A study of skin homeostasis and skin tumorigenesis using transgenic mice

by

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The research reported in this thesis is my own original work except where otherwise stated, and has not been submitted for any other degree.

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Abstract

The ability to engineer mice which carry exogenous genes targeted for expression in specific tissues, or have an endogenous gene functionally switched off has allowed us to examine the role of particular proteins in an *in vivo* model. This technology, in combination with a well-characterised model of chemically-induced skin carcinogenesis in the mouse, has allowed us to start dissecting the changes that occur during a particular pathway of tumorigenesis. TGFβ1 has been shown to act as an inhibitor of both normal cellular proliferation and tumour formation. However, it can also accelerate malignant conversion at later stages of carcinogenesis.

Previously in this laboratory, mice were generated that overexpressed Tgfβ1 in suprabasal layers of skin epidermis under the control of a K10 promoter. These transgenic mice showed an increased proliferation rate in the basal compartment of their epidermis compared to wild-type controls. In this study, it is shown that the change in proliferative index is not dependent on p53, a molecule central to growth control and apoptosis. The K10-*Tgf*β1 mice were crossed on to a Tgfβ1-null background and the proliferative phenotype of the transgene was examined. An increased basal cell proliferative index was observed whether the background was Tgfβ1-null or wild-type.

TGFβ1 has been shown to induce apoptosis and a line of transgenic mice overexpressing BCL-2 in the epidermis became available, providing an opportunity to investigate the role of an apoptotic pathway in the skin. These K10-*BCL*-2 mice were shown to express the transgene at both mRNA and protein levels in suprabasal cells of the epidermis. Normal skin homeostasis, however, was not disrupted by the exogenous BCL-2 and expression of other apoptotic markers was not altered in these mice. Furthermore, K10-*BCL*-2 mice responded to chemical insult by a skin tumour promoter in the same way as wild-type littermates.

K10-*Tgf*β1 mice had demonstrated resistance to papilloma formation during chemical carcinogenesis and tumours on the transgenic mice had also shown a higher rate of malignant conversion in the same experiment. In this study, the effects of epidermally-targeted Tgfβ1 during tumorigenesis were investigated further by crossing the K10-*Tgf*β1 mice to a tumour-developing K5-*RAS* transgenic line. Mice carrying both the K5-*RAS* and K10-*Tgf*β1 transgenes showed no difference in tumour formation compared to those harbouring just the K5-*RAS* transgene.

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Commonly Used Abbreviations

bp	base pair
BCL-2	B-cell lymphoma 2 gene
BrdU	5-bromo-2'-deoxyuridine
CDK	cyclin dependent kinase
cDNA	complementary DNA
DMBA	dimethylbenzanthracene
DNA	deoxyribonucleic acid
DTT	dithiothriotol
EDTA	(ethylenedinitrilo)tetraacetic acid - disodium salt
Gapdh	glyceraldehyde-3-phosphate dehydrogenase gene
kb	kilobase
PCR	polymerase chain reaction
RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
SDS	sodium dodecyl sulphate
TβRII	TGFβ type II receptor
TGF	transforming growth factor
TPA	12-O-tetradecanoylphorbol-13 acetate

Chapter 1
INTRODUCTION

1.1 The Mouse in Cancer Research

1.1.1 Transgenic and Knockout Mice

Transgenic technology, used to stably introduce foreign DNA into the germ line of mice, has had a profound impact on the course, progress and scope of cancer research. Transgenic animals provide a valuable tool for investigation of the complex biological processes which control development of normal and neoplastic cells. The capability to target gene expression to a specific tissue and developmental stage, along with the ability to inactivate genes by homologous recombination, has allowed the development of mouse models for many types of cancer. Neoplasms arising in transgenic mice are heritable, demonstrate reproducible, and therefore predictable, patterns of growth and development, and can be specifically induced in a large variety of tissue types.

The introduction of an exogenous gene driven by a promoter which restricts expression of the transgene to a specific tissue compartment is an approach which particularly lends itself to the analysis of the tumour promoting functions of dominant acting oncogenes. Many mouse cancer models have been developed via this approach (Hanahan, 1989; Bedell *et al*, 1997). Furthermore, the development of gene targeting techniques using mouse embryonic stem cells has allowed the inactivation of one or both alleles of endogenous tumour suppressor genes in order to study their role in cancer promotion. This gene inactivation technology has been applied to study virtually every known tumour suppressor gene originally identified in humans (Bedell *et al*, 1997). There are many advantages to such "knockout" mice. First, the heterozygous version of a tumour suppressor knockout may provide a useful animal model for the corresponding human cancer predisposition syndrome. A second advantage, provided by the homozygous knockout mice, is the insight into the role of these genes in embryonic development. In many cases, mice completely null for a

given tumour suppressor exhibit embryonic lethality and a distinct pattern of organ malformations. Analysis of the biological and molecular bases of the observed defects helps elucidate the function of particular genes in organogenesis, regulation of cell cycle control, differentiation, and apoptosis.

The availability of numerous transgenic and knockout lines has assisted in the analysis of genetic interactions among oncogenes and tumour suppressor genes. Intercrossing of two different gene knockout mice to generate doubly deficient mice, or a gene knockout and a conventional transgenic mouse, can reveal different levels of cooperation or antagonism between the two genes. In some cases embryonic lethality in doubly deficient mice can be accelerated or delayed. One of the most striking examples of this is the rescue of the embryonic lethality of Mdm-2 null mice when crossed on to a p53-null background (Jones *et al*, 1995). This reflected the tight regulation that occurs between these two genes. In other situations, tumour incidence rates can be accelerated and tumour spectra altered by the combined effects of the transgenic and/or knockout alleles.

Another important technology for gene ablation in mice is cell-type specific gene-inactivation. The *Cre-lox* system is one such technique that has been used to knockout genes in specific tissues (Gu *et al*, 1994; Chambers, 1994). One mouse carrying the Cre recombinase enzyme driven by a tissue specific promoter is crossed with a mouse carrying the piece of DNA to be removed between two short *loxP* sites. Offspring of these mice then produce Cre recombinase in the designated tissue which recognises the *loxP* sequences and excises the intervening DNA.

1.1.2 Multistage Skin Carcinogenesis

The mouse skin model is an ideal system in which to study the multistage nature of cancer pathogenesis: it has been well characterised and the target organ is easily accessed and monitored. The majority of human cancers are epithelial in origin with

skin cancer of the epidermis being one of the most prevalent. There are two main types of skin cancer in humans: basal cell carcinoma, in which cells appear as undifferentiated or basal-like cells, and squamous cell carcinoma, in which cells keratinise in an aberrant fashion. Melanoma is also increasingly becoming a major killer. A clearer understanding of the genetic and epigenetic changes which are involved in the process of epithelial carcinogenesis is necessary if significant advances in treatment are to be made. The mouse model is, of course, only a model. Most human skin cancer is UV-induced whereas this model is created by chemical induction. Nevertheless, most genetic changes are very similar in mouse skin and other tumour systems.

1.1.2.1 Biology of the Skin

The skin is the largest organ in the body and consists of two major components. The outer epidermis is a stratified squamous epithelium which forms a protective barrier against ultraviolet radiation and other chemical and biological injury. It is composed of several layers of keratinocytes undergoing terminal differentiation, with the outer cells being constantly shed and replaced. Underlying the epidermis, separated from it by a basement membrane, is the dermis. Whereas the epithelial region is composed of densely packed epithelial cells with relatively little inter-epithelial space, the dermis consists mainly of an intercellular matrix, within which are occasional scattered cells of various types and through which runs a network of both blood vessels and nerve fibres. This matrix contains rich networks of minute fibres of elastin, collagen and reticulin, all produced by fibroblasts. Together the fibres provide the mechanical strength of the skin and give it some elasticity.

Throughout the dermis specialised regions of skin can be seen. These include the hair follicles, which consist of infolds of the epidermis. There are also various glands in the skin including the sebaceous glands, which secrete sebum into the hair follicle canal, and sweat glands. Other cell types found in the skin include melanocytes (which produce melanin, the pigment involved in protection from ultraviolet radiation), adipocytes (involved in the regulation of temperature), and other cells involved in immune and inflammatory responses such as Langerhans' cells and granulocytes.

All the superficial cell layers and the functional integrity of the epidermis are ultimately dependent on the proliferative compartment, the basal layer. It is this layer which replaces the cells lost through surface desquamation or through damage. The basal cells in human epidermis are cuboidal or columnar cells with their long axis perpindicular to the surface plane of the skin. In mouse the basal cells are similar in many areas but in epidermis on the back of the mouse they tend to be much thinner, flatter cells. Lying above the basal layer is the suprabasal of cells which consist of the spinous and granular layers. The number of spinous cell layers is variable, depending on the site, but may be as little as a single layer, as in the mouse. In comparison with the basal cells the suprabasal cells tend to be thinner and have a higher cytoplasmic-nuclear ratio. The outermost layer is the stratum corneum consisting of corneocytes or squames. All internal organs have disappeared and the cells are metabolically dead. These therefore represent the final, mature, terminally differentiated cells.

The epidermis is under a constant state of proliferation, commitment, differentiation, and elimination so that the functional integrity of the tissue is maintained. The intact epidermis has the ability to respond to diverse environmental stimuli by continuous turnover to maintain its normal homeostasis throughout an organism's life. This is achieved by a tightly regulated balance between stem cell self-renewal and the generation of a population of cells that undergo a limited number of more rapid (amplifying) transit divisions before giving rise to nonproliferative, terminally differentiating cells. Normal human stratified epidermis has a temporally and spatially coordinated programme of proliferation and differentiation which is characterised by sequential changes in gene expression. One class of genes which shows such changes is the cytokeratin gene family encoding the major cytoskeletal proteins present in keratinocytes. The actively proliferating keratinocytes of the basal layer co-express cytokeratins K5 and K14. As basal cells commit to terminally differentiate, they exit the cell cycle, and begin to move outward towards the surface of the skin, in conjunction with a loss of contact with the basement membrane. As the cells migrate upwards into the suprabasal or spinous layers, proliferative capacity is lost, K5 and K14 expression is suppressed and the synthesis of K1 and K10 is induced. The keratinocytes change morphology, flatten out and change the nature of contacts between neighbouring cells. As the next stage of differentiation occurs and

keratinocytes move into the granular layer, K1 and K10 expression is downregulated and involucrin, loricrin and keratinocyte transglutaminase are upregulated. This is associated with synthesis of filaggrin which aggregates with keratins 1 and 10 to form large, complex bundles of fibres thus adding more strength to the epidermis. Involucrin and loricrin are crosslinked by transglutaminase. At this stage nuclei and cytoplasmic organelles are lost and in cells of the cornified layer a permeable inner membrane envelope of highly crosslinked proteins replaces the nuclear membrane. Mature squames that reach the surface and are sloughed off, consist merely of keratin macrofibrils in a filaggrin matrix within the cornified envelope.

Other proteins of the epidermis that are important for epidermal architecture and intercellular signalling include integrins and desmosomes. Desmosomes consist of a variety of proteins which serve, amongst other things, as adhesion molecules keeping cells tightly bound and organised (reviewed in Garrod, 1995). They are essential for cell architecture as they provide the anchoring point for the keratin intermediate filament network of the keratinocytes. Integrins are a family of adhesion molecules (Varner and Cheresh, 1996) which mediate cell-extracellular matrix and cell-cell interactions. They are potent signalling molecules and are thought to regulate cell growth and differentiation (Dedhar, 1995) and differences in integrin expression and function can be used to differentiate epidermal stem cells from transit amplifying cells (Jones and Watt, 1993).

1.1.2.2 Mouse Model of Skin Carcinogenesis

Application of carcinogens and tumour promoting agents to mouse skin showed that the development of fully malignant tumour cells involves several stable intermediate stages (Yuspa, 1994). In this mouse skin model the development of malignant squamous cell carcinomas can be divided into distinct stages: initiation, promotion, premalignant progression, malignant conversion, malignant progression, invasion and metastasis. The development of each stage is accompanied by a variety of morphological, biochemical and genetic changes. These changes come about due to quantitative and qualitative alterations in certain cellular genes including changes in gene structure and/or loss of normal expression. An attraction of this carcinogenesis

model is that mouse keratinocyte cell cultures which represent each of the intermediate stages have been well characterised *in vivo*. The genetic changes occurring during multistage carcinogenesis have thus been studied by a combination of both *in vivo* (i.e. mouse skin) and *in vitro* (cultured cell lines) approaches.

Initiation

In the mouse skin carcinogenesis model used in our lab initiation occurs as a result of a single topical application of a mutagen such as dimethylbenzanthracene (DMBA) to the dorsal skin of the mouse. Initiated cells can remain dormant in the skin for a considerable period of time until activated by tumour promotion (Loehrke *et al*, 1983). Initiated keratinocytes display a very subtle change in phenotype. For example, in culture, they have an altered response to signals for terminal differentiation such as calcium (Yuspa and Morgan, 1981). *In vivo,* initiated keratinocytes are indistinguishable from normal ones. The target of DMBA initiation is the activation of the Ha-*Ras* gene by specific point mutations (Balmain *et al*, 1984; Bizub *et al*, 1986; Harper *et al*, 1987). These mutations are associated with papilloma formation and can be found in initiated skin before the appearance of tumours (Nelson *et al*, 1992). The nature and amino acid position of *Ras* mutations depends on the carcinogen (Brown *et al*, 1990). In human cancer, *RAS* gene mutations, which have presumably been induced by UV light, have been found in both benign and malignant skin tumours although at a much lower frequency than that obseved in DMBA-initiated mouse skin tumours (Leon *et al*, 1988; Ananthaswamy *et al*, 1989). When the v-Ras^{Ha} oncogene is introduced into normal mouse keratinocytes, the recipient cells form papillomas when grafted to nude mice (Roop *et al*, 1986), and transgenic mice expressing a *Ras* oncogene in the epidermis develop papillomas (Bailleul *et al*, 1990), showing that this single genetic change is sufficient to produce the initiated phenotype. *In vitro*, mouse keratinocytes expressing v-Ras^{Ha} have a high proliferation rate and fail to terminally differentiate (Cheng *et al*, 1990). Hyperproliferation is caused by overexpression and autocrine response to transforming growth factor α (Tgfα) (Glick *et al*, 1991).

Promotion

Repeated treatment of initiated mouse skin with a tumour promoter causes the selective clonal expansion of initiated cells to produce benign squamous cell

papillomas (Iannaccone *et al*, 1987). These papillomas are characterised by an increase in the number of proliferating cells along with a decrease in the rate of differentiation and cell death. The most potent exogenous skin promoters are the phorbol esters such as tetradecanoylphorbol acetate (TPA) which activates protein kinase C (PKC). This enzyme activity accelerates epidermal terminal differentiation (Dlugosz and Yuspa, 1993). Initiated cells are relatively resistant to this signal and so proliferation of the initiated subpopulation is favoured. There is some dispute as to whether exogenous promotion directly produces genetic alterations that lead to tumour progression. Yuspa (1994) argues that exogenous promotion is likely to be epigenetic since papillomas are diploid when they first emerge, a single genetic change in normal keratinocytes is sufficient to produce a papilloma phenotype, and most promoting agents are not mutagens. Yet, the observed chromosomal aberrations (see below) could potentially be due to the genotoxic activity of TPA (Kinsella *et al*, 1978; Petrusevska *et al*, 1988) and would argue against a purely epigenetic mechanism for promotion in this model system.

Premalignant progression.

Whether exogenous promotion is a purely epigenetic process or not, sequential, non-random chromosomal aberrations correlate with premalignant progression of mouse skin papillomas (Aldaz *et al*, 1987) and this stage is the main time-dependent component of cancer pathogenesis. Molecular (Kemp *et al*, 1993a) and cytogenetic (Aldaz *et al*, 1989) studies have demonstrated frequent trisomy of chromosomes 6 and 7 in papillomas. Trisomy of chromosome 7 involves the duplication of the chromosome which carries the mutant *Ras* allele, suggesting that an increase in mutant Ras level may be required for early selection of initiated cells.

Malignant conversion.

5-10% of papillomas spontaneously convert to cancers (Aldaz *et al*, 1987) and so malignant conversion of benign tumours is relatively rare. This conversion, although spontaneous, is not thought to be random as not all papillomas have an equal chance of conversion. Subpopulations of papillomas with a high risk for malignant conversion have been identified (Hennings *et al*, 1990) and the crucial events in the conversion process appear to be genetic since the conversion frequency can be significantly

increased by exposing animals bearing benign tumours to mutagens (Aldaz *et al*, 1987). The subclass of high-risk papillomas is also more sensitive to mutagen-induced conversion and so the factors that determine the risk of conversion must increase susceptibility to the induced or spontaneous genetic changes. Differences in phenotypic markers such as Tgfβ, α6β4 integrin, keratin 13 and Rarα expression levels have been used to distinguish between high and low risk papillomas at an early stage (Glick *et al*, 1993; Cui *et al*, 1994; Tennenbaum *et al*, 1993, Nischt *et al*, 1988; Darwiche *et al*, 1996).

Several genetic alterations have been associated with malignant conversion. Frequent loss of the remaining wild-type Ha-*Ras* allele is often observed (Bremner *et al*, 1990). Furthermore, mutations in the *Tp53* gene, while rarely found in papillomas, are frequently detected in carcinomas (Burns *et al*, 1991; Kemp *et al* 1993a). Those carcinomas that showed loss of heterozygosity on chromosome 11 (where the *Tp53* gene resides) also had mutations in the remaining allele of *Tp53* (Burns *et al*, 1991). Kemp *et al* (1993b) found that papillomas produced on p53 null mice by chemical carcinogenesis underwent much more rapid malignant progression than those on wild-type mice. Progression rate was also greater in heterozygous mice and was associated with loss of the remaining wild-type allele.

Spindle Conversion

A further stage in mouse skin carcinogenesis is the progression from squamous carcinoma to the more aggressive spindle cell tumour, in which the markers of differentiation such as E-cadherin are lost (Navarro *et al*, 1991; Diaz-Guerra *et al*, 1992). This conversion is also associated with a further increase in the ratio of mutant to normal Ha-*Ras* genes (Buchmann *et al*, 1991). Deletion or altered regulation of *p16Ink4a* and *p15Ink4b* has also been shown to occur in tandem with the loss of differentiation associated with this stage (Linardopoulos *et al*, 1995). In this lab, Cui *et al* (1996) have shown that overexpression of Tgfβ1 in the epidermis of transgenic mice can accelerate spindle tumour formation during mouse skin carcinogenesis and, more recently, that the conversion event is mediated directly via the Tgfβ signalling pathway of the tumour cell (Portella *et al*, 1998).

1.1.3 Effects of Genetic Background on Tumour Susceptibility and Transgenic Phenotype

Similar to the human situation, phenotypic variability is seen in mice when particular spontaneous or induced mutations of interest have been placed on different strain backgrounds. Several recent examples illustrate the importance of strain-specific modifier genes on the phenotype of mutations in mice. Tumour models provide a good example of a genetic background effect. Although unusual and unique neoplasms may arise that are specific for the genetic alteration, the overall tumour spectra in genetically altered mice tends to follow that of the strain background. For example, *Tp53* knockout mice on a 129/Sv background develop tumours sooner on average than do their counterparts of predominantly C57Bl/6 backround (Donehower *et al*, 1995b). Moreover, in C57Bl/6 *Tp53* null mice, the predominant tumour type is lymphoma, whereas in the null 129/Sv strain, testicular teratomas are as common as lymphomas.

Strain background effects have also produced variability in phenotypes other than neoplasia. One example is that found with epidermal growth factor receptor (Egfr) knockouts in which the time of lethality for one mutation ranged from peri-implantation to post-partum depending on the strain background (Threadgill *et al*, 1995). Post-natal disease severity is another phenotype that may be modified by genetic background. Examples such as the cystic fibrosis transmembrane conductance regulator (*Cftr*) knockout mouse model of cystic fibrosis (Rozmahel *et al*, 1996) demonstrate how modulation of the phenotype of induced mouse mutations by modifier genes can more closely mimic the polygenic or multifactorial nature of important human disease.

Usually the modifying loci of the genetic background are undefined. Increasingly, however, modifier loci are being mapped (Balmain and Nagase, 1998). In *Tgf*β1 knockout mice (which will be discussed later), Bonyadi *et al* (1997) were able to map a major genetic modifier of embryonic lethality using the difference in survival rates

between C57Bl/6 and NIH strains. Similarly, in a line of *Apc*-deficient mice called *Min* (Multiple intestinal neoplasms) mapping studies identified a modifier locus *Mom1* (Modifier of *Min* 1) (Dietrich *et al*, 1993). These genetic approaches to the mapping and eventual cloning of genetic modifiers add another dimension to the analysis of disease resistance and susceptibility.

1.2 TRANSFORMING GROWTH FACTOR β

In many cases tumour cells develop when normal progenitor cells lose control of signalling pathways that regulate responses to soluble growth regulatory factors. The transforming growth factors beta (TGFβs) constitute one such family of negative growth regulators that play an important role in carcinogenesis.

1.2.1 TGFβ Family and Biochemistry

The TGFβs are part of a large superfamily of growth factors, which play pivotal roles in regulating broad aspects of cell behaviour, including regulation of cell division, survival, differentiation and specification of developmental fate, in a variety of organisms (Roberts and Sporn, 1990; Cui and Akhurst, 1996). The TGFβ superfamily comprises more than thirty distinct members that are grouped in several subfamilies, including the TGFβ family, the activin/inhibin family, bone morphogenetic proteins (BMPs) and the *Drosophila decapentaplegic* (*Dpp*) gene product, Mullerian inhibiting substance, and the *Xenopus laevis* Vg-1 and Vgr-1.

Three mammalian TGFβ isoforms exist termed TGFβ1, β2 and β3. *In vitro*, all three isoforms have similar qualitative effects on cells, though the sensitivity of certain cell types to different isoforms is remarkably different (Cheifetz *et al*, 1990). TGFβ1 is a 25kDa homodimeric peptide. It is synthesised as a 390 amino acid precursor (pre-pro-TGFβ) with an N-terminal short-signal peptide but undergoes post-translational processing leaving a peptide of 112 amino acids. Processed TGFβ is secreted as a

biologically inactive or latent form in a non-covalent complex with the latency associated peptide (βLAP).

As previously mentioned, TGFβ is a multifunctional growth factor, but the nature of its action on a particular target cell is critically dependent on many parameters including the cell type and its state of differentiation. The most studied property of TGFβ is as a regulator of cell growth and this will be discussed in more detail later. Other functions include stimulation or inhibition of differentiation, regulation of extracellular matrix, and contol of the immune response.

TGFβ is an important regulator of extracellular matrix (ECM) deposition, composition, and cell-substratum interactions. The ECM can affect cell proliferation, differentiation, morphology, adhesion, migration, and interaction between cells and so TGFβ may indirectly affect cell proliferation and differentiation through modulation of ECM. TGFβ promotes ECM formation in general, by stimulating fibroblastic cells to synthesise and secrete various ECM ligands and receptors such as numerous collagens, proteoglycans and fibronectin, as well as several members of the integrin class of ECM receptor and cell adhesion molecules (for review see Roberts and Sporn, 1990). In addition, TGFβ inhibits ECM degradation by stimulating the expression of some ECM protease inhibitors, like the tissue inhibitor of metalloproteinase (TIMP) and plasminogen activator inhibitor type I (PAI-I), and by down-regulating the expression of the ECM proteases themselves (Roberts and Sporn, 1990).

TGFβ is also a potent regulator of the immune system and inflammatory responses, generally functioning as an immunosuppressor (Wahl, 1992). It can inhibit proliferation of thymocytes (Ristow, 1986) and lymphocytes (Kehrl *et al*, 1986), and can suppress B cell proliferation and immunoglobulin secretion (Kehrl *et al*, 1991). TGFβ can also suppress the synthesis and activity of some cytokines, such as interleukins (Wahl *et al*, 1988), interferon-γ (Czarniecki *et al*, 1988) and tumour necrosis factor α (TNFα). Early in the inflammatory response, TGFβ acts as a potent chemoattractant, stimulating monocyte migration and macrophage production (Wahl *et*

al, 1987). However, later in the response, it exhibits anti-inflammatory effects such as down-regulation of macrophages and antagonism of TNFα function.

