves the duplication of the chromosome harbouring the mutant H-*ras* allele induced by initiation.

Malignant conversion of benign tumours is a relatively rare event. The risk of conversion is variable between benign tumours; subpopulations of papillomas with a much higher risk of malignant progression have been identified. Differences in phenotypic markers such as TGFβ1, $\alpha_6\beta_4$ integrin and keratin expression have been used to distinguish

between high and low risk papillomas. (Glick *et al,* 1993; Tennenbaum *et al,* 1992; Gimenaz *et al,* 1990) Genetic alterations associated with malignant conversion include the loss of the remaining normal H *ras* gene and mutations in the p53 gene (Bremner *et al,* 1990). A further stage in mouse skin tumorigenesis is progression to spindle cell tumour, this is associated with an increase in the ratio of mutant to normal H *ras* genes (Buchman *et al,* 1991).

P53 loss has also been shown to be involved in tumour progression *in vitro.* Grafting experiments showed that mice grafted with p53 wild type keratinocytes harbouring oncogenic H *ras* develop papillomas, whereas mice grafted with p53 null keratinocytes with the same ras mutation develop squamous cell carcinomas. P53 heterozygous keratinocytes with mutant H *ras* caused rapidly growing papillomas of which more than 50% progressed to carcinomas in a short space of time. Thus *in vitro* the loss of p53 in combination with mutant H *ras* is associated with the malignant conversion stage of epithelial carcinogenesis (Weinberg *et al* , 1994).

The number and nature of events before p53 loss becomes causal has been assessed through transgenic mating experiments. p53 null mice were bred to HK1.ras mice, which harbour an oncogenically active form of H *ras* targeted to the epidermis. In newborn HK1.ras/$p53^{-/-}$mice no accelerated progression of papillomas to malignancy due to p53 loss was observed. Instead, tumorigenicity was repressed. HK1. ras/ p53 $^{-/-}$ mice developed no skin tumours even after multiple applications of TPA however HK1.ras/$P53^{+/+}$ mice were extremely sensitive to TPA promotion, developing large tumours following only two or three applications of TPA, as did their hemizygous p53 progeny. The hemizygous epidermis exhibited rapid hyperplasia/hyperkeratosis with the beginnings of papilloma formation following one or two TPA applications. $P53^{-/-}$ epidermis on the other hand, exhibited only the acute TPA associated hyperplasia similar to that of normal skin following TPA treatment. (Greenhalgh, 1996)

It seems likely, based on the *in vitro* and *in vivo* evidence that p53 loss does not appear to have an effect on H *ras* expression in the early stages of papillomagenesis. This is consistent with a role for p53 loss late in mouse skin tumorigenesis and is in agreement

with the results of Kemp *et al,* who looked at the occurrence of chemically induced skin tumours in normal mice compared to p53 knock out mice or hemizygous mice. They observed that papilloma yield was similar in wild type and hemizygous mice, but was reduced in p53 null mice. They also noted that the rate of carcinoma development was increased in p53 null and heterozygote mice compared to wild type mice. In addition, they found that carcinoma development in heterozygous mice was associated with the loss of the remaining wild type p53 allele (Kemp, *et al* 1993).

Thus loss of p53 does not increase the incidence or shorten the latency of papilloma development, however it does enhance the rate of malignant progression. It was proposed that, in the absence of p53 cell cycle regulation, the initiated cells progress into S phase with a burden of unrepaired DMBA induced mutations, which acts to prevent successful completion of the cell cycle and results in cell death, corresponding with a reduction in the number of tumours observed in p53 null mice. In support of this idea TPA promotion of DMBA initiated null mouse skin has been shown to display areas of necrotic tissue and epidermal cell loss. This scenario is unlikely to apply to the HK1 transgenics as only a single genetic hit is present, therefore putative DNA damage is reduced.

The results from the mouse skin model of carcinogenesis lend support to the idea that p53 is involved in tumour progression. Loss of p53 and its guardian of the genome function, probably allows the faster accumulation of additional genetic lesions in tumours. However loss of p53 may have a more subtle affect on tumour progression. The ability of tumours to grow is dependent on their ability to develop new blood supplies. Angiogenesis, the formation of new blood vessels, is controlled by the balance between stimulatory and inhibitory influences. Wild type p53 expression results in the secretion of inhibitors of angiogenesis (Dameron, *et al* 1994). This may represent one way in which the presence of wild type p53 inhibits tumour progression. Low oxygen or hypoxic conditions, such as those in a tumour cell with inadequate blood supply, induce accumulation of p53 protein.(Graeber *et al* 1996) Furthermore mutant but not wild type p53 has been shown to synergize with PKC in inducing the expression of the angiogenic vascular endothelial growth factor (VEGF) (Kieser *et al* 1994) In this way mutation of the p53 gene may be a critical event in tumour progression not only for allowing

aberrant cell growth due to lack of cell cycle control and altered growth factor response but also for regulating tumour expansion by stimulating vascularization.

1.15 Mouse skin cell populations and chemically induced mouse skin tumorigenesis.

The skin is composed of two parts, the epidermis and dermis separated by the basement membrane. The dermis is composed of mainly fibroblasts. The epidermis is a stratified squamous epithelium, whose primary cell type is the keratinocyte. As well as keratinocytes and fibroblasts there are more than 20 other cell types in the skin. A large number of Langerhans cells, lymphocytes, granulocytes, macrophages, and eosinophils either permanently reside in the skin, or can be recruited to it. The epidermal cells in the skin can be divided into cells of the hair follicle and cells of the interfollicular epidermis.

Multiplying keratinocytes, as identified by ^{3}H thymidine labelling are located in the basal layers of the epidermis, as well as in the matrix cell population. Distinct populations of dividing keratinocytes differing in their growth capacity are thought to be responsible for the renewal of the epidermis. These distinct populations consist of a population of skin keratinocytes with extensive growth capacity (stem cells) and an alternative population with a limited proliferative capacity (transit amplifying cells). Further evidence for proliferative heterogeneity has come from studies of cultured human keratinocytes. Autologous grafts of cultured keratinocyte sheets persist on the recipient for many years, which suggests stem cells are not lost in culture (Gallico *et al.;* 1984; Compton *et al.*; 1989). In culture keratinocytes are classified in their capacity for self renewal as 1)holoclones with high reproductive capacity; 2) paraclones, in which all cells quickly undergo terminal differentiation; 3)meroclones which are intermediates between holoclones and paraclones in their behaviour (Barrandon and Green, 1987). Holoclones are most likely founded by stem cells (Barrandon and Green 1987) and paraclones by transit amplifying cells. The proof that colony forming cells are indeed stem cells, that is that they have extensive growth potential cannot be examined in rodents since rodent cells routinely immortalise after a short length of time in culture.

Mice initiated with DMBA can be promoted even after a time gap of more than one year after the initial insult, leading to the conclusion that initiation must involve a population of long lived cells, i.e. stem cells present in the skin (Loehrke *et al,* 1983). It seems likely that the stem cells responsible for populating the interfollicular epidermis are also capable of populating the hair follicle. When epidermal tissue is transplanted from hairless sites on the body on to the dermis from a hair producing site the transplanted epidermis starts to produce hair. A system that operates via multipotential stem cells has the advantage of reducing the size of the stem cell compartment. The size of a stem cell population is believed to be critical. Stem cells are by definition long lived. This may render them sensitive to carcinogenic transformation. The smaller the stem cell pool the fewer the number of targets in which this transformation can occur.

Cell cycle kinetic data has provided indirect evidence that stem cells reside in the basal layer (Sheremer et al 1886), the outer root sheath (Lenoir et al., 1988) and bulge region of hair follicles (Cotsarelis et al., 1990). In mouse skin, suprabasal keratinocytes are arranged in columns (Mackenzie, 1970; Christophers, 1991). It has been proposed that one stem cell lies at the base of each column, and is surrounded by transit amplifying and post mitotic cells (Potten, 1974, 1981). The stem cells of the skin probably reside in an optimal microenvironment, and it seems likely that behaviour of stem cell progeny is controlled by environmental factors.

1.16The hair follicle and development of skin cancer.

It is widely accepted that a population of hair follicle cells with stem cell like characteristics are the target cells that interact with carcinogens and eventually undergo cellular transformation. The hair follicle forms a bulb around the specialised mesenchymal structure, the dermal papilla. The relatively undifferentiated matrix cells give rise to concentric rings of differentiated cell types. The hair is surrounded by two root sheaths. The inner root sheath is composed of three cell types: the inner root sheath cuticle, Huxley's layer, and Henle's layer. The outer root sheath is composed of a few layers of a single cell type. The hair and inner root sheath extend to the dermal papilla. The outer root sheath cells do not come into direct contact with the dermal papilla. They are contiguous with the basal layer epidermis, but they are distinct and self propagating. (Reviewed in Lavker *et al,* 1993)

Early work suggesting a role for hair follicles in skin cancer development was carried out in haired versus hairless mouse skin (Lacassagne and Latarjet, 1946). Scarification of the backs of mice showed that scars that healed without hair follicles, did not develop tumours after treatment with carcinogens. Only the periphery of the scar region where hair follicles were present developed tumours. Later tumour development was examined in strains of hairless and haired mice (Giovanella *et al,* 1970). These studies concluded tumours could arise from hairless skin, probably because rudimentary follicles studied were present in the skin of the hairless mice.

Administration of ^{3}H-thymidine for one week twice a day from birth is sufficient to label cells in the basal layer and in the hair follicle. Four weeks later label retaining cells can be found in the bulge area of the outer root sheath of the hair follicle. No label retaining cells are visible in the matrix area of the hair near the dermal papilla. This observation led to the proposal that the slow cycling hair follicle cells are located in the bulge area of the outer root sheath and that the cells surrounding the hair bulb near the dermal papilla belong to a transit amplifying population. Similar results have also been observed in two specialised hair structures- vibrissae and eyelash follicles. Slow cycling cells are localised to the outer root sheath of the vibrissa follicle in an area analogous to the bulge region. Similarly, in the eyelash slow cycling cells are exclusively observed in the outer root sheath in a region corresponding to the bulge (Cotsarelis *et al,* 1990; Lavker *et al,* 1991). No slow cycling cells are present in the matrix keratinocytes.

Bulge cells have been distinguished as carcinogen retaining cells, and are also stimulated to divide by the application of TPA (Cotsarelis *et al,* 1990). Cells in the bulge area of the outer root sheath express few differentiation markers. They have a high nuclear:cytoplasmic ratio and sparse expression of keratin proteins. Outer root sheet cells are also capable of shifting their differentiation to form cells with an interepidermal phenotype when cultured under suitable conditions. It is possible that the basal layer stem cells are temporary stem cells which are replaced by the division of cells in the follicle. There are obvious advantages of positioning stem cells in the bulge area of the outer root sheath. The bulge area is well vascularised which may help maintain the stem cells in a stable, well nourished environment (Schofield, 1983). The bulge is also a

permanent structure which does not undergo cyclic degradation during the catagenic phase of the hair follicle, unlike the cells of the bulb near the dermal papilla.

At the beginning of the growing phase (anagen), the normally slow cycling cells of the bulge area undergo transient proliferation, giving rise to transiently amplifying (TA) cells, which subsequently form the new downgrowth (Andreasen, 1953; Sun *et al,* 1991). The formation of tumours during chemical carcinogenesis is strongly correlated with the stage of the hair cycle the mice are in at the start of carcinogen treatment. Tumour number increases when the treatment is started in the growing phase of the cell cycle when the cells of the outer root sheath are more accessible to carcinogenic insults (Argyris, 1963). This further supports the idea that a high number of tumours derived chemical carcinogenesis come from the hair follicle.

Although many studies have identified the outer root sheath as the location of stem cells in the hair follicle, others have suggested alternative locations within the follicle. Reynolds *et al* proposed that stem cells of the skin were located in the cell layer directly opposite the dermal papilla, known as the germinative layer (Reynolds & Jahoda *et al,* 1991b). Cells from this layer can proliferate and form organotypic structures when cultured with dermal papilla cells. Outer root sheath cells do not possess this ability. However, the only slow cycling cells found in the hair follicle are in the outer root sheath, and germinative cells are not located in the permanent portion of the hair follicle. Outer root sheath cells are thought to be capable of regenerating the population of germinative cells when the lower end of the follicle is removed (Jahoda and Reynold, 1993). Germinative cells however are not thought to be capable of differentiating towards the interfollicular phenotype. It seems unlikely that germinative cells are the stem cell for both the follicle and the interfollicular epidermis. The germinative cells may be a transit amplifying population derived from the outer root sheath cells.

The existing data from diverse approaches support the bulge location of follicular stem cells. During early anagen the normally slow cycling bulge stem cells may be activated by the abutting dermal papilla cells to undergo transient proliferation giving rise to keratinocytes of the lower follicle. This hypothesis has received further support from labelling studies which clearly show the bulge cells proliferating in early anagen.

1.17 Cells outwith the follicle stem cell compartment and the development of skin cancer.

Barrandon *et al* showed that relatively differentiated cells from renewing epithelia such as skin can acquire immortality in culture. Moreover telomerase activity, which is closely related to the ability of a cell to survive in culture, is not present in the vast majority of adult human cells but it can be detected in a large proportion of human tumour biopsies. Based on these observations it seems reasonable to propose that cells which no longer reside in the stem cell compartment and are in a more differentiated state would be capable of giving rise to malignant tumours providing they encounter sufficient genetic changes. In a further extension of this proposal it may be the case that a continuum of target cells may exist in a tissue whose stage of differentiation determines the number of genetic hits required for malignancy to develop.

Brown *et al* (In Press 1997) showed that the expression of mutant H *ras* within the interfollicular compartment of mouse skin induced the formation of benign papillomas which very rarely progressed to malignancy However when the same H *ras* construct was targeted to the hair follicle it stimulated the development of regions of acanthotic areas in newborn skin, later in development the mice developed papillomas and keratoacanthomas which showed a high frequency of malignant transformation to squamous and spindle carcinomas. The authors concluded from these transgenic mouse experiments that the nature of the target cell in chemically induced mouse skin tumorigenesis is a major factor in the malignant potential of the resulting tumour.

Perhaps the most logical interpretation of the data available is that that a continuum of target cell populations exists in the epidermis. The most primitive stem cells, probably situated in the bulge region, are likely to be capable of giving rise to the most malignant tumour types. This risk is minimised by the small number of these cells and their maintenance in a protected environment. The cells furthest removed from the stem cells in terms of differentiated phenotype, may be capable of undergoing transformation resulting in low risk benign tumours which very rarely progress to malignancy. The role of the basal stem cells and their capacity for malignant transformation remains unclear. In this model, proposed by Brown *et al* the stem cells of the basal layer may undergo

transformation giving rise to a less malignant tumour than one derived from the hair follicle stem cell.

Chapter 2. Materials and Methods

2.1 Materials

Restriction enzymes were obtained from Boehringer Mannheim, Lewes, East Sussex; or from Gibco BRL, Paisley. Taq polymerase was purchased from Promega, Southampton. Deoxynucleotides were from Pharmacia. All radioisotopes came from Amesham International P.L.C. Amersham, Buckinghamshire.

DNA size markers for gel electrophoresis, were supplied by Gibco P.L.C. Paisley. As was the Ultrapure tm agarose. Nitro-cellulose was supplied by Sartororious Instruments Ltd., Belmont, Surrey; and Amersham International.

Phenol was delivered as a water saturated liquid from Rathburn Chemicals Ltd., Walkerburn, Peebleshire. Ethanol was supplied by James Burroughs (F.A.D) LTD., Witham. Essex. All other chemicals were obtained from BDH Laboratory supplies, Poole, or Sigma Chemical Cp .Ltd., Poole, Dorset.

Serum, media (excluding KBM) and supplements for cell culture were obtained from Gibco BRL, Paisley. Plastic-ware for cell culture was supplied by Nunc Intermed, Roskilde, Denmark; Sterlin Ltd., Feltham, Middlesex; or by Falcon tm, Becton Dickinson, Dublin. Mice were from Harlan Olac Ltd., Bicester,Oxon. KBM medium was obtained from Clontech (USA)

2.2 Oligonucleotide synthesis.

Oligonucleutides were synthesised on an Applied Biosystems 381A Synthesiser, using the manufacturer's protocol and reagents. After deprotection in ammonia at 55^{o}C overnight, the oligonucleotides were precipitated and used without further purification.

2.3 Restriction enzyme digest of DNA

Genomic DNA was digested overnight with 10 units/ug DNA. Digestions were analysed for completeness, by gel electrophoresis.

2.4 Agarose gel electrophoresis

Agarose gel electrophoresis of DNA was performed using the appropriate horizontal gel electrophoresis apparatus, with concentrations of agarose concentrations between 0.7-0.2% w/v, depending on the desired fragment resolution. The agarose was cast in TAE buffer (40mn Tris.Cl pH 7.8; 20mn Sodium Acetate; 1mn EDTA) which was also used as the running buffer. Ethidium Bromide 1μg/ml was either added direct to the molten agarose, or in the case of southern blotting, the gel was soaked in an Ethidium Bromide solution, prior to UV illumination and photography.

2.5 Purification of fragments from Agarose Gels

The fragment of interest was excised under UV illumination The geneclean technique (Stratech Scientific Ltd) was used under manufactures instructions.

2.6. General culture of mammalian cells.

2.6.1. Medium and Supplements

Cell lines were grown routinely in Special Liquid Medium (SLM, GibcoBRL) supplemented with 10% foetal calf serum and 4mm glutamine. Cells were grown in 25, 75, or 125 cm^2 flasks or 9cm dishes gassed with 5% CO^2 and placed in 5% CO_2 37°C incubators. Cells were washed in phosphate buffered saline or PBS (0.14mm NaCl ; 27mm KCl ; 10mm Na2HPO4; and 15mm K2HPO4). Trypsin for cell disaggregation was used at 0.025% w/v in PE (1mm EDTA pH 7.8 in PBS). Trypsin was inactivated with 10- fold volume of serum containing medium. Cell numbers were estimated using a Coulter Counter.

2.6.2. Freezing and storage of cell lines.

Cells to be frozen were grown to 90% confluence, trypsinised and centrifuged at 1000 rpm for 5 minutes. The cells were the resuspended in equal proportions of growth medium and freezing medium (25% foetal bovine serum; 20% DMSO; 55%growth medium). Cells were stored in 1ml Nunc TM vials in liquid Nitrogen.

2.6.3. Preparation of retroviruses and infection of cultured cells.

Virus producer cell lines were grown to 70% confluence. The medium was replaced with a minimal volume of fresh medium -ensuring the culture surface was completely covered. After 24hrs the medium was removed and filtered through several 0.2 micron syringe filters. The cells to be infected were seeded in $25cm^2$ flasks (5×10^5) 24hrs before viral infection. 50 µls of viral supernatant, 150µl of polybrene (0.8mg ml^{-1}) and 5mls of growth factor medium was added to each flask. 24hrs later the cells were expanded in to $75cm^2$ flasks.

2.6.4. Production of fibroblast primary cultures and cell lines.

Dorsal skin was removed from three day old mice. The skins were placed in 0.05% Worthington Trypsin in PBS and placed at 4% overnight. The dermis was then separated from the epidermis using scalpels. The dermis was chopped into small pieces and placed in $9cm^2$ dishes and left to adhere to the tissue culture plastic for 10 minutes. SLM supplemented with 20% FBS was then added. The medium was changed every three days until fibroblast outgrowth from the pieces of tissue could be seen. The cells were then expanded into $50cm^2$. After two weeks the remaining cells were single cell cloned.

2.6.5. Production of keratinocyte cell lines and primary cultures.

Dorsal skin was removed from three day old mice. The skins were placed in 0.05% Worthington Trypsin and placed overnight at 4^oC The epidermis was removed from the dermis using scalpels. The epidermis was then washed three times with SLM. The SLM was then removed and the epidermis was finely chopped using scalpels. Fresh SLM was then added and the pieces of epidermis drawn through a wide bore syringe needle three times. The cell suspension was then centrifuged at 600RPM for 3 minutes. After suspension in SLM the cells were again passed through a wide bore needle tree times. After further centrifugation the cells were plated in $9cm^2$ dishes and left for seven days before fresh KBM was added. After 10 days. Keratinocyte colonies were ring cloned and expanded into small flasks. After several weeks in culture KBM medium was replaced with SLM and the cells were then routinely cultured in SLM medium.

2.6.6 Isolation of carcinoma cell line.

Carcinomas were removed and placed in PBS. The tissue was washed several times in serum containing medium. The tumours were then cut into small pieces and allowed to adhere to $9cm^2$ tissue culture dishes. Serum containing medium was then added to the dishes. After a week the medium was changed. The cells were allowed to grow out from the tissue pieces. Individual colonies were ringcloned.

2.6.7 Radiation of cell lines

Cells for irradiation were grown to 70% confluence before radiation exposure using a CO^{60} source. (Dose rate $0.96Gy/min^{-1}$) After this the cells were harvested as required.

2.7 Section preparation.

Tissue samples were fixed overnight in 4% phosphate buffered formalin. The samples were then washed in PBS, 50%PBS/ethanol, then 70%PBS/ethanol (30mins each).The fixed tissue samples were then sent to the Pathology Department at Glasgow University Veterinary School. They were dehydrated, and embedded in paraffin wax. For histological examination sections were cut from the samples before being dewaxed and stained with Eosin. The sections were mounted and visualised by light microscopy.

2.7.1 P53 Staining.

Immuohistochemistry was performed as described using CM5 as the primary antibody (see Midgeley, *et al* 1995)

2.7.2 Apoptotic analysis.

Sections were prepared as above and stained with Hematoxylin and Eosin. Apoptotic cells were identified using the characteristic markers of apoptosis; cell shrinkage, loss of cell contact, and condensation of chromatin. Additional apoptotic analysis was performed using a fluorescent *in situ* apoptosis detection kit from Boeheringer Mannheim according to manufacturers instructions.

2.7.3 Bromodeoxyuridne (BrdU) analysis

One hour before sacrifice the mice were injected intraperitonally with 50µl of BrdU stock solution (25µg/ml). Samples were removed and fixed in phosphate buffered formalin overnight. After this the samples were washed in PBS, 50% ethanol /PBS and 70% ethanol /PBS (30mins in each) BrdU detection was carried out using BrdU antibody (Amersham RPN202) and horseradish peroxidase labelled second antibody.

2.7.4 TPA treatment of mouse skin.

The dorsal skin of the mice was shaved 2 days prior to treatment. Two hundred microliters of 10^{-4}m TPA solution in acetone was then applied to mice in anagen phase of the cell cycle based on hair regrowth.

2.7.5 Whole body irradiation of mice

The resting hair follicles of adult p53 wild type and knockout NIH mice were stimulated into activity by shaving. This was not required for two week old mouse skin since most of the follicles are in anagen. Five days after shaving the mice received 4Gy ^{60}Co with a dose rate of 0.96Gy/min^{-1}. Skins were removed and sections cut to give good longditudinal sections of hair follicles. The H&E stained sections were examined and counted under a X20 objective lens. Non irradiated animals served as controls.

2.7.6 Staining frozen sections

Frozen sections were cut by Ian Millan in the Pathology Department at Glasgow University. The frozen sections were washed in 0.02% glutaraldehyde, 2mm $MgCl_2$, 5mm EGTA, and 0.02% NP-40 in PBS for 5 minutes. The samples were then washed twice with 0.02% NP-40 in PBS. The sections were then stained with 1mg/ml-1 X-GAL, 5mm$K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.6H_20$ and 2mM MgCl, 0.01%sodium deoxycholate, and 0.02% NP-40 in PBS. The staining was carried out for 24hrs in a humid 37^{oc} incubator in the dark. After staining the samples were stained with Eosin, dehydrated and mounted. The tissue sections were visualised by light microscopy.

2.8 Southern blot analysis

DNA fragments were separated by agarose gel electrophoresis then transferred onto nylon membranes (Hybond-N TM) (as described by Rigaud et al 1987). The agarose gel was soaked in ethidium bromide before being photographed. The gel was then emersed in 1.5M NaCL, 0.5M NaOH for 2 x20 minutes, and then in 1M NH_4OAc / 0.02 MNaOH for 2 x 30 minutes. Transfer of the gel onto nylon membrane was achieved by capillary transfer overnight in NH_4OAC/NaOH transfer solution. The membrane was fixed by baking for 80°C for 2 hours and hybridised as described. Membranes were pre hybridised for a minimum of 4hrs at 42°C in glass bottles with pre heated solution. Radio labelled probe was denatured by boiling for 10mins and added directly into the pre hybridisation solution. Membranes were hybridised for a minimum of 16hrs. After this membranes were washed on a rotating shaker in 1xSSC / 0.1%SDS, followed by 0.5xSSC / 0.1%SDS for 15 minutes each at room temperature. Membranes were finally washed with 0.1xSSC / 0.1%SDS at 65°C until background levels fell to the expected levels as measured by a Geiger counter. The filters were exposed to Kodak X-OMAT AR film at -70°C in cassettes containing intensifying screens

2.9 Preparation of radiolabelled probes

The p53 probe was as descibed in Kemp, *et al* 1993. It was labelled by random priming (Feinberg and Vogelstein 1984) 50ng of purified insert DNA was boiled for 10 minutes to allow denaturation and was subsequently labelled in a total volume of 50ul which contained 10µl OLB buffer, 2ul of BSA (10µl g/ml stock). Finally $1.85x10^6$ MBequerel of α 32P dCTP and 5 units of klenow enzyme were added. The reaction was incubated at room temperature for 6hr. Unincorporated nucleotides were removed by running the probe through a nick column (Pharmacia) and the specific activity estimated using a scintillation counter.

Oliginucleotide buffer

62µl 1M Tris pH 7.4

6µl 1M MgCL2

1µl 2- Mercaptoethanol

2µl 15mM dATP

2ul 15mM dGTP

2ul 15mM dTTP

125ul 2M HEPES

50µL 150od/ml Calf Thymus Hexanucleotides

stored in aliquots at -200c

2.10 Ras western blot analysis.

Protein samples were prepared as described. The protein content was estimated using the Bio-rad assay (see manufacturers instructions). For detection of H *ras* specific bands the samples were first treated with 50% IgPAS (Protein A Sepharose), and then immunoprecipitated with antibody 172 (H-ras specific antibody Oncogene Science Inc),as described below 1mg/ml of protein was made up with lysis buffer. 100µl of 50% IgPAS was spun down, and the lysates added directly onto PAS. The samples were incubated on a rotator at 4^{o}C for 4hrs. The supernatant was removed and respun to remove residual PAS. A dilution of 1:100 of antibody 172 and 1mg/ml f/c BSA was added to the pre cleared lysates this was incubated overnight at .4^{o}C 100µl of 50%PAS was added to the lysates and incubated for 2 hours at 4^{o}C on a rotator. The tubes were spun and the final pellets resuspended in a volume of 35µlof western sample buffer (10% B-mercaptoethanol; 2%(w/v) SDS; 30%(w/v) glycerol; 0.025% bromophenol blue, a few crystals of methyl green in 0.05M Tris-Hcl pH 6.8). The samples were boiled for 5mins and the supernatant transferred to a fresh tube. The samples were run on a vertical 17.5% polyacrylamide gel (17.5%acrlyamide; 375mMTris-HCL pH 8.8; 0.1% SDS; 0.1% Ammunium Persulphate; 1µl/ml TEMED with a 5% polyacrylamide stacking gel) in Tris glycine running buffer (1 litre 3.025g Tris base; 14.4g Glycine; 1g SDS pH 8.5) Amersham rainbow protein molecular weight markers (2350-46000 molecular weight) were used.

The gel was cut to size (using the molecular markers as a guide) and placed in an electroblotter. Six sheets of whatman paper cut to size soaked in Anode solution 1 (O.3 Tris / 20%methanol pH10.4)

2.11 p21, BCL2 and BAX western blot analysis

Cells were lysed for 20mins on ice in 50mM HEPES (pH 7.0, 250mMNaCl, 0.5% NP40, 5mM EDTA, 50mM NaF, 200 μM sodium orthovanadate, 2mM benzamidine, 50mM B- glycerolphosphate, 1mM PMSF,10μg/ml leupeptin, and 1μg/ml soyabean trypsin inhibitor. Lysates were spun at 14000 rpm at 4°C for 5mins. Protein concentration determination was carried out by diluting the samples 1:10 in copper sulphate, bicinchoninic acid solution (Sigma) 20ug of extract was fractionated by SDS-PAGE before being transferred to immobilon-P filters (Millipore). The filters were then blocked in 5% Marvel solution in the presence of 0.1% Tween 20. The filter was then probed with appropriate antibodies. Goat anti-rabbit IgG was used as a secondary antibody at 1/5000 dilution.

$p21^{waf}$ antibody was used at 1/1000 dilution in the blocking solution
(Santa Cruz cat no.397)
BAX antibody was used at 1/1500 dilution in the blocking solution
(Santa Cruz cat no. 578)
BCL2 antibody was used at 1/500 dilution in the blocking solution
(Santa Cruz cat no 526)
Visualisation of resulting bands was achieved using an ECL detection kit from Boeheringer Mannheim according to manufacturers instructions.

2.12 Tritiated Thymidine incorporation assay

Cells were trypsinised and plated out at a density of $2x10^4$ cells/well into 96well plates and allowed to attach overnight in control medium The next day the cells were incubated for 24hrs with various concentrations of TGFβ1 in SLM. The cells then received a pulse for four hours of 0-33μCi/well ^3H thymidine (26Ci/mmol;Amersham Corp., Buckinghamshire UK). After which the cells were trypsinised and harvested using a Pharmacia Cell Harvester. ^3H Thymidine incorporation was measured with a Pharmacia liquid scintillation counter.

2.13 Clonogenic assay

Clonogenic assays were performed as follows: Cells were seeded into 25cm^2 flasks and irradiated in suspension. Cell numbers were chosen to give approximately 100 colonies in each flask. Plates were prepared in triplicate for each dose point. Both NK and C5N cells required 10-14 days to form colonies. The cells were stained by carbol fushin (Lamb London) after removal of the medium. Colonies were counted microscopically, using the standard definition that a colony consists of 50 or more cells. Survival Fraction (For dose of X Gy) = PE irradiated (X Gy) divided by PE unirradiated where PE is defined as the percentage of cells plated which formed colonies.

2.14 Long term effects of TGFβ1 on keratinocyte proliferation.

Cells were plated in 35mm culture dishes in triplicate in SLM medium. After 24hrs the medium was replaced with either fresh medium in the untreated controls or medium containing TGFβ1. Medium with or without TGFβ1 was replaced every 48hrs until the end of the experiment.

2.15 Long term mouse skin tumorigenesis

Six week old female p53 wild type and knockout mice maintained in the CRC Beatson Labs on an NIH background were shaved and initiated with DMBA two days later. The mice were subsequently treated with 5X10 $^{-5}$mM TPA solution in acetone twice a week for twenty weeks Papilloma and carcinoma development was recorded weekly.

2.16 PCR analysis of p53 LAC Z Δ FOS, mice

DNA was amplified according to Saiki *et al* (1988). A 50μl reaction usually contained 1ug of genomic DNA in commercial reaction buffer (Promega) : 50mM KCL; 50mM Tris-Cl pH 9.0, 7.5mM $MgCl_2$; 0.01% gelatin w/v; 0.1% Triton X-100; each DNA amplimer was at adjusted to a concentration of 140μg/ml^{-1} ; dNTPs were added as follows: 2.5mM stock mix, 4μl in 50μl reaction were combined in a 0.5ml PCR tube. 2.5 units of thermostable DNA polymerase from themus aquaticus (Taq polymerase), was added to the mixture.Thermal cycling was controlled by a programmable heating block (Perkin Elmer Cetus, DNA thermal cycler).