1.2.2 Regulation of Cell Growth

TGFβ is a pluripotent hormone that can positively or negatively regulate proliferation of a variety of target cells. Although TGFβ was originally identified by its ability to induce a transformed phenotype in normal rat kidney fibroblast cells (Moses *et al*, 1981; Roberts *et al*, 1981) consequent studies have shown that the predominant action of TGFβ on cell proliferation is inhibitory. All three TGFβ isoforms possess reversible growth inhibitory activities on cell types including epithelial, endothelial, haematopoietic, fibroblastic, lymphoid, and neuronal cells (for review see Roberts and Sporn, 1990; Massague, 1990). TGFβ can also display some apparently conflicting growth stimulatory effects. For example, TGFβ1 stimulates the growth of NRK cells in the presence of EGF in soft agar, but both inhibits the growth and antagonises the mitogenic action of EGF on the same cells in monolayer culture (Roberts *et al*, 1985). The growth promoting effect may be indirect, due to the enhanced expression of the extracellular matrix (ECM) proteins, fibronectin, type I collagen and proteoglycans, and increased integrin-mediated adhesion to these molecules (Ignotz and Massague, 1986). Others have proposed that TGFβ may act more directly to modulate the components of the cell cycle as part of its mitogenic activity

TGFβ is a potent growth inhibitor of keratinocyte cell lines (Reiss and Sartorelli, 1987), and the inhibition of Mv1Lu epithelial cells and HaCaT keratinocytes has been linked to the suppression of RB phosphorylation and to the inhibition of c-*MYC* transcription (Pietenpol *et al*, 1990; Laiho *et al*, 1990). This suggests that the antiproliferative activity of TGFβ might be mediated by inhibition of c-MYC activity and/or down-regulation of specific CDK components. Indeed, TGFβ has been shown to down-regulate CDK4 protein expression in Mv1Lu cells (Ewen *et al*, 1993) and cyclin E and CDK2 protein levels in keratinocytes (Geng and Weinberg, 1993). Furthermore, it was initially proposed that the CDK inhibitor $p27^{kip1}$ might mediate TGFβ-dependent growth inhibition of epithelial cells (Polyak *et al*, 1994). However, cell lines derived from $p27^{kip1}$ knock-out mice are still growth-arrested by TGFβ

(Nakayama *et al*, 1996) indicating that $p27^{kip1}$ is not absolutely essential for the antiproliferative action of TGFβ *in vivo*, although it is possible that other CDK inhibitors such as $p57^{kip2}$compensate for the loss of $p27^{kip1}$ in the knockout mice.

1.2.3 TGFβ Receptors and Signalling

TGFβ signals by contacting two distinct but related transmembrane serine/threonine kinases called receptors I and II. It binds directly to receptor II which is a constitutively phosphorylated kinase. It seems that bound TGFβ is then recognised by receptor I which is recruited into the complex and becomes phosphorylated by receptor II at the GS domain (a region highly conserved in type I receptors across species). This allows receptor I to generate a phosphorylation signal for the first step of a signalling cascade. It is generally accepted that both receptors are essential in order to signal (Wrana *et al*, 1994).

Much progress in identifying signalling events downstream of the type I receptor has been made with the discovery of the SMAD family of proteins as TGFβ signal transducers. The first known member of this family, Mad, was identified by genetic analysis of a Drosophila BMP-like signalling pathway, the Dpp pathway (Sekelsky *et al*, 1995). Mad (Mothers against decapentaplegic)-related genes, called Sma2, Sma3, and Sma4, were identified by genetic analysis of the related Daf4 pathway in *Caenorhabditis elegans* (Savage *et al*, 1996). Vertebrate homologues, called SMADs (for Sma and Mad homolgue) were identified shortly thereafter (see Kretzschmar and Massague, 1998 and references therein).

Based on the most recent studies on the SMAD family of proteins (see Kretzschmar and Massague, 1998 for review), a model of TGFβ/SMAD signalling can be suggested. TGFβ binds to and activates type I and type II receptor serine threonine kinases leading to transient association with specific receptor-activatable SMADs (SMAD2, SMAD3). These SMADs become phosphorylated by the activated type I receptor, then associate with a co-SMAD (e.g. SMAD4) and move into the nucleus. In the nucleus the SMAD complexes associate with a whole variety of different DNA-

binding transcription factors such as FAST-1 (Liu *et al*, 1997) leading to active transcriptional complexes and stimulation of target gene expression.

1.2.4 *Tgf*β Knockout and Transgenic Mice

1.2.4.1 TGFβ in Development and *Tgf*β1 Knockout Mice

The widespread expression of the *TGF*β genes and protein throughout mammalian development suggests that TGFβ plays an important role during post-implantation embryogenesis (Heine *et al*, 1987; Lehnert and Akhurst, 1988; Gatherer *et al*, 1990). Using *in situ* hybridisation *Tgf*β1 mRNA can be detected by 7.5 days post-coitum in blood islands of the yolk sac, the allantois and in the precardiac mesoderm of the embryo (Akhurst *et al*, 1990).

As previously mentioned, the ability to generate knockout mice has become a very powerful tool for investigating the function of a gene *in vivo*. *Tgf*β1 knockout mice were generated by two independent groups (Shull *et al*, 1992; Kulkarni *et al*, 1993) and have been used to determine TGFβ1's role during embryogenesis. Over 50% of mice, null for Tgfβ1, reached birth. However, these animals developed a fatal wasting syndrome, beginning at about 2 weeks of age, caused by multifocal inflammation. The Tgfβ1 null mice died by around 3-4 weeks and the massive inflammation was attributed to an autoimmune-like disorder brought about by the immunosuppressing actions of Tgfβ1. More detailed examination has shown that death in these mice was usually due to cardiopulmonary failure following severe inflammation of the heart and lungs (Kulkarni *et al*, 1995).

Of the 30-40% of Tgfβ1 null mice that did not make it through to parturition, it was thought that either there was an under-representation of Tgfβ1 null gametes (due to reproductive abnormalities in heterozygous animals) (Shull and Doetchman, 1994), or that Tgfβ1 null conceptuses were dying *in utero* (Dickson *et al*, 1995). On a mixed genetic background, Dickson *et al* (1995) showed that, although normal Mendelian genotype ratios of wild-type, heterozygous, and null conceptuses were present at 9.5

days of gestation, the number of viable Tgfβ1 null embryos was reduced to 50% of expected levels by 11.5 days of gestation. It was found in the Tgfβ1 null conceptuses that the vascular network, which develops from yolk sac blood islands, had failed to form properly. More specifically, there was lack of endothelial tube formation and the establishment of weak endothelial connections probably due to inadequate endothelial differentiation. The other phenotype of the Tgfβ1 null yolk sac was severe anaemia, which is perhaps surprising since Tgfβ1 has been reported to be an inhibitor of haematopoiesis. However, both these facts probably point towards the primary function of Tgfβ1 in early embryogenesis as a modulator of cellular differentiation and phenotype, and not as a negative growth regulator.

Tgfβ2 and Tgfβ3 have also been knocked out in transgenic mice. Tgfβ3 null animals reach parturition with no intrauterine death but they all die within hours of birth due to posterior clefting of the secondary palate. The cellular defect is one of epithelial cell migration out of the midline seam (Proetzel *et al*, 1995).

It became clear during studies on *Tgf*β1 knockout mice in this lab that the percentage of *Tgf*β1 null prenatal lethality depends on the genetic background of the mice. Similarly, the severity of the *Tgf*β3 null phenotype depends on the genetic background of the mice (Proetzel *et al*, 1995). Bonyadi *et al* (1997) used the differences between survival of *Tgf*β1 nulls on a NIH genetic background (70-80% survival) and survival on a C57/Bl6 background (0% survival) to map a potential genetic modifier of the *Tgf*β1 knockout phenotype. A major codominant modifier locus of embryo lethality was identified on proximal mouse chromosome 5. This gene accounted for approximately three-quarters of the genetic effect between strains.

1.2.4.2 *Tgf*β Transgenic Mice

There have been numerous attempts to make transgenic mice which overexpress Tgfβ but many of these have failed due to absence of transgene expression or embryonic lethality (Sporn and Roberts, 1992). However, several laboratories have generated Tgfβ1-expressing transgenic mice, using tissue-specific gene promoters to

restrict the expression of Tgfβ1 to specialised organ systems. Mice which overexpress Tgfβ1, or related proteins, in skin will be discussed in section 1.2.5. Here, the effect of ectopic expression of Tgfβ1 in mammary gland and pancreas are summarised.

The mammary gland is attractive as a model system to study since the development of this tissue takes place mostly postnatally and is therefore readily accessible. Silberstein and Daniel (1987) had originally shown the reversible inhibitory effects of Tgfβ on mouse mammary gland development by implanting slow-release pellets containing Tgfβ next to developing epithelial end buds. Two groups subsequently made transgenic mice ectopically expressing Tgfβ1 in the mammary gland. Jhappan *et al* (1993) used the whey-acidic protein (*WAP*) gene promoter to direct expression to the alveoli, while Pierce *et al* (1993) targeted Tgfβ1 expression to the mammary ductal epithelial cells using the mouse mammary tumour virus (*MMTV*) promoter. *WAP-Tgf*β1 transgenic females displayed significant inhibition of lubuloalveolar formation and therefore defective lactation. These mice were shown to have increased rates of apoptosis in the mammary epithelium at all stages of mammary development (Kordon *et al*, 1995). The *MMTV-Tgf*β1 mice however exhibited hypoplasia of the mammary ductal tree during mammary development, with no effect on alveolar outgrowths during pregnancy. The different phenotypes of these two transgenic lines highlight the difference of using two different promoters directed at the same tissue.

More recently, Bottinger *et al* (1997a) overexpressed a dominant-negative mutant Tgfβ type II receptor (DN-TβRII) in the mammary gland with the *MMTV* promoter. They showed that older virgin transgenic mice had an increased incidence and degree of lobulo-alveolar side-branching in the mammary gland when compared with their wild-type littermates. All this evidence suggests that endogenous Tgfβs are important in maintaining normal mammary epithelial homeostasis. Another report by the same group highlighted the importance of Tgfβ in the pancreas (Bottinger *et al*, 1997b). Targeting of a dominant-negative TβRII to the exocrine pancreas caused increased proliferation of pancreatic acinar cells and also showed perturbed acinar differentiation.

1.2.5 TGFβ in Skin and Multistage Carcinogenesis

1.2.5.1 Expression and Role of TGFβ in Skin Homeostasis

As discussed in section 1.2.2 TGFβ has a profound inhibitory effect on keratinocyte proliferation in culture (Reiss and Sartorelli, 1987). *In vivo*, the phorbol ester TPA was shown to induce high levels of *Tgf*β1 mRNA in mouse epidermis (Akhurst *et al*, 1988). This appeared surprising in view of the fact that initated keratinocyes and early papillomas responded to Tgfβ by growth arrest, but nevertheless underwent clonal expansion in response to treatment with TPA. To elucidate this apparent inconsistency, an investigation of *Tgf*β mRNA and protein synthesis was carried out by a combination of *in situ* hybridisation and immunocytochemistry using isoform specific antibodies to Tgfβ1 (Fowlis *et al*, 1992). TPA treatment of adult mouse skin induced Tgfβ1 protein in suprabasal keratinocytes i.e. that sub-population of epidermal cells which had previously been shown to produce the highest levels of *Tgf*β1 mRNA. The extracellular or processed form of Tgfβ1 was located mainly in the dermis suggesting that it may be involved in a paracrine mechanism of regulatory comunication between epidermal and mesodermal tissues. More recently, Glick *et al* reported basal cell hyperproliferation in the normal epidermis of *Tgf*β1 knockout mice with the observation of a 3- to 5-fold increase in the BrdU labelling index without evidence of suprabasal proliferation or hyperplasia (Glick *et al*, 1993).

To further investigate the role of Tgfβ1 in the epidermis, two different laboratories generated transgenic mice which use keratin promoters to drive Tgfβ1 expression mainly in the suprabasal layer of the epidermis (Sellheyer *et al*, 1993; Cui *et al*, 1995; Fowlis *et al*, 1996). Sellheyer *et al* (1993) targetted expression using a human keratin 1 (K1) promoter driving a porcine *Tgf*β1 cDNA. All of the founder mice died within 24 hours of birth due to complete inhibition of epidermal development. There was complete inhibition of DNA synthesis in the epidermis and a reduced number of hair follicles. Cui *et al* (1995) generated viable lines of transgenic mice using the K10 promoter constitutively expressing bioactive simian Tgfβ1 in suprabasal cells.

Surprisingly, these mice had a two- to threefold increase in epidermal DNA labelling index over control mice, in the absence of hyperplasia. The marked difference in phenotype between the mice of Cui *et al* (1995)and Sellheyer *et al* (1993) is probably due to the level of transgene expression. The K1 promoter is a stronger promoter than that of the *K10* gene. This suggested that Tgfβ1 not only increased keratinocyte proliferation *in vivo*, but also increased keratinocyte turnover in a compensatory manner. In these same transgenic mice however, the transgene acted as expected, as a negative regulator of cell growth, when hyperplasia was induced by treatment with the phorbol ester TPA. Analysis of expression of the Tgfβ type II receptor (TβRII) was consistent with these observations. In quiescent epidermis, TβRII expression was barely detectable, but increased rapidly in response to TPA treatment (Cui *et al*, 1995). Taken together, all this data suggests that Tgfβ1 and TβRII act in concert to regulate epidermal homeostasis *in vivo*.

A similar result was seen when Tgfβ1 was expressed in suprabasal cells under the control of an inducible promoter, K6 (Fowlis *et al*, 1996). On treatment of these transgenic mice with topical TPA, the transgene was "switched on" and, as with K10-*Tgf*β mice (Cui *et al*, 1995), epidermal BrdU labelling index was higher in transgenics than control mice.

Tgfβ's role in maintenance of skin homeostasis was confirmed when a dominant-negative TβRII was expressed in the epidermis of transgenic mice (Wang *et al*, 1997). Targeting expression with a loricrin promoter produced mice with a thickened and wrinkled skin showing hyperplasia and hyperkeratosis. *In vivo* labelling with BrdU showed a 2.5-fold increase in labelling index over controls, with labelled cells occurring in both basal and suprabasal cells of transgenic epidermis. However, a similar transgenic line using the K5 promoter produced mice with undisturbed normal skin (Amendt *et al*, 1998). The K5 promoter targets only basal and follicular cells while the loricrin promoter drives expression in both basal and suprabasal cells. Therefore the different phenotypes of these two mice may indicate a more significant role for Tgfβ signalling in suprabasal keratinocytes than basal cells for maintaining

skin homeostasis. Alternatively, it may be a case of different levels of expression: the weaker K5 promoter may not be a complete knockout of functional TβRII.

1.2.5.2 TGFβ and Tumour Suppression

TGFβ is a potent inhibitor of epithelial proliferation, can affect epithelial differentiation, and can induce apoptosis. These properties suggest that disruption of TGFβ metabolism might play an important role in multistage carcinogenesis. Conflicting findings from various studies have promoted the idea that TGFβ might have biphasic effects during epithelial carcinogenesis. In this section, TGFβ's tumour-suppressing properties will be discussed, and in the following section its activities as a potential promoter of carcinogenesis will be summarised.

TGFβ can inhibit proliferation of several human and rodent transformed and carcinoma cell lines *in vitro* (Manning *et al*, 1991; Arteaga *et al*, 1990; Haddow *et al*, 1991) and it has been shown that the growth inhibitory effect can be reversed using anti-TGFβ antibodies (Arteaga *et al*, 1990). Many transformed keratinocytes and cell lines derived from skin tumours have lost their response to TGFβ (Haddow *et al*, 1991). Using a panel of cell lines derived from benign and malignant mouse skin tumours, it was shown that the loss of growth control by TGFβ occurs at a relatively late stage of carcinogenesis and furthermore, that it is independent of *Ras* gene activation. The question of how this ties in with the action of endogenous TGFβ during tumour progression has been tackled in a number of *in vivo* studies.

Wu *et al* (1992) showed that transfection of a TGFβ-responsive colon carcinoma cell line with a TGFβ1 antisense-expression vector enhanced its tumorigenicity *in vivo*. More direct evidence that TGFβ expression can suppress tumour growth *in vivo* has come from studies on transgenic mice. Pierce *et al* (1995) demonstrated mammary tumour suppression by transgenically targeted Tgfβ1. In our own lab, tumour formation was investigated using mice which express recombinant Tgfβ1 in the epidermis (Cui *et al*, 1996). Several transgenic lines of mice, which expressed active Tgfβ1 in suprabasal keratinocytes either constitutively (Cui *et al*, 1996) or in response

to hyperplasia (Fowlis *et al*, 1996) were subjected to chemical carcinogenesis, involving initiation with dimethylbenzanthracene (DMBA), followed by repeated application of the tumour promoter, TPA, to the skin. The transgenics were more resistant to induction of benign skin tumours than controls due to a prolonged latency and significantly reduced tumour number per mouse.

In accordance with Tgfβ's tumour suppressing activities, loss of Tgfβ expression in benign tumours is correlated with a high risk for malignant conversion (Glick *et al*, 1993; Cui *et al*, 1994). As mentioned earlier, populations of benign papillomas have different rates of premalignant progression and malignant conversion. Glick *et al* (1993) demonstrated an association between the loss of expression of Tgfβ proteins within tumour cells and a high risk of conversion to the malignant state, and Cui *et al* showed that, in benign tumours from p53 null mice, Tgfβ1 protein staining is prognostic for a low probability of malignant conversion (Cui *et al*, 1994). The importance of Tgfβ loss in progression was further demonstrated by reconstituting papillomas in a skin-grafting model system using combinations of v-Ras^{Ha}-initiated keratinocytes and dermal fibroblasts from Tgfβ knockout mice or the wild-type littermates (Glick *et al*, 1994). The v-Ras^{Ha}-transduced wild-type keratinocytes produced benign papillomas in grafts whereas the transduced Tgfβ-null keratinocytes formed either very dysplastic papillomas or squamous cell carcinomas.

1.2.5.3 TGFβ as a Stimulator of Malignant Progression

Tgfβ has also been shown to have positive effects on malignancy during multistage carcinogenesis (Arteaga *et al*, 1993; Glick *et al*, 1994; Cui *et al*, 1996). When tumour cells become TGFβ-resistant, TGFβ production may be increased because there is no longer a selection for down-regulating its production. For example, malignant skin carcinomas, which are no longer responsive to Tgfβ had increased *Tgf*β1 mRNA expression (Krieg *et al*, 1991). Using transgenic mice which overexpress Tgfβ1 in the epidermis, Cui *et al* (1996) found that Tgfβ1 has apparently biphasic effects during multistage carcinogenesis, working in an inhibitory manner at early stages, when

normal growth responses to Tgfβ are active, but accelerating the conversion to malignancy and increasing the incidence of spindle cell carcinomas at later stages.

As previously discussed, TGFβ actively promotes angiogenesis and endothelial cell spreading, wound healing and immunosuppression. These properties could all serve a role to enhance tumour growth. Welch *et al* (1990) showed that TGFβ increased surface lung metastases following inoculation of a TGFβ-resistant mammary adenocarcinoma clone into syngeneic rats. A 6-fold increase in collagenolytic activity and a rise in heparanase activity accompanied this increased invasive potential. Portella *et al* (1998) demonstrated that Tgfβ1 can act directly on keratinocytes *in vivo* to induce the epithelial-to-mesenchymal conversion of a malignant invasive cell line. Further evidence of the importance of the Tgfβ signalling pathway for induction and maintenance of invasiveness and metastasis was demonstrated by Oft *et al* (1998). Expression of a dominant-negative type II TgfβR in Ha-*Ras* transformed mammary epithelial cells blocked the epithelial-to-mesenchymal transition. Furthermore, the same TgfβRII dominant-negative, when expressed in a highly metastatic colon carcinoma cell line, inhibited their *in vitro* invasiveness and completely abolished metastasis formation (Oft *et al*, 1998). Recently, Hojo *et al* (1999) showed that TGFβ induced by cyclosporine could enhance metastasis in vivo and the effect was independent of the host's immune cells.

1.3 P53 and *Tp53* Knockout Mice

1.3.1 The P53 Tumour Suppressor

More than 50% of human primary tumours contain *TP53* mutations (Hollstein *et al*, 1991) and it is well accepted that inactivation of P53 is one of the central events leading to malignant transformation. It has been described as the "guardian of the genome" (Lane, 1992) because of its crucial role in the cell's response to genotoxic stress. In normal cells under physiological conditions the P53 protein is expressed at low levels and has a short half-life due to rapid turnover mediated by ubiquitination and proteolysis. The protein becomes stabilised and activated in response to stressful stimuli including exposure of cells to DNA damaging agents, hypoxia, nucleotide

depletion, or oncogene activation. The response to these insults takes the form of either cell-cycle arrest or apoptosis and it is thought that the tumour suppressing function of P53 is to preserve genomic integrity (Lane, 1992). Cell cycle arrest at the G1 stage allows cells time to repair DNA damage instead of fixation as mutations, whilst apoptosis serves to eliminate potentially oncogenic cells which are mutated.

The P53 protein is a DNA-binding transcription factor that enhances the rate of transcription of several known genes that carry out, at least in part, the P53-dependent functions of a cell. Through analysis of the degeneracy of the P53 DNA binding site, it has been suggested that there may be as many as 200-400 P53 target genes (El-Deiry *et al*, 1992). These include mediators of cell cycle arrest such as $P21^{CIP1}$ (El-Deiry *et al*, 1993) and *GADD45* (Zhan *et al*, 1998) and mediators of apoptosis like *BAX* (Miyashita and Reed, 1995) and *FAS/APO1* (Owen-Schaub *et al*, 1995). Some targets of P53 appear to negatively regulate its function through regulation of the half-life of P53. The *MDM2* gene encodes a P53-binding protein that conceals the P53 transactivation domain thereby inhibiting its function as a transcriptional activator. MDM2 plays a crucial role in targeting P53 for degradation *via* ubiquitination and proteolysis (Haupt *et al*, 1997; Kubbutat *et al*, 1997).

1.3.2 *Tp53* Knockout Mice

Knockout mouse studies have provided invaluable insight into the role of p53 in tumorigenesis. p53 null mice were generated by several groups, all resulting in similar conclusions (Donehower *et al*, 1992; Clarke *et al*, 1993; Jacks *et al*, 1994). The majority of mice deficient in p53 develop normally, however, these animals are prone to the spontaneous development of a variety of neoplasms. The two major tumour types that develop are lymphomas and sarcomas. Other rare tumour types observed include mammary and lung adenocarcinomas, medullablastoma, glioblastoma, testicular tumours, and hepatomas. The majority of mice lacking p53 die by 6 months of age with a mean time to tumour onset of only 20 weeks. *Tp53* heterozygous mice also succumb to tumour development but with a delayed onset and a difference in tumour spectrum compared to p53-null mice (Harvey *et al*, 1995). By 12 months of age only 8% of the heterozygote mice develop tumours, but from 12 months onwards

the rate of tumour development accelerates such that by 18 months of age, 50% of *Tp53* heterozygote mice acquire tumours. Heterozygous mice develop more sarcomas than lymphomas. Thus it is clear that loss of one or both copies of *Tp53* can facilitate tumour development.

Differences in tumour phenotype have been observed in p53 null mice of different genetic backgrounds. For example, 35% of the pure 129/Sv strain null for p53 develop testicular tumours as compared to 9% in p53 null mice on the 129/Sv-C57Bl/6 hybrid strain (Harvey *et al*, 1993). As mentioned above, most p53 null mice are viable and fertile but some developmental abnormalities became visible upon closer inspection (Sah *et al*, 1995). In crosses between *Tp53* heterozygote animals, 25% of the offspring should be null for p53 but this ratio is reduced. Some female embryos were found to display defects in neural tube closure resulting in exencephaly. The increased proliferative potential of the neural ectoderm in mice lacking p53 is in keeping with p53 functioning as a growth suppressor.

Kemp *et al* (1994) showed that p53 null mice were susceptible to radiation-induced tumourigenesis. The irradiated null mice showed a much decreased latency for tumorigenesis compared to irradiated wild-type controls. Another finding was that during chemically-induced carcinogenesis of the skin in *Tp53* knockout mice, loss of p53 did not increase the initiation rate or number of papillomas, but had a marked effect on enhancing malignant conversion to carcinomas.