Chapter 3. Results

3.1 The role of p53 in mouse skin cells.

As discussed in the chapter 1 p53 has previously been implicated in, amongst other cellular activities, TGFβ1 signalling, cell cycle arrest and /or apoptosis after DNA damage in a wide variety of cell lines and tissues both *in vitro* and *in vivo*. Loss of any of these functions is proposed to enhance the development of tumours. In chemically induced mouse skin tumours, loss of p53 is thought to be a major event in the development of malignant tumours. This observation prompted the present study into the role of p53 in mouse skin keratinocytes. By using wild type or p53 knockout keratinocyte cell lines and skin tissue the effect of loss of p53 in mouse skin keratinocytes on TGFβ1 signalling, and on cellular out come after DNA damage was investigated.

3.2 The role of p53 in cell immortalization.

Unlike normal embryonic stem cells that have the ability to permanently divide, normal somatic cells are endowed with a limited doubling capacity when placed in culture (Bradley *et al.*, 1984; Suda *et al.*, 1987a). The period of limited proliferation for normal cells varies from species to species and from cell type to cell type (Todaro, 1964; Hayflick, 1961).

Results from cell fusion experiments are consistent with the hypothesis that immortalization involves changes in specific identifiable genes. (Pereira- Smith & Smith, 1983, 1988). The tumour suppressor gene p53 has been implicated in the immortalization process on the basis of a number of observations. Mutations are common in both cDNA and genomic clones of p53 from immortal rodent cells and also many human cancer cells (Finlay *et al*, 1988; Nigro *et al*, 1989). p53 also has the ability to inhibit growth or induce apoptosis when induced into established cell lines (Baker *et al*, 1990; Yonish-Rouach *et al*, 1991). Furthermore, it has been shown that rodent cells can be immortalised by genes whose protein products bind to wild type p53 (Rovinski & Benchimol, 1988).

Rather than look at p53 mutations in immortal cells the aim of this study was to investigate the characteristics of otherwise normal cells which lack p53. We chose to

look at populations of keratinocytes which were derived from either wild type, p53 knockout, or heterozygous newborn mouse skin. (Donehower *et al*, 1992).

The p53 status of newborn mice obtained from a crossing of p53 heterozygous mice was established by Southern blotting (Figure 3.1). Primary keratinocytes were isolated from individual mouse pups as described in chapter 2 . After two days in culture, cells derived from all three genotypes flattened out on the tissue culture surface and started to divide. Primary keratinocytes derived from $p53^{-/-}$ and $p53^{+/-}$ mice had a similar morphology to keratinocytes derived from $p53^{+/+}$ mice. The keratinocytes grew exponentially for 7-8 days before entering crisis. Although keratinocytes from all three genotypes entered a phase of cell death after 8 days, $p53^{-/-}$ and $p53^{+/-}$ cells showed an increased ability to emerge from the death phase and become established as immortal cell lines. This phenomenon was more pronounced in $p53^{-/-}$ cultures. Healthy keratinocyte colonies were visible in cultures derived from $p53^{-/-}$ mice skins after two weeks in culture, this was also the case for cultures derived from $p53^{+/-}$knockout mouse skins. Very few cells could be seen in $p53^{+/+}$ cell colonies at the same two week time point. Healthy keratinocytes visible in the $p53^{-/-}$ cultures were ringcloned and passaged routinely. Each colony became an established immortal cell line. This was also the case for surviving colonies from $p53^{+/-}$ cells. No cell lines could be established from $p53^{+/+}$ cultures. Fibroblasts from $p53^{-/-}$ mice also showed increased ability to become immortal cell lines compared to fibroblasts derived from wild type mice. The keratinocytes isolated from each genotype of mice were non tumorigenic when injected into nude mice.

Several carcinoma cell lines were isolated from chemically induced mouse skin cancers from $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ mice (see chapter 2). Carcinoma cells from $p53^{+/-}$ and $p53^{-/-}$ mice became immortal more readily than cells from $p53^{+/+}$ carcinomas. All the cell lines established from carcinoma explant cultures were highly tumorigenic when injected into nude mice. When normal primary keratinocytes from $p53^{+/+}$, $p53^{+/-}$, and $p53^{-/-}$ mice were injected into nude mice no tumours developed. Since the initiation of this work similar results have been published by other groups. (Tuskada *et al,* 1993; Harvey, *et al* 1993)

3.3 In vitro analysis of the effect of loss of p53 on the growth inhibitory actions of TGFβ1 .

Having isolated p53 $^{-/-}$ keratinocytes the link between p53 and TGFβ1 signalling was investigated. As previously discussed in Chapter 1, loss of p53 has been implicated directly and indirectly in the loss of responsiveness to TGFβ1 (Blaydes *et al*,1995 ; Reiss *et al* , 1993). The relationship between loss of p53 function and loss of responsiveness to TGFβ1 is particularly interesting in chemically induced mouse skin tumour development since loss of p53 function during the latter stages of the mouse skin tumorigenesis system is closely linked with loss of responsiveness of cells to the growth inhibitory effects of TGFβ1 (Haddow, *et al* 1992).

The response of keratinocyte cell lines from p53$^{+/+}$ (C5N) and p53$^{-/-}$(NK) mice to TGFβ1 was analysed. The cell line C5N is an established skin keratinocyte cell line which contains wild type p53 (Burns *et al* 1991). At the onset of this study C5N was considered to be a suitable p53 $^{+/+}$ cell line with which to compare p53 $^{-/-}$ NK cells. Previous studies within this group had identified C5N as being sensitive to the growth inhibitory effects of TGFβ1 (Haddow *et al,* 1992). Additionally C5N is capable of blocking in G1 after exposure to ionising radiation, which is indicative of functionally active wild type p53 (I. Ganly, unpublished results).

In long term tissue culture there was no evidence to suggest that loss of p53 alters the response of cells to TGFβ1 (Figure 3.2). Both cell types were equally inhibited by the presence of 1ng/ml TGFβ1 in the tissue culture medium. The short term response of the cell lines to varying concentrations of the growth factor (0.01ng/ml, 0.1ng/ml, and 1ng/ml) was also investigated. Again both p53$^{-/-}$ and p53$^{+/+}$ cell lines were equally inhibited in ability to incorporate tritiated thymidine in the presence of TGFβ1 (Figure 3.3 A&C).

Although the p53 status of C5N and NK cells was known, the presence of any additional mutations or deletions that in the these cells could not be ruled out. Such changes could alter the response of these cells to TGFβ1 independent of p53 status. With this in mind, the initial study was extended to examine the response of p53 $^{-/-}$, p53$^{+/-}$ and p53$^{+/+}$

primary cells to TGFβ1. Short term assays were used in this investigation since long term analysis would have been complicated by the onset of primary cell death after 7 days.

$p53^{+/+}$,$p53^{+/-}$, and $p53^{-/-}$ primary keratinocytes were equally inhibited in their ability to incorporate tritiated thymidine in the presence of 0.01ng/ml, 0.1ng/ml and 1ng/ml TGFβ 1 (Figure 3.4). However the degree of response to the growth factor was less than that observed for established cell lines with comparable p53 status. This may have been due to the presence of other cell types such as melanocytes or fibroblasts in the primary cultures.

These results suggest that p53 is not critical for a cell to respond to the growth inhibitory effects of TGFβ1. However, our results do not rule out the possibility that p53 plays a role in TGFβ1 signalling which can be completely compensated for by other cellular proteins in its absence.

3.4 The effect of loss of p53, combined with mutant *H ras* on growth inhibition by TGFβ1.

Having ruled out a direct role for p53 in TGFβ1 mediated signalling we went on to look at the additive effect of mutant *H ras* combined with lack of p53 in TGFβ1 signalling. There are many conflicting results as to the role of *H ras* in the TGFβ1 signalling pathway. It has been shown *in vitro* that activation of H *ras* by DMBA treatment does not alter the response of epithelial cells to TGFβ1. (Haddow *et al*, 1992) In contrast Filmus *et al*, (1993) showed oncogenic H *ras* provided rat intestinal cells with increased resistance to the growth inhibitory actions of TGFβ1

Initial attempts to transfect primary keratinocytes with mutant *H ras* were unsuccessful. Primary keratinocytes were also toxic to infection with *H ras* virus. This may have been due to the toxic effect of polybrene on the lipid membranes of primary cells. Toxicity of primary cells to polybrene has previously been demonstrated in rat glial cells (S. Barrnett unpublished results).

NK and C5N cells were infected with mutant *H ras* virus. Both cell lines showed high levels of H RAS protein after virus infection as measured by western blot analysis using an H RAS specific antibody 172 from Oncogene Science (Fig 3.5). Equal amounts of protein was added to each lane.(Figure 3.5). In ^{3}H-thymidine incorporation assays, both NK and C5N cells showed a small drop in responsiveness to TGFβ1 after infection with mutant *H ras* virus (Figure 3.3 B&D).

Both cell lines showed increased tumorigenicity when injected subcutaneouly into nude mice.That is, both cell types yeilded tumours of between 1-1.5cm within a period of six weeks, compared to the uninfected cell lines which showed no tumorigenicity after injection into nude mice.

These experiments do not rule out a role for oncogenic *H ras* in the TGFβ1 pathway. The slight difference observed between the infected and uninfected cultures may have occurred as a result of unequal virus production in individual cells. Virus titration studies were not included at the time of experimentation, this made it difficult to draw definite conclusions as to the role of mutant ras in TGFB1 signalling. A more definitive answer to the role of p53 and oncogenic H *ras* in the TGFβ1 signalling pathway of mouse skin keratinocytes is likely to be provided by analysis of primary skin keratinocytes produced by crossing p53 knockout mice with mice which contain with oncogenic *H ras* targeted to the skin.

3.5 The role of p21^{waf1} in the response of cells to TGFβ1

After the conclusion of the initial *in vitro* studies, p21^{waf1} was reported as being a downstream target gene of p53. p21^{waf1} is thought to contribute to DNA damage induced G1 arrest by preventing phosphorylation of the pRb gene product. This function had been reported to be under the control of wild type p53. (El - Diery *et al*, 1993; Harper *et al* , 1993; Poon *et al* , 1994).

The p53 knockout keratinocyte cell lines provided an excellent model system in which to study the regulation of p21^{waf1}. As a result of p21^{waf1} being identified as a cell cycle kinase inhibitor we looked at the regulation of p21^{waf1} after TFGβ1 treatment in p53

knockout and wild type keratinocyte cells. Western blotting analysis showed that p21 ^{waf1} was present at increased levels in both p53$^{-/-}$ and p53 $^{+/+}$ keratinocytes after exposure to TGFβ1 (Figure 3.6). Basal levels of p21^{waf1} in p53$^{-/-}$ keratinocytes were lower than basal levels of the protein in p53$^{+/+}$ keratinocytes. This is probably a direct result of lack of normal p53 activity in the knockout keratinocytes. Equal amounts of protein were loaded in each lane, and results were consistent in duplicate experiments. These findings suggest that p21^{waf1} is implicated in growth control via a p53 independent mechanism. This conclusion has also been reached in experiments using HaCat cells. These cells contain two mutant alleles of p53 which are unable to activate transcription from the p21 ^{waf1} promoter. Even so, HaCat cells show an increase in p21^{waf1} levels after exposure to TGF β1 (Datto *et al,* 1995).

3.6 The effect of p53 status on the response of cultured keratinocytes to ionising radiation.

Having investigated the role of p53 in TGFβ1 signalling in mouse skin keratinocytes the relationship between p53, p21, BAX, and BCL2 and the cellular response to ionising radiation was examined. *In vitro* studies had shown that certain cell types respond to ionising radiation with either cell cycle arrest or apoptosis. Fibroblasts derived from p53 $^{+/+}$and p53 $^{+/-}$ mice respond to DNA damage with growth arrest, whilst fibroblasts from p53 $^{-/-}$ mice fail to show any arrest. However thymocytes from p53 $^{+/+}$ and p53 $^{+/-}$ undergo apoptosis in response to radiation whilst thymocytes from p53 $^{-/-}$ mice do not (Clark *et al,* 1993; Lowe *et al,* 1993).

The response of p53 $^{+/+}$ and p53$^{-/-}$ knockout keratinocytes to a range of ionising radiation doses was analysed. Standard radiobiology survival assays were used to assess sensitivity to increasing doses of ionising radiation (see Chapter 2). Again, the onset of cell death in primary keratinocyte cultures precluded their use in this long term experiment. Both NK p53$^{-/-}$ and C5N p53$^{+/+}$ cells lines were equally sensitive to cell killing by ionising radiation (Figure 3.7). Both types of cells were relatively resistant to low doses of ionising radiation. However, this resistance diminished at higher doses. This suggests that cells selected for outgrowth from primary cultures of skin epidermal cells do not require p53 function in order to survive relatively low levels of ionising

radiation the short term. Alternatively, the results may reflect loss of apoptotically active p53 protein in C5N cells. This a may have occurred in the serial passaging of C5N cells

3.7 Regulation of *bax,* and *bcl2* by p53 after exposure of keratinocytes to ionising radiation.

Changes in protein levels of the cell survival gene *bcl2* and the cell death gene *bax* in $p53^{-/-}$ and $p53^{+/+}$ keratinocytes after exposure to radiation were analysed. Both of these genes have been implicated in the cellular response to DNA damage. *bcl2* is known to be transcriptionally repressed by p53 whilst *bax* is known to be transcriptionally activated by the p53 protein. Relative amounts of BAX and BCL2 are thought to control cellular fate after DNA damage (For review see White, 1996).

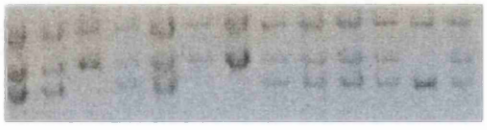
BAX levels remained constant in both $p53^{-/-}$ and $p53^{+/+}$ keratinocytes after they were exposed to 4Gy ionising radiation. The apparent decrease of BAX levels in NK cells was not consistent in repeat experiments. BCL2 protein levels reproducibly decreased in C5N cells 24hrs after exposure to 4Gy ionising radiation. In contrast, BCL2 protein levels remained constant in NK cells after exposure to ionising radiation (Figure 3.8 A&B). Later time points (up to 72hrs post radiation) showed no changes in either BAX and BCL2 levels in either cell type compared to control samples.

These results suggest that the lack of cell death observed previously in cell survival assays with p53 wild type and knockout keratinocytes is due to lack of increase of BAX protein after DNA damage. Interestingly a difference in electrophoretic migration of the BAX protein species between NK and C5N cells was detected. This may be due to differences in phosphorylation of the protein in the two cellular environments. Mutations in *bax* have recently been reported in human tumours (Rampino, *et al* 1997). The possibility that the difference in electrophoretic mobility of the BAX protein in NK and C5N cell lines is due to a mutation is being investigated currently.

3.8 Regulation of $p21^{waf1}$ by p53 in response to ionising radiation.

$p21^{waf1}$ protein levels increase 12hrs after exposure to 4Gy in $p53^{+/+}$ keratinocytes. No change was observed in $p53^{-/-}$ keratinocytes. It was also apparent that basal levels of $p21^{waf1}$ in $p53^{+/+}$ keratinocytes are increased in $p53^{+/+}$ keratinocytes compared to $p53^{-/-}$ keratinocytes.(Figure 3.8). This suggests that, in contrast to $p21^{waf1}$ regulation in response to TGFβ1 signalling, radiation induced $p21^{waf1}$ induction is critically dependent upon p53. This result also goes some way to explaining our previous results regarding cell survival and exposure to ionising radiation. Rather than induction of cell death after exposure to ionising radiation, cells selected in culture from p53 wild type newborn mouse skin initiate a block in the cell cycle via p53 mediated induction of $p21^{waf1}$. The present finding is in agreement with previous studies showing that $p21^{waf1}$ mRNA levels increase in cultured cells in response to ionising radiation in a p53 dependent manner (Xiong *et al,* 1993; Dulic *et al* , 1994)

Figure 3.1



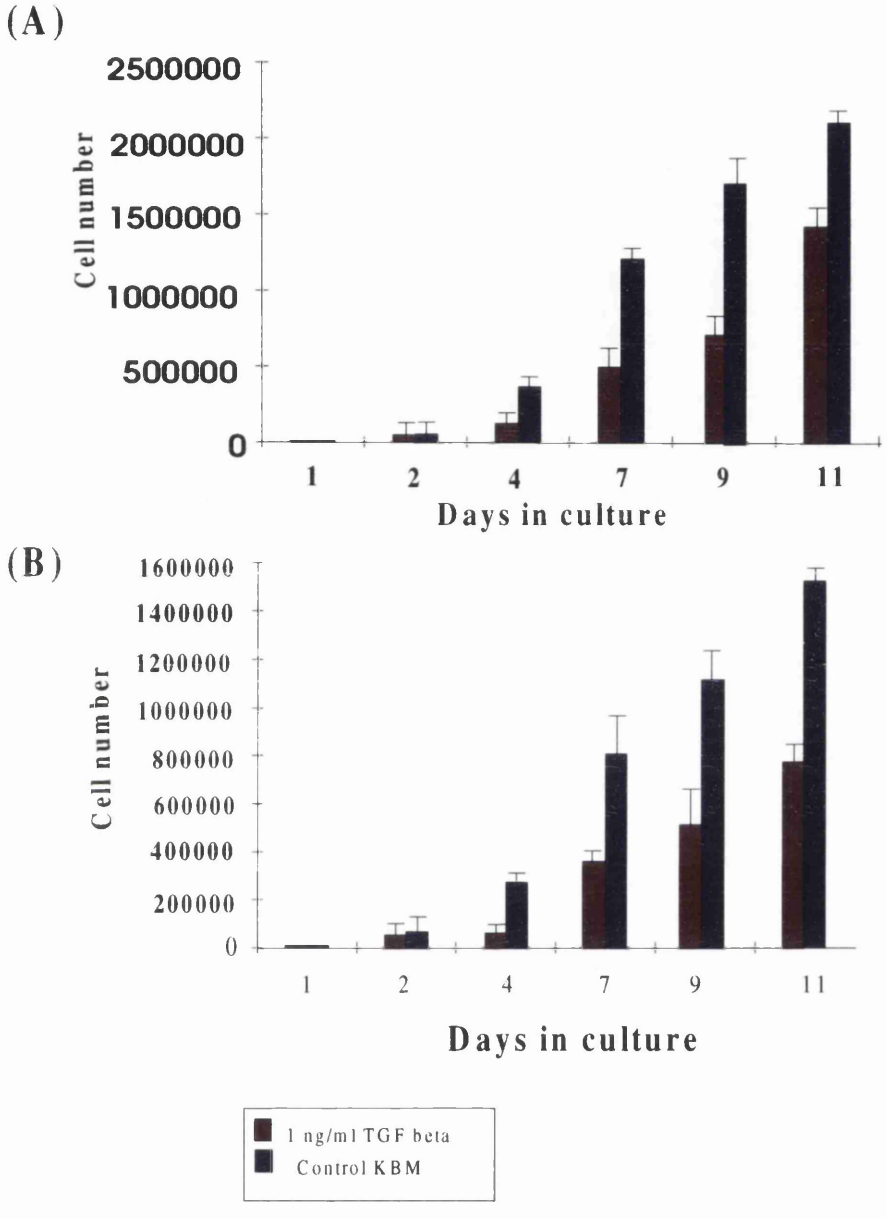
Southern blot analysis of tail tip DNA from a p53 knockout heterozygote litter.

Table 1

	Keratinocyte cell lines	Fibroblast cell lines	Carcinoma cell lines
p53 knockout	5	2	7
p53 heterozygote	1	0	5
p53 wild type	0	0	2

Table of cell lines established from p53 wild type, p53 knockout, and p53 heterozygote mice.

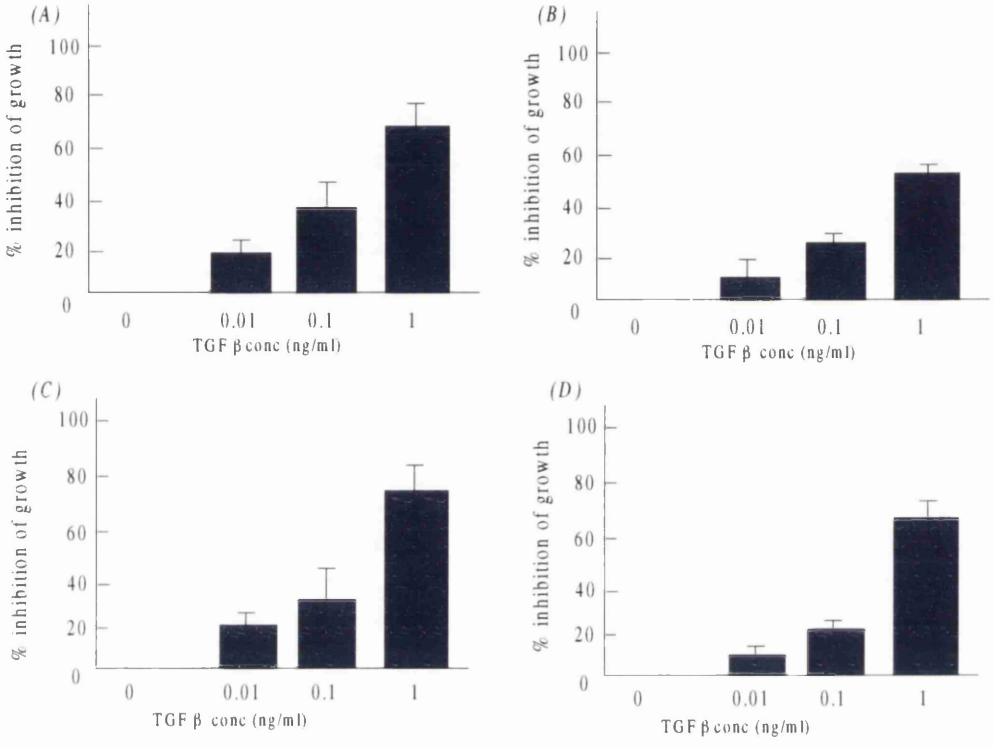
Figure 3.2 *In vitro* growth analysis of p53 wild type and p53 knockout mouse skin keratinocytes in long term culture in the presence of TGFβ1.



(A) p53 wild type keratinocytes, C5N.
(B) p53 knockout keratinocytes, NK.

Both p53 wild type and knockout keratinocytes are equally inhibited byTGFβ1 in long term culture. Three samples were analysed per time point.

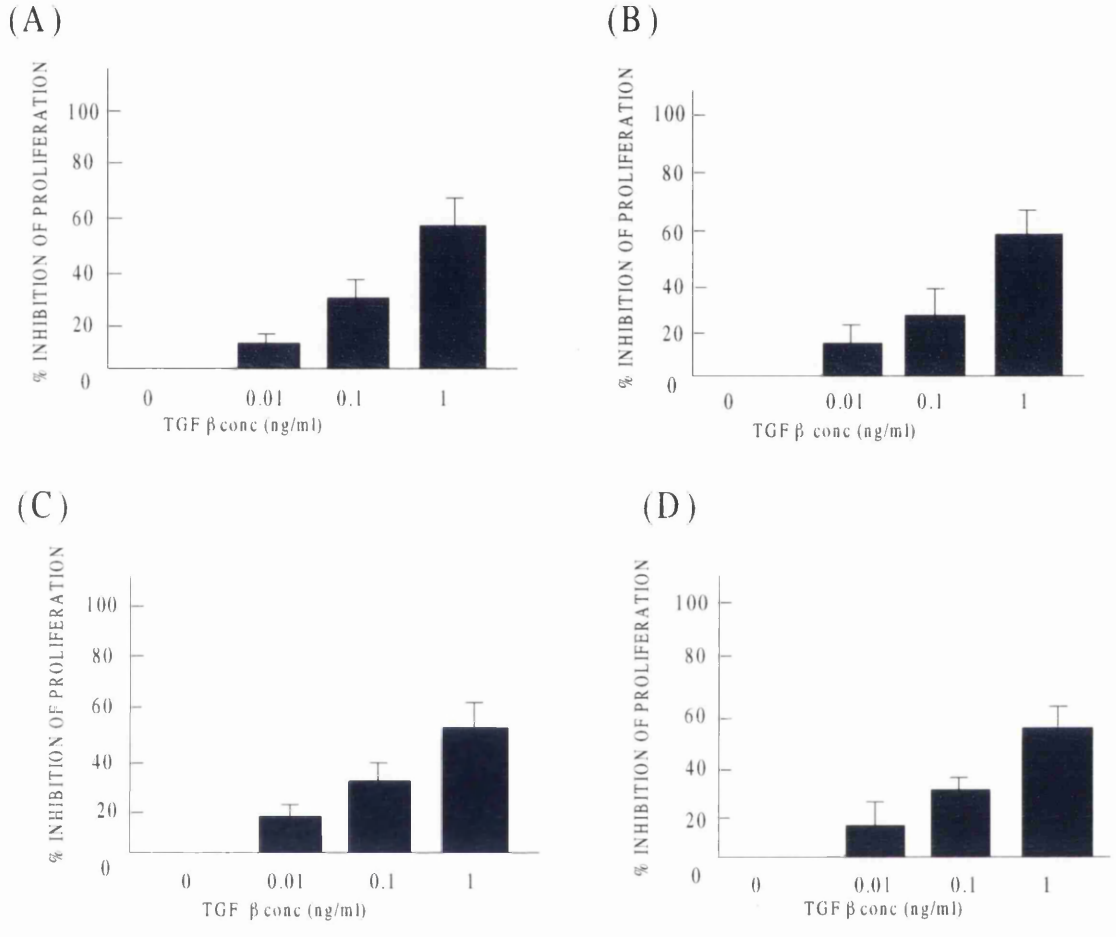
Figure 3.3 Tritiated thymidine incorporation in the presence of TGFβ1 of p53 wild type and knockout cell lines before and after infection with mutant *H ras* virus



(A) C5N
(B) C5N + mutant H *ras* virus
(C) NK
(D) NK + mutant H *ras* virus

Both C5N and NK are equally inhibited by TGFβ1
Both cell lines show a drop in responsiveness to the
growth factor when mutant H *ras* virus is introduced via infection.
Cells were exposed to TGFβ1 for 24hrs.
Tritiated thymidine incorporation assays were carried out as described in chapter 2

gure 3.4. Incorporation of tritiated thymidine by p53 wild type and knockout, and heterozygote rimary keratinocytes in the presence of TGFβ1.

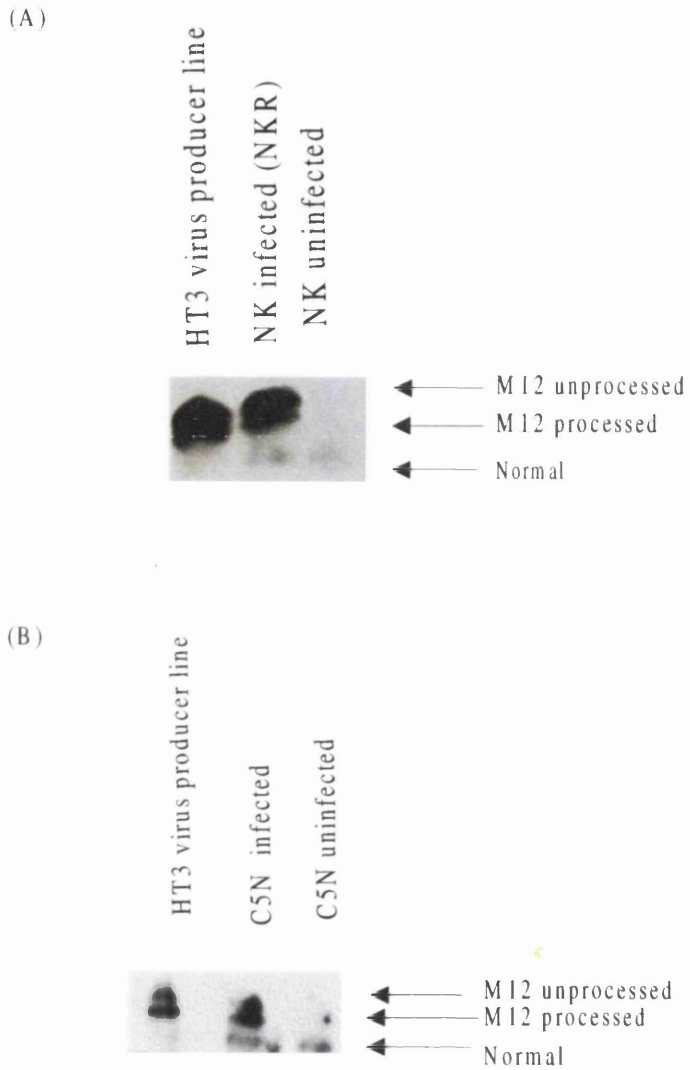


(A) p53 wild type primary keratinocytes
(B) p53 knockout primary keratinocytes
(C) p53 wild type primary keratinocytes
(D) p53 heterozygote primary keratinocytes

p53 wild type, knockout and heterozygote primary keratinocytes are equally inhibited by varying concentrations of TGFβ1 as measured by tritiated thymidine uptake 24hrs after addition of TGFβ1 to the culture medium.

Tritiated thymidine assays were carried out as described in chapter 2.

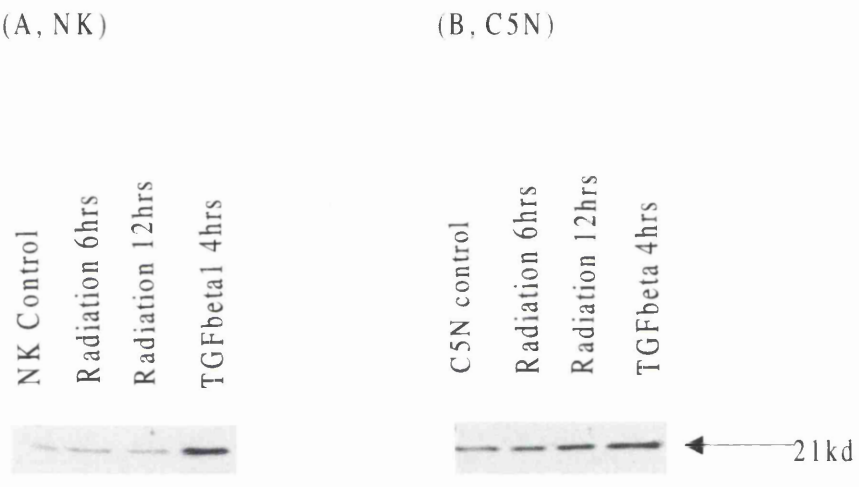
Figure 3.5 Western blot analysis of H-RAS protein levels in NK and C5N cells infected with mutant *Hras* virus.