The *Tp53* knockout mice have been very useful in analysing cooperating effects with other genes during tumorigenesis due to the ability to generate mice with two distinct genetic alterations by breeding. A line of mice that carries a truncated form of SV40 large T antigen, which can bind and inactivate Rb (retinoblastoma gene product) but not p53 (Symonds *et al*, 1994), slowly develop choroid plexus tumours. On breeding these mice to a p53 null genetic background, the animals developed tumours more rapidly. There was also a significant decrease in survival of mice, with a focal emergence of aggressive tumour nodules. The aggressive phenotype corresponded to a decrease in the levels of apoptosis (Symonds *et al*, 1994). Other transgenic and knockout lines in which crossing on to a p53-null background caused a change in

tumour phenotype or latency include *Rb* knockout (Williams *et al*, 1994), *Apc* knockout (Clarke *et al*, 1995), *Atm* knockout (Westphal *et al*, 1997), MMTV-*Wnt1* transgenics (Donehower *et al*, 1995b) and MMTV-*Ras* transgenics (Hundley *et al*, 1997). All these lines showed a reduction in tumour latency on a p53 null background compared to a wild-type p53 background.

Recently, the importance of one of the key negative regulators of p53, Mdm2, was demonstrated in a cross between mice carrying null alleles for these two genes (Montes de Oca Luna *et al*, 1995; Jones *et al*, 1995). Mice null for Mdm2 are not viable and die shortly after implantation at 5.5 days of development. Surprisingly, when crossed on to a p53 null background, the early embryonic lethal phenotype was completely rescued. This data supported two important concepts: (1) the dividing cells of the early embryo make functional p53 and, (2) p53 must be down-modulated by Mdm2 for cell division to proceed.

1.3.3 P53 and TGFβ

TGFβ can inhibit cell proliferation by causing growth arrest in the G1 phase of the cell cycle (Massague, 1990). Progression through G1 is dependent on the sequential formation, activation and subsequent inactivation of the G1 cyclin-CDK complexes, primarily cyclin D-CDK4 and cyclin E-CDK2 complexes (Sherr, 1993). TGFβ has been shown to down-regulate the synthesis of CDK4 and inhibit the activity of cyclin E-CDK2 (Ewen *et al*, 1993; Koff *et al*, 1993). As mentioned above, the activity of the CDKs is negatively regulated by a number of small proteins including $p21^{CIP1}$, $p27^{KIP1}$, $p15^{INK4b}$ and $p16^{INK4a}$. Datto *et al* (1995) showed that in the HaCaT keratinocyte cell line TGFβ could induce $p21^{CIP1}$. $p21^{CIP1}$ is mediator of P53-induced cell cycle arrest and TGFβ's effects on these CDK inhibitors has raised the possibility of P53 being a mediator of TGFβ signalling.

The involvement of P53 as a potential mediator of TGFβ response seems to depend very much on the cell line being analysed. Blaydes *et al* (1995) found that introduction of a dominant negative P53 mutant into TGFβ-responsive thyroid epithelial cells

containing wild-type P53 did not reduce responsiveness, demonstrating that P53 function is not necessary for TGFβ response. However, expression of a temperature-sensitive *TP53* gene in a partially responsive rat line demonstrated a significant modulation of TGFβ response (Blaydes *et al*, 1995). Datto *et al* (1995) showed that $p21^{CIP1}$ induction by TGFβ in HaCaT cells was p53-independent while in Mv1Lu mink lung epithelial cells, Ewen *et al* (1995) demonstrated that TGFβ down-regulated CDK4 translation in a P53-dependent manner. Analysis of apoptotic pathways in the M1 myeloid leukaemia cell line suggested that P53-induced apoptosis and that induced by TGFβ were working through different pathways (Selvakumaran, 1994). Ectopic expression of BCL-2 blocked TGFβ-induced apoptosis but only delayed P53-induced apoptosis. Thus, the role, if any, of P53 in a TGFβ-induced arrest or apoptotic pathway is dependent on the tissue or cell line being analysed.

1.3.4 P53 and Skin

Mutations of the *TP53* tumour suppressor gene have been observed in over 90% of non-melanoma skin cancers (Brash *et al*, 1996), including human cutaneous squamous cell carcinoma (SCC) (Burns *et al*, 1993), UVB-induced mouse SCC (Kanjilal *et al*, 1993) and human basal cell carcinomas (Rady *et al*, 1992). These observations suggest that P53 plays an important role in the pathology of skin tumorigenesis and a number of groups have investigated the role of P53 in normal skin and its carcinogenesis. In normal quiescent skin, functional P53 is not a vital component of growth control as demonstrated by the fact that the skin of p53-deficient mice (Donehower *et al*, 1992) does not display any abnormalities. Hall *et al* (1993) found that no P53 antibody staining was seen in normal human skin but it appeared within 2 hours of UV exposure and peaked at 24 hours. The role of p53 in UV-induced apoptosis in the skin was demonstrated by Ziegler *et al* (1994) that the apoptosis rate was significantly lower in p53 null mice than the wild-type controls. This result differed from the findings of Li *et al* (1996) who reported that skins of transgenic mice carrying a *Tp53* mutant allele had a slight increase in apoptosis rates following UV irradiation. This may be due to the presence of functional wild-type p53 in these mice. Interestingly, the *Tp53* mutant-

carrying mice showed a reduction in repair of UV-induced DNA damage (Li *et al*, 1996).

In terms of skin tumorigenesis it appears that p53 plays different roles in the development of UV-induced and chemical-induced skin cancers. Li *et al* (1995; 1997) used two different mouse models to look at skin tumour induction by UV irradiation. In a *Tp53* transgenic line that carries both wild-type and mutant *Tp53* alleles, they found that these mice developed more tumours and more multiple tumours than control mice, but the latent period for skin tumour development was similar in *Tp53* transgenic and control groups (Li *et al*, 1995). In the second experiment, again using UV irradiation, *Tp53* knockout mice were analysed: they noted that p53 null mice had a higher incidence and a shortened latent period for UV-induced skin tumours compared to wild-type controls. In contrast to this, the tumour incidence of chemically-induced skin tumours was similar in wild-type and heterozygous *Tp53* knockout mice and decreased in *Tp53* null mice (Kemp *et al*, 1993b). The latency of chemically-induced tumours was also similar in wild-type, heterozygous, and homozygous p53-deficient mice; however, the tumours induced in p53 null mice were more malignant, and the loss of *Tp53* alleles enhanced the rate of malignant progression (Kemp *et al*, 1993b).

Thus, it is likely that loss of p53 function is an important step in the initiation of UV-induced skin cancers, but is a rate-limiting causal step in malignant progression of chemical-induced tumours. More recently, under a chemical carcinogenesis regimen, a transgenic line expressing a mutant *Tp53* in the epidermis (K1-$Tp53^{m}$) was shown to undergo accelerated papilloma formation, malignant conversion, and metastasis compared to wild-type mice (Wang *et al*, 1998a). In a follow-up study, cells of papillomas from the K1-$Tp53^{m}$ line were shown to have a significantly higher number of centrosomes than those from p53 null mice, suggesting a gain of function for this mutant *Tp53* (Wang *et al*, 1998b).

1.4 BCL-2

1.4.1 BCL-2 Functions and Biochemistry

One of the most prominent regulators of apoptosis is the proto-oncogene *BCL-2* (B-cell lymphoma 2). As implied by its name this gene was discovered because of its involvement in B-cell malignancies, where chromosomal translocations activate the gene in the majority of follicular non-Hodgkin's B-cell lymphomas (Tsujimoto *et al*, 1985). BCL-2 is now known to belong to a growing family of apoptosis-regulatory gene products, which may be either death antagonists including BCL-2, BCL-X_L, BCL-w, MCL-1, and A1 and death agonists BAX, BAK, BCL-X_S, BAD, BID, BOK, BIK, BIM, KRK, and MTD (Kroemer, 1997).

The members of this BCL-2 family differ in their tissue- and activation-dependent expression patterns, as well as in structural features. Most members possess a carboxy-terminal transmembrane region but some, for example BID and BAD, do not, thereby influencing their subcellular distribution. In addition, they possess variable amounts of BCL-2 homology (BH) regions (BH1 to BH4), which determine their capacity to interact with each other or with other unrelated proteins. The BH1 and BH2 domains are required for BCL-2 and BCL-X_L to interact with BAX and to suppress apoptosis. However, mutations in BCL-X_L can prevent hetero-dimerisation with BAX or BAK but still maintain anti-apoptotic activity, suggesting that the anti-apoptotic proteins can also function independently to regulate cell survival (Cheng *et al*, 1996). Furthermore, using Bax- and Bcl-2-deficient mice, Knudson and Korsmeyer (1997) showed that these molecules could also function independently of each other.

The BH3 domain of proapoptotic proteins such as BAX, BAK or BAD is sufficient but not required for their binding to BCL-2 or BCL-XL and to promote apoptosis (Chittenden *et al*, 1995). In fact, this domain probably plays a dominant role since introduction of the BH3 domain of BAX in BCL-2 is sufficient to convert BCL-2 to a killer protein (Hunter *et al*, 1996). In addition there are proteins such as BID, BIK, and

BIM that have BH3 domains but lack identifiable BH1 and BH2 domains and which are nevertheless efficient proapoptotic proteins, confirming the key role of the BH3 domain in triggering apoptosis.

A fourth domain, BH4, is found in the N-terminal region of antiapoptotic proteins only (apart from the proapoptotic BCL-X_S that also contains a BH4 domain). This domain has been shown to bind to several proteins including RAF-1 (Wang *et al*, 1996) and CED-4 (Wu *et al*, 1997a). Mutants of BCL-2 lacking the BH4 domain not only lose their antiapoptotic activity but behave like killer proteins (Cheng *et al*, 1997). Proteolytically modified forms of BCL-2 lacking the BH4 domain have been shown to be produced through caspase cleavage during apoptosis and were found to accelerate the apoptotic process (Grandgirard *et al*, 1998).

BCL-2 family proteins can undergo posttranscriptional changes. Two modifications, phosphorylation and proteolysis, have been described and shown to have regulatory function on the activity of some family members. BCL-2 has been shown to be phosphorylated following exposure of cells to Taxol or paclitaxel and this may be via a Jun N-terminal kinase (JNK) pathway (Yamamoto *et al*, 1999). The proapoptotic protein BID has been shown to be a substrate for caspase 8 (Li *et al*, 1998). Activation of FAS leads to the activation of caspase 8 which cleaves BID generating a C-terminal fragment of the protein. This fragment can trigger cytochrome c release leading to cell death.

The BCL-2 family members have been shown to be both membrane associated and cytosolic. BCL-2, which appears to be exclusively localised to membranes, has been reported to be associated with ER, mitochondrial membranes, and the nuclear envelope (Krajewski *et al*, 1993). In contrast, BAX and BCL-X_L are found in the cytosol. It is thought that the protein localisation changes during apoptosis. For example, BAX was found to move from the cytosol to the mitochondria when apoptosis was induced (Hsu *et al*, 1997). Insertion of BCL-2 family members such as BAX into the mitochondrial membrane can trigger cytochrome c release (Jurgensmeier et al, 1998) leading to downstream apoptotic events.

1.4.2 Bcl-2 Knockout and Transgenic Mice

Mice deficient in Bcl-2 were created by gene targeting in embryonic stem cells (Veis *et al*, 1993; Nakayama *et al*, 1994). Mice homozygous for the *Bcl-2* mutation complete embryonic development and appear normal during the first week after birth. After this however, the mice begin to show growth retardation and many die young. These mice have massive apoptosis in the embryonic kidney and hypoplastic renal development, which progresses to severe polycystic kidney disease. Initially, *Bcl-2* mutant mice have normal hair pigmentation but, at five to six weeks, with the second hair follicle cycle, the hair turns grey. This is a result, at least in part, of the loss of melanocytes. Over time, Bcl-2-deficient mice develop fulminant apoptosis of the thymus and spleen, and an almost complete loss of lymphocytes (Veis *et al*, 1993). Thus, Bcl-2 may have its most dramatic role in maintaining homeostasis in adult tissues.

A number of transgenic mouse lines have enabled researchers to investigate which processes rely on a Bcl-2-sensitive death programme. Targeted expression of Bcl-2 to lymphoid cells leads to an increase in the numbers of mature resting B cells and potentiates their longevity (McDonnell *et al*, 1989). Affected T cells are markedly resistant to the cytocidal effects of radiation, glucocorticoids and anti-CD3, but thymic censorship appears normal. These *Bcl-2* transgenic mice go on to develop a low incidence of malignant lymphoma. Lymphoid-specific expression of *Bcl-2* transgenes allowed a novel subset of pre-B cells to accumulate in *severe combined immunodeficiency* (*Scid*) mutant mice and in mice lacking recombination activating genes Rag-1, Rag-2, or immunoglobulin μ heavy chain (Strasser *et al*, 1994; Young *et al*, 1997), and this has implications for signalling via the pre-B cell receptor and the B cell receptor. The physiological significance of apoptosis during spermatogenesis has also been established because ectopic expression of Bcl-2 or Bcl-X_L in mouse testis results in male sterility (Furuchi *et al*, 1996; Rodriguez *et al*, 1997a).

The Bcl-2 protein has been shown to be capable of preventing apoptosis in a number of other cell types through overexpression in transgenic mice. These include neurons

of the central nervous system (Martinou *et al*, 1994), hepatocytes (Lacronique *et al*, 1996), retinal photoreceptors (Chen *et al*, 1996) and oocytes (Morita *et al*, 1999). Apoptotic cell death was also prevented in the mammary gland by expression of Bcl-2 under the control of the whey acidic protein (WAP) promoter (Humphreys *et al*, 1996; Jager *et al*, 1997). Jager *et al* (1997) also showed that Bcl-2 could contribute to mammary carcinogenesis by inhibiting apoptosis. When these mice were crossed to MMTV-*Myc* transgenic mice, the Bcl-2 overexpression led to an accelerated development of Myc-induced mammary tumours.

1.5 Growth Control and Apoptosis

1.5.1 Tissue Homeostasis

Tissue homeostasis is regulated by a balance between proliferation, growth arrest and programmed cell death (or apoptosis) (Korsmeyer, 1995). Up until relatively recently, studies of tumorigenesis tended to concentrate on the regulation of cell proliferation. It is now clear that negative growth control (including apoptosis) must also be understood to elucidate how appropriate cell numbers within a tissue are maintained, and how changes to the equilibrium can contribute to malignancy.

One way in which a tissue maintains negative growth control is through terminal differentiation. During this conversion of proliferating undifferentiated cells into non-proliferating, highly differentiated cells, two interrelated cellular processes are invoked; the regulated progression of cells through successive stages of cell differentiation, and growth inhibition which leads to growth arrest. In tissues where there is a rapid turnover of cells, terminal differentiation may culminate in apoptosis of the cell. Therefore, differentiation-inducing cytokines can be considered as a type of negative regulator of cell growth. TGFβ is a strong inhibitor of proliferation of most epithelial, endothelial, and haematopoietic cells as has been discussed previously. It has also been shown to induce apoptosis (Oberhammer *et al*, 1992) and differentiation (Zentella and Massague, 1992) in various cell types.

Cell cycle regulatory genes, proto-oncogenes and tumour suppressor genes are being shown to play major roles in processes such as positive and negative regulation of cell growth, differentiation and survival. Progression through the cell cycle is governed by the cyclin-dependent kinases (CDKs) and their associated subunits, the cyclins (Sherr, 1993; Sherr, 1994). One way in which the CDKs are known to exert positive control on the cell cycle is by hyperphosphorylation of and inactivation of negative cell cycle regulators such as RB (retinoblastoma gene product) (Sherr, 1994). CDK inhibitors such as $p21^{CIP1}$, $p27^{KIP1}$, $p15^{INK4b}$ and $p16^{INK4a}$ have been implicated in negative growth control (Elledge and Harper, 1994). The tumour suppressor P53 is also closely involved in cell cycle regulation, mediating delay or arrest at checkpoints along the cell cycle following DNA damage (Lane, 1992). It has also been shown to play a vital role in the induction or mediation of various apoptotic pathways (Oren, 1994). The product of the proto-oncogene *BCL-2* is known to play a role in promoting cell survival and inhibiting apoptosis (Oltavai and Korsmeyer, 1994) and its connection to the cell cycle is now being elucidated.

1.5.2 Apoptosis

Apoptosis is a physiological form of cell death that plays a critical role in development, tissue homeostasis, and immune defence in multicellular organisms (Wyllie *et al*, 1980; Vaux *et al*, 1994). During apoptosis, a cell activates an intrinsic suicide mechanism that systematically destroys the cell. Its surface membrane begins to bleb and express pro-phagocytotic signals, the cell shrinks and severs contact with its neighbours, chromatin becomes condensed and cleaved, and eventually the whole cell fragments into membrane-bound vesicles that are rapidly ingested by neighbouring cells (Fraser and Evan, 1996; Korsmeyer, 1995). This cell suicide is programmed by genetically controlled pathways (Wyllie *et al*, 1980). Defects of genes that control this pathway that save cells normally destined for destruction may underlie both cancer and autoimmune diseases, while defects that promote excessive cell death may contribute to neurodegenerative disease and immunodeficiency.

1.5.3 TGFβ and Induction of Apoptosis

Initiation of apoptosis can be induced by the absence of incoming survival signals from the environment or signalled directly as a result of cell damage or through cytokine-mediated pathways. Several studies have provided evidence that TGFβ might act as an inducer of apoptosis in some cell types. Levels of *TGF*β1 mRNA were increased during castration-induced regression of the prostate, a process where apoptosis is of major importance (Kyprianou and Isaacs, 1989). In cultures of primary rat hepatocytes and human Hep 3B hepatoma cells, TGFβ1 appeared to induce cell death with apoptotic features, including DNA fragmentation, condensation of chromatin, cellular rounding and fragmentation, and phagocytosis by neighbouring cells (Oberhammer *et al*, 1991; 1992; Lin and Chou, 1992). Similar events have also been observed in cultures of primary rabbit uterine epithelial cells and endometrial stromal cells treated with TGFβ (Rotello *et al*, 1991; Moulton, 1994). Jurgensmeier *et al* (1994) cocultured transformed fibroblasts with normal fibroblasts and found that, in synergy with TGFβ, normal fibroblasts were able to eliminate transformed cells by induction of apoptosis.

The mechanisms by which TGFβ exerts its apoptotic effect are still poorly understood but recently some studies have been addressing this question. Bies and Wolff (1995) found that overexpression of B-MYB accelerated apoptosis in TGFβ1-treated M1 myeloid leukaemia cells, and overexpression of SMAD4, a signal transducer of the TGFβ family, caused MDCK cells to undergo apoptosis (Atfi *et al*, 1997). Caspase family proteases were activated in response to TGFβ treatment of the Hep3B hepatoma cell line (Chen and Chang, 1997) and, in the same cell line, the PI3-kinase/AKT pathway was shown to protect against TGFβ-induced apoptosis by inhibiting a step downstream of SMAD but upstream of caspase-3 (Chen *et al*, 1998).

1.5.4 P53 and Apoptosis

It is believed that P53-dependent apoptosis is one of the central pathways of somatic cells to secure the elimination of cells with abundant DNA damage. Under circumstances where DNA damage is irreparable, survival factors for the cells are limiting, or an activated oncogene is forcing the cell into a replicative cycle, P53-mediated apoptosis prevails over growth arrest. P53's involvement in apoptosis was realised when it was noted that over-expression of wild-type P53 could result in rapid loss of cell viability, in a manner characteristic of apoptosis (Yonish-Rouach *et al*, 1991). Indisputable evidence for the role of P53 in apoptotic processes was provided by the study of *Tp53* knockout mice. *Tp53*-/- thymocytes (Lowe *et al*, 1993; Clarke *et al*, 1993), and *Tp53*-/- stem cells of the small and large intestine (Clarke *et al*, 1994) are much more resistant to radiation-induced apoptosis than the corresponding wild-type cells. However, these studies also made clear that only a subset of apoptosis-inducing events are P53-dependent. For example, immature thymocytes lacking p53 die normally when exposed to glucocorticoids or to components that mimic T-cell receptor engagement (Lowe *et al*, 1993; Clarke *et al*, 1993).

The mechanism by which P53 stimulates apoptosis is still relatively unknown. Studies of $p21^{CIP1}$-deficient fibroblasts revealed the significant contribution of p21 induction to p53-mediated G1 arrest; however, the same studies also showed clearly that p21 was not required for p53-mediated apoptosis (Deng *et al*, 1995; Brugarolas *et al*, 1995). The first P53 target gene identified to encode a candidate effector of P53-mediated apoptosis was BAX (Miyashita and Reed, 1995). The relative contribution of BAX to p53-mediated apoptosis appears to be cell type-dependent: bax is not required for radiation-induced, p53-dependent apoptosis of thymocytes (Knudson *et al*, 1995). However, it does contribute to apoptosis of oncogene-expressing mouse fibroblasts, as shown by the attenuated apoptosis of such cells when derived from bax-deficient mice (McCurrach *et al*, 1997). Furthermore, in a transgenic mouse tumour model, it was shown that p53-induced bax expression is necessary for efficient inhibition of tumour growth, attributed to apoptotic elimination of the tumour cells (Yin *et al*, 1997). Other p53 target genes implicated in apoptosis include *FAS/APO1* (Owen-Schaub *et al*, 1995) and more recently *DR5/KILLER* (Wu *et al*, 1997b), p53-induced genes (*PIGs*)

(Polyak *et al*, 1997) and the IGF binding protein 3 (*IGF-BP3*) (Buckbinder *et al*, 1995).

1.5.5 BCL-2, Apoptosis and Skin

As discussed in section 1.1.2.1, the skin is a layered organ in which a differentiation programme exists that initiates within the basal layer, continues upward through the spinous layer, and terminates within the granular layer. Despite the constant generation of basal keratinocytes, the cellularity within each differentiation layer remains stable. Thus, the rate of cell loss or death in the differentiating suprabasal layers is in equilibrium with the rate of proliferation observed in the basal layer. Studies of the skin reveal that the morphological features seen in the granular and corneal layers are similar to the cellular changes described for apoptosis (Gavrieli *et al*, 1992). Furthermore, constant exposure to environmental hazards such as UV light dramatically increases the probability that an epidermal stem cell may acquire a harmful mutation. To protect the organism from accumulating damaged or aberrant cells, basal keratinocytes *in vivo* seem to be susceptible to apoptosis in response to genotoxic damage (Kraemer, 1997). In this way, apoptosis helps maintain homeostasis within the skin.

The identification of proteins from the BCL-2 family in skin suggests that these proteins may play a regulatory role in epithelial cell homeostasis (Rodriguez-Villanueva *et al*, 1995, Krajewski *et al*, 1994). There is some controversy as to which layer of the epidermis the protein is expressed in. A number of studies located BCL-2 expression exclusively in the basal layer of the epidermis (Hockenbery *et al*, 1991; Cerroni and Kerl, 1994; Verhaegh *et al*, 1995) while others found expression also in the spinous and granular layers (Krajewski *et al*, 1994; Stenn *et al*, 1994). Plettenberg *et al* (1995) on the other hand found that, within normal human epidermis, melanocytes were the only cells that expressed BCL-2 constitutively. In skin tumours, two different studies have revealed that basal cell carcinomas and malignant melanomas express BCL-2 while squamous cell carcinomas do not (Verhaegh *et al*, 1995; Morales-Ducret *et al*, 1995).

Recently, transgenic studies have investigated a role for Bcl-2 in skin and skin tumorigenesis. Rodriguez *et al* (1997b) found that overexpression of Bcl-2 in female mice could prevent vaginal opening by blocking apoptosis in this tissue. Targeting of human BCL-2 to the epidermis under control of a human keratin-1 (K1) promoter caused aberrant expression of keratin 6 (K6), regions of multifocal hyperplasia, and an increased susceptibility to skin tumour formation by chemical carcinogenesis (Rodriguez-Villanueva *et al*, 1998). Transgenic mice expressing Bcl-X_L or Bcl-X_S under control of the human keratin 14 (K14) promoter were also generated (Pena *et al*, 1997). The Bcl-X_L transgenics showed an increased resistance to UV-irradiation, whereas the Bcl-X_S transgenic mice showed an increased sensitivity. These studies suggest that BCL-2 and related proteins may play a role in influencing cell survival in the epidermis.