(A) NK
(B) C5N

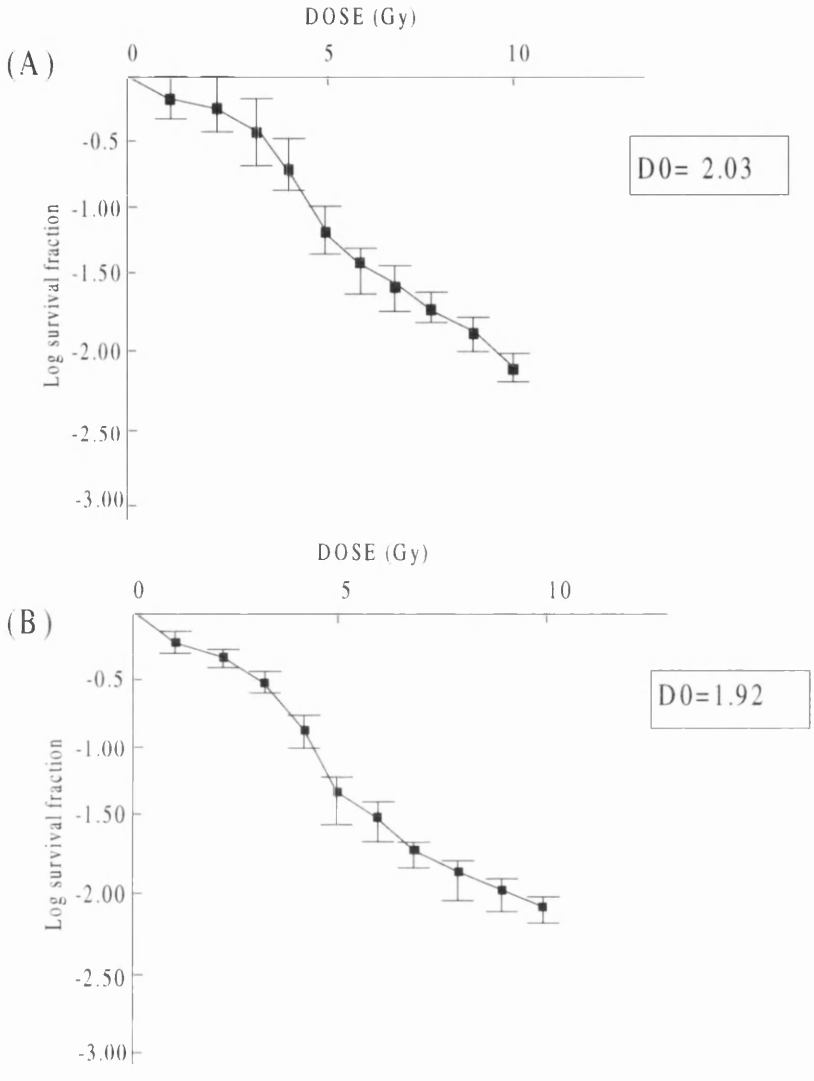
Both C5N and NK cells infected with mutant H *ras* virus produce high levels of mutant H-RAS protein. Equal amounts of protein was added to each lane.

Figure 3.6. Western blot analysis of p21*waf 1* protein levels in p53 wild type and knockout keratinocyte cell lines after exposure to 4Gy and TGFB1.



p21*waf1* protein levels rise 12hrs after radiation in C5N cells, no change is observed in irradiated NK cells. Both cell lines show an increase in p21*waf1* levels 4hrs after exposure to TGFB1.
Equal amounts of protein was added to each lane.
Pre incubation of antibody with cognate peptide resulted in no immunoreactivity.

Figure 3.7. Survival of p53 wild type and knockout cells in response to increasing levels of ionising radiation.



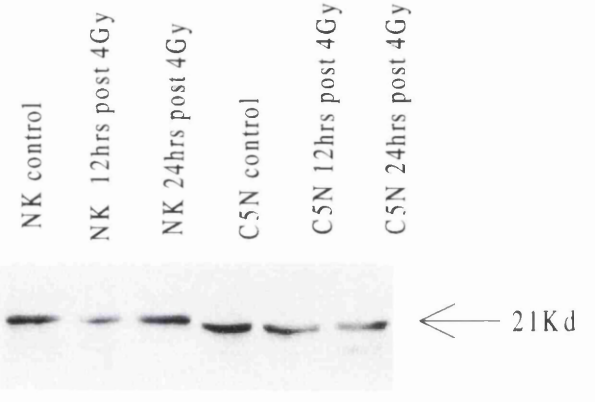
(A) NK
(B) C5N

C5N and NK cells were exposed to increasing doses of ionising radiation. Cell survival was assessed as described in chapter 2.
Do values are similar for the two cell lines
Both NK and C5N cells are equally sensitive to increasing doses of radiation.

Three samples were analysed per time point.

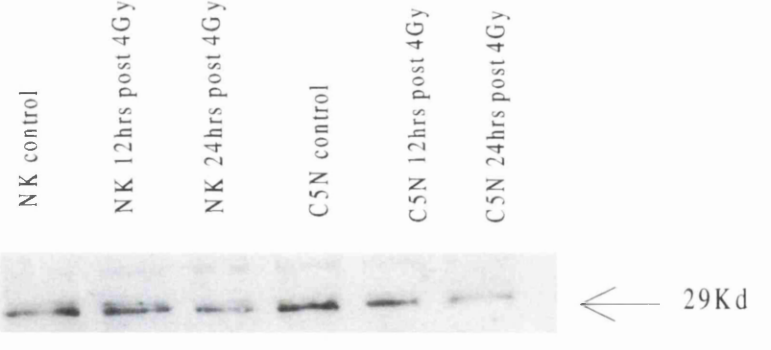
Figure 3.8 Western blot analysis of BAX and BCL2 protein levels in p53 wild type and knockout cell lines before and after exposure to 4Gy.

(A)



(A) Western blot analysis of BAX protein levels after exposure of NK and C5N cells to 4Gy. There is no significant change in BAX protein levels inNK and C5N cells after exposure to 4Gy.

(B)



(B) Western blot analysis of BCL2 protein levels after exposure of NK and C5N cells to 4Gy. There is no significant change in BCL2 protein levels in NK cells after 4Gy. There is a slight reduction in BCL2 protein levels in C5N cells 24hrs after 4Gy.

Pre incubation of both antibodies with respective cognate peptides resulted in no immunoreactivity. Protein loading determination was performed as described in chapter 2.

Chapter 4. Results

4.1 The effect of ionising radiation *in vivo* on mouse skin

Having examined the response of cells *in vitro* to ionising radiation, the response of cell *in vivo* to ionising radiation was analysed using mouse skin as a model. A number of accounts of cell type specific responses to radiation have been reported. Midgely *et al,* (1995) showed accumulation of p53 protein in mouse splenocytes, thymocytes and osteocytes, but not in hepatocytes of irradiated mice. The same authors demonstrated that cell type specific apoptosis, as a result of DNA damage, occurred *in vivo*. They found that thymocytes entered the apoptotic pathway after radiation but osteocytes did not.

4.2 p53 expression and apoptosis in adult mouse skin after exposure to 4Gy.

Using immunohistochemistry we found very little p53 protein in any of the cells of p53 wild type adult mouse epidermis. However high levels of p53 were detectable in a large proportion of cells in the epidermis of p53 wild type mouse skin 4hrs after exposure to 4Gy. p53 protein levels remained elevated for up to 72hrs post radiation. (Figure 4.1). As expected no evidence of p53 protein in p53 knockout mouse skin was found either before or after radiation treatment (Figure 4.2).

Characteristic darkly staining apoptotic bodies were present in H&E stained, p53 wild type adult mouse skin sections 4hrs after radiation exposure. Unlike the induced p53 expression pattern, the apoptotic bodies were restricted to a population of cells in the matrix region of the hair follicle (Figure 4.3). The increase in apoptotic bodies observed in p53 wild type adult mouse skin after radiation exposure was transient and returned to normal 12hrs after radiation exposure (Figure 4.5A). Exposure of p53 wild type mouse skin to 10Gy produced similar numbers apoptotic bodies than to 4Gy (data not shown).

Analysis of p53 knockout mouse skin did not result in an increase in apoptotic bodies at any time point after radiation exposure in any population of skin cells, even after exposure to increased radiation doses (Figure 4.4A & 4.5B). p53 heterozygote mouse skin responded to radiation with an increase in apoptosis roughly 75% of the increase observed in p53 wild type mouse skin (Figure 4.6).

4.3 p53 induction and apoptosis in two week old mouse skin

Following the initial observations in adult mouse skin, p53 induction and apoptosis in two week old mouse skin was investigated. Two week old mouse skin has a larger matrix cell population than adult mouse skin. Based on the previous results, a greater degree of apoptosis in the two week old follicles than in the adult follicles was anticipated.

p53 was present at elevated levels in the epidermis 4hrs after exposure of two week old p53 wild type mice to 4Gy (Figure 4.7 A&B and 4.8 A&B). Similarly, there was an increase in apoptotic bodies 4hrs after exposure to 4Gy in the matrix region of the hair follicle (Figure 4.9 A&B). No change, however, was observed in the interfollicular cell population (Figure 4.10 A&B). In contrast, no apoptotic bodies were present in either the follicular or interfollicular cell populations of p53 knockout mouse skin after exposure to 4Gy (Figure 4.9 C&D and Figure 4.10 C&D)

The increase in apoptosis observed in p53 wild type two week old mouse hair follicles after 4Gy was more dramatic than the increase observed in adult mouse skin hair follicles. The number of apoptotic bodies remained elevated for up to 24hrs post radiation treatment in contrast to the transient increase observed in irradiated adult mice 4hrs after irradiation (Figure 4.11).

To confirm the presence of apoptotic cells in the hair follicle, a fluorescent *in situ* DNA strand labelling kit (see chapter 2) which detects DNA strand breaks characteristic of apoptosis, was used. The position of the fluorescence signal in skin sections taken 4hrs after exposure to 4Gy correlated almost exactly with the position of the apoptotic bodies observed in the H&E stained sections (Figure 4.13). No increase in staining was observed in p53 knockout hair follicles after irradiation (Figure 4.14). This confirmed the initial observations that apoptosis occurs in the matrix region of the hair follicle after exposure to ionising radiation.

An unexpected pattern of background fluorescence staining was observed in both control and irradiated sections. In the hair follicles, long elongated cellular bodies appear very strongly stained. The true identity of these bodies remains unclear however the

strong fluorescent signal may be a result of particularly high keratin levels in these specific cells

The results demonstrate that apoptosis occurs *in vivo,* in not only a tissue specific manner but also a cell type specific manner. It may be the case in the skin, as in the intestinal epithelium, that it is the more stem like cells that exhibit the highest sensitivity to radiation induced apoptosis. This is discussed in more detail in a later chapter where the role of the radiation sensitive cells in skin tumorigenesis is investigated.

4.4 Entry into apoptosis and cell cycle status.

The increase in apoptotic bodies observed in p53 wild type two week old mouse skin was accompanied by a decrease in the number of mitotic cells visible in H&E stained sections (Figure 4.11 A). This prompted an examination of the possibility that a proportion of apoptotic bodies present in the sections were derived from cells in mitosis, rather than in G1.

Two week old mice were prelabelled with BrdU for 1hr (as described in chapter 2) before being exposed to 4Gy. The mice were subsequently sacrificed at various time points (Table 2). Since cells in S phase at the time of labelling would incorporate the BrdU label, any BrdU labelled apoptotic bodies present by 4hrs following radiation exposure are likely to have come from cells in S, G2 or M of the cell cycle. It is considered unlikely that a cell would progress through S, G2 and M and encounter the G1/S checkpoint of the cell cycle, in a period of less than 4hrs.

A number of BrdU labelled apoptotic bodies in mouse skin pre labelled with BrdU before exposure to radiation were identified (Fig 4.12). The number BrdU labelled apoptotic bodies peaked 4hrs after exposure of the skin to radiation. Table 3 summarises these results. Based on these findings, it is likely that at least 15-20% of the apoptotic bodies which are present in two week old mouse skin exposed to radiation originate from cells in the G2/M phase of the cell cycle rather than in G1/S. No increase in apoptotic bodies, or alterations in mitotic cell numbers were observed at any time point in p53 knockout two week old mouse skin following exposure to various doses of ionising radiation (Figure 4.11 B).

4.5 p53 induction and cell cycle arrest in adult mouse skin

Having observed a cell type specific response to p53 induction and subsequent entry into apoptosis, we decided to look at cell cycle arrest and p53 induction *in vivo* in mouse skin. Skin sections were taken at various time points from irradiated adult mice. One hour before being sacrificed, each mouse was injected with BrdU (as described in chapter 2). Subsequent immunohistochemistry with anti BrdU antibodies made it possible to evaluate BrdU incorporation in irradiated and control mouse skins.

A reduced level of BrdU incorporation was observed in $p53^{+/+}$ adult mouse skin 24hrs after exposure to ionising radiation, compared to unirradiated control $p53^{+/+}$ skin. The drop was not detected 48hrs post radiation treatment (4.15A). The drop in BrdU labelled cells after 24hrs was not restricted to a specific cell population of adult mouse skin. p53 heterozygous mice also showed a drop in BrdU incorporation 24hrs after exposure to radiation. The drop was roughly 25% of that observed in p53 wild type mouse skin (Figure 4.16). p53 knockout adult mouse skin showed no change in BrdU labelling after radiation exposure (Figure 4.15B). Exposure to increasing levels radiation failed to induce a change in BrdU incorporation in p53 knockout mouse skin at any time point examined.

4.6 p53 induction and cell cycle arrest in two week old mouse skin.

To establish a clearer link between cell type specific cell cycle arrest in response to radiation, two week old mouse skin was used, since it contains more readily distinguishable cell populations than adult mouse skin. This is due to the large size of the follicles present in young mouse skin. p53 wild type two week old mouse skin treated with 4Gy showed a drop in BrdU incorporation after 4hrs in the matrix follicular cells compared to unirradiated control skin. This result may have been a consequence of the matrix cells entering the apoptotic pathway after DNA damage rather than entering cell cycle arrest. The interfollicular cells of two week old mouse skin also showed a drop in BrdU labelling. However this occurred 24hrs after exposure to 4Gy (Figure 4.17A). No change in BrdU labelling occurred in p53 knockout two week old mouse skin after exposure to 4Gy ionising radiation (Figure 4.17B).

4.7 P53 induction and cell cycle arrest in proliferating adult mouse skin.

Results from the two week old mouse skin experiments led to an investigation of the effect of proliferation rate on cell cycle arrest and apoptosis after DNA damage. The possibility that matrix cell were more likely to enter the apoptotic pathway than less proliferative cell types was examined. Adult mouse skin stimulated to proliferate with TPA (as described in Chapter 2) was used to investigate the effect of proliferation on cell cycle arrest and apoptosis after exposure to ionising radiation.

TPA treatment induced the proliferation in the interfollicular cells of p53 wild type adult mouse epidermis. This proliferation was maximal 36hrs post TPA treatment and returned to basal levels 72hrs post TPA treatment. Identical results were obtained for p53 knockout animals treated with TPA (Figure 4.18 A&B).

p53 wild type adult mouse skin treated with TPA followed 36hrs later by exposure to 4Gy shows a drop in BrdU incorporation after 4hrs. This decrease was restricted to the proliferating cells of the TPA stimulated interfollicular epidermis. The slower cycling cells of the adult hair follicle show a drop in BrdU incorporation 24hrs after exposure to ionising radiation. (4.15 A). Adult mouse skin treated with TPA and irradiated after 72hrs shows a universal drop in BrdU incorporation 24hrs after exposure to 4Gy (Fig 4.18B). p53 knockout adult mouse skin treated with TPA before exposure to 4Gy failed to exhibit a reduction in BrdU incorporation 4hrs post radiation (Figure 4.18 B).Thus it was possible to clearly identify the drop in BrdU incorporation as being a consequence of cell cycle arrest rather than apoptosis, since apoptotic bodies are almost never observed in the interfollicular epidermis of mouse skin.

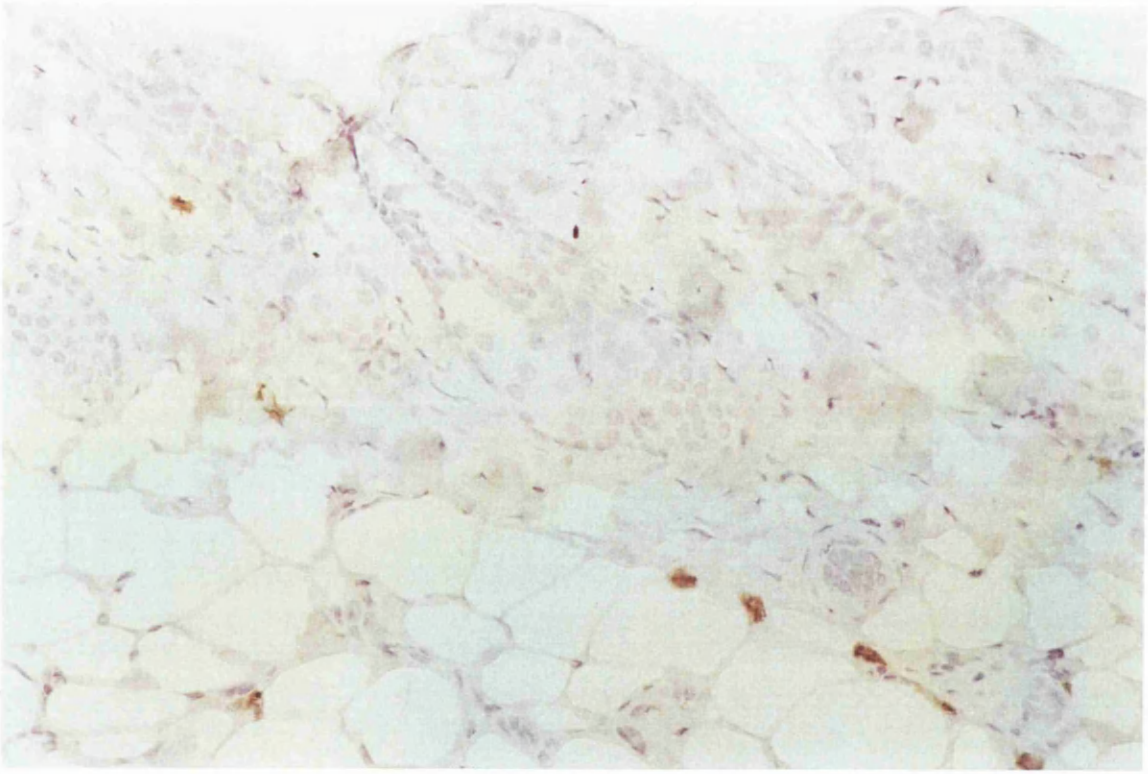
In a reversal of the above experiment, p53 wild type and knockout mice were exposed to 4Gy followed 4hrs afterwards by treatment with TPA for 36hrs. The mice were labelled with BrdU and skins taken 36hrs post TPA treatment.

Both p53 wild type and knockout mouse skin showed an increase in proliferation due to TPA, which was maximal 36hrs after treatment. No significant difference in TPA induced proliferation was detected between p53 wild type and p53 knockout mice

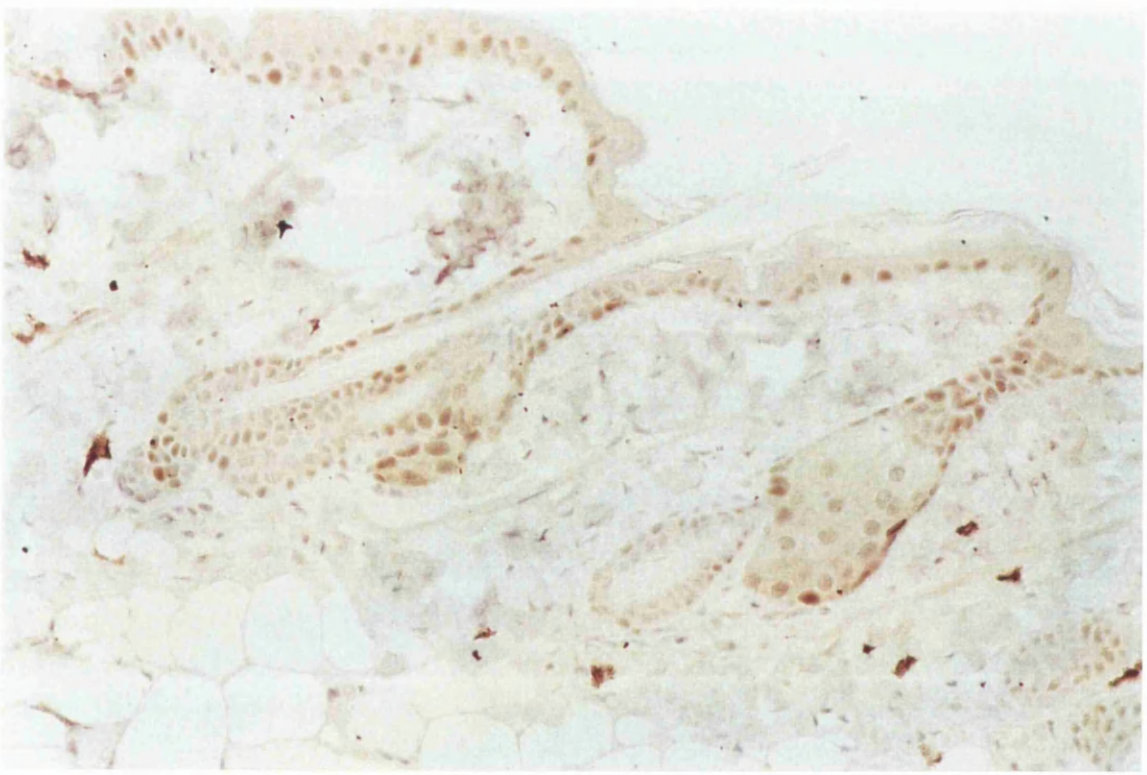
In brief, these findings suggest that different cell types respond to radiation with either cell cycle arrest or apoptosis. From these initial results it seems that cellular proliferation rate does not influence the entry of mouse skin cells into the apoptotic pathway, but can affect cell cycle arrest.

Figure 4.1 CM5 stained p53 wild type adult mouse skin before and 4hrs after exposure to 4Gy

(A)



(B)

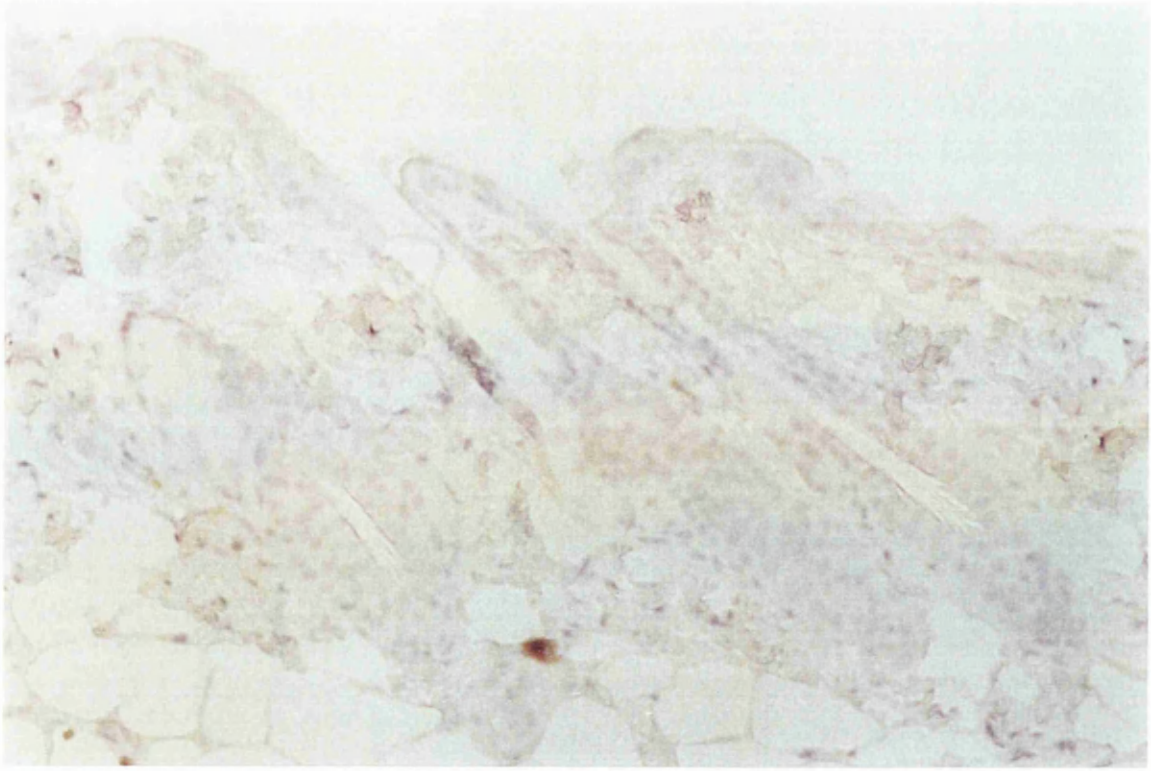


(A) CM5 stained p53 wild type adult mouse skin before exposure to 4Gy.
(B) CM5 stained p53 wild type adult mouse skin 4hrs after exposure to 4Gy.

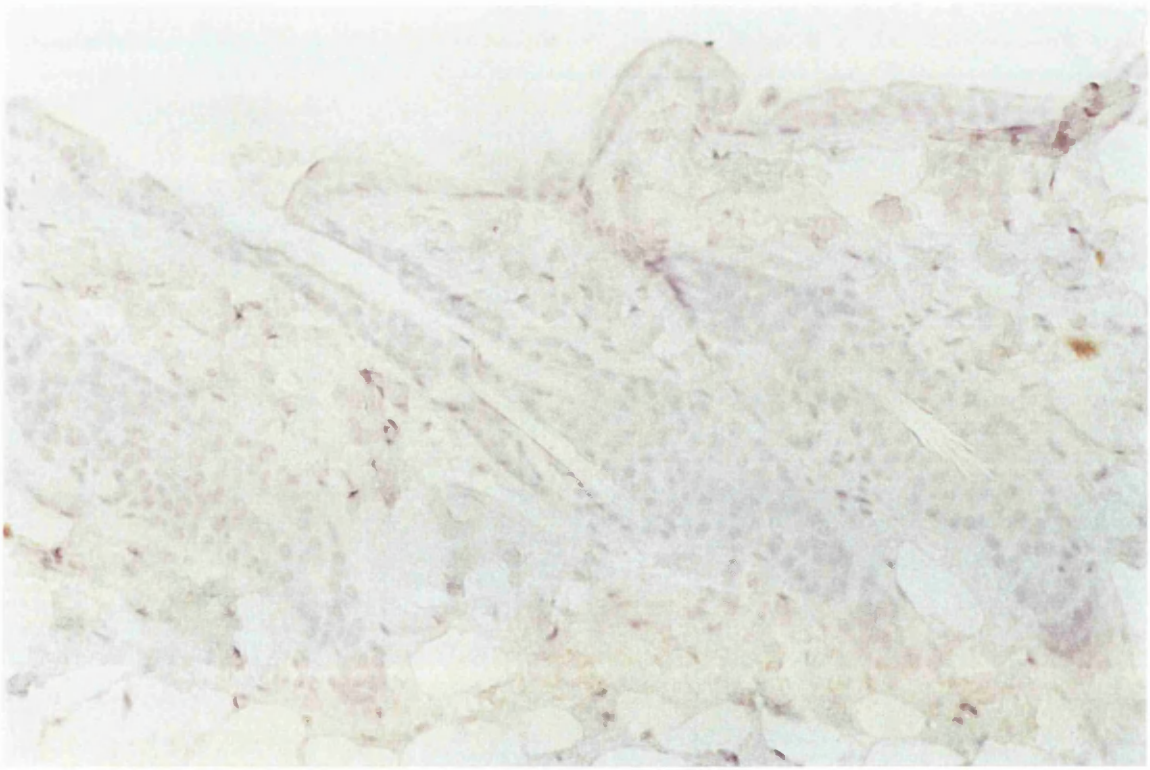
4hr after exposure to 4Gy p53 protein levels rise in the follicular and interfollicular epidermis of p53 wild type adult mouse epidermis.
CM5 staining was performed as described in materials and methods.

Figure 4.2 CM5 stained p53 knockout adult mouse skin before and 4hrs after exposure to 4Gy

(A)



(B)



(A) CM5 stained p53 knockout adult mouse skin before exposure to 4Gy.
(B) CM5 stained p53 knockout adult mouse skin 4hrs after exposure to 4Gy.

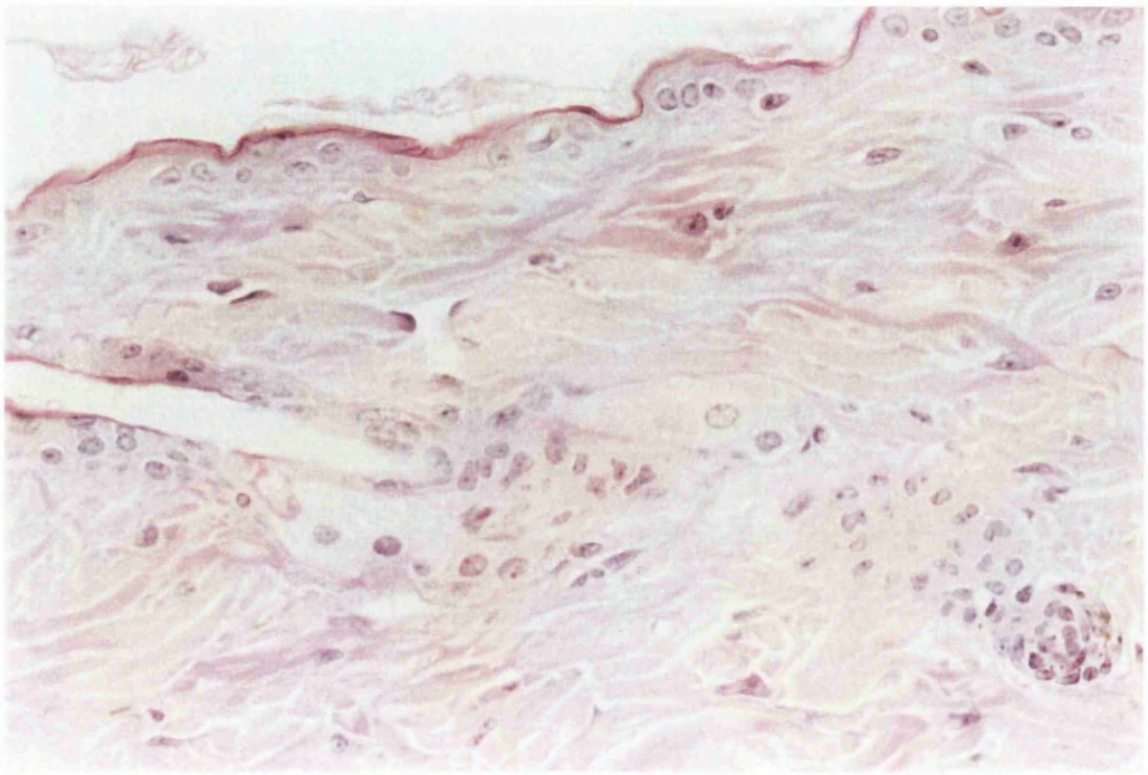
CM5 staining was performed as described in materials and methods.