Chapter 2
MATERIALS AND METHODS

2.1 Mouse Procedures

2.1.1 Animal Husbandry

All mice were housed under a 14-hour light to 10-hour dark cycle. Animal technicians handled food, water and bedding requirements. All mouse procedures were conducted in accordance with Home Office Regulations and as detailed in the covering project licence. Unless otherwise stated, I conducted the procedures as covered by my Home Office personal licence.

2.1.2 Observation and Monitoring of Mice

In accordance with procedures as described in the Home Office project licence, all animals susceptible to the development of neoplasia were monitored at least twice a week for signs of ill-health and discomfort. Any animal suffering or deviating from a normal health status was sacrificed without delay. Mice which developed tumours were observed more closely and the position of the tumours, size of tumours and speed of growth of the tumours were recorded. If a single tumour reached 10mm in diameter or if 10% of the mouse body surface was affected by neoplasia, the mouse was sacrificed and relevant tissues were collected.

2.1.3 Identification and Tail Biopsy of Transgenic Mice

At 19-21 days of age mice were weaned and males and females were separated. The mice were given a number in consecutive sequence and identified by ear punching. They were put under brief anaesthesia with a halothane/oxygen mixture in an

anesthesia chamber. A small tail-tip biopsy (0.5-1.0cm) was taken using a hot, sterile scalpel blade to ensure cauterisation.

2.1.4 Collection of Tissues from Mice

When skin was to be collected, mice were shaved 48 hours before collection to check that the mouse was in the resting telogen phase of the hair cycle. Mice that exhibited signs of hair growth at the time of tissue collection were excluded from the experiment. If whole skin was being collected for histological analysis mice were injected with BrdU as described in Section 2.2.5. Animals were sacrificed by cervical dislocation. A strip of skin was cut from the shaved area, part of which was put in 4% paraformaldehyde (Sigma), and the rest snap frozen in liquid nitrogen for –70°C storage.

When only epidermal cells were required for RNA or protein extraction, the skin was treated with a hair-removing agent (Immac^{TM}) prior to sacrifice. The strip of skin was then immersed in water at 60°C for 30 seconds followed by immersion in ice-cold water for 30 seconds so that the epidermal cells could be scraped off using a scalpel blade. These cells were snap frozen in liquid nitrogen.

For collection of tumour samples, the mouse was injected with BrdU one hour before sacrifice. Tumour samples were split in two with one half being fixed in 4% paraformaldehyde and the other frozen in liquid nitrogen and stored at -70°C. A piece of adjacent dorsal skin was also taken from those affected mice for comparison.

2.1.5 Injection of Bromodeoxyuridine (BrdU) into Mice

BrdU is a potent carcinogen and so all procedures were carried out in a fume hood. Bromodeoxyuridine (Sigma) was dissolved at a concentration of 125mg/ml in water by heating the solution to 60°C. This was stored as a stock solution at 4°C and from this a working solution of 25mg/ml in sterile saline (0.85% NaCl) was made. Each mouse was weighed and injected intraperitoneally with the equivalent of 2µl BrdU solution

per gram of mouse (i.e. 50μg BrdU per g mouse). Exactly one hour later the mouse was sacrificed and the appropriate tissues fixed in 4% paraformaldehyde.

2.1.6 Chemical Carcinogenesis Treatment of Mice

All handling of TPA (12-O-tetradecanoylphorbol-13 acetate) was conducted in a fume hood. Animals were aged between 10 and 14 weeks at time of treatment and any animal found to be in the telogen phase of the hair cycle was excluded. TPA was made up as a stock solution using acetone as a solvent and stored at –20°C. From this, a working solution of $5x10^{-5}$ M was prepared and each application consisted of 200μl of this TPA solution being dropped on to a shaved area of dorsal skin. 200μl of acetone alone was used as a negative control. After treatment, mice remained in the fume hood for a minimum of 1 hour to ensure the chemicals had completely dried. Exactly one hour before skins were due to be harvested, mice were injected with BrdU as described above.

Mice were treated with TPA under two different regimes – a multi-application chronic treatment and a single-application acute treatment. Three mice of each genotype were treated for each timepoint. Under the acute treatment, mice received one application of TPA and skin was harvested at 0 hours, 6 hours, 12 hours, 24 hours, 48 hours, 4 days 7 days, and 10 days after the application. Chronic TPA treatment consisted of 4 applications of TPA to the dorsal skin: an initial one followed by applications at 3 days, 6 days and 9 days after the first. Skins were harvested at 12 days into the treatment.

2.2 Different Transgenic Mouse Lines Utilised

2.2.1 *Tgf*β1 knockout mice

Mice carrying a disrupted non-functional *Tgf*β1 gene were initially provided by Kulkarni *et al* (1993). Targeted deletion of some of the first exon and first intron was carried out by insertion of a neomycin-resistance gene to render the *Tgf*β1 gene non-functional (figure 2.1A). The mice were analysed on both the original mixed genetic background (50% NIH, 37.5%C57Bl6, 12.5%129SV) and also, after breeding through four generations with NIH mice, on a 93% NIH background.

These mice were genotyped, following a tail-tip biopsy, by polymerase chain reaction (PCR) - see below. Wild-type alleles were identified with nested primers from the first exon and intron of the mouse *Tgf*β1 gene (95,96,97,98). Null alleles with a primer from exon 1 (TGF) and from the phosphoglycerate kinase 1 (PGK1) promoter of the neomycin resistance cassette (PGK). Table 2.1 lists sequences of primers with annealing temperatures used.

2.2.2 Tgfβ1 transgenic mice

Transgenic mice engineered to overexpress a constitutively active simian Tgfβ1 protein were utilised. The two lines used in this investigation were the H-line (Cui *et al*, 1995) and the M2-line (Fowlis *et al* 1996) as shown in figure 2.1B. The H-line transgene consists of the bovine cytokeratin VI (K10) promoter driving the activated simian *Tgf*β1 cDNA with the SV40 small t intron and polyadenylation signal from SV40. This targets transgene expression to the suprabasal layer of skin epidermis. The M2-line transgene consists of the inducible bovine K IV (K6) promoter driving the same activated simian *Tgf*β1 and with the same SV40 polyA tail. Again, this targetted expression to the suprabal layer of epidermis, but only in response to a promoter such as TPA. Both lines of mice were on a 93% NIH genetic background.

Both these lines of mice were screened to detect the presence of the transgene either by PCR and/or by Southern Blotting. The PCR reaction was carried out using primers

from the SV40 polyA region of the transgene (SV1 and SV2, see Table 2.1). The cycle was as described in section 2.4.2 with an annealing temperature of 50°C. Southern blot analysis used a SV40-specific hybridisation probe to detect the transgene and a mouse cystic fibrosis transmembrane conductance regulator gene probe (mCftr, Tata *et al* 1991) as an internal DNA loading control was used to estimate hemi- or homozygosity of the transgene.

2.2.3 *Tp53* knockout mice

Mice carrying a disrupted, non-functional *Tp53* gene were initially provided by Donehower *et al* (1992). They had been derived by homologous recombination in an ES cell line of 129/Sv genetic background rendering the *Tp53* gene non-functional as shown in figure 2.1C. In this lab, they were bred by crossing them to NIH mice through 4 generations or transgenic mice with a NIH background.

The mice were genotyped by PCR. Wild-type alleles were identified with primers around the locus of exon 5 (P53W1 and P53W2) and null alleles with a primer from a *Tp53* intron (P53M1) and a primer from the inserted cassette (P53M2). The PCR was carried out as described in section 2.4.2 with an annealing temperature of 55°C.

2.2.4 Ras transgenic mice

K5-*RAS* mice were generated at the Beatson Institute for Cancer Research by K. Brown *et al* which consisted of the keratin 5 (K5) mini-gene driving a constitutively active human Ha-*RAS* cDNA as shown in figure 2.1D (Brown *et al*, 1998).
The mice were bred through 3 generations onto at least a 82% NIH genetic background. Genotyping was carried out by PCR either using the SV40 polyA PCR (described above for the *Tgf*β transgenic mice) or using primers specific for the mutated *RAS* gene itself (tcr1 and tcr2). When carrying out this PCR an internal control was also used by amplification of the thyroxine stimulating hormone beta (*Tsh*β) gene

(primers TSH1 and TSH2). The PCR reaction was carried out as described in section 2.4.2 and an annealing temperature of 55°C was used.

2.3 Genotyping of Transgenic Mouse DNA

2.3.1 Genomic DNA Extraction from Tail Tip Biopsy

The mouse tail tip was placed in 700µl of tail lysis buffer (100mM Tris-HCl pH8.5, 5mM EDTA, 0.2% SDS, 200mM NaCl) containing 0.1mg/ml proteinase K (Sigma) and was incubated at 55°C until the tissue was fully digested (usually overnight). Following lysis, the mixture was shaken and centrifuged in a bench top Eppendorf microcentrifuge at 13,000rpm for 10 mins to pellet the tail debris. The supernatant was decanted into a fresh 1.5ml eppendorf, and an equal volume of isopropanol was added. This mixture was shaken until precipitation was complete; the precipitate was removed with a sterile pipette tip, dried in a fresh eppendorf tube and the DNA was dissolved in 100µl 1xTE buffer.

2.3.2 Genotyping by PCR

Oligonucleotides were synthesised on an Applied Biosystems 391 DNA synthesiser. 1 µl of the genomic DNA preparation was mixed with 10-25pmol sense and antisense primers, 200µM each of dCTP, dATP, dTTP and dGTP (Boehringer Mannheim), 1 unit of Taq polymerase (Cambio) and 1x PCR reaction buffer (Cambio) in a total volume of 30µl. The reaction was overlaid with mineral oil (Sigma) to prevent evaporation and the PCR was carried out using a DNA Thermocycler (Perkin Elmer Cetus). 30 - 35 cycles of amplification were carried out, each cycle consisting of 3 sequential steps: denaturation (95°C for 1 min), annealing (50 - 62°C, 1 min), and elongation (72°C, 2 mins). PCR products were stored at 4°C until analysed by agarose gel electrophoresis on a 1.2 - 1.5% gel.

2.3.3 Genotyping by Southern Blotting

The concentration of genomic DNA was determined by measuring OD at 260nm. 1µl of the DNA solution was dissolved in 999µl dH_2O. A solution containing 50µg/ml double-stranded DNA has an absorbance of 1 at 260nm.

10µg genomic DNA was digested by an appropriate restriction enzyme. 4 units of enzyme (Gibco BRL) per µg DNA were used in a total volume of 40µl containing the appropriate reaction buffer (Gibco BRL) and the digestion was carried out at 37°C overnight. The digested DNA was separated in a 0.8% agarose gel at 80V for 4-5 hours. The gel was then denatured in 0.5M NaOH, 1.5M NaCl for 30 mins followed by neutralisation in 1M Tris-HCl pH8.0, 1.5M NaCl for 30 mins.

The gel was placed on a sheet of 3MM Whatman paper which was soaked in 10xSSC and which acted as a wick in a tray containing a reservoir of 10xSSC. A piece of hybridisation membrane (Hybond-N, Amersham) cut to the size of the gel was placed on top of the gel followed by two pieces of 3MM Whatman paper. Finally, a stack of paper towels was laid flat on the top and covered with a glass plate and a light weight (1kg). The transfer took place overnight at room temperature. The Hybond-N membrane was removed, dried and the DNAs were fixed by placing it on a UV transilluminator for 4 minutes.

The Hybond-N membrane was pre-soaked with 2xSSC then rolled up with a mesh and placed in a hybridisation bottle containing pre-hybridisation solution (6xSSC, 5xDenharts, 100µg/ml salmon sperm DNA, 0.5%SDS). The membrane was pre-hybridised by incubation at 65°C for 4 hours. The radioactive probe (see section 2.5.4) was denatured, added to the hybridisation mix (as pre-hybridisation mix + 10% Dextran Sulphate) at 2 x 10^6 cpm per ml and hybridisation was carried out overnight at 65°C.

Membranes were washed in the bottle at 65°C for 10 mins with 2xSSC, 0.1%SDS and in a sandwich box at 65°C with shaking for 15 minutes with 0.2xSSC, 0.1%SDS. The membrane was wrapped in clingfilm and exposed to Kodak X-Omat autoradiographic film overnight before being developed in a Fuji X-ray film processor RGII.

2.4 DNA Analysis

2.4.1 Agarose Gel Electrophoresis

A 0.8-2.0% agarose solution was made up in 1 x TAE buffer (0.04M Tris-acetate, 1mM EDTA pH8.0) and dissolved in a microwave; ethidium bromide (Sigma) was added to a final concentration of 0.5μg/ml. The agarose mixture was poured into a horizontal gel mould and allowed to set at room temperature. DNA samples were mixed with 10% volume of gel-loading buffer (50% glycerol, 1% bromophenol blue, 10mM sodium phosphate pH7.0) and loaded into wells. Gels were run at approximately 7V/cm inter-electrode distance in TAE buffer. An appropriate size marker (Gibco BRL) was run alongside the PCR samples, the bands were visualised on a UV transilluminator and photographed.

2.4.2 Preparation of Plasmid DNA

All preparations were performed under sterile conditions. 20-50ng of plasmid DNA containing the required insert was added to 100μl of *E. Coli* DH5α competent cells on ice before heat-shocking at 56°C for 1.5 minutes. Incubation on ice for 2 minutes was followed by addition of 0.5ml of sterile L-broth (1% bacto-tryptone, 0.5% yeast extract, 1% sodium chloride, pH 7.0). The cells were shaken for one hour at 37°C in an orbital incubator (x 225rpm), before plating out at 200, 100, and 50μl volumes onto agar plates (L-broth plus 1.5% bacto-agar and 50mg/ml ampicillin), and incubated at 37°C overnight in an inverted position.

The following day, a single bacterial colony was picked and used to inoculate 5ml of L-broth containing 50mg/ml ampicillin. This was cultured overnight at 37°C in an orbital shaker at 225rpm. 1.5ml of the overnight culture was microcentrifuged for 5 minutes, the supernatant discarded and the pellet resuspended in 100μl of Solution 1

(50mM glucose, 25mM Tris-HCl, 10mM EDTA pH8.0). 200µl of fresh Solution 2 (0.4M NaOH, 1%SDS) was added and inverted gently until the mixture cleared. The lysed cells were incubated on ice for 5 minutes. The solution was neutralised by the addition of 150µl Solution 3 (3M KOAc pH4.8), the tubes mixed gently, and then placed on ice for 30 minutes to precipitate the protein. The samples were microcentrifuged for 2-3 minutes before removing and retaining the supernatant. An equal volume of isopropanol was added to the solution and the tubes vortexed, then centrifuged at 14000rpm to precipitate and pellet the DNA. The supernatant was removed and the pellet resuspended in 50µl TE plus 50µg/ml RNase A. This was incubated at 37°C. After 30 minutes an equal volume of phenol/chloroform (50/50) was added to the sample, mixed thoroughly and centrifuged for 5 minutes in a microcentrifuge. The aqueous phase was retained, a 1/10th volume of 3M sodium acetate and an equal volume of isopropanol were added, and DNA was precipitated at -20°C for 1 hour. Microcentrifugation at 14000rpm for 15 minutes pelleted the DNA which was washed 2x in 70% ethanol, air-dried, then resuspended in 30µl TE.

2.4.3 Generation of DNA Fragments for Probes

To isolate a DNA insert, approximately 20µg of plasmid DNA was incubated with the appropriate restriction enzyme(s) (Gibco BRL), at a concentration of 2-3 units/µg DNA, in a total volume of 100µl with buffer (Gibco BRL), at 37°C for at least 1 hour. An aliquot of the digest was analysed with respect to an uncut sample by agarose gel electrophoresis, to confirm complete digestion. 1µg/ml 1kb DNA ladder (Gibco BRL) was run alongside the samples to confirm band sizes.

To isolate the excised DNA fragment, the remaining volume of the digestion was run in a 1% low melting-point agarose gel (Metaphor). The agarose gel was visualised by a UV transilluminator and the band of the correct size was cut from the gel with a sterile scalpel blade and weighed. To extract the DNA from the gel a GeneClean II kit (Bio 101 Inc.) was used. Three volumes of 6M sodium iodide per volume of agarose gel were mixed together and incubated at 50°C for 5 minutes to solubilise the gel. 5µl of glassmilk (silica matrix) per 5µg DNA was added, followed by vortexing, and

incubating on ice for 5 minutes with occasional mixing. The glassmilk/DNA was then pelleted by microcentrifugation at 14000rpm for 5 seconds before washing in 10-50 volumes of NEW Wash^{TM} (sodium chloride, Tris, EDTA, ethanol mixture in kit). The glassmilk/DNA was pelleted again, supernatant removed, and the washing step was repeated twice. The pellet was resuspended in an equal volume of distilled water and incubated at 50°C for 2-3 minutes to release the DNA from the glassmilk. The glassmilk was pelleted, and the water containing the DNA was removed to a fresh eppendorf. 1µl of the DNA sample was run in a 1% agarose gel to check integrity and estimate the concentration.

2.4.4 Preparation of ^{32}P-labelled Probes

DNA inserts to be used as probes were labelled with α-^{32}P dCTP (Amersham) using a random primed labelling kit (Boehringer Mannheim). 100ng DNA was resuspended in 23µl H_20, denatured at 100°C for 10 minutes and quenched on ice. 100mM of each dNTP (except dCTP), 4µl reaction buffer (Boehringer Mannheim), 1 unit of Klenow enzyme and 0.5µCi α-^{32}PdCTP was added to DNA for a final volume of 40µl and the reaction incubated at 37°C for 60 minutes. The labelled probe was collected by passing the sample through a Nick column^{TM} (Pharmacia), followed by two 400µl aliquots of TE buffer. The second 400µl aliquot, containing radiolabelled DNA, was collected. The specific activity of the probe was determined using a Texas Instuments scintillation counter.

2.5 RNA Analysis

2.5.1 Extraction of RNA from Frozen Tissue

All glassware, tips, eppendorfs, and solutions (except those containing Tris or organic solvents) were treated with 0.01% diethylpyrocarbonate (DEPC) (Sigma) before sterilisation to prevent the degradation of the RNA by RNAses. A commercial solution, TRIzol™ (Biogenesis Ltd, based on the method described by Chomczynski and Sacchi 1987), was used to extract RNA from frozen tumours, skin and other tissues. If necessary, tissues were partially ground using a mortar and pestle then transferred to a glass homogeniser. 2ml TRIzol™ per 100mg tissue was added to the homogeniser. The tissue was disaggregated and incubated at room temperature for 5 minutes. 0.2ml chloroform / ml TRIzol™ was added, the sample shaken and incubated for a further 3 minutes at room temperature. Samples were then centrifuged at 10,500rpm in a refrigerated Sorvall centrifuge (SM24 rotor) for 15 minutes at 4°C. The aqueous layer was removed to a fresh tube and the RNA was precipitated by addition of 0.5ml isopropanol per ml TRIzol™ and incubated at room temperature for 10 minutes. Samples were re-centrifuged and the supernatant discarded. The RNA pellet was washed once with 75% ethanol and resuspended in 30-50µl DEPC water.

2.5.2 Northern Blotting

RNA was run in a 1.5% denaturing agarose gel containing 16.6% formaldehyde and 1x MOPS buffer (200mM MOPS Sodium salt, 50mM sodium acetate, 10mM EDTA pH7.0). 50% formamide, 2.2M formaldehyde, and 1x MOPS was added to 5µl of RNA to a total volume of 10µl, and incubated at 55-60°C for 10 minutes before quenching on ice. 1/6th volume of 10x loading buffer (50% glycerol, 1mM EDTA pH8.0, 0.25% bromophenol blue, 0.25% xylene cyanol) was added, and the samples loaded into wells. E.coli RNA ran alongside the samples and was used as size markers. Electrophoresis occurred at 50-100V for 4-8 hours in circulating 1x MOPS to prevent depletion of ions at the anode.

After electrophoresis, the gel was washed for 20-30 minutes 2-3 times in Milli-Q water to get rid of formaldehyde. Marker lanes were cut off and stained for 10 minutes in 1.5μg/ml ethidium bromide before destaining in water. The rest of the gel was placed on a piece of 10x SSC soaked 3MM Whatman paper, acting as a wick in a reservoir of 10x SSC. The RNA was blotted overnight onto Hybond-N membrane (Amersham) as described above for Southern Blotting.

2.5.3 Reverse-Transcription of RNA to cDNA

7μl of RNA (0.5 - 1μg) was incubated at 90°C for 5 minutes before quenching on ice. 1μl (50ng) of oligo d(T) was added, followed by incubation at 65°C for 10 minutes. After quenching on ice, 4μl of 5x RT buffer (250mM Tris HCl pH8.3, 375mM potassium chloride, 15mM magnesium chloride), 5μl of 2.5mM dNTPs, 1μl 0.1mM DTT, 1.2μl Moloney Leukemia Virus Reverse Transcriptase (MMLV RT) (Gibco BRL), and 0.8μl of RNA Guard (RNAse inhibitor) were added and the reaction incubated at 37°C for 1 hour. The reaction was stopped by heating at 95°C for 5 minutes, then quenched on ice. The mixture was stored at –20°C until required.

2.5.4 Reverse-Transcription PCR

1μl of the 20μl cDNA mixture resulting from reverse transcription was combined with 15mM magnesium chloride, 1x buffer (Cambio), 50pmol of primers BC1 and BC2 (see Table2.1), 200mM dNTPs, and 1 unit of Taq polymerase (Cambio) in a total volume of 50μl. The reaction was overlaid with mineral oil (Sigma) to prevent evaporation and the PCR was carried out using a DNA Thermocycler (Perkin Elmer Cetus). 35 cycles of amplification were carried out, each cycle consisting of 3 sequential steps: denaturation (95°C for 1 min), annealing (55°C, 1 min), and elongation (72°C, 2 mins). PCR products were cleaned using a 'Wizard' Clean-up kit (Promega) and the DNA was redissolved in 30μl TE buffer. Digestion of the PCR products was carried out as described above.

2.5.5 Radioactive In Situ Hybridisation

2.5.5.1 Radioactive Riboprobe Synthesis

In order to generate a ^{35}S-labelled riboprobe, the plasmid containing the insert of choice (see Table 2.2) was linearised using appropriate restriction enzymes with respect to the T7 and T3 promoter, ethanol precipitated and resuspended in clean 1x TE at a known concentration. 1µg DNA template in 1µl TE was incubated at 37°C for 1.5 hours with the appropriate polymerase in reaction buffer (0.04M Tris-HCl pH8.0, 8mM $MgCl_2$, 1mM spermidine, 0.025M NaCl) (Gibco BRL) containing 0.1mM DTT, 1mM ATP, CTP and GTP, 7.5mM UTP-S, 0.3mg/ml BSA (RNase and DNase free) (all Pharmacia), 75µCi ^{35}S-UTP (Amersham) (dried into a RNase-free 1.5ml eppendorf tube), and 30 units RNAguard (Pharmacia) in a total volume of 10µl. The reaction was terminated by incubation with 0.15 units DNaseI dissolved in 1x DNase buffer (0.1M NaOAc pH5.0, 5mM $MgCl_2$, 10mM DTT (added fresh)), 50 units RNAguard and 0.05mg polyA. The reaction mix was separated through a G50 sephadex Nick^{TM} column (Pharmacia). The column was first equilibrated with 3ml column buffer (0.3M NaOAc pH5.0, 10mM Tris-HCl pH7.5, 1mM EDTA pH8.0, 0.1% SDS, 10mM DTT. The probe mixture was eluted through the column with 2 volumes column buffer and the final fraction retained. An equal volume of phenol pH5.0 was added, mixed and microcentrifuged for 10 minutes. The aqueous phase was retained and extracted with an equal volume of chloroform : isoamyl alcohol (24 : 1). 2.5 volumes of ethanol were added to precipitate the riboprobe by incubating on dry ice for 30 minutes. The RNA was pelleted by microcentrifugation for 20 minutes, the pellet air-dried and resuspended in 10µl DEPC-treated water. 73µl alkali digestion buffer (40mM $NaHCO_3$ pH10.2, 60mM Na_2CO_3, 10mM DTT) was added and the riboprobe was alkali-digested for a predetermined time at 60°C.

The length of alkali-digestion was determined by the following equation (Cox *et al*, 1984):

$$\text{Incubation time (minutes)} = (Lo - Li) / (0.11 \times Lo \times Li)$$

where Lo = original length of riboprobe (kb)

and Li = required length of riboprobe (kb)

The alkali-digested probe was quenched on ice and 10µl 0.1M NaOAc pH6.0, 0.05mg PolyA added before the sample was separated using a G50 sephadex Nick™ column (Pharmacia). The eluted sample was extracted with phenol and chloroform : isoamyl alcohol before ethanol precipitation, as described above.

The pellet was resuspended in an appropriate volume of 50mM DTT calculated so that a 1 in 10 final dilution would result in a solution at 3 x 10^4 cpm/µl. The 10x stock solution of riboprobe was stored at –20°C for up to 14 days before use.

2.5.5.2 Pretreatment of Tissue Sections

All pre- and post-hybridisation washes were performed using 250ml glass troughs (Solmedia). Slides were held in a glass microscope slide carrier. All washes took place at room temperature unless otherwise stated.

Slides were dewaxed twice in Histoclear™ for 10 minutes then rehydrated in 3x 2 minute washes of 100% ethanol, followed by 2 minute immersions in an ethanol series comprising 90%, 80%, 70%, 50% then 30% ethanol in Milli-Q water. The slides were equilibrated in 0.85% saline for 5 minutes and then immersed in PBS for 5 minutes. The tissue was refixed in fresh 4% PFA/PBS for 20 minutess then washed twice in PBS for 5 minutes. The sections were incubated with 40µg/ml proteinase K in PKB (50mM Tris-HCl pH7.5, 5mM EDTA pH8.0) for 7.5 minutes then washed for 3 minutes in PBS. This was followed by further fixation in 4% PFA/PBS for 5 minutes. The tissue was acetylated in 0.1M triethanolamine with freshly added 0.2% acetic anhydride for 10 minutes then washed for 5 minutes in PBS followed by 0.85% NaCl. The sections were dehydrated by 2 minute washes in an ascending etanol series (30%, 50%, 70%, 80%, 90%, 100%). The slides were left to air-dry under cover at room temperature.

2.5.5.3 Hybridisation of Probe to Sections

The riboprobe was applied to selected tissue sections at a 1 in 10 dilution (3 x 10^4 cpm/ml) in a freshly made hybridisation mix comprising 0.3M NaCl, 10mM Tris-HCl pH8.0, 5mM EDTA, 10mM $NaPO_4$ pH6.8, 10% dextran sulphate, 1x Denhardts, 0.5mg/ml tRNA, 0.5mg/ml PolyA, 50mM DTT. The hybridisation mixture and probe were mixed and denatured at 80°C for 3 minutes and quenched on ice. 3-7µl of the riboprobe mixture was carefully pipetted onto individual tissue sections then covered with a coverslip. Coverslips were cut using a diamond pen in order to fit individual tissue sections. They were placed carefully onto the sections to prevent air bubble formation and to protect the tissue. The slides were placed in a humid chamber containing a tissue soaked in 4x SSC, 50% formamide and sealed with tape for hybridistion overnight at 55°C.

2.5.5.4 Post-hybridisation Washes

Slides were transferred quickly to a glass slide holder, then washed sequentially in the following: 5x SSC, 0.1% β-mercaptoethanol for 15 minutes at 50°C; 2x SSC, 50% formamide, 1% β-mercaptoethanol for 20 minutes at 65°C; 4x 10 minute washes at 37°C in RNase buffer (10mM Tris-HCl pH8.0, 5mM EDTA, 0.5M NaCl) followed by a 30 minute incubation in RNase buffer plus 20µg/ml RNase A at 37°C, then a 15 minute wash in RNase buffer; 2x SSC, 50% formamide, 1% β-mercaptoethanol at 65°C for 20 minutes; 2x SSC at 50°C for 15 minutes; 0.1x SSC at 50°C for 15 minutes. The sections were dehydrated in an ethanol series in Milli-Q water (50%, 70%, 90%, and 100%) for 2 minutes each. The slides were allowed to dry in a dust-free environment then dipped for 10 seconds in a 0.1% gelatin, 0.01% chrome alum solutionand allowed to dry before emulsion (Ilford) was applied.

2.5.5.5 Autoradiography

All autoradiographic procedures were carried out in darkroom conditions illuminated with Kodak 904 filtered light. Slides were dipped in a 45°C, 50% solution of Ilford K5 emulsion / 1% glycerol. The emulsion was allowed to dry for 1.5-2 hours in a light-tight environment. Dry slides were stored in light-tight boxes with dessicant at 4°C prior to developing. The period of storage (7-14 days) was governed by the expression levels of the mRNA under investigation.

After the sections had been exposed for an appropriate period of time they were developed at room temperature by agitation in 20% Phenisol (Ilford) for 3 minutes, followed by 30 seconds in 1% acetic acid, then 30 seconds in Milli-Q water and fixed in a fresh 30% solution of sodium thiosulphate for 5 minutes. The developed slides were washed in cold running water for a minimum of 1 hour before staining.

2.5.5.6 Staining Radioactive In Situ Slides

Slides were stained in filtered Harris' haematoxylin (Gurr) for 30 seconds and rinsed in running tap water for 1 minute. The stain was fixed in Scott's Tap Water (20g $MgSO_4$, 20g$NaHCO_3$/ litre of water) and the slides rinsed in running tap water for 1 minute. The slides were then prepared for mounting in Gurr's DePeX Neutral Mounting medium (BDH).

2.6 Protein Analysis

2.6.1 Extraction of Protein from Frozen Tissue

Protein was extracted from tissues which had been snap frozen in liquid nitrogen and stored at –70°C. Tissue was first ground with a mortar and pestle, then transferred to a 15ml (Falcon 2059) tube containing 1ml lysis buffer (25mM Tris pH7.4, 150mM NaCl, 1% Nonidet P40(Sigma), 1mM EDTA, 2mM EGTA, 10mM NaF, 1mM DTT plus the protease inhibitors leupeptin, aprotinin, and PMSF, all 50µg/ml (Sigma)). The tissue was incubated in lysis buffer on ice for 30 minutes. Lysis mixture was transferred to eppendorfs and centrifuged in a benchtop microfuge at 14000rpm for 10 minutes. Supernatant was collected and stored at –20°C until needed.

Protein concentration was determined using the Lowry method. A series of bovine serum albumin (BSA, Sigma) standards were made up in water (0 – 500µg/ml). 80µl of protein sample (diluted 1:25 in water) or BSA standard was added to 800µl Solution A (2% Na_2CO_3 in 0.1M NaOH, 1% $CuSO_4$ in water, 2% NaK tartrate in water, combined freshly in a ratio of 98:1:1 respectively) and incubated at room temperature for 10 minutes. 80µl Folin's reagent (freshly diluted 1:1 with water) was added, samples were mixed and incubated in the dark at room temperature for 45 minutes. Samples and BSA standards were measured spectrophotometrically at OD_{750} and protein concentration was determined from the ensuing standard curve.

2.6.2 SDS-PAGE Separation of Proteins

SDS-PAGE separation of proteins was carried out using a 10% separating gel and a 5% stacking gel. The separating gel consisted of 6.5ml 30% acrylamide/bisacrylamide (Sigma), 5.1ml solution A (1.5M Tris, 0.4% SDS, pH8.8), 7.7ml sterile water, 195µl fresh 10% ammonium persulphate (APS), 19.5µl TEMED (Sigma). Stacking gel contained 1.9ml 30%acrylamide/bisacrylamide, 3.2ml solution B (0.5M Tris, 0.4%

SDS, pH6.8), 6.8ml water, 60µl 10% APS, 12µl TEMED. A 1mm-thick gel was poured and allowed to set for at least 1 hour. 40-50µg total protein in a total volume of 60µl was added to the 2x sample buffer (160mM Tris-HCl pH6.8, 4% SDS, 20% glycerol, 0.02% bromophenol blue, freshly added 100mM DTT) and heated at 85°C for 5 minutes. Samples were loaded on to the gel alongside a Rainbow molecular weight marker (Amersham) and electrophoresed in running buffer (0.025M Tris, 0.2M glycine, 0.1% SDS, pH8.3) at 125V until the samples had stacked at the stacking gel / separating gel interface. The voltage was then increased to 200-250V until bands had separated as desired.

2.6.3 Western Blotting

The separated proteins in the SDS-PAGE gel were transferred onto a membrane (Hybond ECL, Amersham) in transfer buffer (0.025M Tris, 0.192M glycine, 20% methanol). A Biorad electroblot apparatus was used to transfer at 30V overnight in the cold room followed by 1-2 hours at 60V the following morning. The membrane was then rinsed in Tris-buffered saline (25mM Tris, 125mM NaCl, pH8.0) before probing.

The membrane was incubated in blocking buffer (0.01M Tris pH7.5, 0.1M NaCl, 0.1% Tween 20 (Sigma), 2% BSA) for 2 hours at room temperature on a rocking platform. Primary antibody at the required dilution was added to dilution buffer (0.01M Tris pH7.5. 0.1M NaCl, 0.1% Tween 20, 1% BSA) (see table 2.3) which was incubated with the membrane for 1 hour at room temperature. The membrane was given three sets of washes: 2x 5minutes in wash 1 (0.01M Tris, 0.1M NaCl, 0.1% Tween 20), 2x 5 minutes in wash 2 (0.01M Tris, 0.5M NaCl, 0.1% Tween 20) and 2x 5 moinutes in wash 1 again. The secondary horseradish peroxidase (HRP)-conjugated antibody was diluted in dilution buffer (ususally 1:5000) and applied to the membrane for 1 hour. Washes were repeated as before and the labelled proteins were detected using an Amersham ECL kit where the HRP-catalysed oxidation of luminol results in emission of light. Membranes were wrapped in clingfilm and exposed to Amersham Hyperfilm ECL for between 10 seconds and 1 hour before being developed in a Fuji X-ray film processor RGII.

2.7 Histochemistry

2.7.1 Preparation of Slides

Microscope slides were dipped in a 2% 3-Aminopropyl-triethoxysilane (TESPA, Sigma) in acetone solution for 5 seconds followed by two washes in acetone and two in Milli-Q water. The slides were baked dry at 42°C before storing in boxes at room temperature.

2.7.2 Preparation and Sectioning of Paraffin-Embedded Tissues

Tissues were fixed overnight in 4% PFA at 4°C with continuous rotation. The following day, the sample was washed in PBS (30 mins), ethanol : 0.85% saline mix (1 : 1) (2 x 15mins) and 70% ethanol (2 x 15mins). The tissue could be stored at this stage for an indefinite period. Processing of the tissues was done through a Shandon Citadel Automatic Processor which consisted of 3 washes in methanol, 1 wash in 100% ethanol, 3 washes in xylene and 2 washes in paraffin wax. The tissues were embedded in paraffin wax in a suitable orientation and stored at 4°C.

Tissues embedded in paraffin wax blocks were placed on a microtome and 6μm serial sections cut. These were floated onto Milli-Q water at 42°C. TESPA-coated slides were placed under the floating sections and used to pick them up. The slides were then covered and left to dry at 42°C. Sections were stored at 4°C.

2.7.3 Pretreatment of Sections before Staining

Dewaxing and rehydration of the sections was performed before any staining procedure. Slides were placed in microscope slide holders, and soaked in Histoclear™ for two 7 minute periods. This was followed by two 2 minute immersions in 100% ethanol, then a single immersion for 2 minutes in each of 90%, 80%, 70%, and 50% ethanol dilutions. The sections were then ready for staining.

2.7.4 Dehydration and Mounting of Sections

Following staining, the slides were dehydrated through an ethanol series (30%, 50%, 70%, 90%, 100% and 100%) for 2 minutes each, followed by two soaks in Histoclear™ the first 2 minutes, then 7 minutes. Coverslips were mounted onto the sections using Gurr's DePeX Neutral Mounting medium (BDH) in a fume hood, and left to dry overnight.

2.7.5 Haematoxylin and Eosin Staining

Following dewaxing and rehydrating, sections were stained in filtered Harris' haematoxylin (Gurr, BDH) for 10-30 seconds, then rinsed in running tap water for 1 minute. The stain was fixed in Scott's Tap Water (20g $MgSO_4$, 20g$NaHCO_3$/ litre of water). Following a further rinse in running water, sections were stained in filtered eosin (5g eosin (Gurr), 50ml saturated acetic picric acid, 400ml water, 2.5g potassium dichromate, 50ml ethanol, added in that order / 500ml stock; the stock was diluted 1 in 4.5 with water for a working concentration) for 30 seconds, then rinsed briefly in running water. Sections were then rehydrated and mounted as described above.

2.7.6 Antibody Staining

Sections were dewaxed and rehydrated as described above. The sections were washed three times in PBS, 5 mins each wash, before blocking endogenous peroxidase by incubation with 1.2% hydrogen peroxide in methanol for 20 mins. Three 5 minute washes in PBS were followed by incubation with proteinase K (20 μg/ml) in 50mM Tris pH 8.0 for 15 minutes. After three 5 minute washes in PBS, 20-50 μl blocking solution was applied to the sections for 30 mins (0.1% gelatin, 0.1% BSA and sheep serum in PBS). Most of the blocking solution was removed and primary antibody was applied. If dilution of the antibody was required, dilution was done in blocking solution (as above) at the dilutions shown in table 2.3. Slides were incubated in a humidified chamber for either 2 hours at room temperature or overnight at 4°C. Small pieces of coverslips were laid on top of the sections to spread antibody uniformly over the section and to prevent the section from drying out.

Coverslips and antibody were removed by washing three times with PBS containing 0.1% sheep serum and 0.1% BSA (5 minutes per wash). A biotinylated secondary antibody was diluted in blocking solution (see above) and applied to the sections for 60 minutes at room temperature. The sections were washed 3 times as before and incubated with an avidin-biotin complex (ABC kit, Dako) for 30 minutes. After washing, 3,3'-diaminobenzidine tetrahydrochloride (DAB, Sigma) was applied for 1-6 minutes until the brown signal appeared. The sections were then placed in water, and rinsed under a running tap. Sections were counterstained with Harris' haemotoxylin (Gurr) for 30 seconds and after washing in tap water they were dehydrated and mounted as described above.

2.8 Statistical Methods

When it was impossible to determine whether the data being analysed followed a Gaussian (or normal) distribution, non-parametric statistical tests were carried out. The

Mann-Whitney rank sum test was employed when comparing two data groups and, for three or more data groups, the Kruskal-Wallis test was performed.

In comparing tumour formation in two different K5-*RAS* transgenic lines, the data was plotted as a Kaplan-Meier survival curve with the endpoint being age at onset of tumour (as opposed to death). A log-rank test was performed to compare the sastistical significance of difference between the two 'survival curves'.

Statistical tests were carried out using GraphPad Prism version 3.0a for Macintosh.

Table 2.1 Sequences of primers used in polymerase chain reactions

Allele	Primer Name	Primer Sequence 5' to 3'	Annealing temperature
*Tgf*β1 WT	95	GCGGACTACTATGCTAAAG	
	96	GGTCACCCGCGTGCTAATGG	58°C
	97	CGTGCGCCTGTCGCTTTCTG	
	98	GAGAGTAAGCCCACTAGAG	
*Tgf*β1 KO	TGF	AGGGAGCTGGTGAAACGGAA	58°C
	PGK	TCCATCTGCACGAGACTAGT	
SV40 polyA	SV1	GGACAAACTACCTACCTACAGAGATTTA	58°C
	SV2	ATTCATTTTATGTTTCAGGTTCAG	
Tp53 WT	P53W1	GTGTTTCATTAGTTCCCCACCTTGAC	55°C
	P53W2	CTGTCTTCCAGATACTCGGGATAC	
Tp53 KO	P53M1	GGGACAGCCAAGTCTGTTATGTGC	55°C
	P53M2	TTTACGGAGCCCTGGCGCTCGATGT	
K5-*RAS*	tcr1	ATGGGGAGACGTGCCTGTTG	55°C
	tcr2	GTCCTGAGCCTGCCGAGATTC	
K10-*BCL2*	K10	TTGGGGCTGGTGATTACTTA	60°C
	Bcl-K	CCGCATCCCACTCGTAGC	
	BC1	GCCTCTGTTTGATTTCTCCTG	54°C
	BC2	CTGATTCGACGTTTTGCC	
*Tsh*β	TSH1	TCCTCAAAGATGCTCATTAG	55°C
	TSH2	GTAACTCACTCATGCAAAGT	

Table 2.2 Templates and probes used for radioactive *in situ* hybridisation

cDNA	Plasmid	Linearised By:	RNA Polymerase	Probe length	Reference
Human *BCL-2*	pBluescript SK+	*Xba* I (antisense)	T7	600bp	Allsopp *et al*, 1993
Human *BCL-2*	pBluescript SK+	*Kpn* I (sense)	T3	600bp	Allsopp *et al*, 1993
SV40 polyA	pBluescript SK-	*Kpn* I (antisense)	T3	900bp	Mulligan *et al*, 1979
SV40 polyA	pBluescript SK-	*Sac* I (sense)	T7	900bp	Mulligan *et al*, 1979

Table 2.3 Antibodies used in Western Blotting and Immunohistchemistry

Antibody	Catalogue no.	Specificity	Western blotting dilution	Immunohistochemistry dilution
BrdU (Amersham)	RPN 202	-	-	No dilution
BAX (Pharmingen)		Human and mouse	1 : 750	1 : 500
BCL-2 (100) (Santa Cruz Biotechnology)	sc-509	Human only	1: 1000	-
BCL-2 (N-19) (Santa Cruz Biotechnology)	sc-492	Human and mouse	-	1:400

Chapter 3
RESULTS

3.1 Introduction

TGFβ1 is an inhibitor of epithelial cell growth and can alter the differentiative properties of keratinocytes. It has also been shown to be an inducer of apoptosis in some cell types (Oberhammer *et al*, 1991; Rotello *et al*, 1991; Lin and Chou, 1992). Our lab showed that *Tgf*β1 mRNA and protein is induced in suprabasal keratinocytes *in vivo* in response to hyperplasia (Akhurst *et al*, 1988; Fowlis *et al*, 1992) which suggests that it plays a role in the regulation of epidermal homeostasis. To investigate this phenomenon, transgenic mice had been generated using a keratin 10 (K10) promoter to drive expression of *Tgf*β1 in the suprabasal keratinocyte compartment. Surprisingly, these mice showed a two- to three-fold increase in epidermal DNA labelling index over control mice (Cui *et al*, 1995). Possible explanations for this include firstly, that at a low concentration, Tgfβ can directly elevate mitosis, perhaps via effects on the extracellular matrix. Secondly, Tgfβ may be inducing the migration of basal keratinocytes into the suprabasal compartment (i.e. induction of differentiation). Finally, there may be an induction of apoptosis by Tgfβ which leads to a compensatory increase in proliferating cells in order to maintain epidermal homeostasis.

3.2 Increased proliferative index in K10-*Tgf*β1 mouse skin is not mediated via p53

One of the molecules most commonly associated with apoptosis (or programmed cell death) is the tumour suppressor p53, and many apoptotic pathways have been shown to occur via a p53-dependent mechanism (Lowe *et al*, 1993; Clarke *et al*, 1994; Lin *et al*, 1995). Also, p53 has been implicated in the control of growth of various systems (Cajot *et al*, 1992; Finlay, 1992). In order to test whether p53 was involved in the elevated proliferation index of K10-*Tgf*β1 mouse epidermis, the K10-*Tgf*β1

transgenic mice were crossed with a *Tp53* knockout line which was available in the lab at the time.

The *Tp53* knockout mice were bred onto a NIH genetic background (from a FVB background) so as to reduce variation due to strain background since the K10-*Tgf*β1 line (H-line) was already on the NIH background. The H-line transgenic mice were then crossed with the *Tp53*-knockout mice to generate H-line mice either wild-type, heterozygous or homozygous null for *Tp53*. Two generations of transgenic breeding were necessary to achieve the desired transgenic genotypes. The breeding protocol is outlined in figure 3.1A. Mice were genotyped by PCR. To determine *Tp53* status, two separate PCRs were carried out as shown in figure 3.1B. One set of primers detected the presence of the endogenous *Tp53* gene giving a band of 385bp. The other reaction used primers which amplified a 512bp fragment indicating the presence of the disrupting knockout cassette (see figure 3.1B). The presence of the H-line transgene K10-*Tgf*β1 was detected using primers which amplified a 580bp fragment of the SV40 polyA fragment of the transgene as in figure 3.1C.

Female mice that were either wild-type ($Tp53^{+/+}$) or homozygous null ($Tp53^{-/-}$) with respect to their *Tp53* genotype were chosen for analysis. At 11-12 weeks of age, the mice were injected with BrdU, 1 hour later the mice were culled and a strip of dorsal skin was taken for paraffin histology. Skin sections were labelled with an anti-BrdU antibody (see figure 3.2), counterstained with haematoxylin and the number of BrdU-positive cells present in the interfollicular epidermis were counted.

The results are presented in Table 3.1 and in graph form in figure 3.3. Firstly, the results show an increased BrdU labelling index when the K10-*Tgf*β1 transgene is present (compare p53+/+, H-ve column with p53+/+, H+ve column) which is as expected from previous experiments (Cui *et al*, 1995). The difference reported previously was a two- to three-fold increase when H-line homozygote mice were compared to NIH controls. In this experiment, the H-line mice are a mixture of heterozygotes and homozygotes (in fact, a 2:1 heterozygote:homozygote ratio) which explains why the difference is not so pronounced.

This experiment suggests, however, that p53 does not play a pivotal role in the mechanism by which increased Tgfβ leads to increased cellular proliferation in these transgenic mice. Comparison with the other two columns in figure 3.3 (H-ve and H+ve on a p53-null background) shows that mice with no functional p53 behave no

Figure 3.1
Breeding scheme and genotyping of *Tp53* and K10-*Tgf*β1 knockout mice

A. Breeding scheme for crossing *Tp53* knockout mice with H (K10-*Tgf*β1) line mice

$p53^{-/-}$ represents a mouse which is homozygous for the *Tp53* knockout allele
$p53^{+/-}$ represents a mouse which is heterozygous for the *Tp53* knockout allele
$p53^{+/+}$ represents a mouse which is wild-type with respect to its *Tp53* status

H/H represents a mouse which is homozygous for the K10-*Tgf*β1 transgene
H/+ represents a mouse which is heterozygous for the K10-*Tgf*β1 transgene
+/+ represents a mouse with no K10-*Tgf*β1 transgene.

Fractions represent the expected Mendelian ratio of each genotype from the cross shown.

B. Determination of *Tp53* knockout genotypes by PCR

M (marker): 1kb DNA ladder (Gibco BRL)

Two separate PCR reactions were carried out on each DNA preparation.
The first reaction detected the *Tp53* wild-type allele.
The second reaction detected the *Tp53* knockout allele.
Therefore, in the gel shown above,
mice 1 and 2 are *Tp53* null: **$p53^{-/-}$**
mice 3 and 5 are *Tp53* heterozygous: **$p53^{+/-}$**
mouse 4 is *Tp53* wild-type: **$p53^{+/+}$**

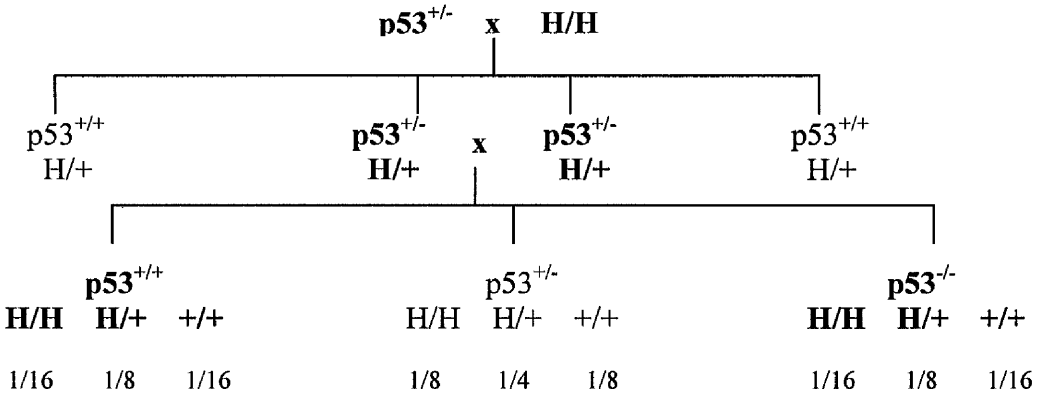
C. Determination of the presence of the K10-*Tgf*β1 transgene by PCR

Primers SVP2 and SVP3 were used to amplify a region of the SV40 polyA fragment of the K10-*Tgf*β1 transgene.