Magnification X20

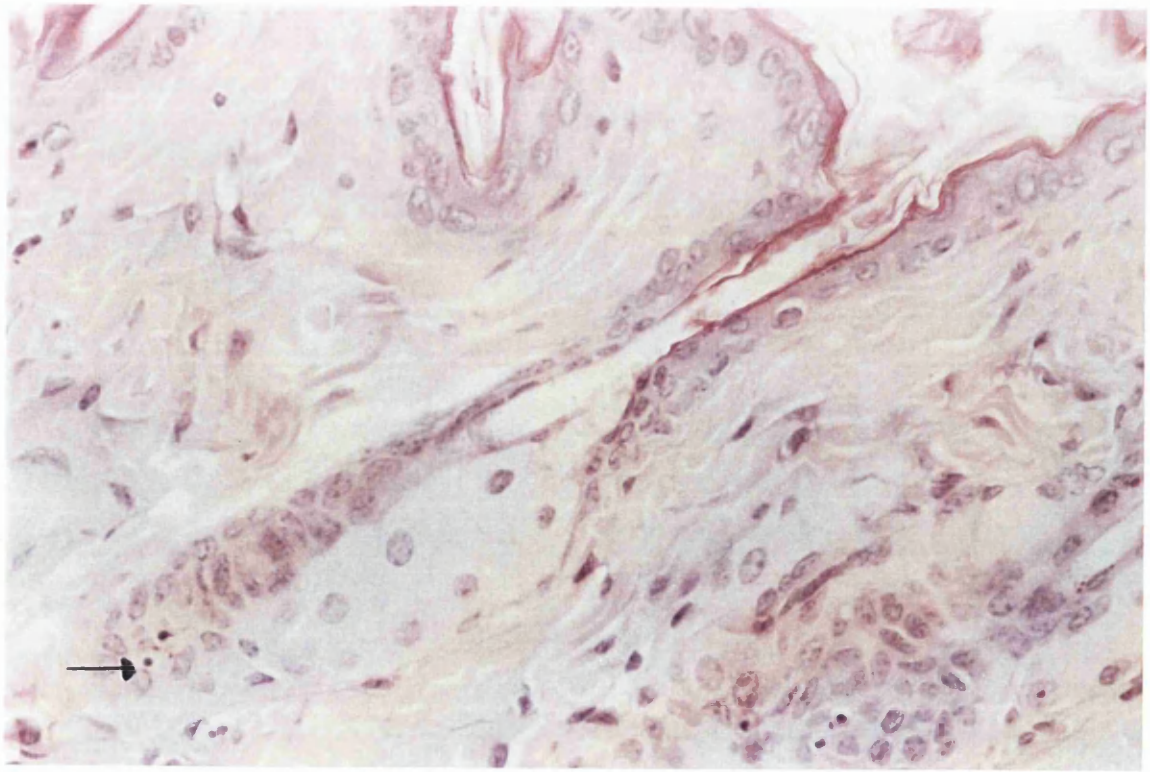
Figure 4.3. The arrow indicates apoptotic bodies in the matrix region of p53 wild type adult mouse skin four hours after exposure to 4Gy.

Figure 4.3 H&E stained p53 wild type adult mouse skin before and 4hrs after exposure to 4Gy

(A)



(B)

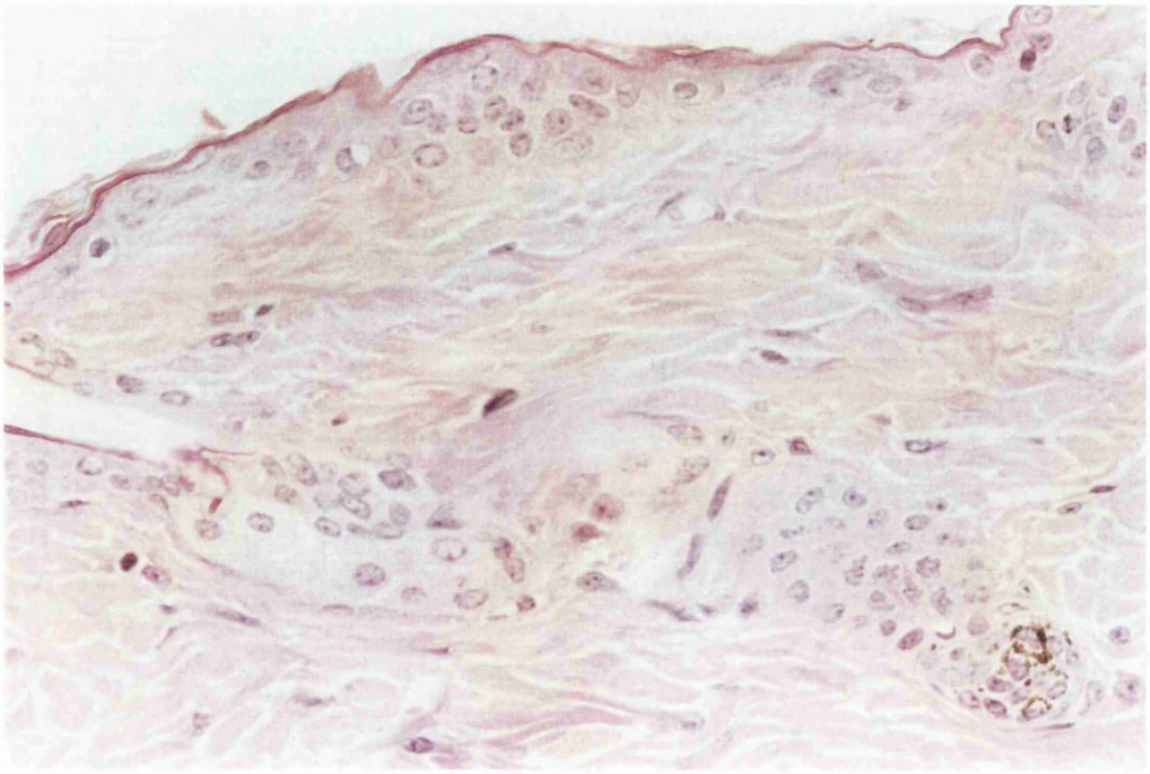


(A)H&E stained p53 wild type adult mouse skin before exposure to 4Gy.
(B)H&E stained p53 wild type adult mouse skin 4hrs after 4Gy.

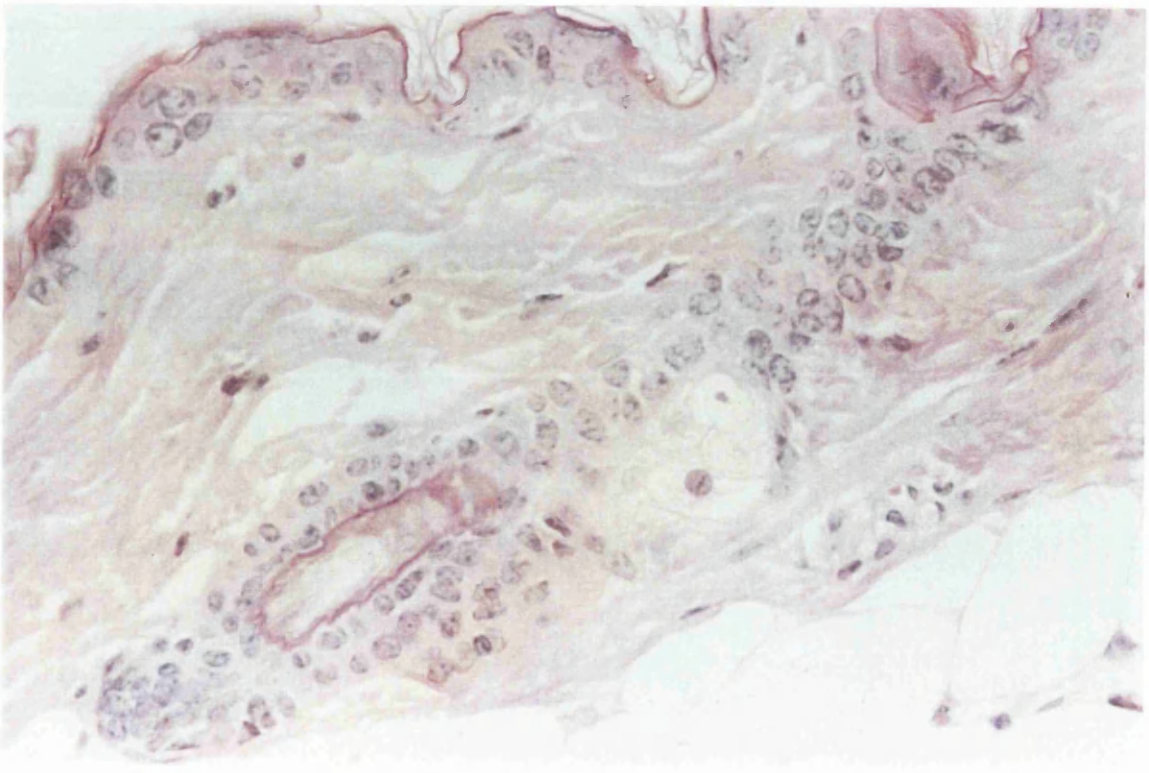
Magnification X40

Figure 4.4 H&E stained p53 knockout adult mouse skin before and 4hrs after exposure to 4Gy

(A)



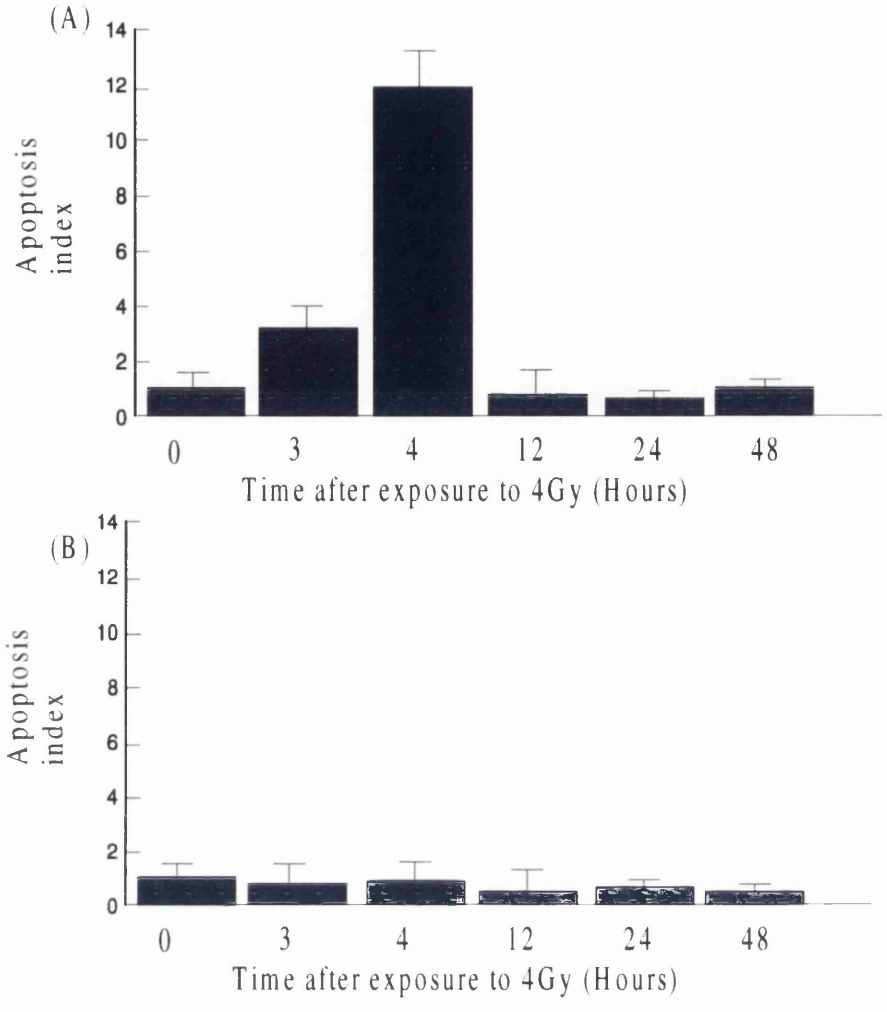
(B)



(A)H&E stained p53 knockout adult mouse skin before exposure to 4Gy.
(B)H&E stained p53 knockout adult mouse skin 4hrs after 4Gy.

Magnification X40

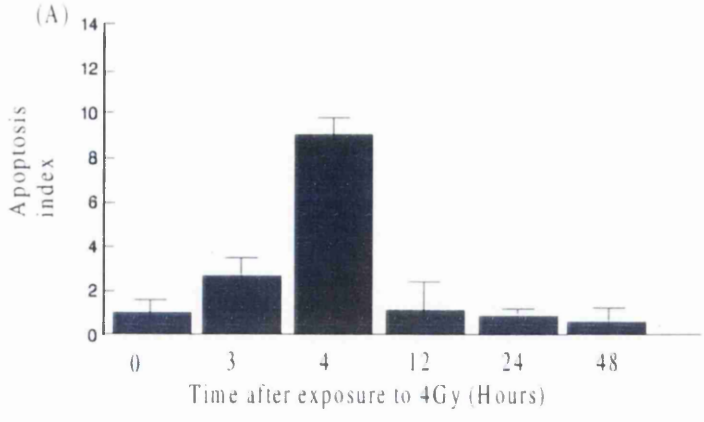
Figure 4.5. Apoptotic analysis of p53 wild type and knockout mouse skin after exposure to ionising radiation.



(A) p53 homozygous wild type adult mouse skin.
(B) p53 homozygous knockout adult mouse skin.

The number of apoptotic bodies increases four hours after irradiation of p53 wild type adult mouse skin. The increase is transient and falls to normal basal levels 12hrs post irradiation. No change is observed in p53 knockout mouse skin after irradiation.
Six skin sections were analysed per time point. Staining was performed in triplicate for all samples.
Apoptotic index = The number of apoptotic bodies per section divided by the length in millimeters of the section.

Figure 4.6 Apoptotic analysis of p53 heterozygote adult mouse skin after exposure to 4Gy.



The number of apoptotic bodies, increases after irradiation of p53 heterozygous adult mouse skin after exposure to radiation. The rise is more than half that observed in p53 wild type mouse skin after radiation.

Six skin sections were analysed per time point.

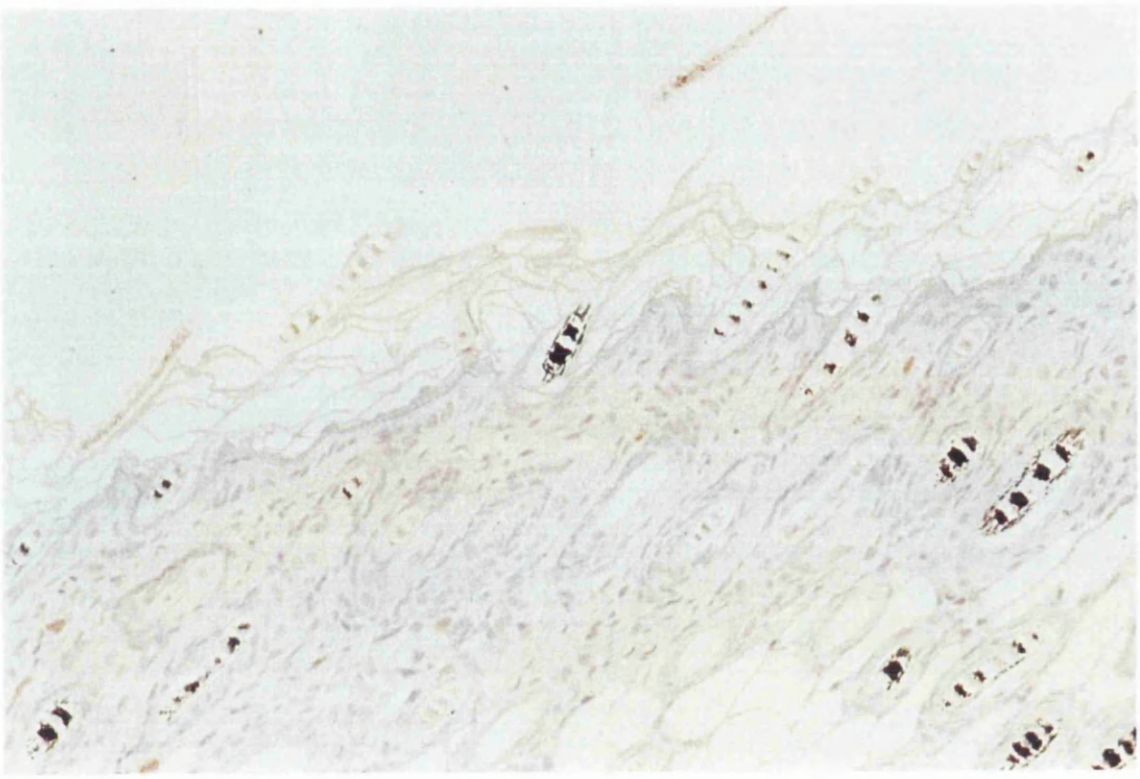
H&E staining was performed in triplicate for all samples

Apoptosis was scored by the presence of darkly staining apoptotic bodies

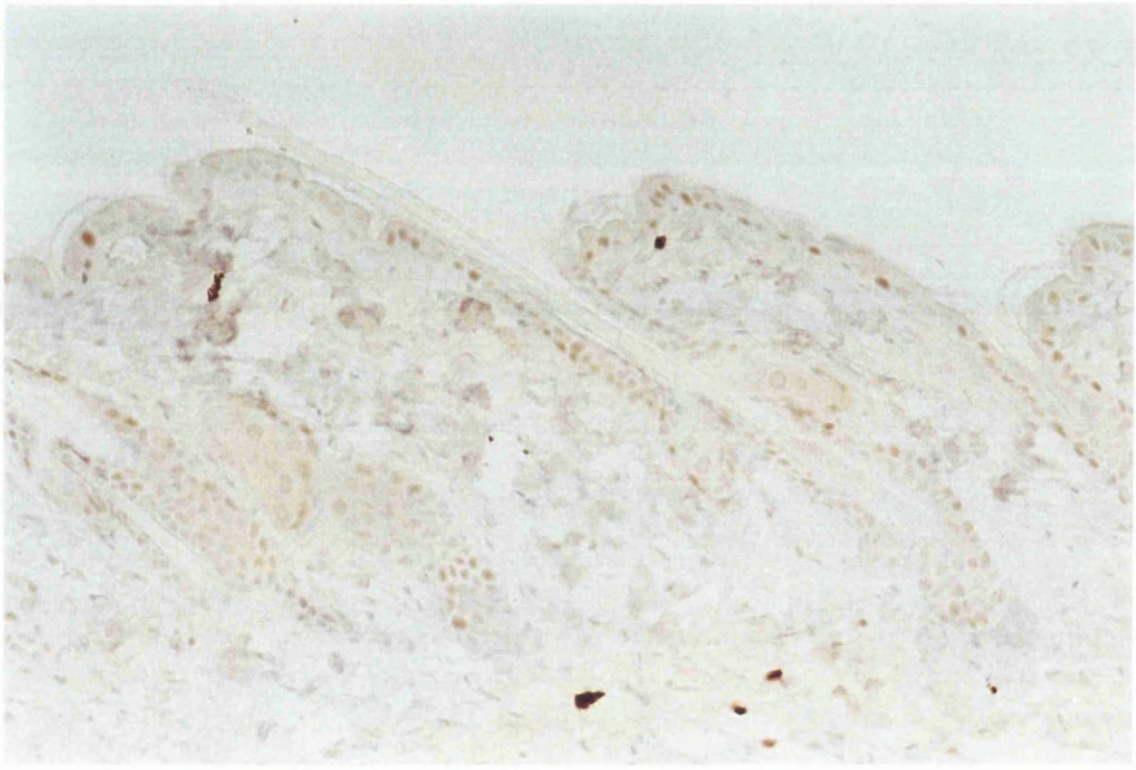
Apoptotic index= The number of apoptotic bodies per section divided by the length in milimeters of the section.

Figure 4.7 CM5 stained two week old mouse skin before and 4hrs after exposure to 4Gy

(A)



(B)



Magnification X20

(A) CM5 stained P53 wild type two week old interfollicular epidermis before exposure to 4Gy

(B) CM5 stained p53 wild type two week old interfollicular epidermis 4hrs after exposure to 4Gy.

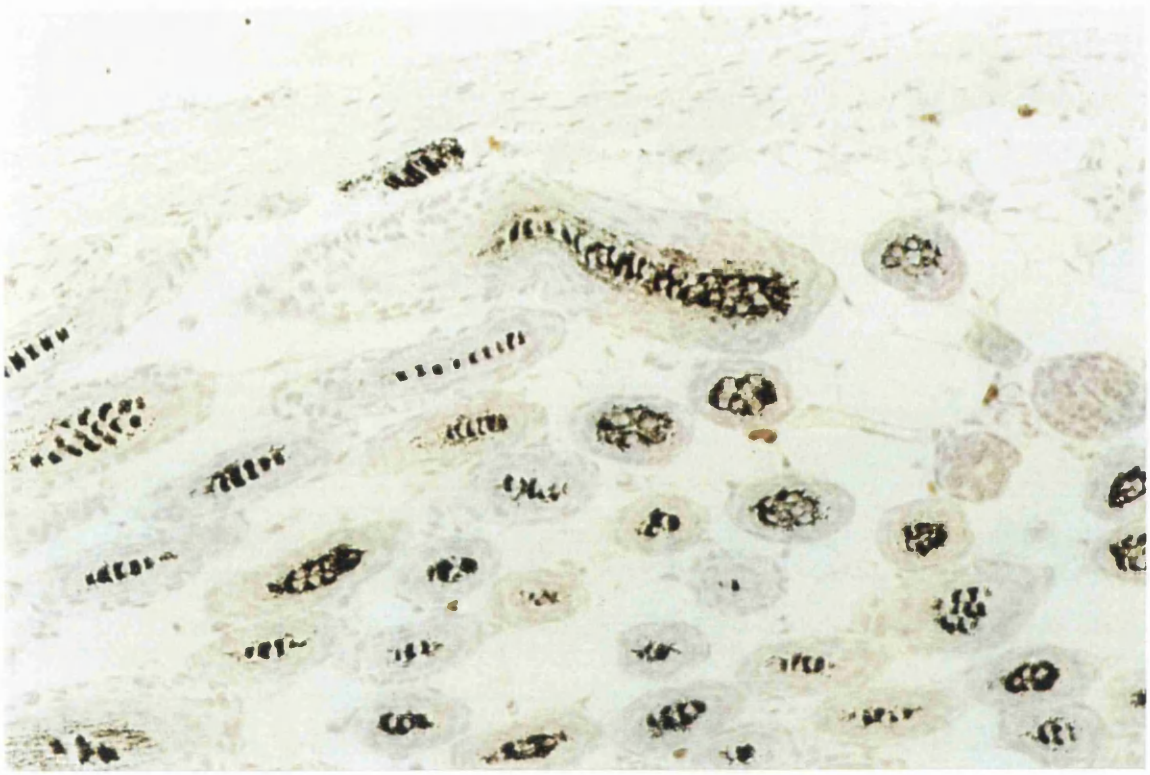
4hrs after exposure to 4Gy p53 protein levels rise in the interfollicular epidermis of p53 wild type two week old mouse skin.

CM5 staining and radiation treatments were performed as described in materials and methods

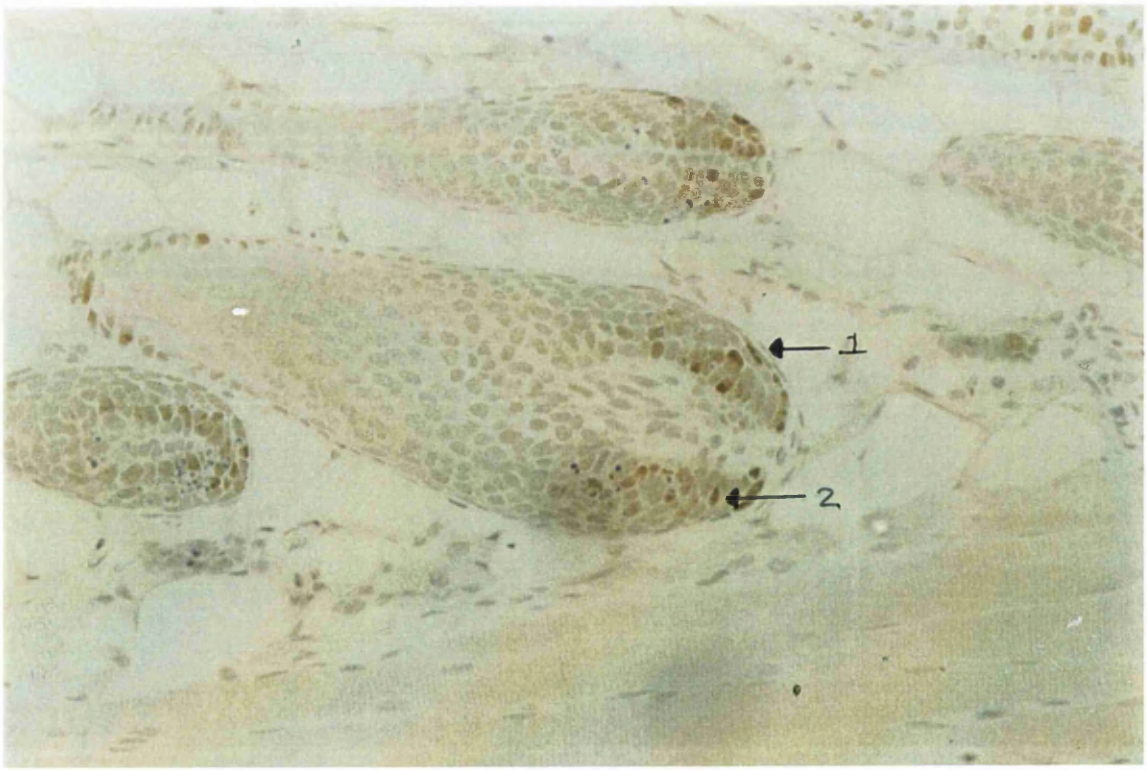
Figure 4.8. Arrow 1 indicates CM5 stained outer root sheath cells Arrow 2 indicates CM5 stained cell in the matrix region of p53 wild type two week old mouse hair follicles .

Figure 4.8 CM5 stained two week old mouse hair follicles before and 4hrs after exposure to 4Gy

(A)



(B)



Magnification X20

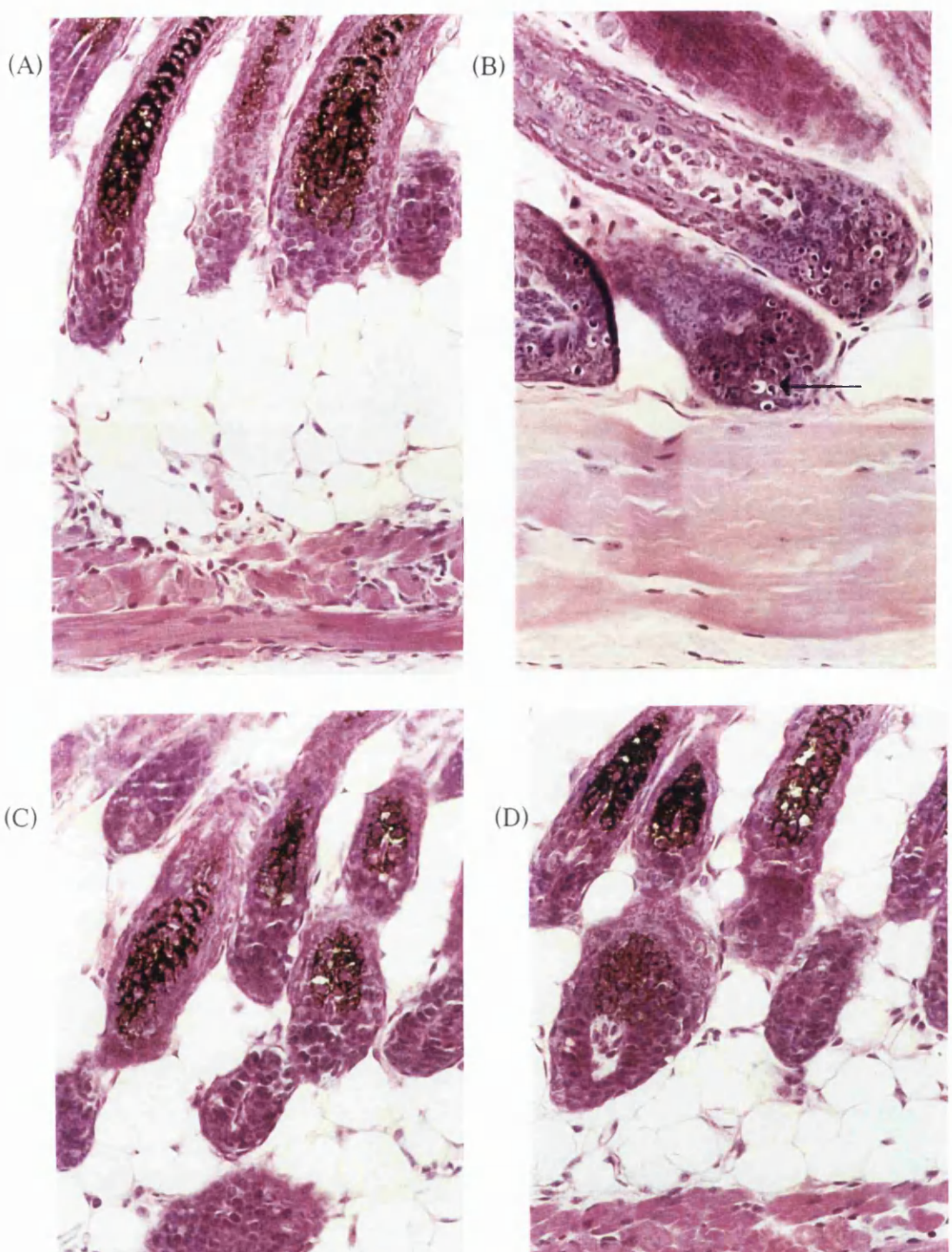
(A) CM5 stained p53 wild type two week old hair follicles before exposure to 4Gy.
(B)CM5 stained p53 wild type two week old hair follicles 4hrs after exposure to 4Gy

4hrs after 4Gy p53 protein levels rise in the hair follicles of p53 wild type adult mouse epidermis.

CM5 staining was performed as described in materials and methods.

Figure 4.9 The arrow indicates apoptotic bodies present in the matrix region of p53 wild type two week old mouse skin 4hrs after 4Gy.

Figure 4.9 H&E stained two week old p53 wild type and knockout hair follicles before and after exposure to 4Gy

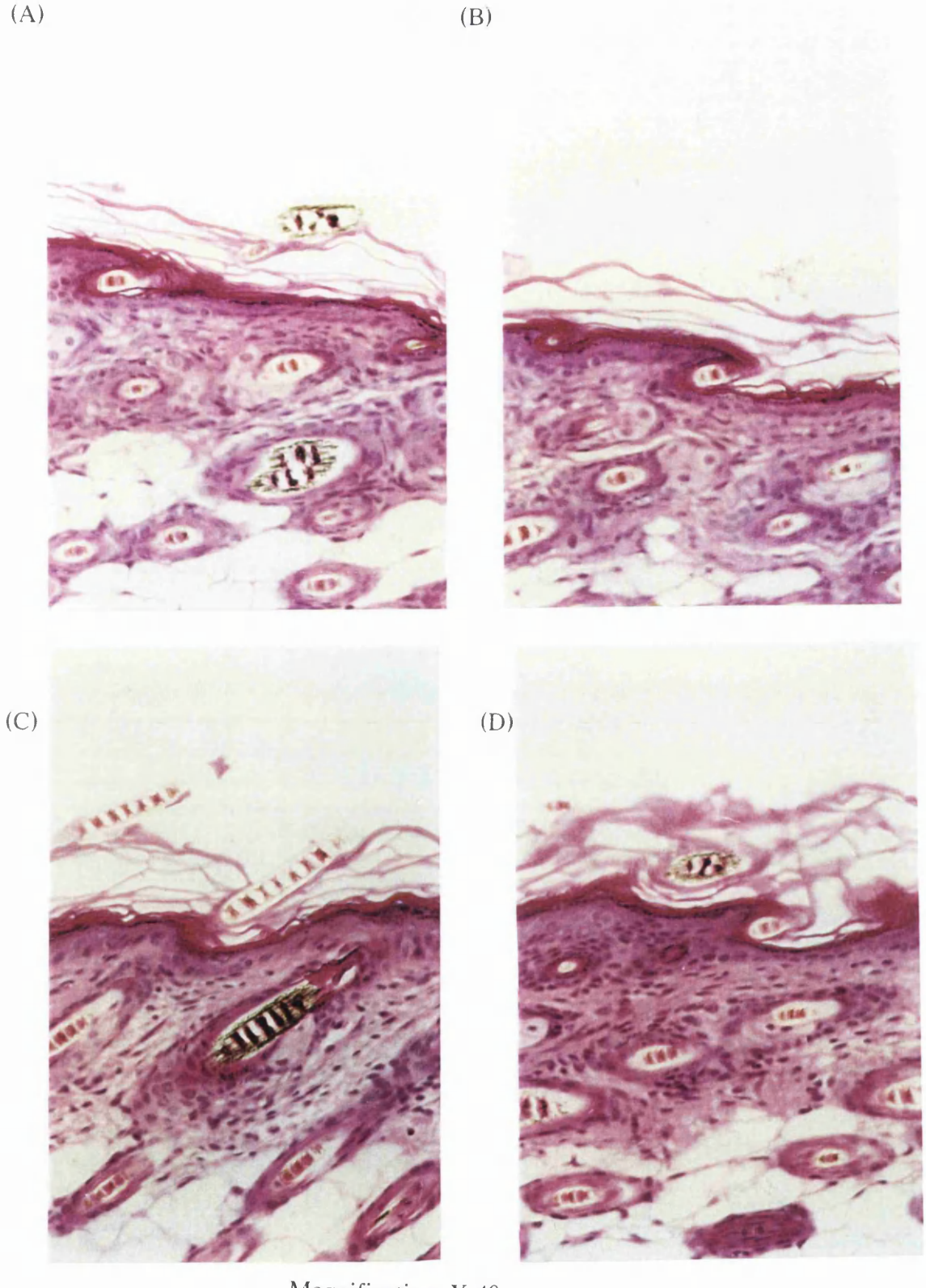


Magnification X 40

(A) H&E stained p53 wild type two week old follicles before exposure to 4Gy.
(B) H&E stained p53 wild type two week old follicles 4hrs after exposure to 4Gy.
(C) H&E stained p53 knockout two week old follicles before exposure to 4Gy.
(D)H&E stained p53 knockout two week old follicles 4hrs after exposure to 4Gy.