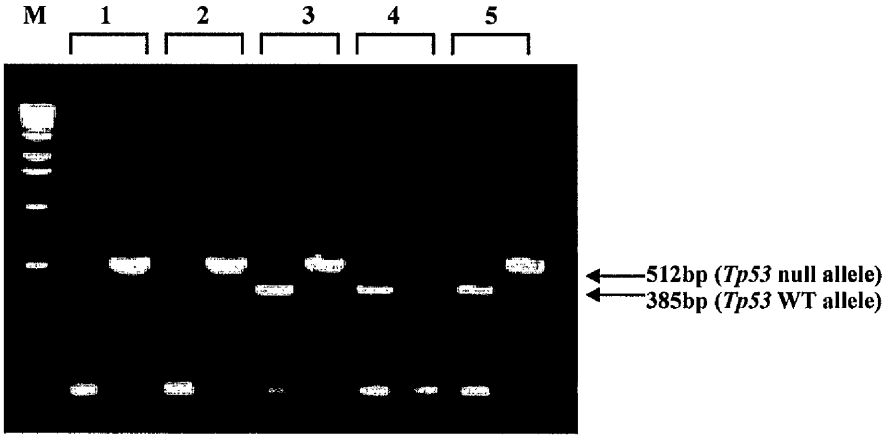
M (marker): 1kb DNA ladder (Gibco BRL)

Lanes 1,2,3,6: K10-*Tgf*β1 positive: **H/+ or H/H**
Lanes 4,5: K10-*Tgf*β1 negative: **+/+**

A


p53+/- x H/H
p53+/+ H/+
p53+/- H/+
x
p53+/- H/+
p53+/+ H/+
p53+/+ H/+ +/+
p53+/- H/H H/+ +/+
p53-/- H/+ +/+
H/H
H/H
H/H
1/16 1/8 1/16
1/8 1/4 1/8
1/16 1/8 1/16

B


M 1 2 3 4 5
512bp (Tp53 null allele)
385bp (Tp53 WT allele)

C

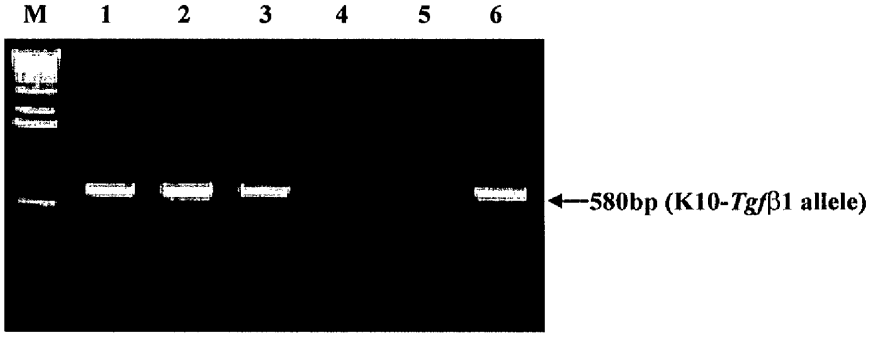
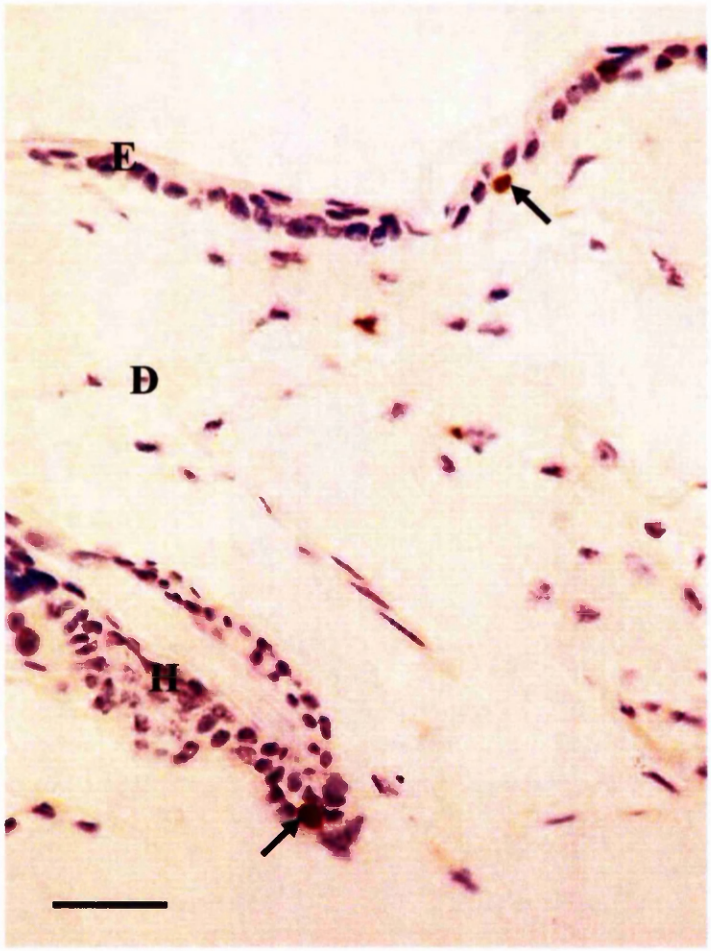

M 1 2 3 4 5 6
580bp (K10-Tgfβ1 allele)

Figure 3.2

Labelling of mouse skin with bromodeoxyuridine (BrdU)



BrdU staining showing a cell of the interfollicular epidermis and a cell of the hair follicle in S-phase. Mice had their dorsal skin shaved 24 hours before administraton of BrdU and only those animals showing no hair regrowth selected, to ensure that the skin was not in a hair-growing cycle (anagen). Mice were injected with 50μg BrdU/g bodyweight one hour before sacrifice. On sacrifice a strip of dorsal skin was taken and fixed in 4% paraformaldehyde and subjected to immunohistochemistry with an anti-BrdU antibody (Amersham).

Arrows indicate BrdU+ve cells - one in the interfollicular epidermis and the other in the hair follicle.
E: epidermis
D: dermis
H: hair follicle

Bar, 100μm

Table 3.1

BrdU labelling indices of interfollicular cells of skin epidermis from mice produced by crossing K10-*Tgf*β1 transgenics with *Tp53* knockout mice

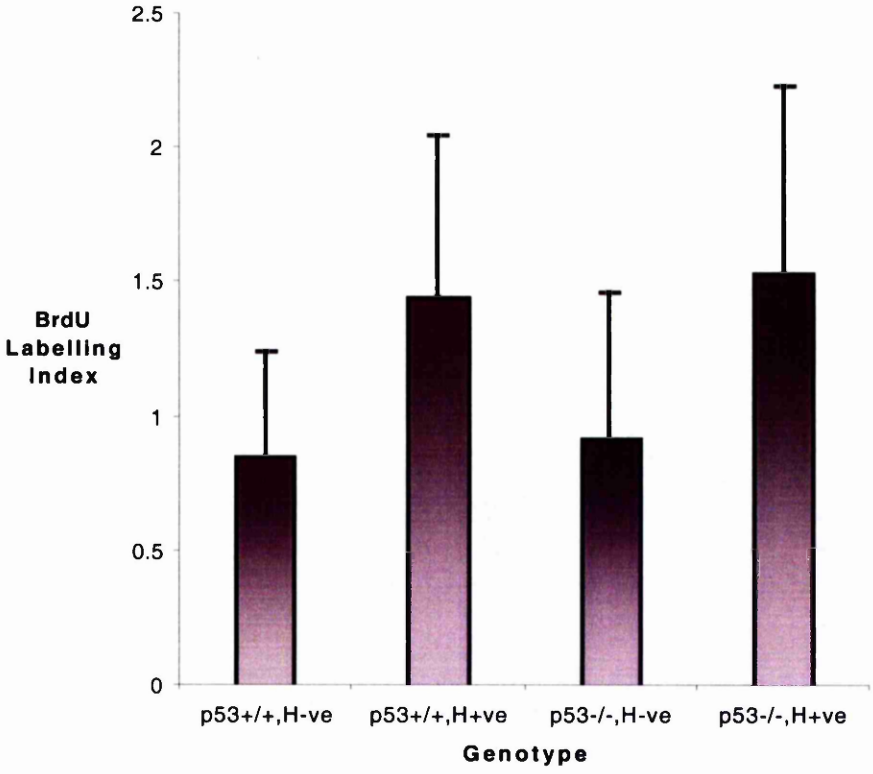
GENOTYPE	P53+/+ , H-ve	P53+/+ , H+ve	P53-/- , H-ve	P53-/- ,H+ve
BRDU Labelling Index	0.65 1.46 0.76 0.67 1.07 1.07 0.87 0.32 0.5 1.44 1.51 0.2 0.98 0.47	1.23 1.44 1.19 2.1 1.78 2.61 2.85 1.1 1.36 1.53 1.2 0.78 0.69 0.69	0.64 0.35 0.94 1.19 0.51 0.57 2.06 1.92 0.62	0.44 1.57 1.3 3.1 1.82 1.68 1.95 2.48 2.39 0.74 1.1 0.58 0.95
MEAN +/-SD	0.855 +/- 0.40	1.45 +/- 0.64	0.92 +/- 0.59	1.54 +/- 0.77
MEDIAN	0.82	1.78	0.64	1.57

H+ve denotes mice that carry the K10-*Tgf*β1 transgene in either the heterozyous or homozygous state.

Each number represents the labelling index of a single mouse. Labelling index shown is the number of BrdU-positive cells per 100 basal cells of interfollicular epidermis. A minimum of 1000 cells was counted for each skin section and two sections were assayed from each mouse.

Figure 3.3

Loss of p53 does not affect the increased BrdU labelling index in the epidermis of H-line (K10-*Tgf*β1) mice



The BrdU labelling indices represent the mean of the scores for each genotype shown in Table 3.2 i.e. the labelling index is the mean number of BrdU+ve cells per 100 basal cells of the interfollicular epidermis.

differently than those wild-type for p53 function in terms of cellular proliferation in the epidermis. In the presence of elevated cellular proliferation, yet no hyperplasia, one must postulate an increase in apoptosis or differentiation whereby cells slough off more rapidly from the skin surface. Whether or not Tgfβ1 induces apoptosis directly or indirectly, this process must be p53-independent.

3.3 Neither increased suprabasal Tgfβ1 nor loss of p53 affects Bax expression

Another important player in many apoptotic pathways is the Bcl-2 family member Bax. Bax has been shown to promote apoptosis in certain cell types (Oltvai *et al*, 1993). Some work suggests that Bax might act downstream of p53 (Selvakumaran *et al*, 1994; Miyashita and Reed, 1995; Yin *et al*, 1997). Similarly, there have been studies which have found that Bax is up-regulated during TGFβ1-induced apoptosis (Teramoto *et al*, 1998). Therefore, epidermal skin sections from the K10-*Tgf*β1/*Tp53* knockout cross were stained with an anti-Bax antibody to assess protein expression in the various *Tp53*/K10-*Tgf*β1 genotypes.

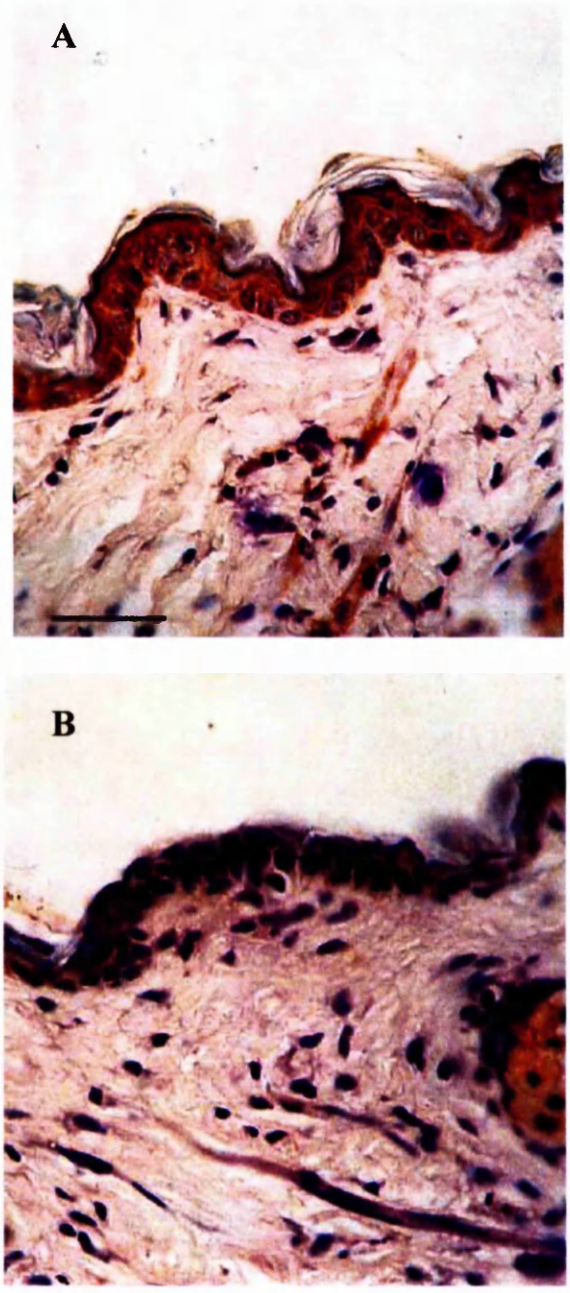
Bax staining was observed throughout the epidermis, in both basal and suprabasal cells, as shown in figure 3.4. However, no difference in Bax levels was found between genotypes, whether *Tp53* null or K10-*Tgf*β1 positive. This shows that firstly, *Tgf*β1 overexpression in skin epidermis does not lead to an increase in Bax levels and secondly, absence of p53 does not affect Bax production in the skin as determined by immunohistochemistry.

3.4 Nullizygosity for *Tgf*β1 does not alter epidermal proliferative index

Previous work in the lab had shown that a line of mice overexpressing *Tgf*β1 in the suprabasal layer of the epidermis had an increased BrdU labelling index in the suprabasal layer of the epidermis. Glick *et al* (1993) had shown that the *Tgf*β1 knockout mice also had an increased BrdU labelling index, which contrasts with the data of Cui *et al* (1995). The results of Glick *et al* could be due to indirect effects of loss of Tgfβ1 since the Tgfβ1 null animals have neither epidermally nor dermally-derived Tgfβ1. However, their data were obtained from only four mice which were of disparate ages between 2 and 5 weeks post-partum. In this type of experiment, with potentially large biological variation, we felt that this was inadequate, especially as the skin undergoes such profound changes in the first 3 to 4 weeks of life. We therefore

Figure 3.4

Immunohistochemical Bax expression in mouse epidermis is affected neither by loss of p53 or overexpression of *Tgf*β1



Skin was fixed in 4%PFA, embedded in wax, cut into 6μm sections and stained with mouse anti-Bax antibody (A) as described in Materials and Methods. bax protein staining is cytoplasmic and is present throughout all layers of the epidermis. There were no qualitative or obvious quantitative differences in staining. (B) shows a no primary antibody control.

Bar: 50μm

decided to examine this issue by re-assessing BrdU labelling index in the colony of Tgfβ1 null mice.

An initial study was carried out assessing how many Tgfβ1$^{-/-}$ mice survived to birth and to what age they survived. Heterozygous *Tgf*β1 knockout mice were intercrossed as in figure 3.5A. Pups were genotyped by PCR on death if before 3 weeks of age, or after tail-tipping at weaning as shown in figure 3.5B. It was found that approximately 60% of the expected *Tgf*β1 null mice were born and the spread of age at death is illustrated in figure 3.6.

It had become clear from concurrent studies in our lab that *Tgf*β1 knockout mice have a variable penetrance of prenatal lethal phenotype which depends on mouse strain. For example, on a C57/Bl6 background, no *Tgf*β1 null mice survive to birth, whereas on a NIH background, 70-80% of null mice are born (see Bonyadi *et al*, 1997 for details). The mice we had were on a mixed genetic background (50% NIH, 37.5% C57Bl/6J, 12.5% 129SV). Some of these mixed background mice were therefore bred through at least 3 generations with NIH stock until the *Tgf*β1 knockout allele was on a 93% NIH background and both mixed and inbred NIH backgrounds were analysed.

Four litters from the mixed genetic background and three litters with the NIH background were injected with BrdU at 21 days of age and dorsal skin was harvested one hour later. A piece of tail was taken for genotyping and BrdU-positive cells in the dorsal interfollicular epidermis were counted.

This experiment showed no significant difference in the number of epidermal cells in S phase between *Tgf*β1 knockout mice and either heterozygote or wild-type littermates (see Table 3.2 and figure 3.7), unlike Glick *et al* (1993), who found a 3- to 5-fold increase in BrdU labelling in skins of *Tgf*β1 null animals compared to heterozgote and wild-type animals.

One possible reason for the discrepancy between these results and the results of Glick *et al* is the difference in genetic background of the two *Tgf*β1 knockout lines. On a mixed genetic background there appeared to be greater variation among BrdU labelling indices compared to mice on a NIH genetic background. The genetic background of the mice analysed by Glick *et al* is not defined but appears to be a mixed background. Even among the two wild-type and four heterozygote *Tgf*β1 knockout mice in their study, labelling index varied between 1.7 +/- 0.4 and 8.2 +/- 2.6 BrdU-positive cells per total basal nuclei. A further difference between the two studies is that, of the three litters

Figure 3.5

Breeding scheme and genotyping of *Tgf*β1 knockout mice

A. Breeding protocol used to generate *Tgf*β1 null mice

tgfb1$^{-/-}$ represents a mouse which is homozygous null for *Tgf*β1
tgfb1$^{+/-}$ represents a mouse which is heterozygous null for *Tgf*β1
tgfb1$^{-/-}$ represents a mouse which is wild-type at the *Tgf*β1 locus

Fractions represent the expected Mendelian ratio of each genotype born assuming no prenatal loss.

B. Wild-type and knockout *Tgf*β1 alleles and PCR reaction used to genotype the mice

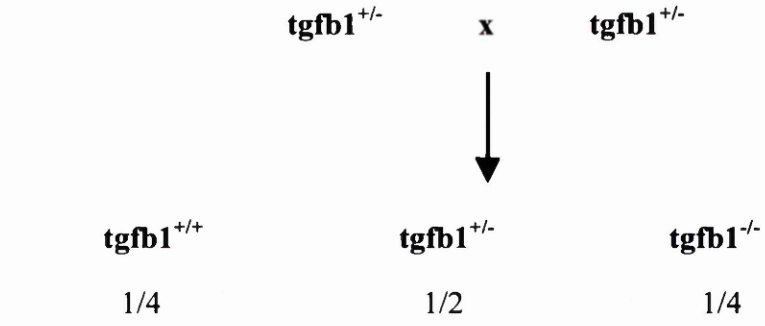
Two separate PCR reactions were carried out on each DNA preparation.
The first reaction, using primers 95-98, detected the *Tgf*β1 wild-type allele.
The second reaction, using primers TGF and PGK, detected the knockout allele.

M (marker): 1kb DNA ladder (Gibco BRL)

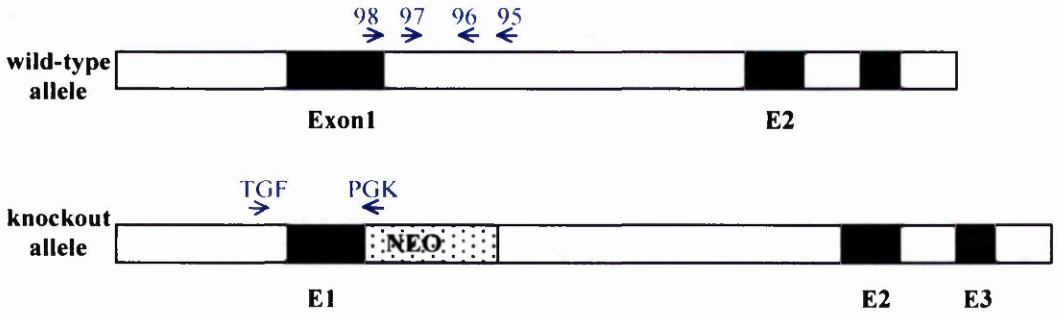
In the gel shown opposite:

mouse 1 is heterozygous **tgfb1$^{+/-}$**
mouse 2 is wild-type **tgfb1$^{+/+}$**
mouse 3 is null **tgfb1$^{-/-}$**
mouse 4 is heterozygous **tgfb1$^{+/-}$**

A


tgfb1+/- x tgfb1+/-
tgfb1+/+
tgfb1+/-
tgfb1-/-
1/4
1/2
1/4

B


98 97 96 95
wild-type allele
Exon1
E2
TGF PGK
knockout allele
NEO
E1
E2
E3

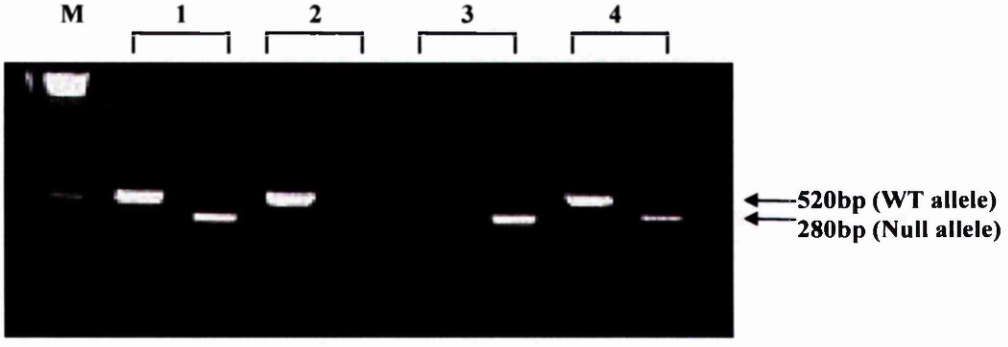
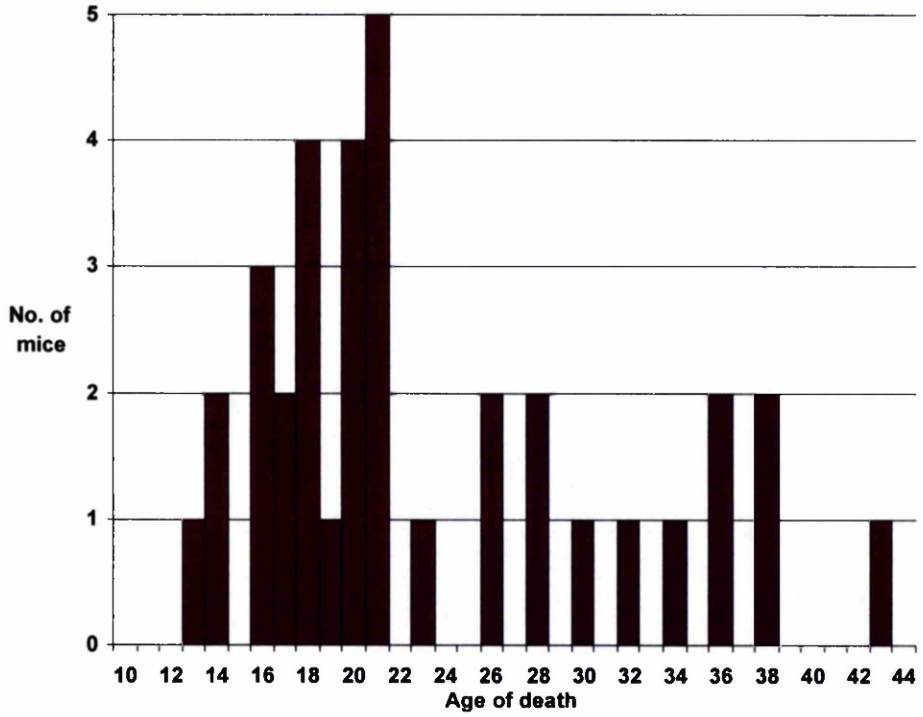