4hrs after exposure to 4Gy p53 wild type two week old mouse skin follicles show an increase in apoptotic bodies. No change is observed in the follicles of p53 knockout two week old mouse skin.

Figure 4.10 H&E stained two week old p53 wild type and knockout mouse epidermis before and after exposure to 4Gy



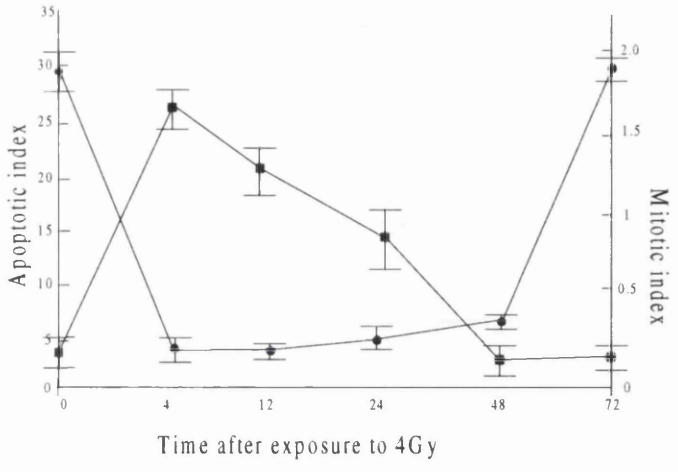
Magnification X 40

(A) H&E stained p53 wild type two week old basal skin before exposure to 4Gy.
(B) H&E stained p53 wild type two week old basal skin 4hrs after exposure to 4Gy.
(C) H&E stained p53 knockout two week old basal skin before exposure to 4Gy.
(D)H&E stained p53 knockout two week old basal skin 4hrs after exposure to 4Gy.

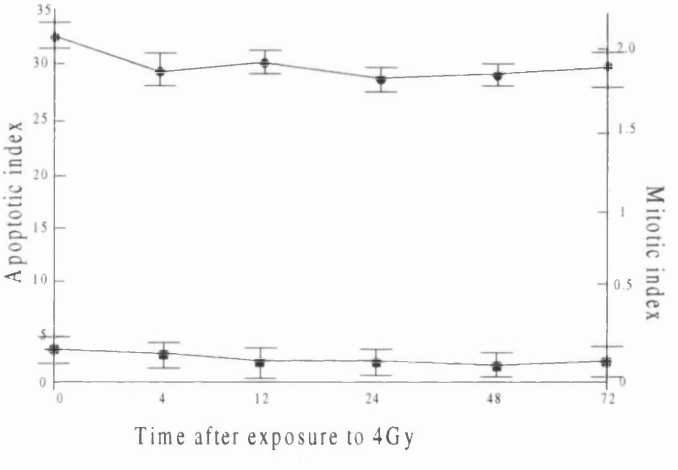
4hrs after exposure to 4Gy p53 wild type or knockout two week old mouse basal skin shows no change in apoptotic bodies.

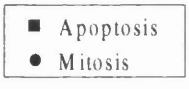
Figure 4.11. Apoptotic and mitotic analysis of p53 wild type and knockout mouse skin after exposure to 4Gy.

(A)



(B)



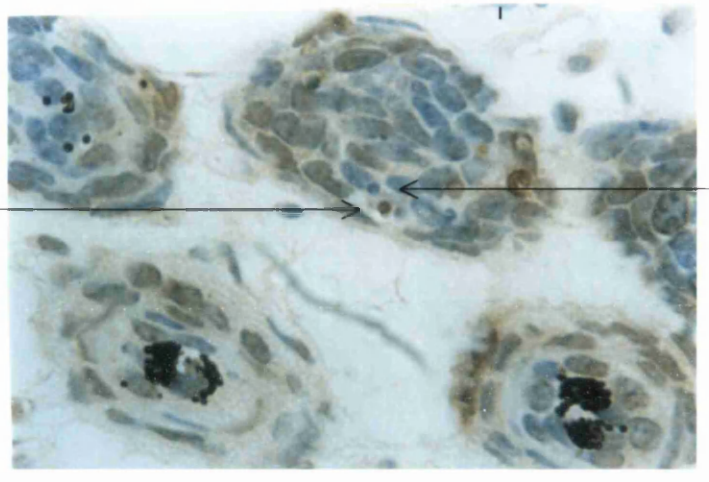


(A) p53 wild type two week old mouse skin
(B) p53 knockout two week old mouse skin
The number of apoptotic bodies present in two week old wild type p53 mouse skin rises after exposure to 4Gy. This is concomitant with a decrease in mitotic cells No change in mitotic cells or apoptotic bodies is observed in p53 knockout mice.

Mice were injected with BrdU as described in methods Skin sections were stained with Haematoxylin & Eosin. Apoptosis was scored by the presence of darkly staining apoptotic bodies. Mitotic cells were clearly visible in the stained sections.

Six skin sections were analysed per time point.
Apoptotic index = The number of apoptotic bodies per section divided by the length of the section in millimetres
Mitotic index = The number of mitotic cells per section divided by the length in millimetres of the section.

Figure 4.12 BrdU labelled apoptotic bodies in the hair follicles of p53 wild type adult mouse skin prelabelled with BrdU before exposure to 4Gy.



Mice were injected with BrdU 1hr prior to irradiation. Sections were taken post radiation. BrdU positive bodies were scored on the basis of brown stain.

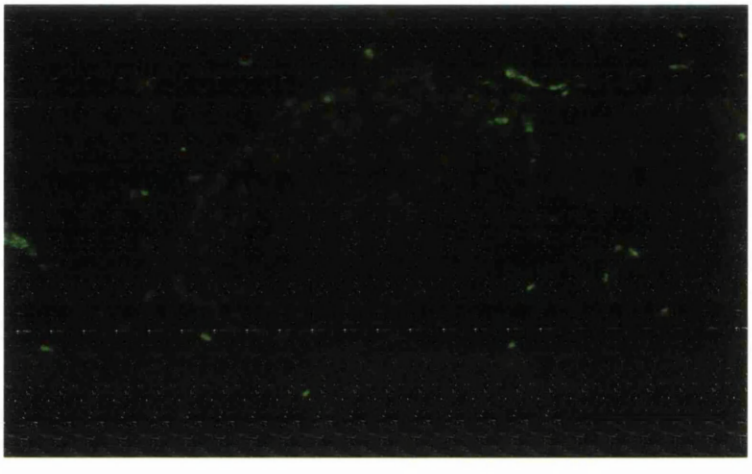
(Magnification X 40)

Table 2 Incidence of BrdU labelled apoptotic cells in p53 wild type adult mouse skin after exposure to 4Gy.

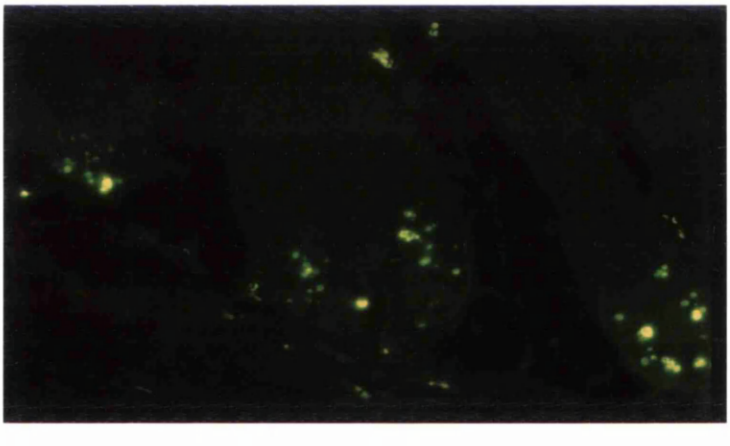
	Percentage of BrdU labelled apoptotic cells in p53 wild type mouse hair follicles after exposure to 4Gy ionising radiation.
0hrs	0% (+/- 0.1)
1hr	0.1% (+/- 0.13)
2hrs	0.1% (+/- 0.2)
3hrs	3% (+/- 0.5)
4hrs	17% (+/- 1.2)

Figure 4.13 *In situ* analysis of DNA strand breaks in p53 wild type two week old mouse hair follicles before and after exposure to 4Gy

(A)



(B)



(A) p53 wild type two week old mouse hair follicles
before exposure to 4Gy
(B) p53 wild type two week old mouse hair follicles
4hrs after exposure to 4Gy
The position of the fluorescence signal correlates with the position of apoptotic bodies visible in H&E stained section. Magnification X40

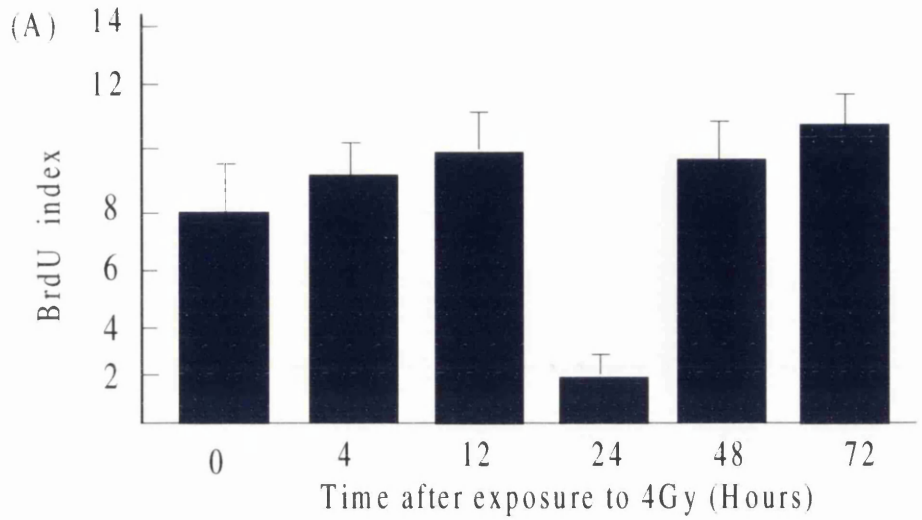
Figure 4.14 *In situ* analysis of DNA strand breaks in p53 knockout two week old mouse skin before nd after exposure to 4Gy.

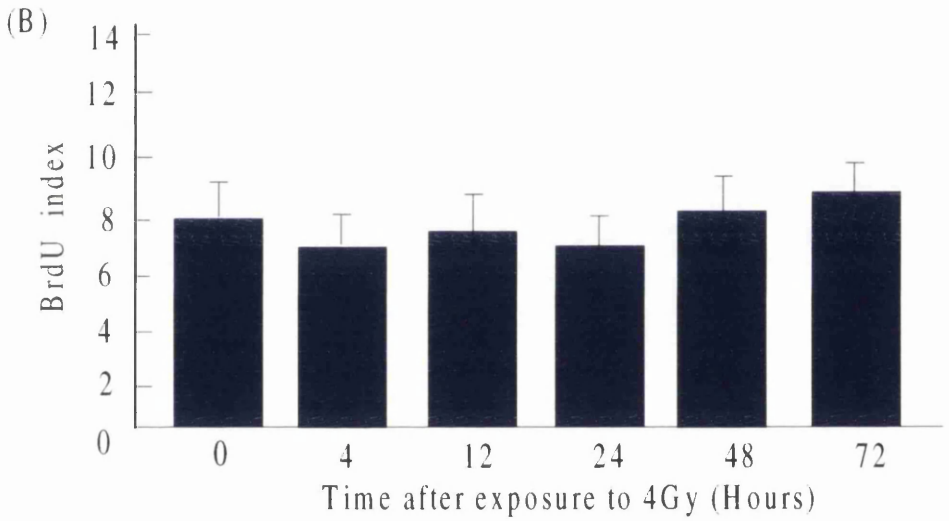
(A)

(B)

(A) p53 knockout two week old mouse hair follicles before exposure to radiation
(B) p53 knockout two week old mouse hair follicles 4hrs after exposure to 4Gy.
No staining was seen in the hair follicles of p53 knockout mouse skin before or after exposure to 4Gy.

Figure 4.15 BrdU incorporation in p53 wild type and knockout adult mouse skin after exposure to 4Gy.

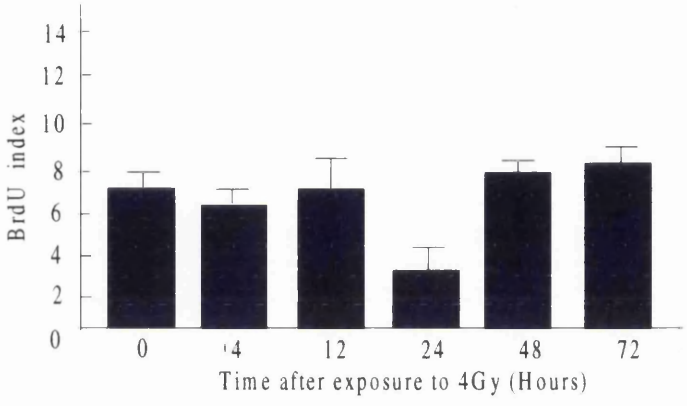




(A) p53 homozygous wild type adult mouse skin.
(B) p53 homozygous knockout adult mouse skin.

BrdU incorporation falls 24hrs after exposure to 4Gy in p53 wild type adult mouse skin,incorporation returns to normal basal levels 48hrs after irradiation. No change in BrdU incorporation is observed in p53 knockout mouse skin.
Six skin sections were analysed per time point. Staining was performed in triplicate for all samples.
BrdU index = The number of BrdU positive cells per section divided by the length in millimeters of the section.

Figure 4.16 BrdU incorporation of p53 heterozygote adult mouse skin after exposure to 4Gy.



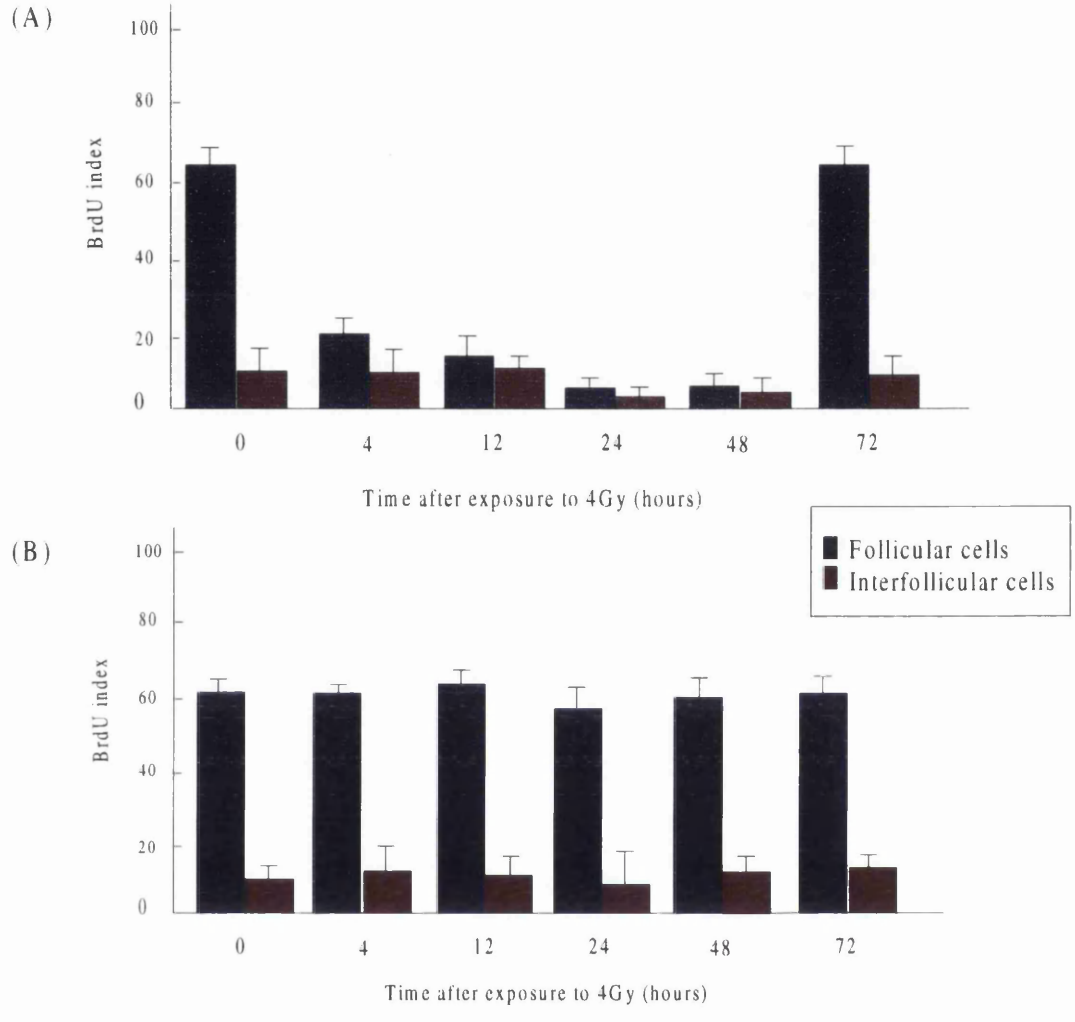
(B) BrdU index of p53 heterozygote adult mouse skin after exposure to 4Gy.

p53 heterozygote mouse skin, like p53 wild type adult mouse skin shows a drop in BrdU incorporation 24hrs after exposure to 4Gy. The changes observed in p53 heterozygote mouse skin after radiation are similar to the changes observed in p53 wild type mouse skin after exposure to radiation however the magnitude of the changes in BrdU incorporation is less than that observed in p53 wild type mouse skin.

(B) BrdU index= The number of BrdU positive cells per section divided by the length of the section in millimeters.

Two samples were analysed per time point. BrdU labelling and detection was performed as described in chapter 2 Staining was performed in duplicate for all samples.

Figure 4.17. BrdU incorporation in p53 wild type and knockout two week old mouse skin before and after exposure to 4Gy.



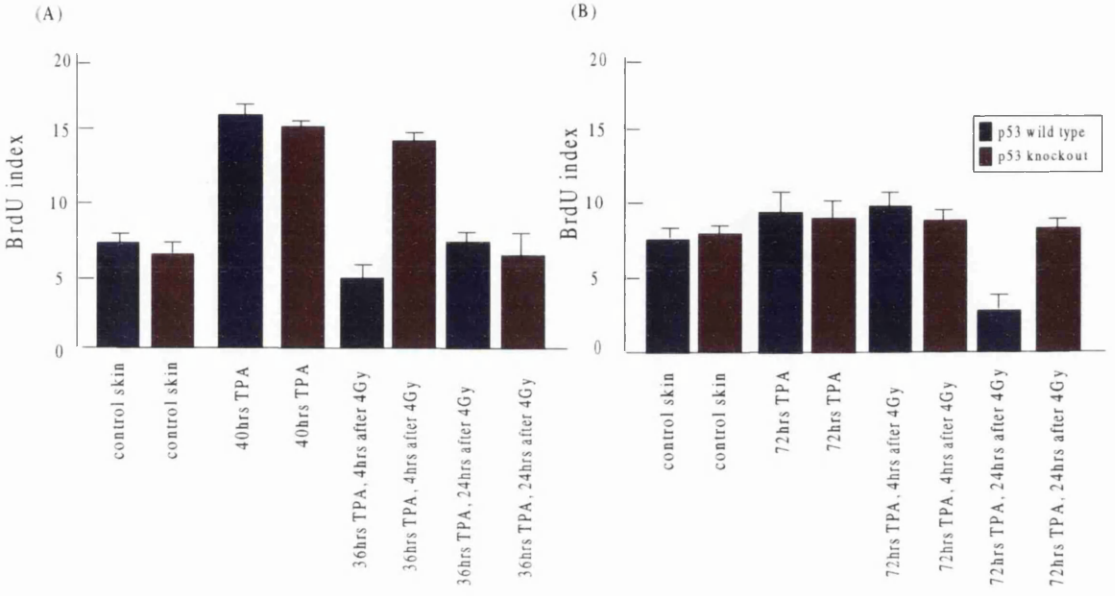
(A) p53 wild type two week old mouse skin
(B) p53 knockout two week old mouse skin

BrdU incorporation falls 4hrs after exposure to 4Gy in the follicular cells of p53 wild type two week old mouse skin. Interfollicular cells show a drop in BrdU incorporation 24hrs after exposure to 4Gy. No change in BrdU incorporation is observed in p53 knockout mouse skin at any time point after exposure to 4Gy.

Six skin sections were analysed per time point. BrdU labelling and analysis was performed as described in chapter 2. Staining was performed in triplicate for all samples.

BrdU index= The number of BrdU positive cells per section divided by the length of the section in millimeters.

Figure 4.18. BrdU incorporation in p53 wild type and knockout adult mouse skin after exposure to TPA and 4Gy.



(A) p53 wild type and knockout adult mouse skin pretreated with TPA for 36hrs before exposure to 4Gy ionising radiation. Skin sections were removed 4hrs and 24hrs after radiation exposure.

(B) p53 wild type and knockout adult mouse skin pretreated with TPA for 72 hours before exposure to ionising radiation. Skin sections were removed 4hrs and 24hrs post radiation treatment.

Both p53 wild type and knockout adult mouse skin respond to TPA treatment with an increase in interfollicular cell proliferation. BrdU incorporation falls after 4hrs in p53 wild type adult mouse skin pretreated with TPA for 36hrs prior to irradiation. p53 wild type adult skin treated with TPA for 72hrs prior to irradiation shows a drop in proliferation 24hrs after exposure to 4Gy. No change in BrdU incorporation is observed in p53 knockout mouse skin pre treated with TPA before being exposed to 4Gy
Three skin sections were analysed per time point. BrdU labelling and analysis was performed as described in materials and methods.
Staining was performed in triplicate for all samples.

BrdU index = The number of BrdU positive cells per section divided by the length in millimetres of the section.

4.8 P53 response to ionising radiation and TPA treatment in adult mouse skin.

Irradiated skin of adult mice shows both apoptosis and cell cycle arrest in distinct cellular locations. The creation of p53-LacZ reporter mice (MacCallum, *et al* 1996) allowed analysis of p53 transcriptional activity in areas of the skin already identified as being in a state of cell cycle arrest or apoptosis

Much of the data relating to the role of p53 mediated transcription comes from *in vitro* assays using cell lines with uncharacterised genetic background, as a consequence of immortalization and routine tissue culture. This includes the work of Yonish-Rouach, *et al* (1995) & Haupt, *et al* (1995) which was carried out on transfected, established cell lines. The first group found that transcriptional activation by p53 was required for p53 dependant apoptosis, whilst the latter found the opposite. The uncertainty of the genetic make up of the cells used in these studies and the effect of unregulated p53 transcription in the cell, may explain the contradictory results. The reporter mice used in the present study provide a simple *in vivo* model with which to study the role of p53 mediated transcription in a variety of normal tissues and cell types.

Initially, our studies concentrated on the analysis of the *LacZ* line RZ2.8, which contains 5-10 copies of the transgene. However similar results have been obtained with a second line of mice again carrying 5-10 copies of the transgene.

The presence of the *Lac Z* transgene was assessed by PCR (Figure 4.19). No *Lac Z* expression could be detected in unirradiated *LacZ* mice or *Lac Z* positive mice which received 4Gy ionising radiation. (Figure 4.20A). It is possible that *Lac Z* expression may occur later than initial p53 induction, since it may take additional time for stabilised p53 protein to turn on the expression of the *Lac Z* gene. However, even 48hrs after radiation exposure no *Lac Z* expression could be detected in either the follicular or interfollicular epidermis of *Lac Z* positive mouse skin (Figure 4.20B).

Preliminary experiments using ultraviolet radiation also failed to induce transgene expression in the skin of the reporter mice.

It is clear from the positive staining results obtained from the skin of transgenic mice containing a keratin 5 gene promoter fused to the *Lac Z* gene (D.Strathdee University of Glasgow, PhD thesis 1996) that *Lac Z* can be used as a marker of gene activity in the skin (Figure 4.21A).

Lac Z staining was observed in a number of other tissues following radiation including the spleen, small intestine, and salivary gland (4.22B). However, expression was absent in the kidney and liver after radiation. This result highlights the cell type specificity of the p53 response and subsequent down stream events *in vivo.*

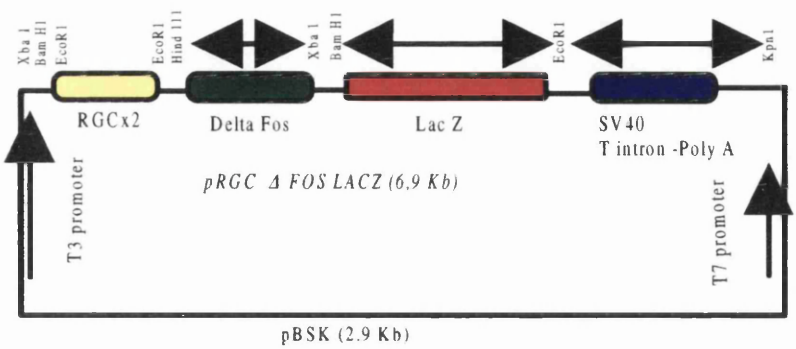
It has been suggested by previous studies that changes in the phosphorylation status of p53 may alter the transcriptional activity of the protein. Phosphorylation by Cyclin A/cdk2 and Cyclin B/cdc2 allows p53 to discriminate between different DNA binding sites (Wang *et al* 1995). This observation led to the suggestion that p53 may have altered transcriptional activities during the S and G2/M phases of the cell cycle when these complexes become active. Based on these observations, it was decided to look at *Lac Z* expression in TPA treated *Lac Z* positive mouse skin since TPA is known to phosphorylate p53.

It was predicted that TPA induced alterations in phosphorylation of p53 would act to alter the transcriptional activity of the protein and mediate the upregulation of $p21^{waf1}$. This would go some way to explain the rapid induction of a cell cycle block in irradiated TPA treated adult mouse skin observed after 4hrs in the BrdU labelling studies. However no *Lac Z* expression was detected up to 48hrs following radiation treatment of TPA treated *LacZ* reporter mouse skins. This was consistent for a wide range of exposure to TPA (36hrs-72hrs) Additional radiation treatment following TPA treatment also failed to induce *Lac Z* expression.

Analysis of this data is open to diverse interpretation. However if these results are a true representation of the skin after DNA damage, our system represents a unique model in which to study cell cycle arrest and apoptosis as a consequence of p53 induction independent of downstream target gene activation.

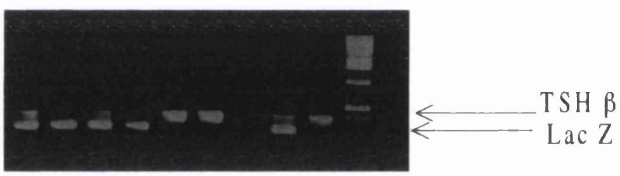
Figure 4.19a. Plasmid map of *pRGC D Fos Lac Z*

(A)



Constructed by T.Frebourg, SH Friends Laboratory,
MGH cancer center Boston M.A
USA
pRGC ΔFos Lac z ; Ribosome
gene cluster containing a p53 binding
site, the murine fos promoter and Lac Z

(B) **Figure 4.19b. PCR analysis of a litter derived from a LacZ 2.2.2, FVB cross**



Primers lz1 CGC TTT AAT GAT TTC AGC C
lz2 AGT TCA ACC ACC ACC GCA TAG

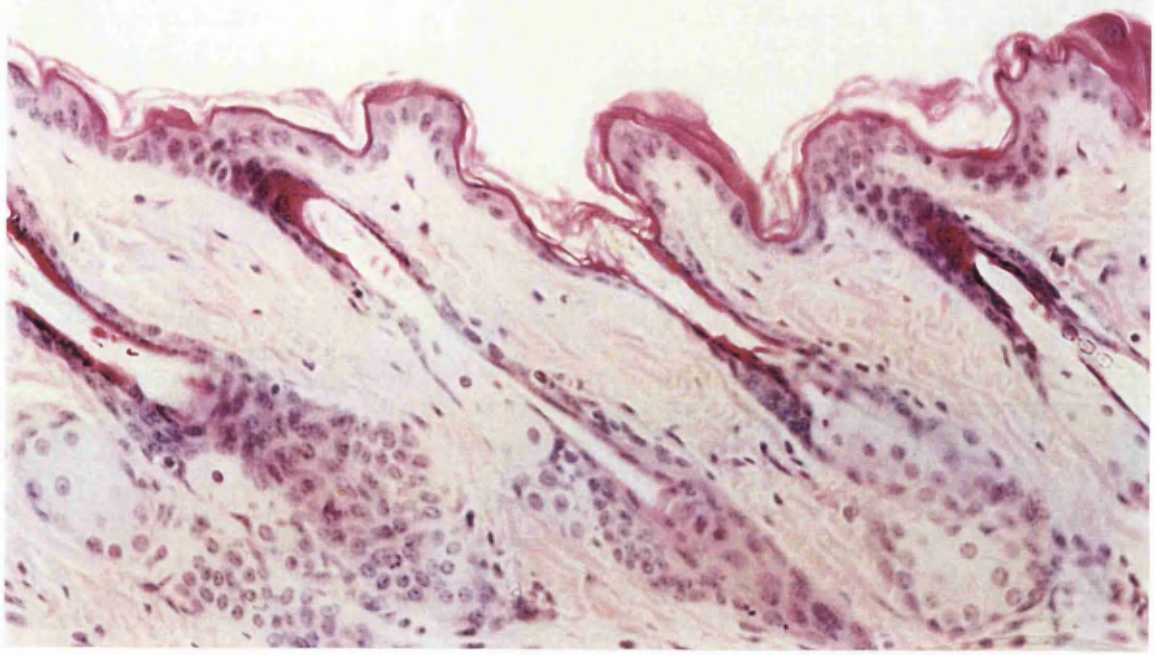
Primers hsp1 TCC TCA AAG ATG CTC ATT AG
hsp2 GTA ACT CAC TCA TGC AAA GT

The PCR reactions for each allele were performed seperately and mixed before running on an agarose gel. The PCR reactions for both alleles were performed under standard conditions on a Perkin Elmer Gene Amp 9600 PCR machine.

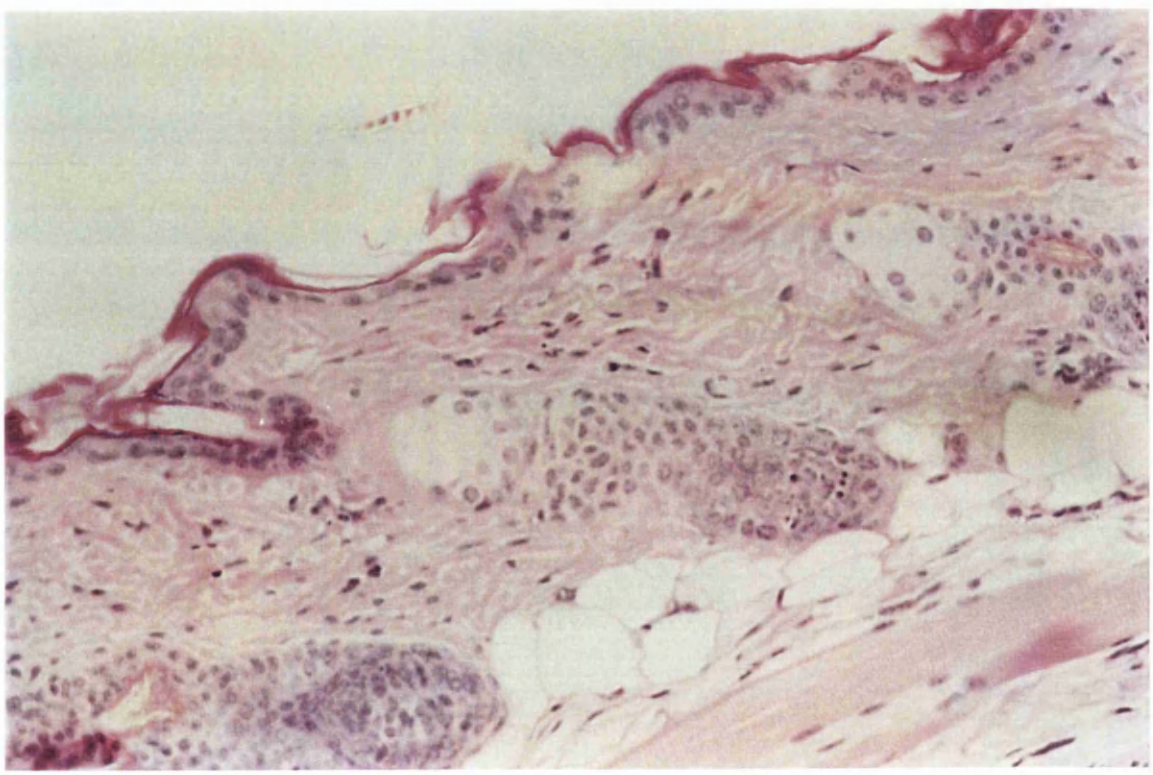
Expected fragment sizes : Lac Z 257
TSHβ 386

Figure 4.20 H&E lac Z stained p53 reporter mouse skin skin before and 4hrs after exposure to 4Gy

(A)



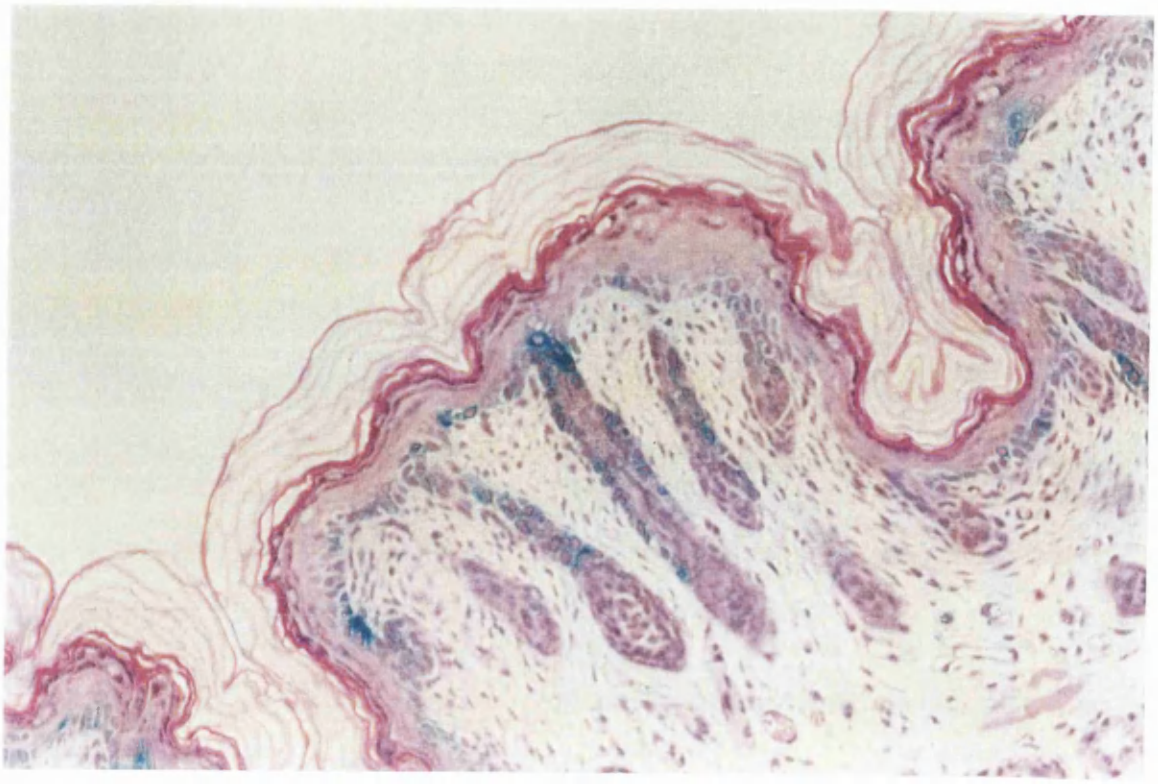
(B)



(A) H&E/ Lac Z stained section of p53 RGC delta Fos Lac Z adult mouse skin before exposure to 4Gy. (Mag X 40)

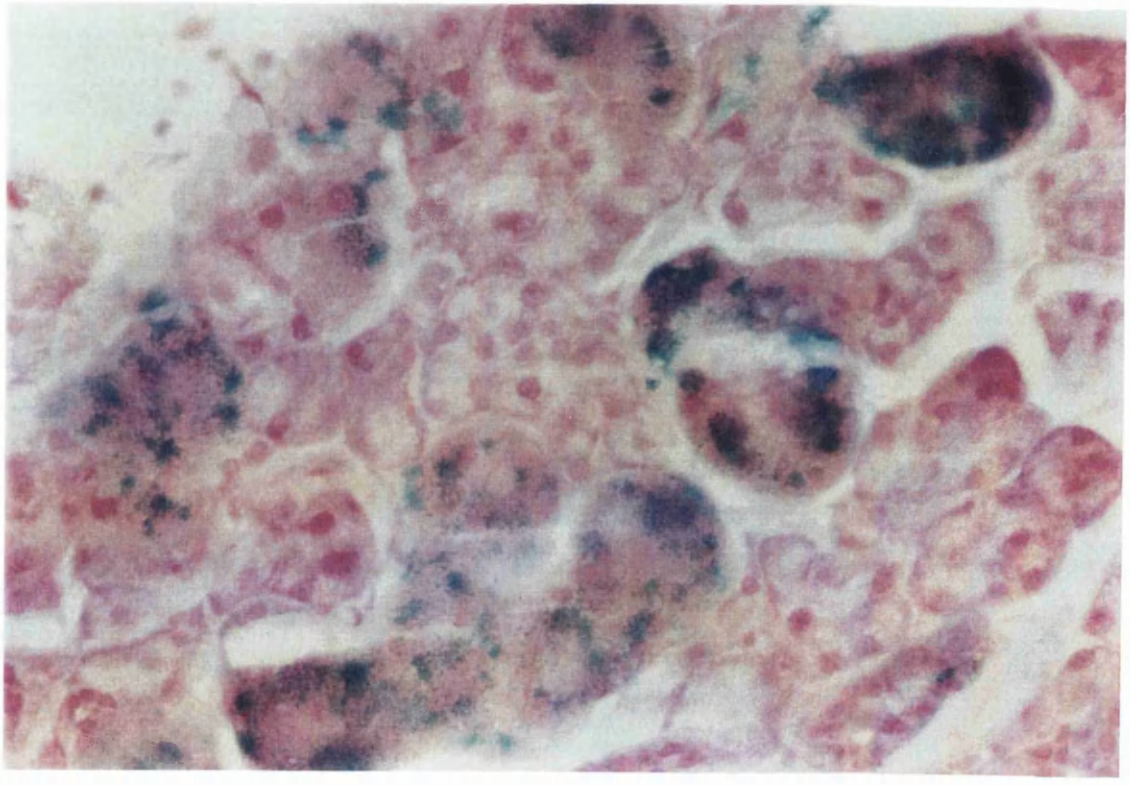
(B) H&E/ LacZ stained section of p53 RGC delta Fos Lac Z adult mouse skin 4hrs after exposure to 4Gy. (Mag X40)

Figure 4.21a H&E stained K5-Lac Z stained skin



Magnification x20

Figure 4.21b H&E stained LacZ reporter mouse salivary gland 18hrs after exposure to 4Gy



Magnification x40

4.9 The effect of radiation on mouse strains with mutations affecting skin morphology.

Nude mice are devoid of hair, although unlike hairless mice, they do have hair follicles (Flannagan 1966). Recent data lends support to the idea that the mutated gene responsible for the nude phenotype codes for a transcription factor which plays a critical role in keratinocyte growth and differentiation. Brissette, *et al* (1996), suggested that the number of keratinocyte stem cells and their differentiated progeny may be altered in nude mouse skin.

We have shown that radiation induced apoptosis occurs in the skin of normal mice in the matrix region of the hair follicle, however the stem cell capacity of hair follicle matrix cells *in vivo* is unclear. This contrasts with the more clearly defined murine intestine model, in which there is an established relationship between intestinal stem cells and susceptibility to p53 mediated radiation induced apoptosis (Merritt *et al.*, 1994). Using mouse skin as a model, the hypothesis of Brissette *et al* was tested utilising radiation induced apoptosis as a marker of stem cell populations.

Following exposure to 4Gy the number of apoptotic bodies in the nude mouse hair follicles increased dramatically, reaching a peak 4hrs after radiation treatment (Figure 4.22). As in all other cases, the increase in apoptosis was restricted to the hair follicles, whilst p53 staining was widespread. The location of apoptotic bodies in the nude mouse follicles was not restricted to the matrix cell population of the follicle, but was spread amongst the inner and outer root sheath cell populations (Figure 4.23). MF1 nude mice carrying one copy of the nude mutation show an increase in apoptosis levels approximately half of that observed in mice carrying two nude mutations (Figure 4.24.). *In situ* DNA labelling was used to confirm the presence of apoptotic bodies in the hair follicles of nude mice following exposure to 4Gy (Figure 4.25). The increase in apoptotic bodies observed in nude mouse skin was not related to increased cell populations in the nude mouse follicle, since wild type mouse and nude mouse follicles displayed on average, similar numbers of cells

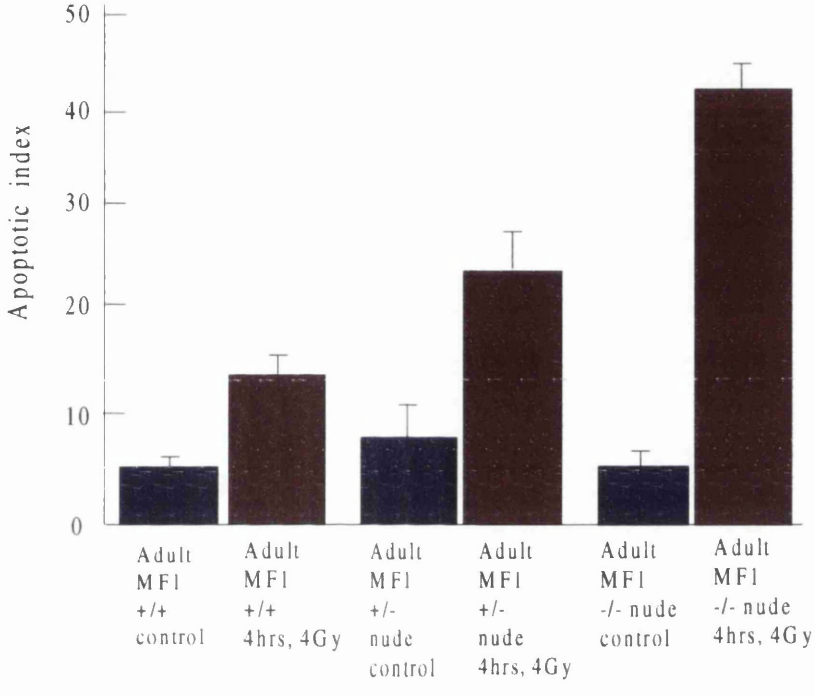
These results suggest that nude mouse skin may have an abnormal pattern of cellular differentiation, and go some way to endorse the proposal that, in the hair follicle, cell

type specific apoptosis occurs after exposure to ionising radiation and this may be related to the differentiation status of the cell. The increased apoptosis observed in nude mouse skin, compared to wild type mouse skin after exposure to radiation may be due to abnormal cell cycle patterns in the nude mice keratinocytes. Further experiments are required to elucidate these proposals

4.10 The hairless mouse.

Hairless mice have skin devoid of hair, Primitive hair follicles are however present in the skin (Gonzalez *et al.*, 1994). We reasoned that, similar to nude mice, hairless mice would have an abnormal follicular cell population, and that this may be reflected in radiation susceptibility. The primitive hair follicle structures of hairless mice show a slight increase in apoptotic bodies 4hrs after 4Gy (Figure 5.4). The irregularity of the follicle structure however, made it impossible to establish a precise location of the apoptotic bodies within the follicles. MF1 hairless mice showed an increase in apoptotic bodies similar to that observed in 129/NIH p53 wild type mice following radiation (Figure 5.4). It is interesting to note the difference in basal levels of apoptosis found in unirradiated MF1 wild type and MF1 hairless 1 mice. Adult hairless mice have lower levels of apoptosis than normal control MF1 mice. This may be the result of having reduced populations of cells which undergo apoptotic cell death in normal circumstances, i.e in telogen phase of the cell cycle, including matrix cells.

Figure 4.22. The apoptotic response in the skin of wild type, heterozygote and homozygous nude MF1 mice to 4Gy.



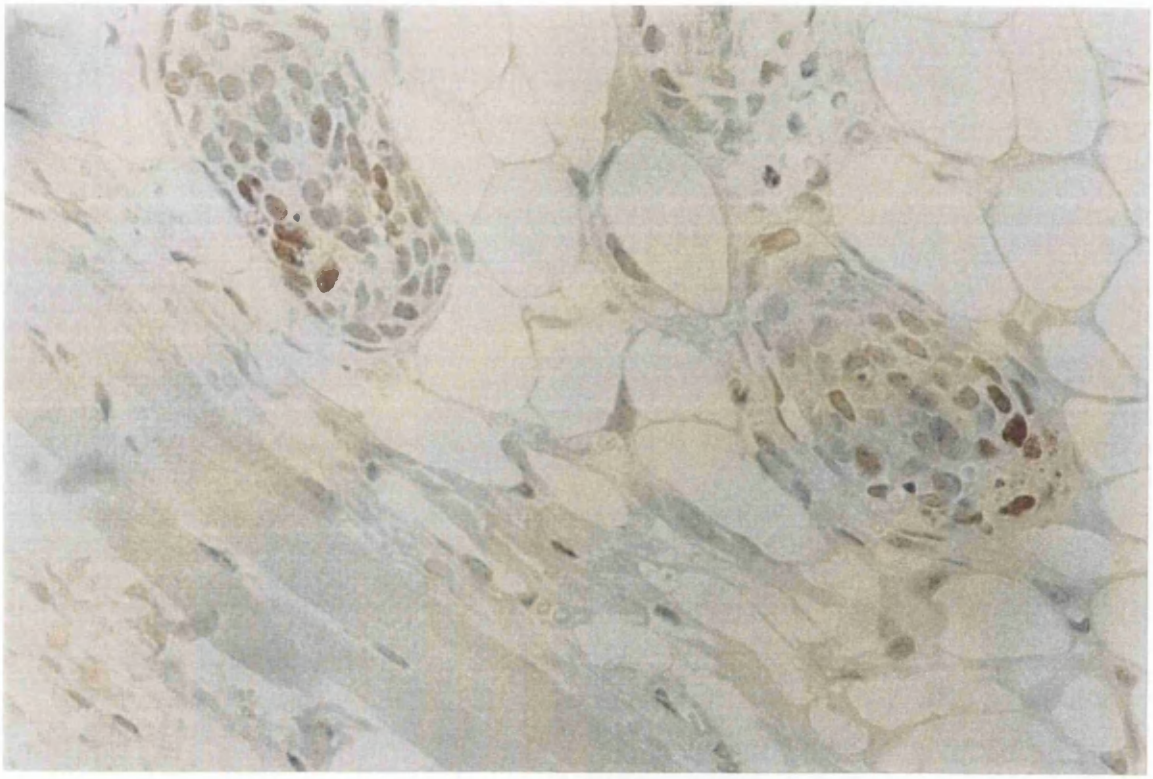
Apoptotic index of MF1 wild type, MF1 +/- nude, and, MF1-/- nude mice 4hrs after exposure to 4Gy.

The increase in apoptotic bodies observed in homozygous nude mice 4hrs after 4Gy is significantly greater than the increase observed in MF1 wild type and MF1+/-nude mice.
Three skin sections were analysed per time point. Skin sections were stained with H&E. Apoptosis was scored by the presence of darkly staining apoptotic bodies.
Apoptotic index = The number of apoptotic bodies per skin section divided by the length of the section in millimetres

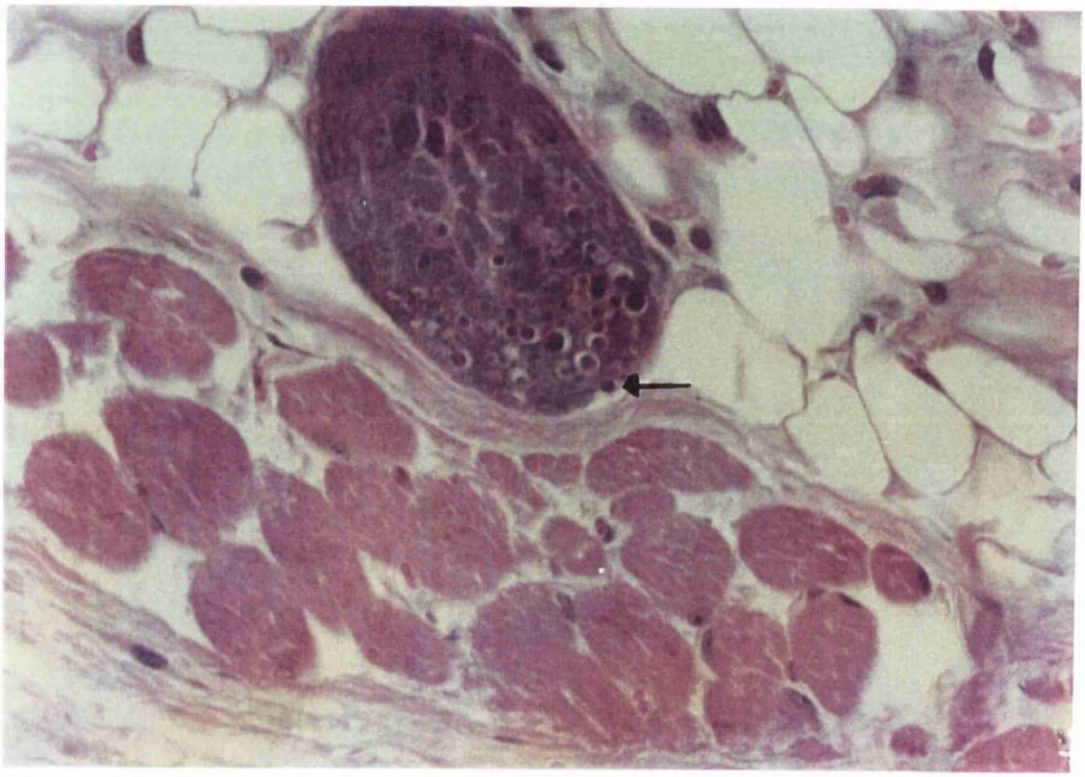
Figure 4.23B. Arrow indicates apoptosis in the outer root sheath of a nude mouse hair follicle 4hrs after exposure to 4Gy.

Figure 4.23 p53 and apoptotic response of nude mouse hair follicles before and 4hrs after exposure to 4Gy.

(A)



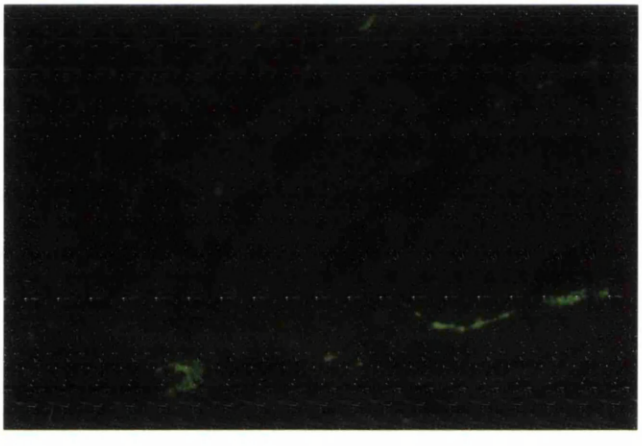
(B)



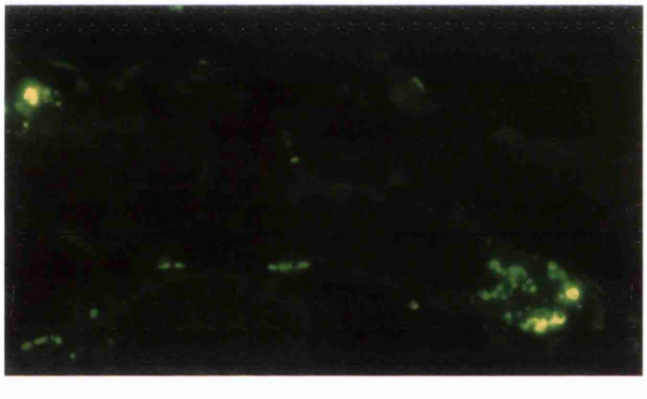
(A) CM5 stained adult nude mouse skin 4hrs after 4Gy.
(B) H&E stained adult nude mouse 4hrs post4Gy.
The hair follicles of nude mouse skin show intense p53 staining and widespread apoptosis 4hrs after 4Gy.

Figure 4.24 ***In situ*** **DNA labelling of homozygous nude mouse hair follicles before and 4hrs after exposure to 4Gy.**

(A)

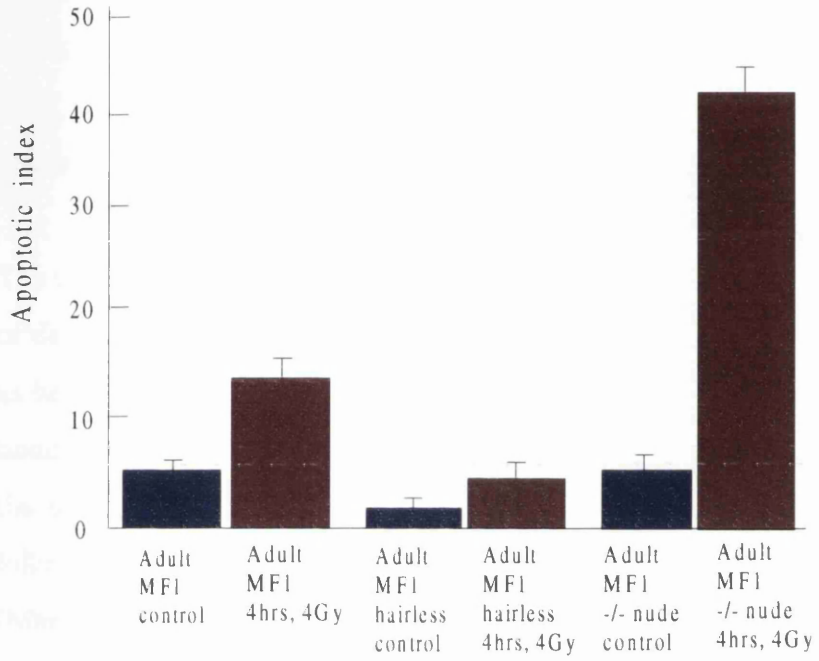


(B)



(A) Homozygous nude mouse hair follicles before exposure to 4Gy
(B) Homozygous nude mouse hair follicles 4hrs after exposure to 4Gy.
The position of the fluorescence correlates with the position of apoptotic bodies visible in haematoxylin & eosin stained sections. Magnification X40

Figure 4.25 The apoptotic response in the skin of wild type, hairless and nude MF1 mice before and after exposure to 4Gy.



Apoptotic index of MF1 wild type, MF1 hairless, and, MF1 nude mice 4hrs after exposure to 4Gy.

The increase in apoptotic bodies observed in homozygous nude mice 4hrs after 4Gy is significantly greater than the increase observed in MF1 wild type and MF1 -/- hairless mice.
Hairless mouse skin shows a modest increase in apoptotic bodies 4hrs after 4Gy in comparison to MF1 wild type control mouse skin

Three skin sections were analysed per time point. Skin sections were stained with H&E. Apoptosis was scored by the presence of darkly staining apoptotic bodies.
Apoptotic index = The number of apoptotic bodies per skin section divided by the length of the section in millimetres

5.1 p53 and apoptosis in DMBA, TPA treated adult mouse skin.

Given the radiation specific p53 response in mouse skin epidermal cells, and the accumulating data linking this cellular phenomenon to stem cells and differentiation status, we felt we should investigate the role of p53 dependent apoptosis in damaged cells, particularly those damaged cells which possess the ability to form tumours upon promotional stimuli.

The mouse skin tumorigenesis model is an ideal system in which to study the progression of damaged cells into malignant carcinomas. Various cellular types have been proposed, as being the cells of the skin that when damaged, escape death and go on to form skin tumours. These include cells of the bulge region of the outer root sheath of the follicle, the matrix region, the interfollicular epidermis, and the inner root sheath of the hair follicle (Sheremer *et al,* 1986; Lenoir *et al,* 1988; Cotsarelis *et al,* 1990). Brown *et al* (Manuscript submitted 1997) recently proposed that a continuum of target cells exist in the mouse epidermis, most primitive cells, giving rise to very malignant tumours, whilst more differentiated cells would give rise to low risk benign skin tumours.

A DMBA/radiation/TPA skin carcinogenesis strategy was devised. This was used to investigate the role of matrix cells and other cell types which are susceptible to p53 induced apoptosis following DNA damage, in chemically induced mouse skin carcinogenesis (Quintanilla *et al* ,1986). p53 wild type and knockout mice were treated with an initiating dose of DMBA and left for one week before being exposed to 4Gy ionising radiation. 24hrs later the mice were promoted with TPA twice weekly for 20 weeks. Our hypothesis was that loss of DMBA initiated cells from cell populations susceptible to p53 mediated radiation induced apoptosis would result in fewer tumours. Lack of p53 in DMBA - irradiated p53 knockout animals would facilitate the uncontrolled proliferation and ultimate tumour formation of cells with malignant potential. Firstly the effect of individual DMBA and TPA treatments on mouse skin was assessed.

Levels of p53 increased in the skin of DMBA treated p53 wild type mouse skin. p53 staining was particularly intense half way up the hair follicle (Figure 5.1B). Apoptotic

bodies were also present in this region of hair follicle five days after DMBA treatment (Figure 5.1 A). No apoptosis was observed in p53 knockout mouse skin treated with DMBA. The number of apoptotic bodies per follicle in p53 wild type and knockout mouse skin five days after treatment with DMBA is summarised in Table 3. p53 wild type mouse hair follicles showed on average 10 fold more apoptotic bodies in the hair follicles than wild type acetone control skin. Levels of apoptosis did not change in p53 knockout mouse skins treated with either DMBA or acetone (Figure 5.2 B).

TPA treatment produced an increase in p53 levels in the interfollicular epidermis of p53 wild type mouse skin (Figure 5.3 A&B). Both p53 wild type and knockout mouse skin responded to TPA treatment with increased interfollicular cell proliferation. No alterations in the levels of apoptosis was detected after TPA treatment between the skins of p53 wild type or knockout mouse skin (Figure 5.4).

In a combination of these treatments, for the reasons outlined above, both p53 wild type and knockout mice were initiated with DMBA. Half of each group of mice then received 4Gy one week later. All animals were then treated twice weekly with TPA for twenty weeks, as described in chapter 2. Tumour development was recorded weekly for the duration of the experiment.

p53 wild type mice that received both radiation and DMBA plus TPA developed more papillomas and carcinomas than their unirradiated counterparts (Figure 5.5 A&B). No change in the rate of malignant progression was observed in the papillomas from each group. Similarly, the irradiated p53 knockout mice developed more papillomas than the p53 knockout mice treated with DMBA/TPA alone (Figure 5.6). The difference in papilloma development between irradiated and unirradiated p53 knockout is greater than that observed between the p53 wild type groups. The reasons for this are unclear however, it should be stressed that the number of knockout mice used in the study was restricted to 10 animals in each group due to the restricted availability of p53 knockout animals. The wild type mouse study was carried out using groups of twenty mice.

We did not detect any difference in the histology of tumours between irradiated and non irradiated sets of p53 wild type mice, as summarised in Table 4. The ratio of poorly

differentiated spindle tumours to the more differentiated squamous tumours was similar in both groups of mice. No carcinomas were available for examination from p53 knockout mice.