M
1
2
3
4
520bp (WT allele)
280bp (Null allele)

Figure 3.6

Average age of death of Tgfβ1 null mice



Graph shows the spread of ages of death of Tgfβ1 null mice.
The mice have a mixed genetic background: 50% NIH, 37.5% C57Bl6, 12.5% 129SV

Total no. of Tgfβ1 null mice examined = 35

Mean age of death: 23.3 days

Median age of death: 20.5 days

Table 3.2

Incorporation of BrdU into proliferating cells of the interfollicular epidermis in 21-day old Tgfβ1-null mice and their wild-type littermates

Mixed Genetic Background (50% NIH, 37.5% C57Bl6, 12.5% 129SV)

	Wild-type	tgfβ1 null
Litter 1	3.6 1.7 2.3 2.0	1.25 1.3
Litter 2	0.8 1.9	1.3
Litter 3	1.65 0.8	3.25 2.25
Litter 4	3.3	2.6 2.1 0.8
Median	2.2	2.0
Mean +/- S.D.	2.0 +/- 0.9	1.9 +/- 0.8

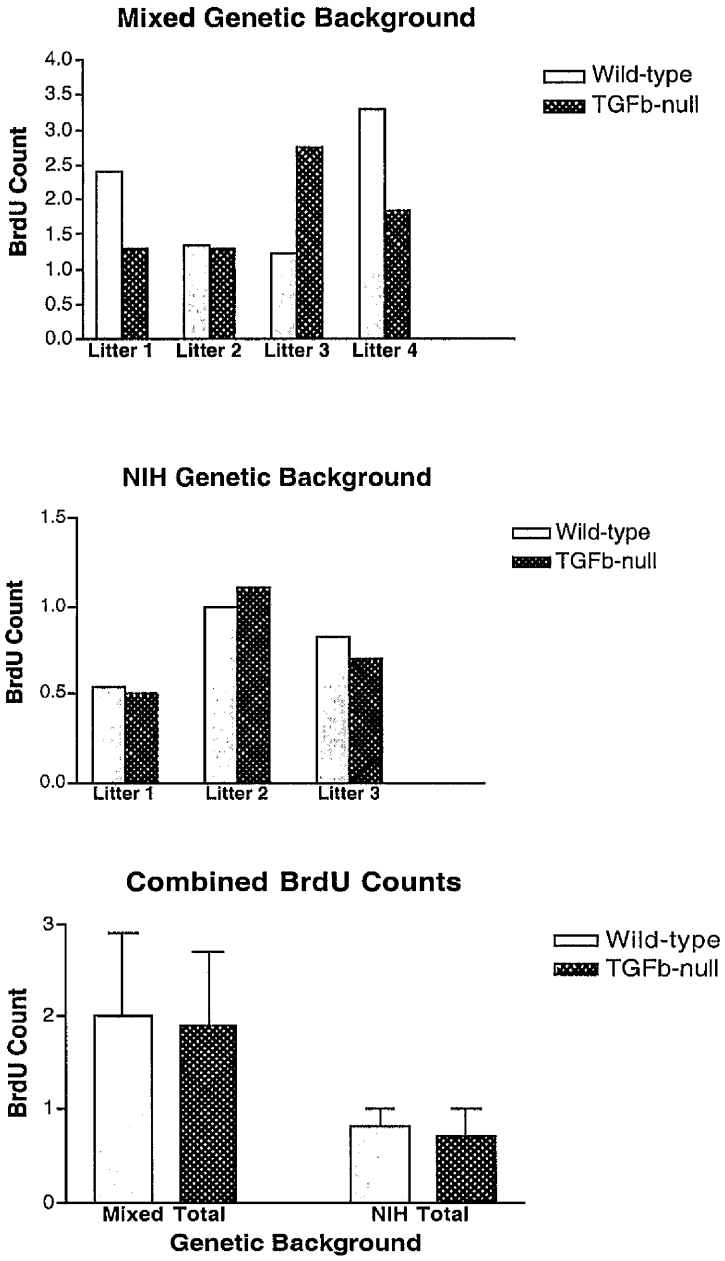
93% NIH Genetic Background

	Wild-type	tgfβ1 null
Litter 1	0.66 0.42	0.4 0.6
Litter 2	0.9 1.1	1.1
Litter 3	0.9 0.75	1.0 0.4
Median	0.76	0.75
Mean +/- S.D.	0.8 +/- 0.2	0.7 +/- 0.3

Each number represents the labelling index of a single mouse. Labelling index shown is the number of BrdU-positive cells per 100 basal cells of interfollicular epidermis. A minimum of 1000 cells were counted for each skin section and two sections were assayed from each mouse.

Figure 3.7

Incorporation of BrdU into proliferating cells of the interfollicular epidermis in *Tgf*β1-null mice and their wild-type littermates



Each bar represents the average BrdU labelling index of all the wild-typeb or Tgfβ1-null mice in each litter shown in Table 3.2. Combined BrdU counts represents the pooled data of the litters within each genetic background.
TGFb-null = *Tgf*β1-null

analysed by Glick *et al*, two were 5 weeks old and one was 3 weeks old whereas mice in this study were all analysed at precisely 21 days, and when the mice were not in a hair-growing phase of the hair cycle.

3.5 Suprabasal Tgfβ1 increases keratinocyte proliferation in both wild-type and Tgfβ1 null mice

To further investigate the action of the K10-*Tgf*β1 transgene, it was decided to cross the transgenics onto a *Tgf*β1-null background. It was a possibility that epidermally targetted Tgfβ1 from the transgenic mice could act in an endocrine manner to rescue *Tgf*β1$^{-/-}$ mice from their lethal phenotypes. This cross would also be interesting in assessing the epidermal proliferation phenotype of the K10-*Tgf*β1 transgene on a Tgfβ1-null background.

Two generations of breeding were necessary to produce animals with the full range of genotypes for littermate comparisons. Firstly, an animal heterozygote for the *Tgf*β1 knockout allele was crossed with a female which was homozygous for the K10-*Tgf*β1 transgene. Offspring were screened for the presence of the *Tgf*β1 knockout allele (as shown in figure 3.5B) and males heterozygous for both the knockout and the K10-*Tgf*β1 alleles were crossed with females of the same genotype (see figure 3.8A).

This cross produced animals with a range of genotypes (see figure 3.8A). In this F2 generation, the K10-*Tgf*β1 allele was screened by Southern blotting in an attempt to distinguish homozygote K10-*Tgf*β1 from heterozygote. An SV40 polyA probe and a probe specific for the cystic fibrosis transmembrane regulator (*Cftr*) gene (Tata *et al*, 1991) were hybridised at the same time to a nylon membrane containing the F2 generation mouse genomic DNAs which had been digested with *Bgl*II. Zygosity was estimated by comparing intensity of SV40 polyA bands using the *Cftr* bands as an internal control (see figure 3.8B).

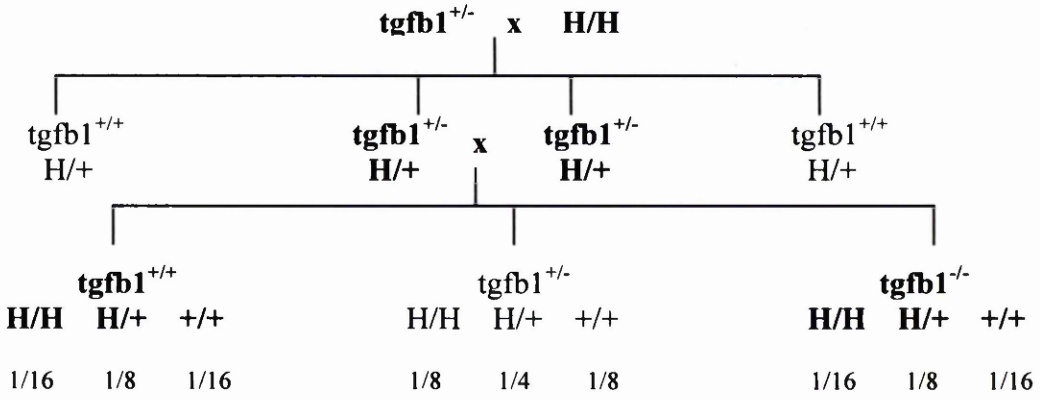
As before, these mice were injected with BrdU and dorsal skins were harvested. Skin sections were labelled with an anti-BrdU antibody and labelled cells in the interfollicular epidermis were counted. The counts are shown in table 3.3.

The first observation was that there was no obvious rescue of lethality of *Tgf*β1$^{-/-}$ mice. Due to small numbers of *Tgf*β1 null mice reaching weaning age, it proved impossible to generate sufficient numbers of all genotypes to achieve statistical

Figure 3.8

Generation and genotyping of *Tgf*β1 knockout X K10-*Tgf*β1 mice

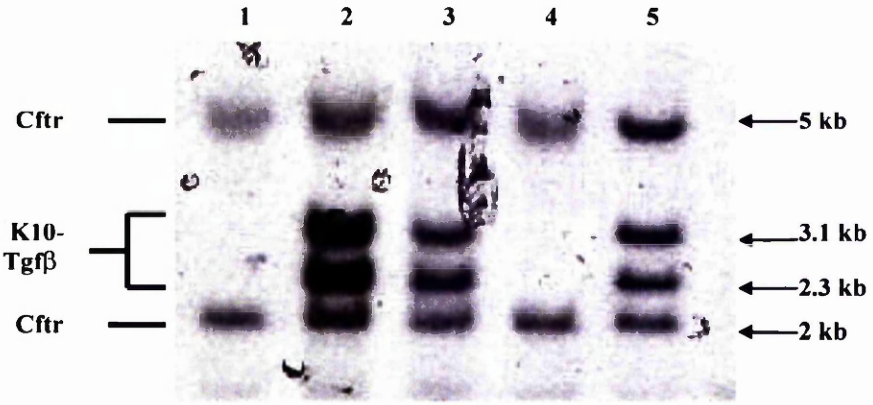
A. Outline of breeeding scheme to generate *Tgf*β1 knockout mice carrying the K10-*Tgf*β1 (H) transgene



tgfb1$^{-/-}$ represents a mouse which is homozygous for the *Tgf*β1 knockout allele
tgfb1$^{+/+}$ represents a mouse which is wild-type with respect to its *Tgf*β1 status.

H/H represents a mouse which is homozygous for the K10-*Tgf*β1 transgene
+/+ represents a mouse with no K10-*Tgf*β1 transgene.

B. Example of southern blot used to screen for K10-*Tgf*β1 transgene



Southern blot analysis of genomic DNA from various *Tgf*β1 knockout x H-line mice using the SV40-specific hybridisation probe to detect the transgene and the *mCftr* probe (Tata et al 1991) as an internal loading control

Lanes 1 and 4: Wild-type **(+/+)**
Lanes 3 and 5: Heterozygous for K10-*Tgf*β1 transgene **(H/+)**
Lane 2: Homozygous for K10-*Tgf*β1 transgene **(H/H)**

Table 3.3

Suprabasal *Tgf*β1 expression increases BrdU labelling index in K10-*Tgf*β1 transgenic mouse epidermis on both a wild-type and *Tgf*β1-null background

GENOTYPE	BRDU LABELLING INDEX
$tgfb1^{+/+}$, +/+	1.1 2.1 1.8
$tgfb1^{+/+}$, H/+	1.7 1.2
$tgfb1^{+/+}$, H/H	3.5 4.9
$tgfb1^{+/-}$, +/+	1.4 1.6
$tgfb1^{+/-}$, H/+	2.1
$tgfb1^{+/-}$, H/H	4.1
$tgfb1^{-/-}$, +/+	1.3 1.8
$tgfb1^{-/-}$, H/+	2.5 1.1 1.0
$tgfb1^{-/-}$, H/H	3.6

Each number represents the labelling index of a single mouse. Labelling index shown is the number of BrdU-positive cells per 100 basal cells of interfollicular epidermis. A minimum of 1000 cells was counted for each skin section and two sections were assayed from each mouse.

significance: even assuming no prenatal lethality, only one in sixteen mice born from the cross would be both null for *Tgf*β1 and homozygote for the K10-*Tgf*β1 transgene. However, from the mouse skins that were analysed, some observations were made. BrdU labelling indices from this cross suggest that the K10-*Tgf*β1 transgene enhances basal cell proliferation even in the complete absence of endogenous Tgfβ1. One possible explanation for this is that the transgene effect is not solely due to a general over expression of Tgfβ1. Instead, the increased basal cell proliferation may be ascribed to the perturbed Tgfβ1 production in the particular cellular compartment of transgene expression. An alternative explanation is that endogenous Tgfβ1 levels are near to zero in the epidermis. Hence, in terms of the epidermis, *Tgf*β1$^{-/-}$ mice are not much different than wild-type mice. However, clearly, increasing Tgfβ1 does have an effect, therefore there must be Tgfβ receptors present.

3.6 Characterisation of K10-*BCL*-2 Transgenic Mice

3.6.1 Introduction

BCL-2 is a central player in research on apoptosis (for review see Hockenbery, 1995; Korsmeyer, 1999). It has been shown to be an inhibitor of apoptosis in various systems, although its relevance in skin is still being determined. Two lines of *Bcl*-2 transgenic mice that had not yet been analysed in terms of expression of their transgenes became available by collaboration with Dr D. Fowlis at the Centre for Genome Research, Edinburgh. The same transgene was utilised to generate both lines and consisted of a bovine K10 promoter, a rabbit beta-globin intron (for efficient RNA processing and nuclear export), a human *BCL*-2 cDNA with some 3′ non-coding sequence and a human growth hormone poly-adenylation signal (see figure 3.9). Therefore, *BCL*-2 was targeted to the suprabasal compartment of the epidermis. Previous reports suggested that endogenous Bcl-2 expression was restricted to the basal layer of the epidermis (Hockenbery *et al*, 1991), and so these mice could provide an *in vivo* model to investigate the consequences of perturbed Bcl-2 expression in the epidermis.

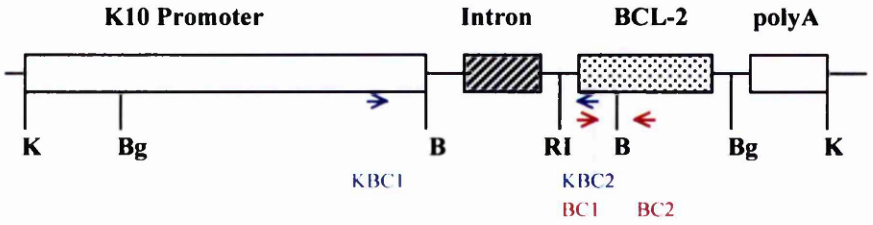
3.6.2 Screening K10-*BCL*-2 transgenic mice

The two transgenic lines originally received were named the B-line and C-line. The genetic background of the mice was [C57Bl6 x CBA]. A set of primers was designed to screen the mice. One primer annealed to the K10 promoter (KBC1), the other to the *BCL*-2 cDNA (KBC2) which together amplified a recombinant fragment of 830bp (see figure 3.9). Using this pair of primers ruled out the possibility of a false positive reaction occurring through human contamination.

This PCR was used to screen some of the original offspring of the B-line founder mouse sent from Edinburgh (figure 3.10A) This revealed a much higher proportion of mice carrying the K10-*BCL*-2 transgene than expected. Therefore, the mice were re-screened by Southern blot using the human *BCL*-2 cDNA fragment of the transgene as a probe. This revealed that some of the B-line offspring contained a low-copy number transgene insert (probably 2-3 copies) and some contained a higher-copy number insert (see figure 3.10B) i.e. the B-line founder had a double insert of the transgene. The high-

Figure 3.9

Structure of K10-*BCL*-2 transgene



K10 promoter: 5.5kb
Promoter region of bovine *cytokeratinVI* gene
Targets suprabasal cells of epidermis

Intron: 650 bp
Intron fragment from rabbit *beta-globin* gene

BCL-2: 1.9 kb
760 bp of human *BCL*-2 cDNA followed by 1.1 kb 3' non-coding sequence

polyA: 630 bp
Polyadenylation cassette from human *growth hormone* gene

Restriction sites: B: *Bam*HI, Bg: *Bgl*II, E: *Eco*RI, K: *Kpn*I

KBC1, 2: Primers used in PCR reaction shown in figure 3.10
BC1, BC2: Primers used in PCR reaction described in figure 3.12

Figure 3.10

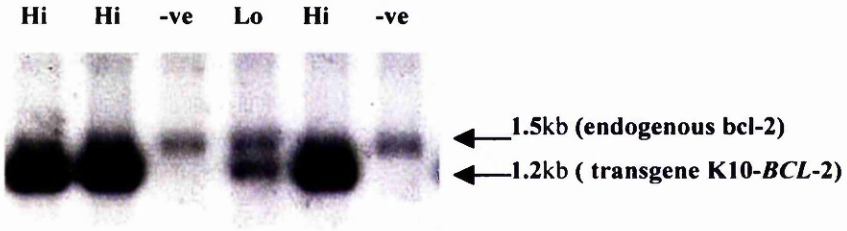
Transgene status and screening of K10-*BCL*-2 transgenic mice

A. PCR reaction to screen K10-*BCL*-2 transgenic mice

Analysis of PCR on tail DNAs using primers KBC1 and KBC2 (see figure 3.9).

L: 1kb DNA ladder
Lanes 1,2,5,7: Mice that are wild-type with respect to K10-*BCL*-2 transgene
Lanes 3,4,6: Mice carrying the K10-*BCL*-2 transgene

B. Southern blot analysis of transgenic K10-*BCL*-2 and wild-type littermate DNAs probed with a human *BCL*-2 cDNA probe



B-line mouse genomic DNAs were digested with BamHI and probed with the human *BCL*-2 cDNA. The probe hybridised as expected to a 1.2 kb fragment in the transgenic DNAs. It also hybridised to a 1.5 kb DNA fragment which corresponds to a *Bam*HI fragment of endogenous mouse bcl-2.

Hi: Transgenic (high-copy) DNA
Lo: Transgenic (low-copy) DNA
-ve: Wild-type DNA

copy insert appeared to have at least 50 copies of the transgene. The B-line was subsequently bred out to produce a new line: BL (low-copy). Attempts were made to differentiate between the mice with both the high copy *and* the low-copy integration and those with just the high-copy: the human growth hormone polyA fragment of the transgene was used as a probe on the Southern blot to check for different *Bam*HI end fragments but this was uninformative. Also, *Bgl*II and *Eco*RI were used in the initial genomic DNA digestion of other Southern blots in the hope of distinguishing different-sized end fragments of the integrated transgenes. However, it was not possible to distinguish high-copy insert DNAs from those with both inserts.

The third line (C-line) had just one insert which contained multiple (>50) copies of the transgene. This was estimated by comparing the relative densities of the endogenous mouse Bcl-2 band (2 copies) with the transgene band.

3.6.3 RNA expression analysis of K10-*BCL*-2 transgenic mice

To test for expression of the transgene, RNA was extracted from dorsal skin epidermis, tail skin epidermis and whole ear as described in Materials and Methods. Reverse transcriptase PCR (RT-PCR) was performed using a pair of Bcl-2 primers (BC1 and BC2), designed to amplify a 300bp fragment of the human *BCL*-2 cDNA. This pair of primers was not species-specific and also amplified the equivalent endogenous mouse *Bcl*-2. The PCR products from the two species are the same size and very similar in sequence, but the mouse product contains a *Sal*I site which the human does not (see figure 3.11A). RNA extractions from the ears of the three transgenic lines and wild-type mice were reverse-transcribed; the resulting cDNA products were amplified with the BC1/BC2 primers (see figure 3.11B) and the products were then gel-purified (Geneclean^{TM}) and digested with *Sal*I. All three transgenic lines retained undigested PCR product whereas RNA from wild-type lines did not (figure 3.11C). Therefore all three transgenic lines seemed to be expressing transgenic RNA. A PCR using the KBC1 and KBC2 primers on the cDNA products ruled out the possibility of contamination of the RNA preparations by genomic DNA.

Northern blot analysis was performed with independent RNA preparations. The strongest signal was seen with C-line ear RNA. The B and BL transgenic lines showed weaker bands of the same size (see figure 3.12). The transgenic transcript is approximately 1.8kb, which is consistent with the size of the human *BCL*-2 insert

Figure 3.11

Detection of human *BCL*-2 mRNA from the K10-*BCL*-2 transgene through RT-PCR

A.PCR products of a reaction using primers BC1 and BC2 yield products for both transgenic and non-transgenic cDNAs

Ear RNA preparations from wild-type and K10-*BCL*-2 transgenic were reverse transcribed as described in Materials and Methods. The resulting cDNAs, as well as transgenic and wild-type genomic DNAs, were subjected to PCR with primers BC1 and BC2 (see figure 3.9).
Genomic DNAs produce a PCR product as the binding sites for primers BC1 and BC2 lie within the same exon.
Contamination of cDNAs with genomic DNA was ruled out by demonstrating the lack of a PCR product sing primers KBC1 and KBC2 (see figures 3.9 and 3.10).

Marker: 1kb DNA ladder
Lane 1: PCR product of genomic C-line DNA
Lane 2: PCR product of genomic wild-type DNA
Lane 3: PCR product of cDNAs from wild-type ear RNA
Lane 4: PCR product of cDNAs from C-line ear RNA

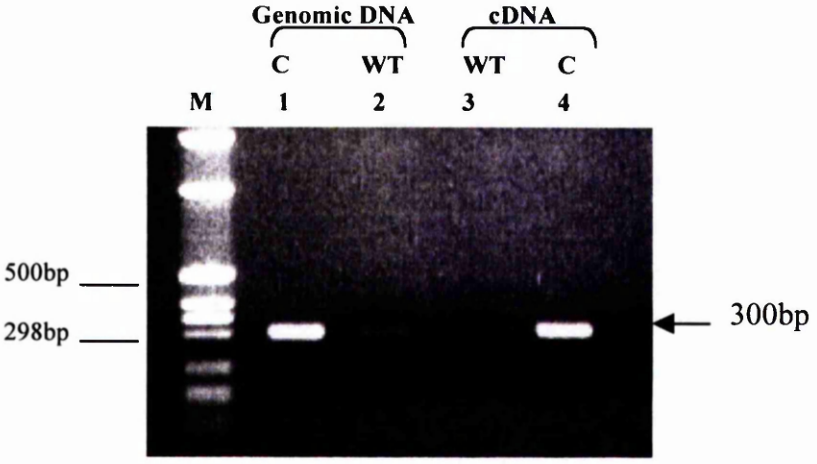
B. Comparison of mouse and human sequences of the *BCL*-2 gene showing *Sal*I site

The pair of primers BC1 and BC2 amplifies both human and mouse BCL-2 cDNAs. The resulting PCR products are the same size and very similar in sequence except for certain single-base differences shown in red. One of these differences results in the presence of a *Sal*I site in the mouse *bcl*-2 sequence which is absent in the human version.

C. Digestion of cDNAs with *Sal*I yields cleaved mouse *bcl*-2 cDNA and uncleaved human *BCL*-2 cDNA

PCR products from A were cleaned, digested with *Sal*I, and digests were run out on a 1.8% agarose gel. PCR product of cDNAs from C-line ear RNA remain uncut, while that of wild-type ear RNA is digested. The same result is obtained with B and BL lines.

A



B

SalI

murine *bcl*-2: ...GGCCACAAGTGAGGTCGACAAACCT...

human *BCL*-2:...GGCCACAAGTGAAGTCAACATGCCT...