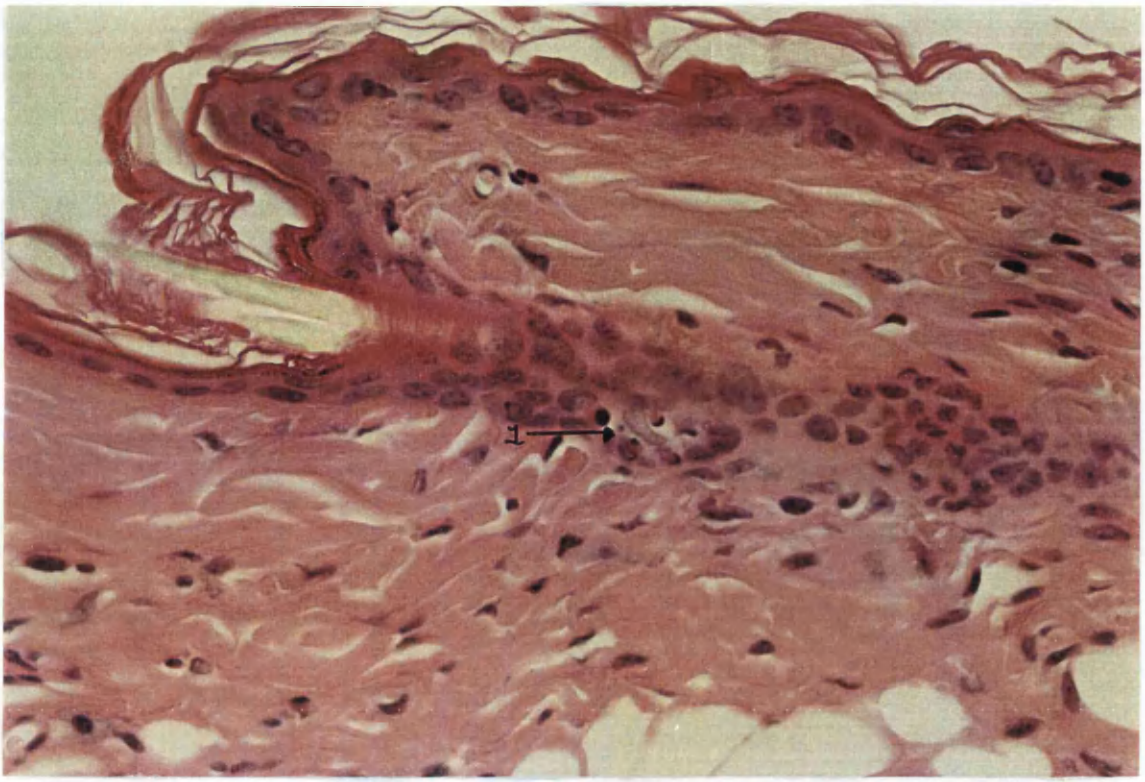
Based on these results, it can be concluded that matrix cells are unlikely to play a part in DMBA/TPA induced mouse skin carcinogenesis. However this may be an over simplistic interpretation of our data, since radiation - induced apoptosis in cells of the immune system, particularly cytotoxic T cells of the skin may, have allowed more initiated cells to evade detection at early stages and become established as tumours. It has been reported previously that dendritic epidermal T cells, which are skin specific members of the tissue type γ δ T cell family in mice, are capable of killing selected tumour cell lines (Mohamadzadeh *et al.*, 1996). This result reinforces the concept that dendritic epidermal T cells represent skin resident killer cells that play a role in local immunosurveilance against tumour development. If this is indeed the case, any effect of radiation- induced matrix cell death on carcinogenesis would be masked by the removal of these cells

Figure 5.1 A.
Arrow 1 indicates apoptotic bodies half way up the hair follicle of p53 wild type adult mice treated with DMBA.

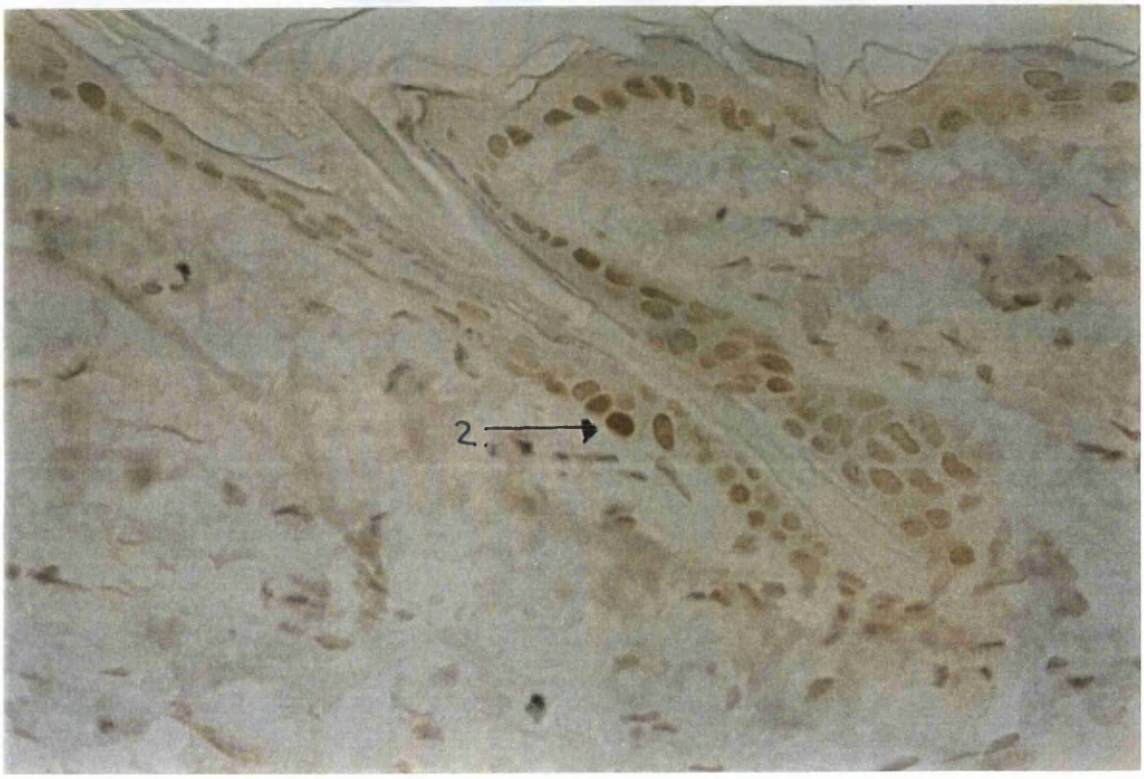
Figure 5.1B
Arrow 2 indicates intense CM5 staining in the same position.

Figure 5.1 CM5 staining and H&E stained adult mouse skin one week after exposure to DMBA.

(A)



(B)



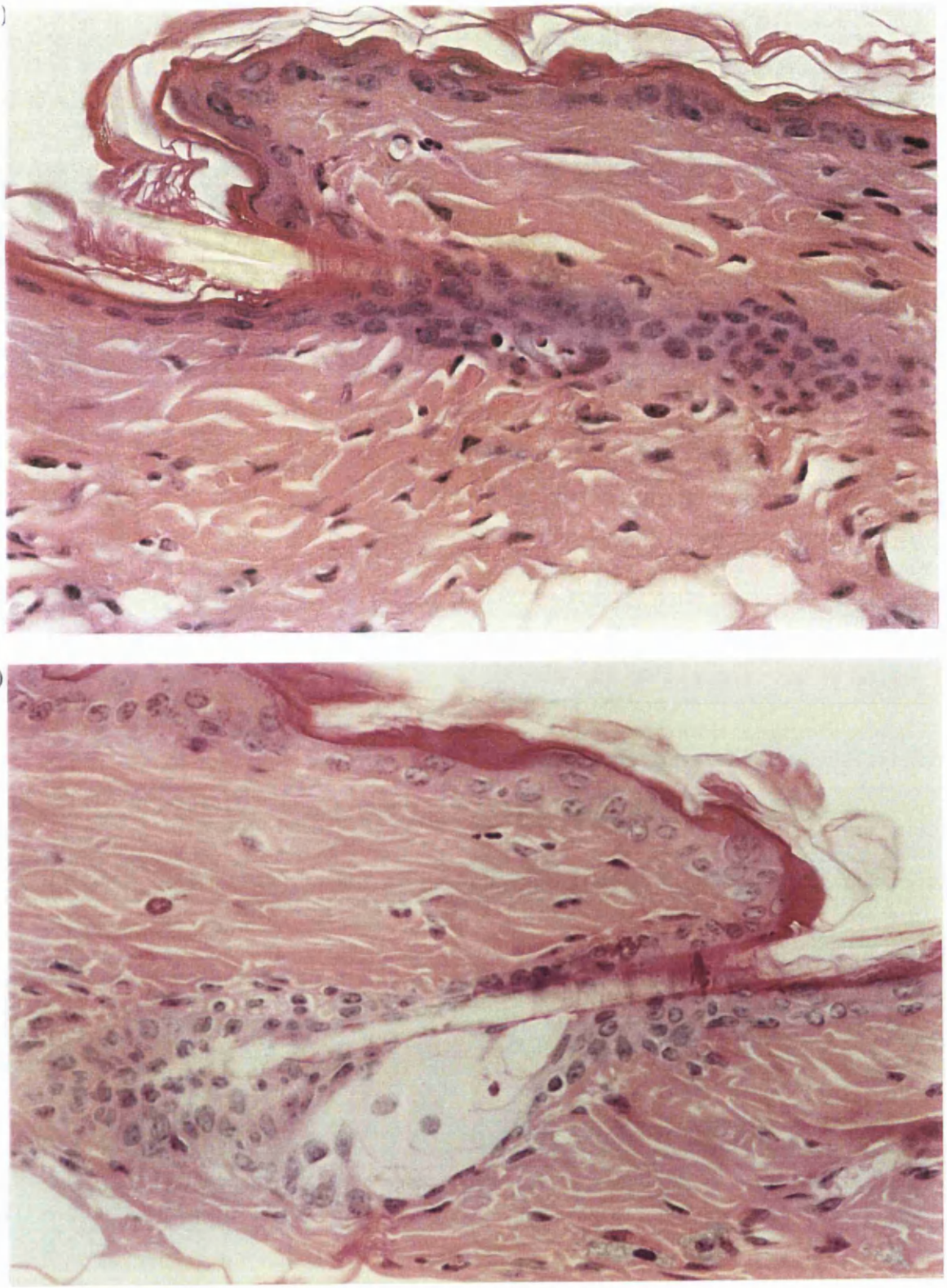
(A) H&E stained P53 wild type adult mouse skin epidermis 1 week after exposure to DMBA
(B) CM5 stained p53 wild type adult mouse skin epidermis 1 week after exposure to DMBA

1 week after exposure to DMBA , CM5 positive cells are present half way up the hair follicles of p53 wild type adult mouse skin. Apoptotic bodies are also present half way up the hair follicle 1 week after DMBA treatment.

CM5 staining and DMBA treatments were performed as described in materials and methods

Magnification X40

Figure 5.2 Comparison of p53 wild type and knockout adult mouse follicles five days after DMBA treatment.



(A) p53 wild type adult mouse skin five days after DMBA treatment.
(B) p53 knockout adult mouse skin five days after DMBA treatment.
Five days after DMBA treatment apoptotic bodies are visible half way up the hair follicles of p53 wild type mouse skin.

Magnification X40

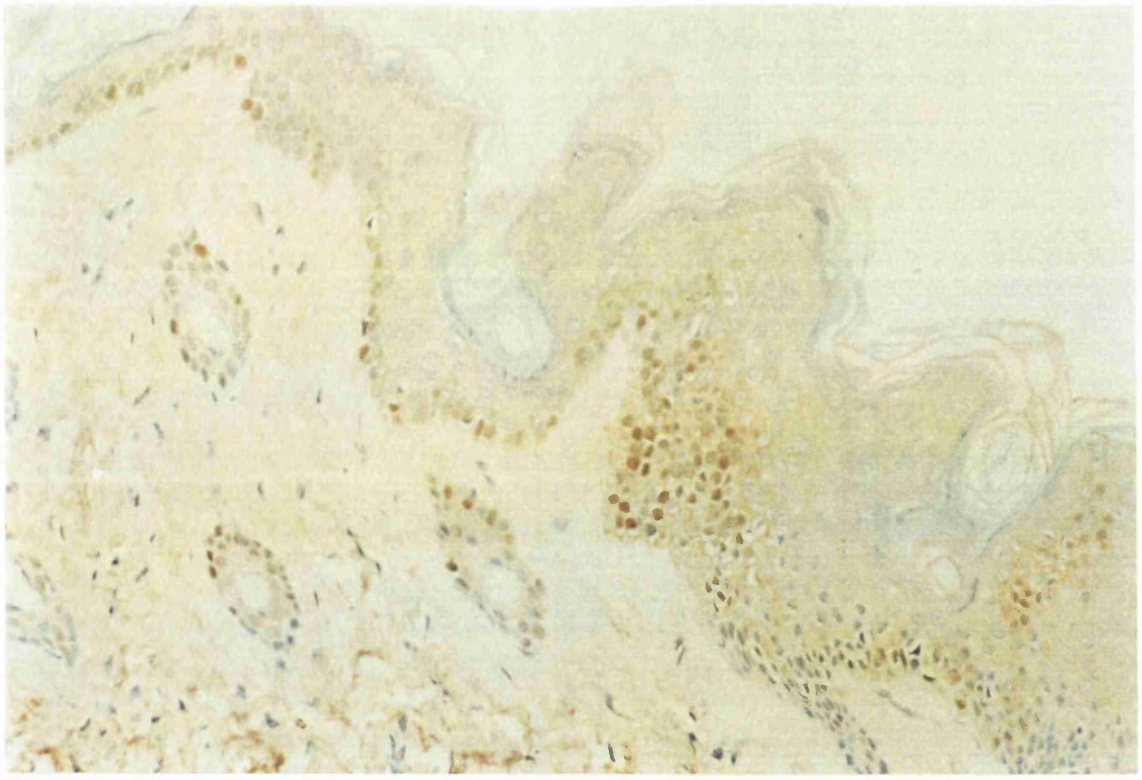
Table 3. Incidence of apototic bodies in p53 wild type and knockout mouse skin treated with an initiating dose of DMBA

	Average number of apoptotic bodies per follicle in p53 wild type mouse skin.	Average number of apoptotic bodies per follicle in p53 knockout mouse skin.
DMBA Treatment	1.3 +/- 0.6	0.4 +/- 0.1
Control skin Acetone alone	0.2 +/- 0.1	0.2 +/- 0.05

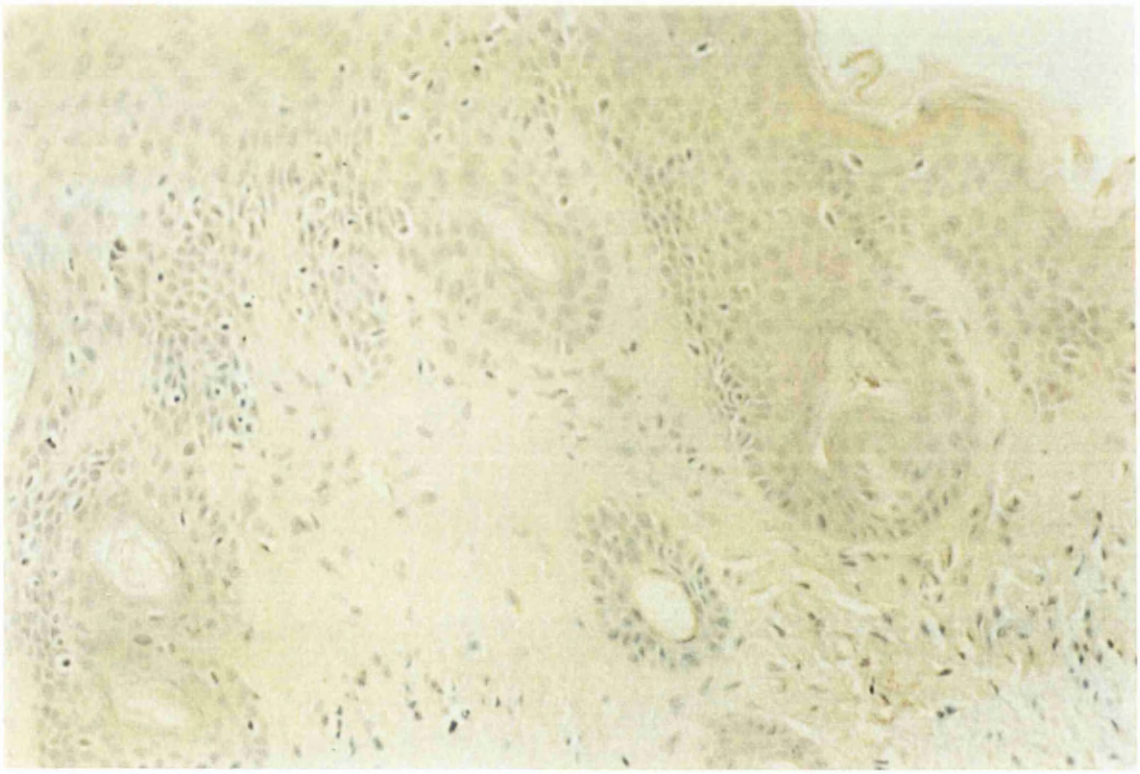
Three mouse skins were counted in each case. Skin sections were stained with Haematoxilin and Eosin. Apoptosis was scored morphologically

Figure5.3 CM5 stained p53 wild type and knockout adult mouse skin 36hrs after TPA treatment.

(A)



(B)



(A) CM5 stained P53 wild type adult mouse skin epidermis 36hrs after two treatments with TPA

(B) CM5 stained p53 wild type knockout adult mouse skin epidermis 36hrs after two treatments with TPA

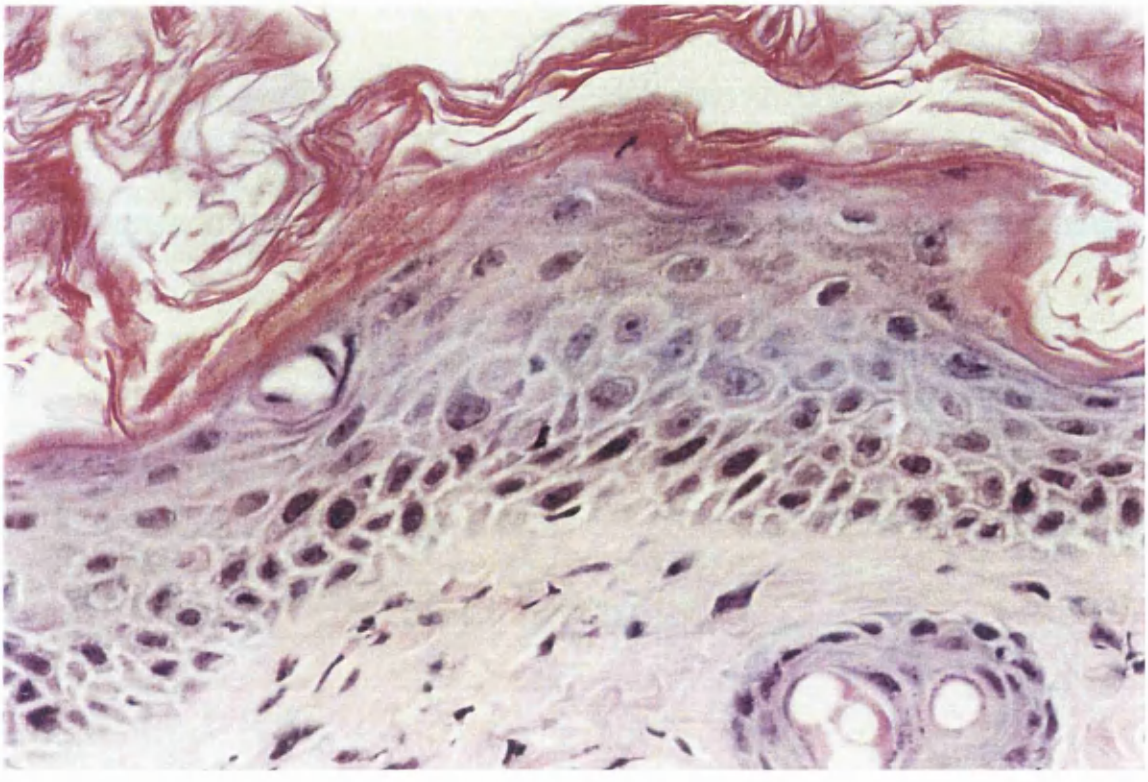
36hrs after exposure to TPA, p53 is induced in both follicular and interfollicular cells of adult wild type mouse skin.

CM5 staining and TPA treatments were performed as described in materials and methods

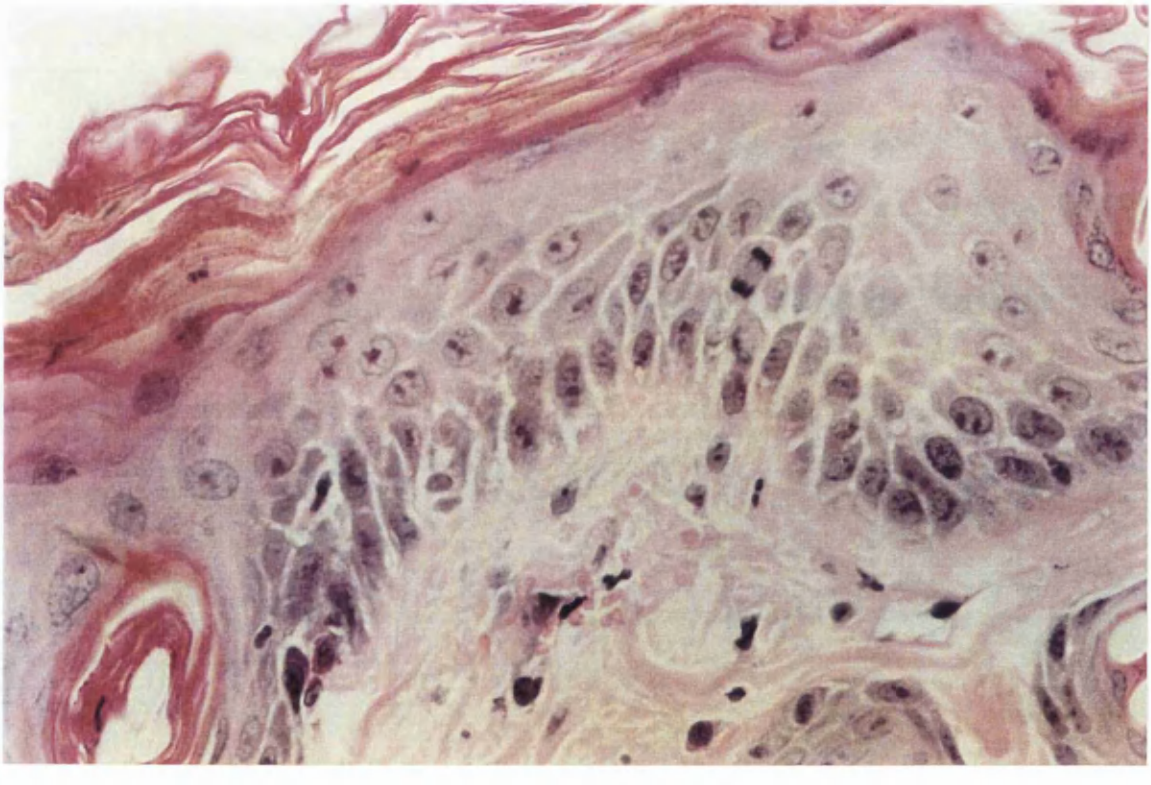
Magnification X40

Figure 5.4 H&E stained p53 wild type and knockout adult mouse epidermis 36hrs after TPA treatment.

(A)



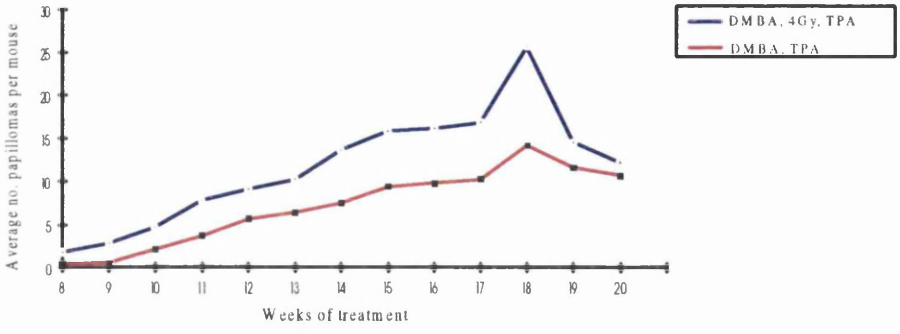
(B)

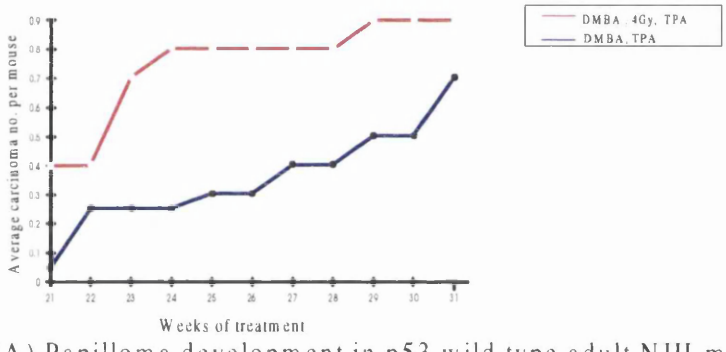


(A) H&E stained p53 wild type adult mouse skin 36hrs post TPA treatment.
(B) H&E stained p53 knockout adult mouse skin 36hrs post TPA treatment.
Both types of mouse skin respond to TPA treatment with increased interfollicular cellular proliferation.

Magnification X40

Figure 5.5. Papilloma and carcinoma development in p53 wild type mice treated with DMBA/4Gy/TPA and DMBA/TPA alone





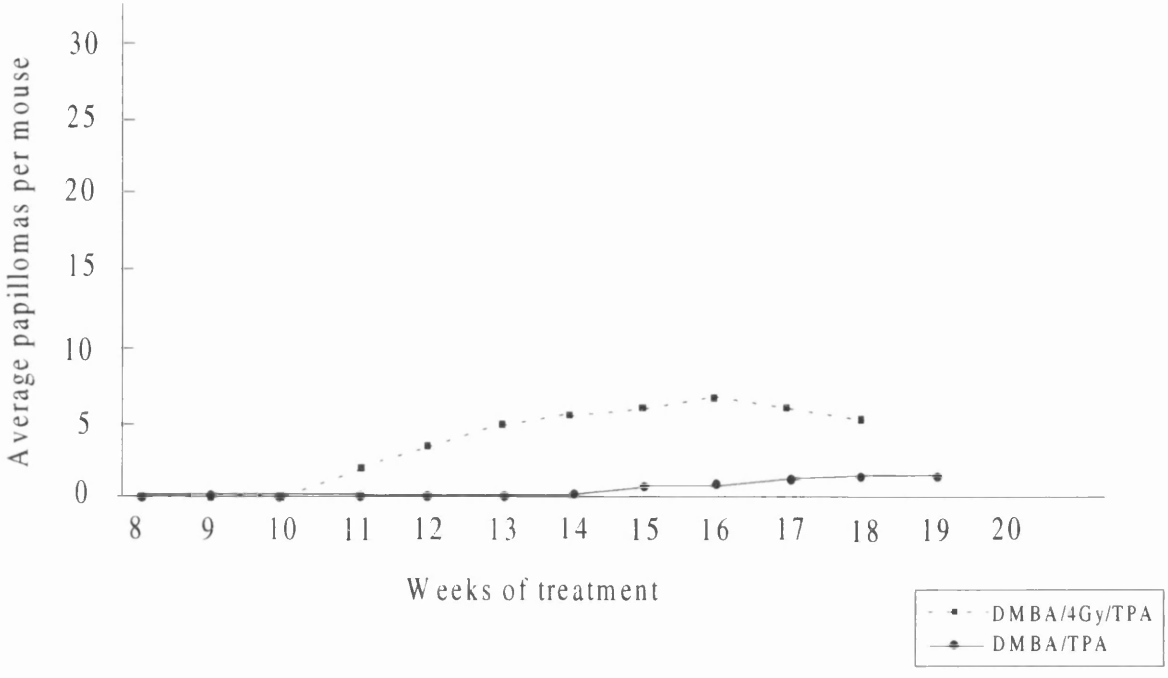
(A) Papilloma development in p53 wild type adult NIH mice treated with DMBA/4Gy/TPA or DMBA/TPA alone.
(B) Carcinoma development in p53 wild type adult NIH mice treated with DMBA/4Gy/TPA or DMBA/TPA alone.
Mice were treated with DMBA/TPA/4Gy as described in Materials and Methods. Papilloma and Carcinoma development was recorded weekly.
Adult mice were treated with DMBA, TPA and 4Gy as described in materials and methods.
Mice treated with DMBA/4Gy/TPA develop significantly more papillomas and carcinomas than mice treated with DMBA/TPA alone

(C) Histology of carcinomas available from p53 wild type DMBA/4Gy/TPA and DMBA/TPA experiment.

	squamous	*spindle*
DMBA 4Gy TPA	11	4
DMBA TPA	10	5

The histology of carcinomas taken from DMBA/4Gy/TPA treated animals is similar to carcinomas taken from DMBA/TPA treated animals

Figure 5.6 Papilloma development in p53 knockout mice treated with DMBA/4Gy/TPA and DMBA/TPA alone.



Papilloma development in p53-/- adult mice treated with DMBA/4Gy/TPA or DMBA/TPA alone.
p53-/- mice treated with DMBA/4Gy/TPA develop more papillomas than mice treated with DMBA/TPA alone.

Adult mice were treated with DMBA/TPA/ 4Gy as described in Materials and Methods. Papilloma development was recorded weekly

Chapter 6. Discussion

6.1 p53 and cellular immortalization

Normal diploid cells have a limited capacity to divide both *in vivo* and *in vitro*. In contrast, genetically transformed cells or cells transformed by chemical or biological agents appear to have lost the regulatory mechanisms of limited growth potential, thus becoming immortal. During routine growth, normal diploid cells undergo a period of rapid proliferation followed by progressive loss of replicative capacity, as reflected in slower population growth rates. The population finally reaches a state of growth arrest and almost all the cells fail to divide. Late passage non proliferating cells are characterised by increased size, cell spreading, and modified organisation of the cytoskeleton and the extra cellular matrix (Holliday *et al*, 1995).

The oncogenic products of DNA tumour viruses, such as the T antigen of SV40 and Polyoma Viruses, the E1A oncoprotein of some adenoviruses and the E7 oncoprotein of types -16 and -18 human papilloma viruses (HPV), together with some retroviral gene products and their activated cellular homologues (e.g. *v*-or *c- myc*, *v*- or *c- fos*) can overcome and cancel the proliferation block of senescent cells in several mammalian cell types, a process which is termed immortalization. As discussed in the chapter 1, p53 serves as a cellular target for the gene products of many DNA tumour viruses. This led to investigation of the role of p53 in cellular immortalization.

It has been reported that cdc2, cyclin A, and PCNA are not induced in senescent cells (Stein *et al.*, 1991), furthermore it has been shown that inactive cyclin E-cdk2 and cyclin D1- cdk2 complexes accumulate in senescent human diploid fibroblasts (Dulic *et al.*,1993). The universal cyclin dependent kinase inhibitor $p21^{waf1}$, which is transcriptionally activated by p53 has been implicated in cellular senescence. $p21^{waf1}$ was shown to be over expressed in senescent cells, which provided a link between p53 and cellular immortalization (Noda *et al.*, 1994). An increase in $p21^{waf1}$ levels would account for the defect in activities of cyclin cdk complexes and the absence of pRb phosphorylation at the G1-S boundary. A single gene, named *hic-5,* has recently been isolated This gene can induce senescent like phenotype in immortal human fibroblasts (Shibanuma *et al.*, 1997). The induction of senescence by *hic-5* is accompanied by an

increase in $p21^{waf1}$ levels. Thus, two genes may work together to accomplish cellular senescence.

p53 mRNA levels do not change as cells age *in vitro*. In contrast, transcriptional activity increases several fold during ageing (Atadja *et a.*, 1995). It would appear that the signals which link DNA damage to increased p53 expression are not activated when human fibroblasts begin to senesce. However DNA damage is widespread in senescing cultures as typified by the high number of chromosomal aberrations which increase during the serial passaging of cells.

The results presented here suggest that lack of p53 in keratinocytes *in vitro* is prohibitive for the increase in $p21^{waf1}$ protein levels observed in a large number of senescent cell populations. As a consequence a greater number of p53 knockout keratinocytes show increased ability to escape senescence compared to p53 wild type keratinocytes.

The role of other cyclin dependent kinase inhibitors which are not under the control of p53 in senescence should be considered in analysing why p53 knockout cells should enter the senescence pathway. These genes include *p15(ink4b/mts-2), p16 (ink4a/mts/cdkn2), p27(kip-1)* and *p57 (kip-2)* . The *p15* and *p16* genes have been located in human chromosomal region 9p21, adjacent to one another (Nobori *et al.*, 1994). Both genes have the ability to act as inhibitors of cdk4 and cdk6 and block proliferation in cells possessing functional RB. *p27* is a potent inhibitor of cyclinD -cdk4 and cyclin A- cdk2 activity, overexpression of which causes G1 arrest in Saos-2 cells (Toyosima *et al,* 1994). The *p57* gene is a potent inhibitor of G1 and S phase cdks and has been found to be overexpressed in terminally differentiated cells (Lee *et al,* 1995). Most of the known cyclin dependent kinase inhibitors are overexpressed in senescent cells. To achieve a complete absence of senescence *in vitro,* it is probable that many genes need to be knocked out in various combinations depending upon the cell type. Our results suggest that p53 is only one of the many cellular products that is likely to be involved in the immortalization of mouse skin keratinocytes.

6.2 p53 and TGFβ1

The TGFβ's are a large family of growth inhibitors for a diverse range of cell types. Interruption of the TGFβ1 signalling pathway can occur through multiple mechanisms, including mutational inactivation of the TGFβII receptor and post receptor changes in genes potentially mediating the TGFβ1 pathway (For review see Brattain *et al.,* 1996). During the late 1980s, it became apparent that most normal epithelial cells were inhibited by exogenous TGFβ1 treatment. Some cancer cells were also inhibited by TGFβ1, but many were refractory to the polypeptide. For example, loss of TGFβ1 sensitivity was demonstrated to be associated with the progression of cultured colon carcinoma cells displaying early malignant properties to cells with late progression properties (Chantret *et al.,* 1988).

It has been reported that some tumour cells not only lose responsiveness to growth inhibition by TGFβ1, but gain the ability to produce the growth factor and be stimulated by it. However, carcinogenesis is not simply the result of losing responsiveness to negative growth regulation. A fully malignant cell must gain the ability to invade and display metastatic potential. TGFβ1 is believed to play a role in the cascade of events which leads to activation of proteolytic enzymes thought to be involved in progression to a more invasive phenotype. Therefore, a tumour cell which has lost responsiveness to the growth inhibitory actions of TGFβ1 and gained the ability to produce the growth factor is placed at a selective advantage for tumour progression.

Three major types of membrane bound TGFβ1 binding proteins have been identified. They are referred to as type I, type II, and type III receptors. Molecular cloning and functional analysis have shown that RI, and RII are serine/threonine kinases which are both necessary for TGFβ1 signalling (Wrana *et al* 1994, and references therein). The direct involvement of RI, and RII in TGFβ1 signalling would suggest that loss of RI, or RII expression could contribute to the loss of TGFβ1 the response, resulting in a growth advantage that could contribute to tumour progression. However, due to the stepwise nature of malignant tumour development, it seems likely that there may be a progressive loss of growth control in by TGFβ1 in cancer development involving membrane receptors, cytoplasmic proteins and nuclear factors involved in the signal cascade.

Both mutations in, and loss of, the p53 gene have been correlated by previous studies with the loss of responsiveness to TGFβ1. (Reiss *et al* 1993, Gerwin *et al*, 1992). However, others have shown that p53 status bears no relationship to the degree of responsiveness of a cell to TGFβ1 (Williams *et al*, 1994) Most studies on TGFβ signalling have used cell lines which commonly have alterations in p53, as well as in other tumour suppressor genes and protooncogenes. This makes the interpretation of such results difficult. The utilisation of our p53 knockout keratinocytes has allowed us to conclude that loss of wild type p53 does not lead to a decrease in responsiveness to the growth inhibition by TGFβ1. Perhaps the most convincing evidence for this is that p53 wild type and knockout primary keratinocytes show no difference in response to TGFβ1. Primary cultures less likely to have additional acquired significant genetic alterations due to the short time they spend in culture.

Since p53 knockout animals develop show no defects in skin development, it seems likely that other cellular pathways exist to compensate for a lack of p53 in growth regulation (Donehower *et al.*, 1992).

Although the signal transduction pathways that mediate TGFβ1 induced growth inhibition have not been fully characterised, TGFβ1 is thought to maintain the product of the Retinoblastoma gene in an hypophosphorylated state and block cells in late G1. Furthermore, TGFβ1 reduces the expression of cdk4 and Cyclin E, but not that of cyclin D. This prevents subsequently activation of both Cyclin D-cdk4 and Cyclin E-cdk2 complexes. Alterations in the levels of the universal cyclin dependent kinase inhibitor p21 ^{waf1} have been proposed to be responsible for changes in cyclin D, and cyclin E kinase complex regulation.

Cellular p21^{waf1}protein levels rise following exposure to TGFβ1. (Li *et al*, 1995) We observed an increase in p21^{waf1} in both p53 knockout and wild type keratinocytes after TGFβ1 treatment. Therefore expression of p21^{waf1}may represent an important mediator of TGFβ1 activity in both p53 wild type and knockout keratinocytes. The lower basal levels of p21^{waf1}in p53 knockout mouse keratinocytes, compared to p53 wild type

keratinocytes, is probably due to the lack of transcriptional activation in the p53 knockout cells. However in p53 knockout keratinocytes, $p21^{waf1}$ levels are capable of rising to levels comparable to those observed in p53 wild type keratinocytes following TGFβ1 treatment. This non p53 mediated pathway has the ability to produce levels of $p21^{waf1}$ capable of blocking cell cycle progression.

Given the broad spectrum of cdks which $p21^{waf1}$ is capable of inhibiting, it is not surprising that more than one level of control exists over its regulation. Although $p21^{waf1}$ appears to be controlled by more than one cellular pathway it is not the only cdk inhibitor to be involved in TGFβ1 arrest. $p27^{Kip1}$ has been shown to be unregulated in certain cell types after TGFβ1 treatment (Ravitz *et al.*, 1996). Again it is hardly surprising that more than one mechanism operates in response to TGFβ1 since growth regulation by this factor is of extreme importance for both normal development and normal tissue homeostasis. Other members of this family may yet be identified. It is unlikely that should any TGFβ1 regulated cdks be discovered, they will be solely under the control of p53.

Our results do not rule out a role for mutant p53 in TGFβ1 signalling. Attempts to introduce a gain of function mutant p53 construct were hampered by its toxic effect on both knockout and wild type keratinocyte cell lines. One attractive possibility is that mutant p53 confers resistance to TGFβ1 by interfering with wild type p53 mediated down regulation of cdk4 leading to the accumulation of unphosphorylated pRb and subsequent cell cycle arrest (Ewan *et al,* 1995). However, in the p53 knockout keratinocytes p53 mediated regulation of cdk4 is absent and other mechanisms of regulation, which may or may not be affected by the presence of mutant p53 are likely to be present. An extension to this study would be to analyse the response of primary keratinocytes from mutant p53 transgenic animals to the growth inhibitory actions of TGFβ1.

The effect of p53 dosage and the presence of mutant *H ras* on TGFβ1 signalling was investigated. This was in response to the growing number of accounts linking *H ras* to the TGFβ1 signalling cascade. Changes in the expression levels of mutant *H ras* have

been linked to loss of responsiveness to TGFβ1 in mouse skin carcinogenesis (Haddow *et al,* 1991). More direct evidence for the role of mutant *H ras* in TGFβ signalling comes from studies on cell lines which show increased H *ras* activity within 10 minutes of exposure to TGFβ1 (Mulder *et al,* 1992).

Curiously, after infection with mutant *H ras* both p53 wild type and knockout keratinocytes displayed cytoplasmic as well as membrane bound forms of mutant H *ras,* (data not shown). This may be a consequence of overexpression of H *ras* protein in these cells or an artefact of the fixation procedure used. After infection with mutant *H-ras* the keratinocyte cell lines showed a limited drop in responsiveness to TGFβ1. Our results do not conclusively elucidate the role of *H-ras* in TGFβ1 regulation, since the infection protocol could not ensure that each cell would produce comparable amounts of H *ras* protein.

It is possible that other cellular oncogenes and tumour suppressor genes cooperate with *H ras* to induce an increased level of resistance to TGFβ1. The MAD-related family of proteins are essential components in the signalling pathways of serine threonine kinase receptors for the TGFβ1 superfamily. It has been shown that the *MADR2* gene is a tumour suppressor gene located on human chromosome 18q alongside *DPC4,* which is yet another potential component in TGFβ1 signalling. Mutations in *MADR2* and/or loss of chromosome 18q are thought to function to disrupt TGFβ1 signalling in human colorectal cancer (Frank, *et al* 1997). The collaborative effect of ras mutation, p53 mutations, and alterations in MAD related genes may well vary from one cell type to another. The availability of a range of knockout animals and skin targeted *H ras* transgenic animals provide an ideal model with which to study the factors that co-operate to induce total resistance to the growth inhibitory effects of TGFβ1 in mouse skin keratinocytes.

In summary, TGFβ1 signalling is a process which transfers instructions from the cell surface to the nucleus. Loss of any part of this signalling cascade is likely to induce a certain degree of resistance to TGFβ1. The relative importance of each individual part of the cascade may differ from cell type to cell type. Such a scenario would go some way

to explain the diverse and conflicting results published as to the role of *H ras*, p53 and $p21^{waf1}$ and the TGFβ1 receptors in the response of a variety of cell types to TGFβ1.

It is likely that wild type p53 is not an important constituent of the TGFβ1 signalling system in mouse skin keratinocytes. However, p53 may play a pivotal role in TGFβ1 signalling in other cell types. This idea is supported by the recent report that a proportion of progenitor cells in bone marrow of p53 knockout mice are unresponsive to growth inhibition by TGFβ1 (Sasaki *et al*, 1997)

6.3 p53 and cell survival after DNA damage.

After exposure to 4Gy radiation, $p21^{waf1}$ protein levels rise in C5N cells. Recent results from this lab have also shown that p53 protein levels rise before detectable changes in $p21^{waf1}$ levels. (I. Ganly, personal communication). There is no alteration in $p21^{waf1}$ protein levels in NK cells after exposure to 4Gy. After radiation exposure, the increased levels of $p21^{waf1}$ probably lead to cell cycle arrest in G1 as a result of inhibition of cdk activity as previously discussed. Fluorescent activated cell sorter analysis has recently been performed on C5N and NK cells, and has revealed the existence of a G1 block in C5N but not in NK cells following exposure to low doses of ionising radiation.

BAX protein levels remain constant in C5N and NK cells after exposure to radiation. However, C5N cells show a drop in BCL2 protein levels 24hrs after exposure to 4Gy. BAX is widely accepted to counteract the anti apoptotic signal of BCL2. It has been proposed that when BAX homodimers predominate in a cell, the cell is susceptible to apoptosis; the opposite being the case for BCL2 homodimer excess (Oltvali *et al.*, 1993). Collectively, these results suggest that cultured keratinocytes do not enter the apoptotic pathway after exposure to 4Gy. In contrast, if p53 is functionally active, keratinocytes arrest in G1 as a consequence of $p21^{waf1}$ induction.

Kastan and colleagues carried out studies using a cell line (BAF) derived from the bone marrow of an adult mouse. As detailed in the chapter 1 the BAF cell line undergoes apoptosis in response to ionising radiation when IL3 is removed from the culture

medium. In contrast, when IL3 is present and the cells are irradiated, they display a cell cycle block rather than entering the apoptotic pathway. No changes were found in BCL2 or BAX to explain this phenomenon. However, $p21^{waf1}$ levels were increased in the cells that entered the growth arrest state compared to the cells that entered the apoptotic pathway (Canman *et al,* 1995). This is to results showing that removal of $p21^{waf1}$ can cause a switch from growth arrest to apoptosis (Polyak *et al,* 1996)

In the context of this study, it would seem that modulation of $p21^{waf1}$ levels dictates the response of p53 wild type keratinocyte cells to ionising radiation by facilitating a cell cycle block which is not evident in p53 knockout keratinocytes. In this regard it seems strange that both cell lines show comparable survival curves after DNA damage. Initially, this result was unexpected since thymocytes and hematopoietic cells from p53 knockout mice had previously been shown to be more radio resistant than cells from p53 wild type mice. The results presented suggest that p53 is not necessary for the relatively short term survival of mouse skin keratinocytes *in vitro* after DNA damage. It is likely that irradiated p53 knockout keratinocytes would show high levels of genetic lesions compared to irradiated p53 wild type keratinocytes after routine subculture.

6.4 p53 induction and cell death *in vivo.*

The stabilisation of p53 protein in response to genetic damage is well documented *in vitro*. p53 accumulation is thought to be part of a signalling pathway that induces either cell cycle arrest or apoptosis (Reviewed in Hansen and Oren 1997). In the present study, the effect of ionising radiation in mouse skin on the accumulation of p53, cell death, and cell cycle arrest was analysed. Early studies on the radiosensitivity of human and mouse keratinocytes suggested that when primary keratinocytes are routinely cultured they display increased radioresistance (Parkinson, *et al* 1986). Furthermore *in vitro* studies have provided evidence that human fibroblasts are more radioresistant than human keratinocytes (Beverstock and Simons, 1982). It has also been reported that human keratinocytes are more radiosensitive than mouse keratinocytes (Dover and Potten., 1983). However the *in vivo* results presented in this study highlight the dangers of collectively labelling skin keratinocytes as more or less radioresistant than other cell types. It would appear that keratinocyte radiosensitivity is dictated by the differentiation status or cellular fate of the cell *in vivo*.

Low dose irradiation of p53 wild type anagen adult and two week old 129/NIH mouse skin resulted in increased levels of p53 in both follicular and interfollicular cells of the epidermis. Furthermore 4hrs after 4Gy p53 dependent apoptosis was present only in the matrix region of the hair follicle. Surprisingly, no hair loss was observed in animals which were alive for up 40 weeks after 4Gy. Others recently reported maximal induction of apoptosis in the follicle matrix cells of adult Balb C mice 12hrs after exposure to radiation. Furthermore, they also reported that induction of apoptosis in the follicles was proportional to the dose of radiation received (Kim *et al,* 1997). In our study there was no significant difference in the extent of apoptosis induced by either 4-10Gy. Maximal apoptosis occurred 4hrs after exposure to radiation, and in p53 wild type two week old mouse skin levels remained high for 24hrs. We are presently re-investigating the effect of exposure to 10Gy with respect to hair loss in a 129/NIH background, since it is possible that failure to observe increased apoptosis after 10Gy may have been due to increased phagocytotic activity of normal cells at increased DNA damage levels. The finding that p53 heterozygote mice display a degree of apoptotic response 75% of that found in wild type mouse skin after radiation suggests that loss of a single allele of p53 does not result in a 50% fall in p53 mediated apoptosis. This may reflects the importance in evolution of retaining the p53 response.

Merritt *et al,* (1994) analysed the response of the gastrointestinal tract of normal and p53 knockout mice to low doses of ionising radiation. The result of these studies led to the conclusion that p53 dependent apoptosis was restricted to the stem cell compartment of the small intestine. These results were soon confirmed in a separate line of p53 deficient mice (Clarke *et al,* 1994). These observations led to the proposal that p53 plays a role in removal of damaged stem cells with the potential for uncontrolled growth and tumour formation.

In mouse skin, matrix cells are not the most favoured candidate for skin stem cells. The current generally accepted location for the stem cells of the skin is in the bulge region of the hair follicle (Cotsarelis *et al,* 1990). However our results suggest that matrix cells are the population specifically removed after radiation. The role of matrix cells in tumour formation will be considered later.

6.5 p53, cell cycle status and entry into apoptosis.

p53 can trigger both cell cycle arrest and apoptosis. An important question is whether p53 induced G1 arrest and apoptosis are independent pathways or whether p53 induced apoptosis is a consequence of, or follows G1 arrest. p53 induced cell death in M1 mouse myeloid leukaemia cells is not preceded by growth arrest, although cells in G1 are preferentially susceptible to apoptosis. In contrast, p53 dependent apoptosis in DP1 murine erythroleukemia cells occurs only after G1 arrest (Guillouf *et al,* 1995). Thus, it seems likely that p53 induced apoptosis is, at least in some cases preceded by G1 arrest, but that this is cell specific. The results generated from *in vitro* studies on cell cycle kinetics and entry into apoptosis may not reflect the true situation *in vivo,* since tissue culture cells often display altered cell cycle patterns as a result of serial passaging.

In our study using BrdU labelling we identified cells that were probably in S, G2 or M at the time of entering the apoptosis pathway were identified. This result suggest that at least a proportion of matrix cells can enter the apoptotic pathway from G2/M rather than G1.

6.6 P53 and cell cycle arrest

The results obtained from BrdU labelling studies led us to conclude that the p53 response in mouse skin varies not just in apoptotic response but also in cell cycle arrest patterns. A block in DNA synthesis 24hrs after exposure of normal adult mouse skin to ionising radiation was observed. This represents a relatively long time gap between initial DNA damage and cell cycle arrest, the reasons for which are unclear. The purpose of the cell cycle block is proposed to enable the cell to repair DNA damage before entering the next round of replication, and thus prevent propagation of genetic lesions (Lane, 1992; Kemp, *et al* 1993; Harvey *et al,* 1993; Greenblatt *et al,* 1994;). Cells entering S phase up to 24hrs after initial exposure must arrest at a downstream phase of the cell cycle to maintain genetic integrity. This raises the possibility that the 24hr block in BrdU incorporation which we observe is mediated by p53 at the G2/or M phase of the cell cycle.It is interesting to note that a specific splice variant of p53 is elevated at

G2/M of the cell cycle. (Lu *et al,* 1994). Analysis of irradiated skin sections with an antibody to this G2/M splice variant of p53 is presently underway.

Alternatively, the delay observed before cell cycle arrest becomes apparent may be as a consequence of a large proportion of skin cells having a long G1 phase, resulting in an extended time period before the cell encounters the G1/S checkpoint. Early work by Potten *et al,* (1988) identified slow cycling cells in mouse epidermis however, little is known about the lengths G1, G2 or S phase *in vivo* This alternative explanation remains a possibility.

By altering the proliferation and differentiation status of cells by TPA treatment in adult epidermis, we were able to induce a change in the pattern of cell cycle arrest after DNA damage. This is in contrast to the lack of change in apoptotic outcome after exposure of a cell to DNA damage. As discussed earlier, $p21^{waf1}$ has been linked to p53 mediated cell cycle arrest at the G1/S border. The change in timing of the proliferation block between irradiated acetone and TPA treated adult mouse skin may be due to p53 dependent changes in either $p21^{waf1}$ level or activity in the skin leading to changes in cell cycle kinetics. Unfortunately we were hindered in our attempts to examine $p21^{waf1}$ levels in our skin sections due to the lack of commercially available mouse specific antibodies for the protein. In this regard irradiation of $p21^{waf1}$ knockout mice should facilitate a greater understanding of the role of $P21^{waf1}$ in mouse skin after DNA damage.

The generation of p53 -*Lac Z* reporter mice allowed an investigation of the role of p53 transcriptional activity in both apoptotic and arrested cells in irradiated mouse epidermis. Cells which contain transcriptionally active p53 are identifiable in reporter mice by measuring beta galactosidase activity. However, the results obtained from the irradiated skin of these mice generated more questions than answers. No evidence of p53 transcriptional activity was detected in the skin of reporter mice up to 48hrs after exposure to 4Gy radiation or 72hrs post TPA treatment. However, both these stimuli resulted in increased levels of p53 protein in mouse skin. Activity of the transgene was clearly detected in other tissues of irradiated mice, in the salivary gland for example. These observations may accurately reflect the transcriptional activity of p53 in the skin. However, it is also possible that the results are due to a unknown skin specific factor

interfering with the regulation of the transgene. The p53 responsive element in the promoter region of the LacZ reporter transgene does not vary from the p53 consensus region of the $p21^{waf1}$ gene. This observation and the results from other irradiated tissues, where transgene induction is detected suggests that the skin may be unique in its response to DNA damage and subsequent p53 stabilisation. TPA is known to stimulate Protein Kinase C, which is thought to play a major role in the phosphorylation of p53, mainly at the C terminus. Alterations in p53 phosphorylation status have been suggested to alter the transcriptional activity of the p53 protein (Meek, 1982). In view of our earlier observation that TPA followed by 4Gy radiation 36hrs later produced a drop in BrdU incorporation evident 4hrs after radiation treatment rather than at 24hrs, we investigated the effect of changes in transcriptional activity following TPA treatment of *Lac Z* reporter mice. No transcriptional activity was detected in *Lac Z* mouse skin treated with TPA and irradiated with 4Gy 36hrs later. This was for consistent up to 48hrs post radiation treatment of the mice. This casts doubt on the earlier proposal that changes in p53 phosphorylation status as a result of TPA treatment caused altered transcriptional activity of the p53 protein, leading in turn to increased levels of $p21^{waf1}$ protein in the epidermis. Okadaic acid, which acts as to block dephosphorylation also to failed to produce increased transcriptional activity in p53 *LacZ* reporter mouse skin.

The idea that p53 can induce apoptosis without transcriptional activation is highlighted in our recent collaborative study with Prof D.Lane, and Prof. P.Hall (research paper enclosed). p53 mediated apoptosis can occur in certain cell types despite the absence of transcriptional activation by p53. p53 mediated cell type specific apoptosis occurs in the spleen of irradiated LacZ reporter mice. Not all of the apoptotic cells in the spleen show p53 transcriptional activity after radiation exposure, although most cells do show elevated levels of p53 protein. Clearly, many levels of control must exist to regulate the p53 apoptosis pathway. These observations rule out a singular role for the cell death gene *bax* which is transcriptionally activated by p53 in all incidences of apoptosis after DNA damage. Instead our results lend support to the idea that p53 induces apoptosis by acting directly on cell death systems or by transcriptional repression of cell survival genes such as *bcl2*.

The majority of studies which have attempted to address the role of p53 transcriptional regulation in the apoptotic pathway have been performed on cell lines *in vitro*. Recently it has been demonstrated that p53 mediated apoptosis does not require transcription function in HeLa cells (Yonish-Rouach *et al* 1994: Haupt *et al* 1995). In contrast it was shown by the White laboratory that p53 mediated transcriptional activation was essential to the induction of apoptosis in their system (Sabbatini *et al*, 1995). These condradictory results may be a consequence of the different cell lines used in the individual studies. The *in vivo* work clearly shows there is cell type specificity in p53 mediated transcriptional activation and apoptosis. This means that individual cell lines derived from whole tissues may display individual specificity in the pathways leading to apoptosis. Additionally, cells in culture commonly acquire genetic lesions through serial culture, such lesions may manifest themselves as mutations in cell death/survival genes such as *bcl2* and *bax*. Deregulation of cellular death/survival genes would be most likely to cause changes in the apoptotic signalling pathway in cultured cells.

If the lack of p53 transcriptional activation in skin, which is known to be cell cycle inhibited, is real and not do to vagaries of transgene expression, it has significant implications for the mechanisms of p53 mediated cell cycle arrest. It is of particular note that $p21^{waf1}$ is not required for the G2 checkpoint response to ionising radiation. Normal cells in G2 respond to ionising radiation - within 1hr the cells cease to enter mitosis (Paules *et al.*, 1995). This being the case, the G2 checkpoint is likely to be transmitted by a protein synthesis independent signal transduction pathway. This is in agreement with our lack of p53 mediated transcriptional activation in normal adult mouse skin following exposure to radiation. Further supporting the idea that the arrest observed in normal mouse skin after exposure to ionising radiation is a G2 arrest. That our results accurately reflect the lack of transcriptional activity by p53 is strengthened by work on similar mice which utilise the *mdm2* rather than the *fos* promoter. Similarly, these mice also show no transgene activity in the skin (Gottlieb *et al*, 1997). The regulation of $p21^{waf1}$ and other downstream effectors of p53 is the subject of ongoing research.

6.7 Hair follicle, stem cells and apoptosis

Upon exposure to radiation, some cells in mouse epidermis die, and some cells enter a period of cell cycle arrest. In murine intestine epithelium, it has been postulated that the

stem cell like cells of the intestinal crypts undergo apoptosis to prevent the repopulation of the intestine with progeny from a genetically damaged stem cell (Merritt *et al,* 1994). We examined the effect of removing matrix cells by radiation induced apoptosis on the development of chemically induced mouse skin tumours. It is widely accepted that a population of hair follicle cells with stem cell like characteristics are the target cells that interact with carcinogens and undergo cellular transformation. The role of matrix cells in this process is unclear as they do not fit the classical description of stem cells, in that they are rapidly proliferating, there are present in large numbers, and they are not long lived. Matrix cells have been proposed to be transit amplifying cells of the skin, equivalent to the committed progenitor cells of the hemopoietic tissue. A more acceptable location for the stem cell population of the skin is in the bulge region of the outer root sheath, where long lived slowly dividing cells have been identified (Laveker *et al,* 1993).

Expression of mutant H *ras* in the interfollicular population of mouse skin can bring about the development of benign papillomas with low progression frequency to carcinomas. When the same gene was targeted to the hair follicle it stimulated the development of tumours with a high frequency of progression to carcinomas. (Brown *et al* submitted 1997). This result led to the proposal that a continuum of target cell populations exist in the epidermis. The most primitive stem cells capable of giving rise to malignant tumours while the more differentiated cells capable of giving rise to low risk benign tumours. Based on this model matrix cells may have the capacity to give rise to tumours low risk skin papillomas. To test this hypothesis a DMBA/radiation/TPA protocol was devised to look at the effect of removal of matrix cells by radiation on mouse skin carcinogenesis.

Irradiation, rather than decreasing the tumour yield, actually increased the rate of both papilloma and carcinoma formation. This effect was observed in both p53 wild type and knockout mice. The possibility that radiation was acting as an additional initiating agent was eliminated by treating mice with 4Gy followed by 20 weeks TPA treatment, this regime produced no tumour development. Similarly DMBA followed only radiation treatment did not result in the formation of tumours, thus ruling out any effect of promotion by radiation. It is possible that matrix cell depletion stimulates the stem cells

of the follicle to divide, thus creating a bigger target of stem cells for the initiating agent to act upon. This, in turn, would be expected to lead to greater tumour development only in p53 wild type mice where the matrix depletion phenomenon occurs. This model does not explain the increased incidence of tumour formation in p53 knockout mice.

Alternatively the result may be explained by death of cytotoxic T lymphocytes that present in the skin that are considered to be part of the skin immune surveillance system which acts to remove genetically damaged cells. This would be a valid model of explanation only if the radiation induced death of these cells is p53 independent. The possible involvement of the immune system makes analysis of our results difficult, as the effect of removal of matrix cells may be masked by the effect of removing the skins natural defence system.

Mice which lack circulating γ δ cytotoxic killer cells as a result of a homologous null recombination are now available (Chandler *et al,* 1995). It would be of interest to examine the effect of matrix cell depletion on skin carcinogenesis experiments in these animals. An alternative way to study matrix depletion, without the involvement of the immune system, would be to analyse the effect in nude mice which have no cytotoxic immune cells. There is however a drawback to using nude mice to answer this question.

In this study we have shown that nude mice have an abnormal number of hair follicle cells that are extremely sensitive to radiation induced apoptosis. The reason for this sensitivity is unclear. The recent discovery by Brissette *et al*,(1997) linking the *HFH11* gene, the gene which gives rise to the nude mouse phenotype, to cellular differentiation status offers a possible explanation. Nude mouse keratinocytes exhibit an altered differentiation behaviour *in vitro*, consistent with the abnormal keratinization process observed in nude mouse skin (Kopf-Maier *et al,* 1990). The altered pattern of differentiation in nude mouse skin under basal conditions is similar to that observed in wild type cells after exposure to TPA. *In vivo* treatment of mouse skin with TPA induces keratinocyte stem cells to divide and produce an increased number of transiently amplifying and more committed populations (Cotsarelis *et al* ,1990). Given the fact that nude mouse keratinocytes may be in a "TPA- primed state", the number of keratinocyte stem cells and their progeny may altered in nude mouse skin. If this is the case, the

similarity of the mouse skin system and the intestinal model becomes more evident. If the nude mouse follicle does contain more stem cells than normal follicles the hypothesis that in the hair matrix, as in the intestinal epithelium, there is cell type specific apoptosis related to differentiation status as a result of radiation is strengthened. However, there is no evidence to suggest that matrix cells are the stem cells of the skin.

Radiation may causes matrix cells to initiate a programme of cell death which they are already primed to undergo, that is programmed cell death in the catagen phase of the hair cycle. This may also be comparable to the apoptotic response of thymocytes to radiation, since a large proportion of thymocytes are deleted through the apoptotic pathway during thymic ontogeny. In previous studies on the haematopoietic and intestinal system it has been shown that cells which have a high proliferation rate also show p53 induction and rapid induction of the apoptotic pathway. In this study we have shown that entry into the apoptotic pathway is independent of proliferation rate, since interfollicular cells stimulated to proliferate with TPA do not enter the apoptotic pathway after exposure to radiation. If the interfollicular cell population were to undergo apoptosis in response to DNA damage the end result would be desquamation, that is, skin falling off in sheets. It seems likely that the differentiation of a cell may play a major role in determining cell fate after DNA damage.

The role of matrix cells in the development of mouse skin tumours is presently being investigated in this laboratory. By targeting mutant *H ras* to the matrix cell population, we hope to be able to establish the role of initiated matrix cells in the development of benign and malignant skin tumours.

No apoptosis was detected in either the basal layer, the outer root sheath, or the bulge area of normal mouse skin after exposure to radiation. All of these regions of the skin have been proposed as likely to contain stem cells. Therefore, there is a strong possibility that the true stem cells of the skin do not enter the apoptotic pathway as a consequence of radiation induced DNA damage. This specificity may be due to the proliferative status of the stem cell, or its location and micro environment in the tissue. Stem cells of the skin may be surrounded by survival factors that prevent their entry into the apoptotic pathway. Resistance of skin stem cells to cell death after radiation exposure may have

evolved to protect the skin from degeneration as a result of constant exposure to radiation. This does not imply that the skin stem cells may be resistant to all types of genetic damage. Highly localised apoptotic bodies and p53 staining are present high in the hair follicle in response to DMBA treatment. This may represent apoptosis of the outer root sheath bulge cells after DMBA damage. If indeed this is the case, the cells which escape death after initiation are likely to be the cells capable of giving rise to highly malignant tumours. Cell specific death was absent in p53 knockout mouse skin treatment after DMBA treatment. Paradoxically, p53 knockout mice do not develop as many papillomas as p53 wild type mice in classical DMBA/TPA mouse skin tumorigenesis. This is unexpected since in the p53 knockout follicle a higher number of damaged cells would escape cell death and pass on potentially lethal mutations to subsequent daughter cells. However, it may be the case that the burden of genetic damage which remains unfixed in p53 knockout cells is too high and the cell dies by necrosis at a later time point. The effect of DMBA on mouse skin is an ongoing study in the laboratory.

In summary, we have shown specific cell populations in mouse skin respond to ionising radiation with either cell cycle arrest or apoptosis. This is in contrast to the results obtained from cell culture studies, which showed mouse skin keratinocytes show a uniform response to radiation by entering a period of G1 arrest. Our *in vivo* results are of significant importance in the evaluation of the sensitivity of individual cell types to DNA damaging agents, and are also relevant to the assessment of therapeutic treatment of tumours of varying origins. Assays of specific tumour responses to radiation may lead to improved therapeutic regimes by individualising tumour treatments based on the cellular origin of a specific tumour.

Chapter 7. Bibliography

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