C

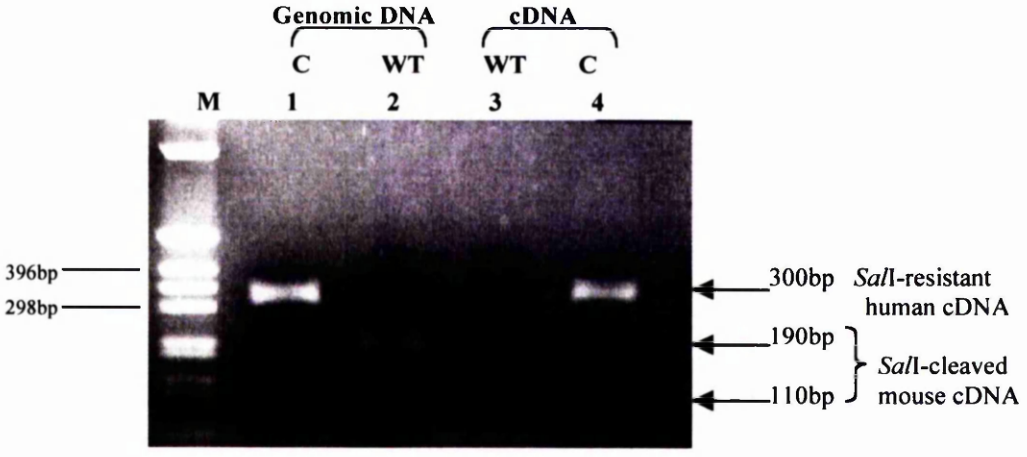
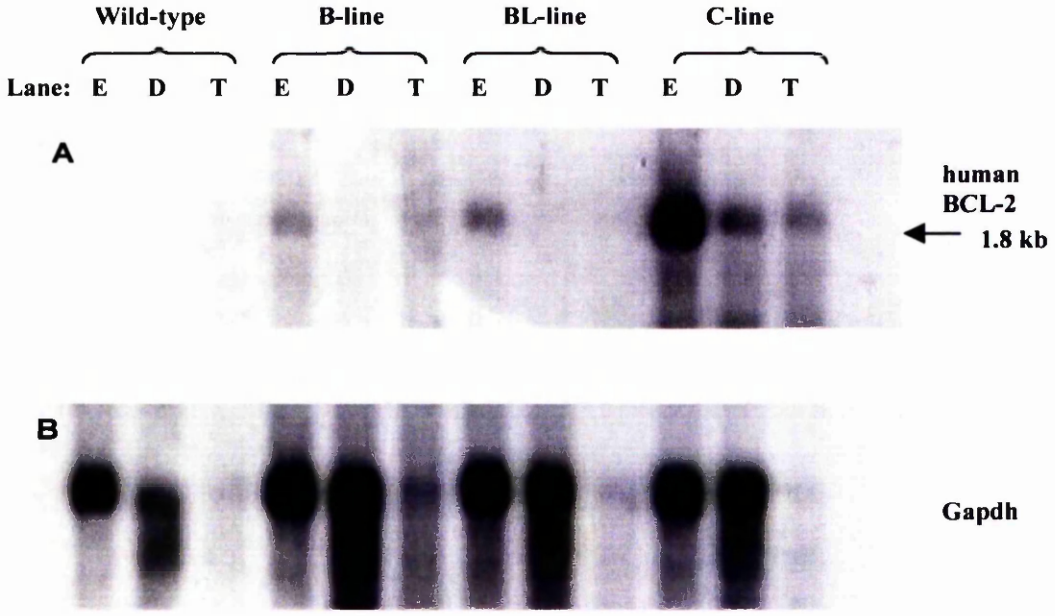


Figure 3.12

Northern blot showing expression of transgenic human *BCL*-2 mRNA in K10-*BCL*-2 transgenic mouse skins



Northern blot analysis of wild-type and transgenic K10-*BCL*-2 mice using 15μg total RNA per lane, probed with human *BCL*-2 DNA and a Gapdh (glyceraldehyde phospho-dehyrogenase) probe to determine loading.

E: RNA from whole ear
D: RNA from dorsal skin epidermis
T: RNA from tail epidermis

All three transgenic lines show expression of transgenic mRNA with the strongest being C-line.
RNA extracted from tail epidermis was degraded.

without transcription of the downstream polyA fragment. This is not surprising, since the transgenic *BCL*-2 fragment contains a long 3' untranslated sequence (1.1kb) which probably contains sufficient processing signals for efficient mRNA production.

The probe used for the blot was the human *BCL*-2 cDNA fragment of the transgene. This fragment has 95% sequence similarity to its mouse equivalent but the probe did not pick up any endogenous mouse *Bcl*-2 mRNA (expect 7.5kb and 2.4kb transcripts (Negrini *et al*, 1987)). This may indicate a relatively low endogenous Bcl-2 expression in the skin. The blot was also probed with *Gapdh* to check loading.

3.6.4 Human BCL-2 protein expression in K10-*BCL*-2 transgenic mice

Having shown RNA expression in all three transgenic lines, the next step was to determine whether the transcript was producing protein. Protein was extracted from ear, dorsal skin epidermis and tail epidermis as described in Materials and Methods. A Western blot was carried out with the skin protein extracts from the three lines. An antibody specific for human BCL-2 (i.e. which does not cross-react with mouse Bcl-2) picked up protein expression in the C-line (see figure 3.13). The size of the protein was as expected - approximately 26kDa.

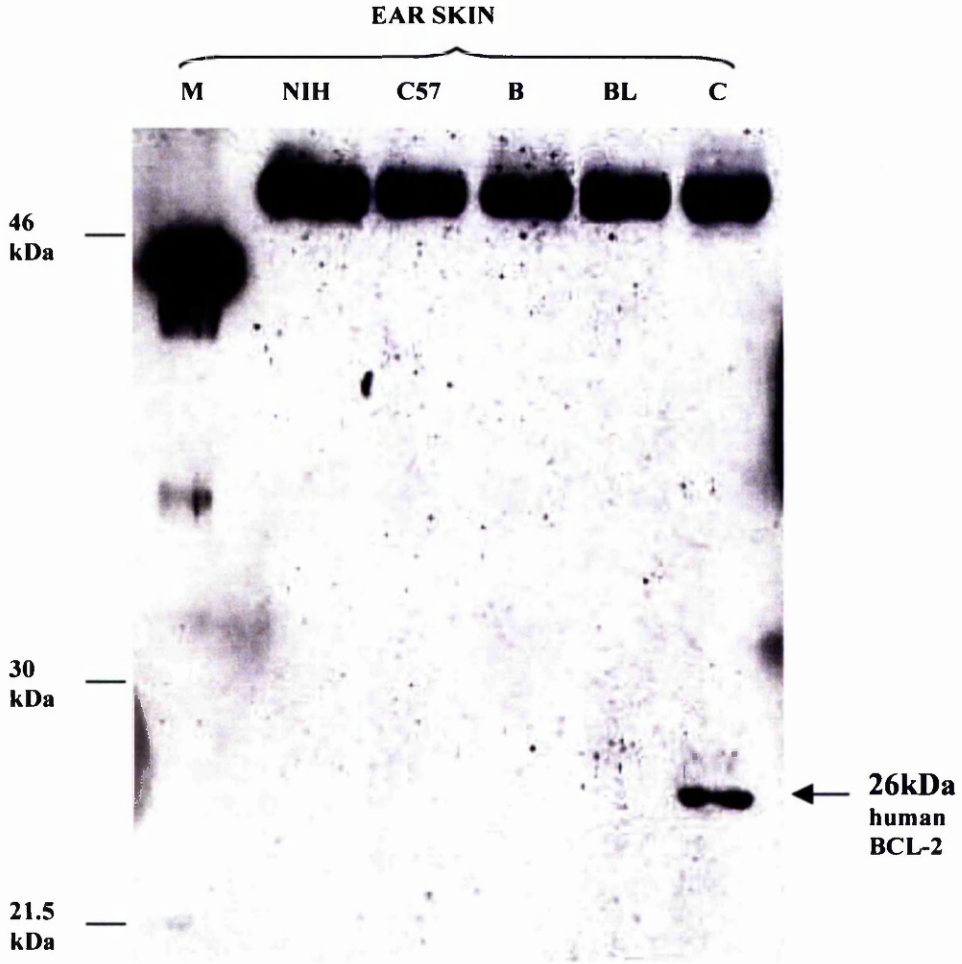
3.6.5 K10-*BCL*-2 transgene RNA expression and localisation by *in situ* hybridisation

To further qualify mRNA expression of the transgene in the C-line, various skin sections were subjected to *in situ* hybridisation. Using this technique it should be possible to identify individual cells which possess the RNA transcript under investigation. A combination of in situ hybridisation and immunohistochemistry can also, in certain cases, show differences between the site of mRNA synthesis and localisation of the final protein.

In the case of BCL-2, previous reports claimed that it was endogenously expressed in the basal layer of the epidermis (Hockenbery *et al*, 1991; Krajewski *et al*, 1994)). The K10 promoter should direct expression of the transgenic BCL-2 to the suprabasal layer. Since adult mice have a relatively thin epidermis in which it can be sometimes difficult to distinguish a suprabasal layer of cells, the skins of transgenic and control neonatal mice were also collected for analysis by in situ hybridisation. The skins of

Figure 3.13

Expression of transgenic human BCL-2 protein by C-line transgenic mice



The antibody used was a human-specific BCL-2 antibody (sc509, Santa Cruz).
50μg total protein was loaded in each lane except the positive control lane (500ng).
Human BCL-2 protein was detected in protein preparations from both ear and skin epidermis of C-line mice. The antibody appears to cross-react with a 50kDa protein – possibly an abundant keratin.

M: Positive control fusion protein (bcl2Δ21, Santa Cruz)
NIH: Wild-type NIH mouse
C57: Wild-type C57/Bl6 mouse
B, BL, C: K10-*BCL*-2 transgenic lines

these pups (2-5 days old) have a thicker epidermis, making identification of different layers of cells more apparent (see figure 3.14).

A 561bp BamHI fragment of the human *BCL*-2 cDNA was subcloned into the pBluescriptII SK^+ plasmid. Antisense and sense templates were generated using appropriate enzymes and polymerases. The templates were used to make probes labelled with ^{35}S-UTP and skin sections of C-line mice were then treated and probed as described in Materials and Methods. Following autoradiogaphy and development of the emulsion, the sections were stained with Harris's Haematoxylin.

Strong but relatively patchy signal was observed in transgenic pup skin with no signal apparent in the negative littermates (see figure 3.15). [Under a high power (x40), the signal appears to be in mainly suprabasal cells of the epidermis (figure 3.15D)]. The signal is not uniform with some cells showing strong mRNA signal and others very little or none. Adult transgenic epidermis also shows expression of the transgene transcript although the signal is weaker than that of neonatal skin.

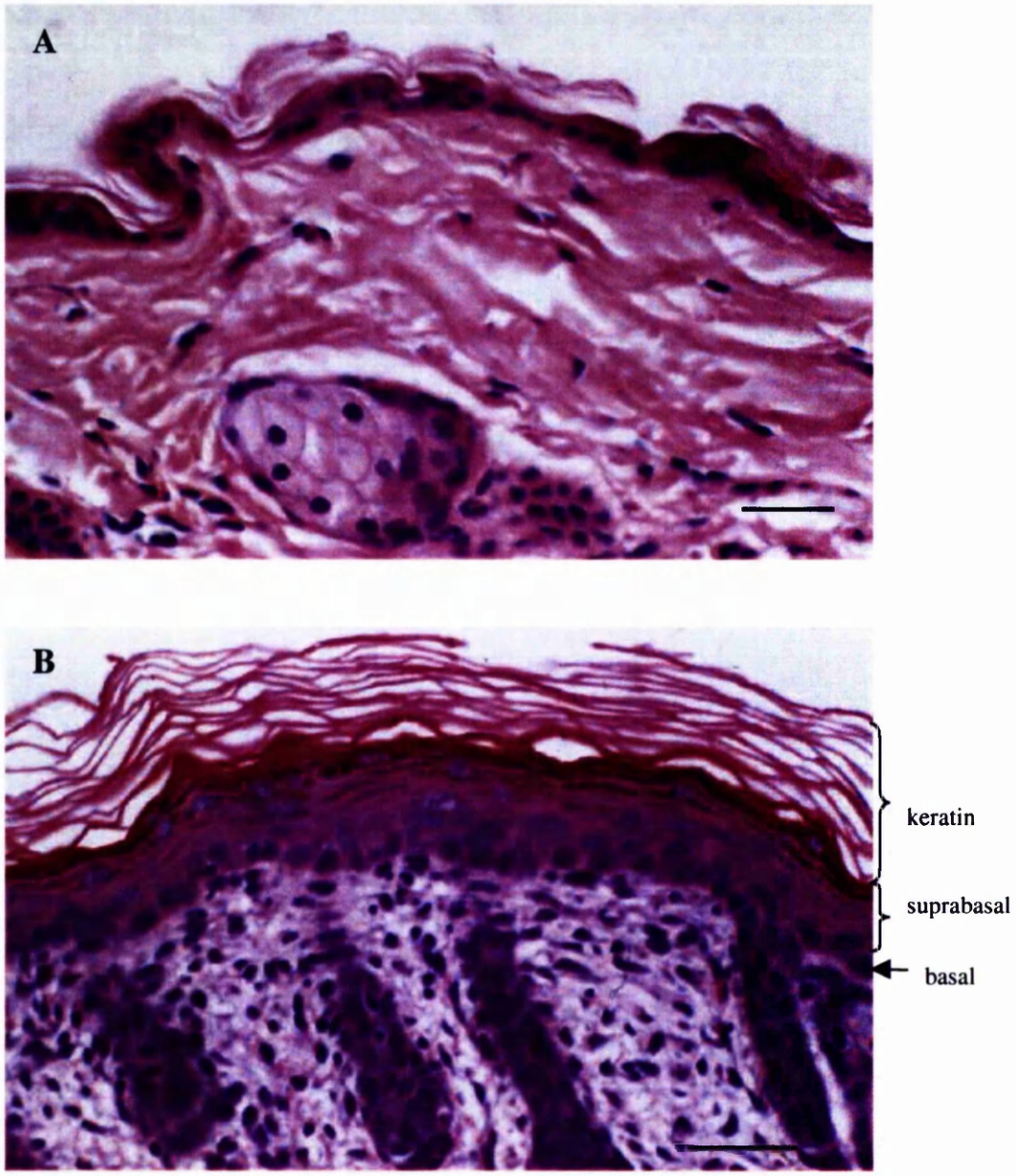
A comparison of transgenic mice with their negative littermates shows that the C-line mice obviously express the human *BCL*-2 mRNA in the epidermis. The data from the neonate mice suggests that expression is suprabasal as expected. A surprising finding perhaps is the lack of any endogenous mouse *Bcl*-2 signal. The human *BCL*-2 cDNA fragment used for the probe has 98% sequence homology with the equivalent mouse fragment and so if their was sufficient mouse *Bcl*-2 mRNA present it is reasonable to assume that the probe would have picked it up. As mentioned previously, several studies report BCL-2 protein expression in the epidermis. One possible explanation is that the protein may have a long half-life, and so relatively little RNA transcript is needed.

3.6.6 Localisation of transgenic human BCL-2 protein in suprabasal cells of the epidermis by immunohistochemistry

Having established that transgenic protein was being produced in the skin it was decided to investigate protein localisation in the epidermis with a BCL-2 antibody. Despite many variations of the labelling technique, it was not possible to detect transgenic protein in paraffin sections of C-line skin with the human-specific BCL-2 antibody that had been used in the Western blot (section 3.2.4). Therefore, a second

Figure 3.14

Epidermis of a neonatal mouse pup is thicker and contains more suprabasal layers than adult mouse epidermis



6μm paraffin sections of skin from adult mouse (A) and 4-day old mouse pup (B) were stained with haematoxylin and eosin. Neonatal epidermis has more pronounced suprabasal and granular layers than adult epidermis.

Bar: 50μm

Figure 3.15

Localisation of *BCL*-2 transgene mRNA to the suprabasal keratinocytes of C-line neonatal mice by *in situ* hybridisation

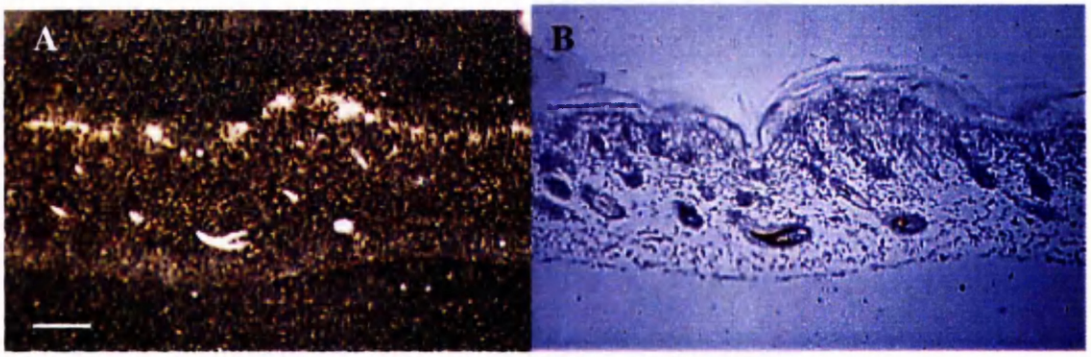
Six-micron paraffin sections of C-line neonatal dorsal skin were subjected to *in situ* hybridisation using ^{35}S-labelled antisense human *BCL*-2 cDNA riboprobe.

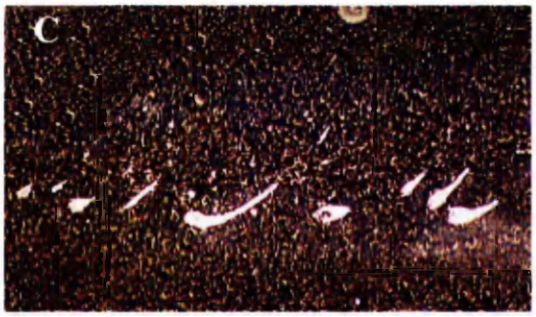
Dark-field (A) and bright-field (B) images of the same section are shown.

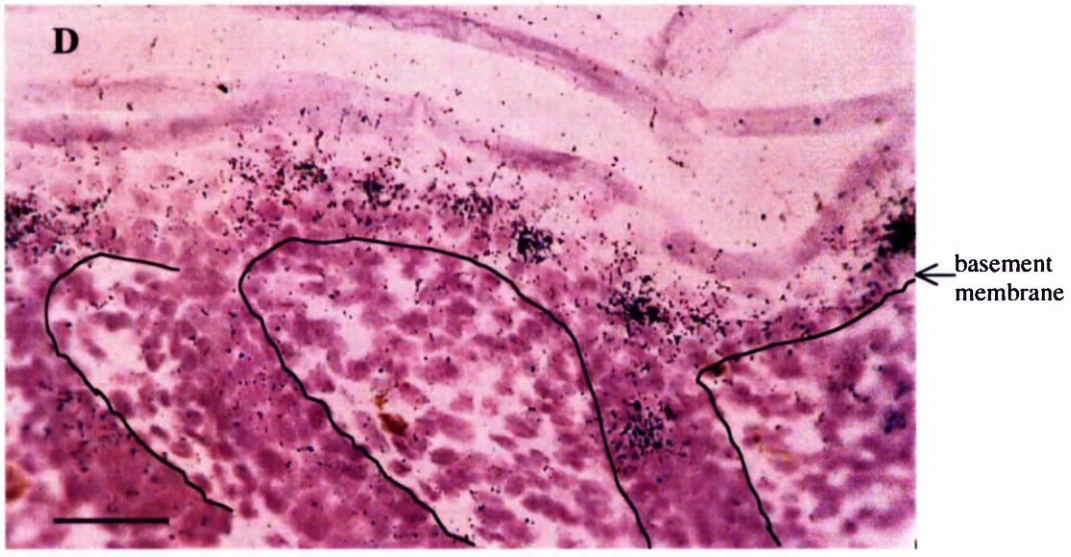
Staining of skin of a negative littermate (C) shows no positive staining.

(D) Higher magnification of B showing suprabasal signal of the transgene mRNA.
In (D) the basement membrane is outlined to show the epidermis:dermis boundary.

Bars: 400μm (A,B,C) or 50μm (D)


A
B


C


D
basement
membrane

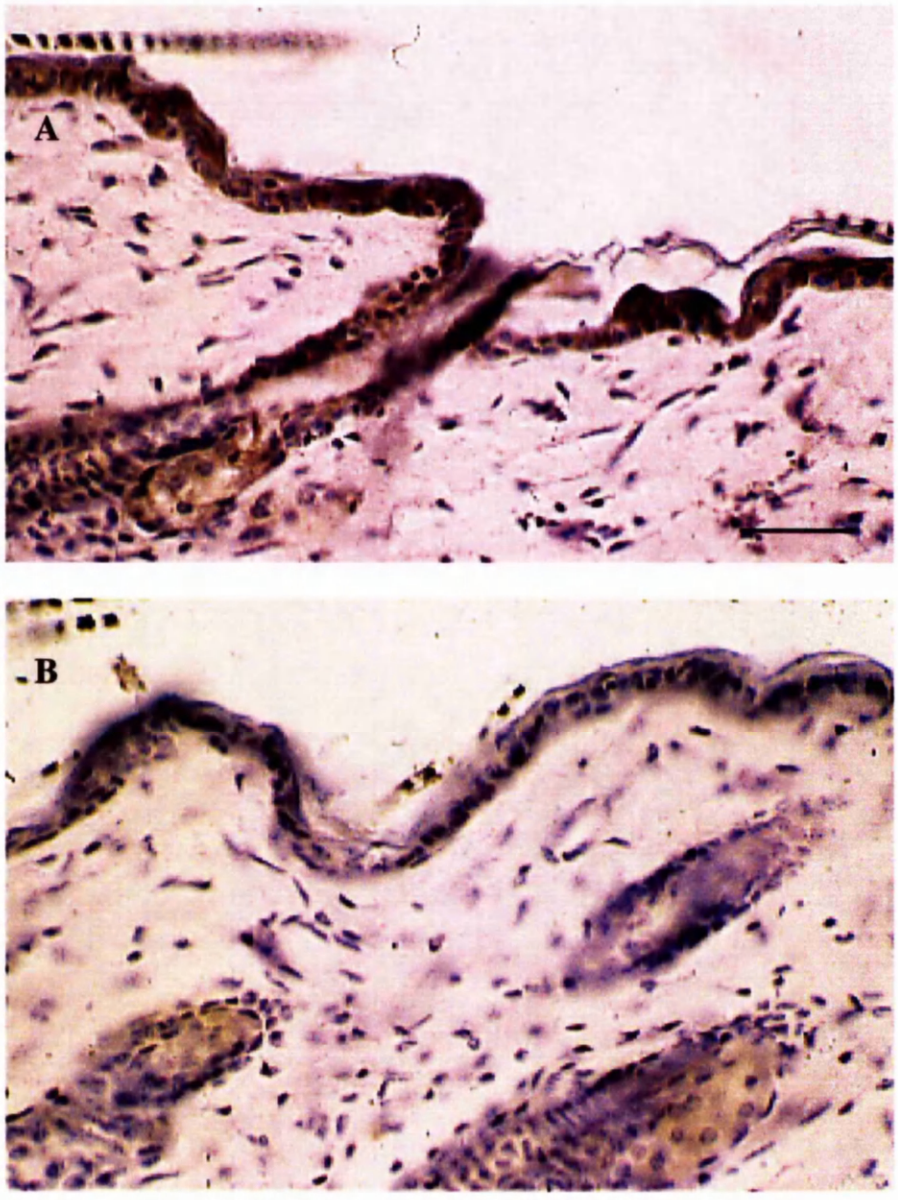
BCL-2 antibody (Bcl-2(N19)) was tried – an antibody, according to the manufacturers, that should detect both human and mouse Bcl-2.

6μm sections of dorsal skin from both adult and neonate C-line mice (both transgenic and wild-type) were cut from paraffin blocks. Sections were labelled with BCL-2(N19) antibody as described in Materials and Methods. Slides were counterstained with Harris's haematoxylin.

The BCL-2(N-19) antibody detected differences between the transgenic and non-transgenic skins in both adult and neonate mice. In adult mice, transgenics showed a distinct staining pattern compared to negatives and this tended to be suprabasal when different epidermal layers were distinguishable (see figure 3.16). Transgenic neonates also had suprabasal staining. With these skins, there was a more noticeable patchy expression of the transgenic protein with some individual cells heavily stained (see figure 3.17)

As mentioned, this antibody should pick up both human and mouse BCL-2 (in this case, transgenic and endogenous). In negative skins there was weak staining of the epidermis, and this tended to be uniform throughout the different layers (figure 3.17B). This disagrees with earlier reports of basal-restricted expression of BCL-2 (Hockenbery *et al*, 1991). More recently however, some studies have detected BCL-2 in suprabasal and granular layers also (Krajewski *et al*, 1994; Stenn *et al*, 1994). The staining reported here would agree with the latter studies.

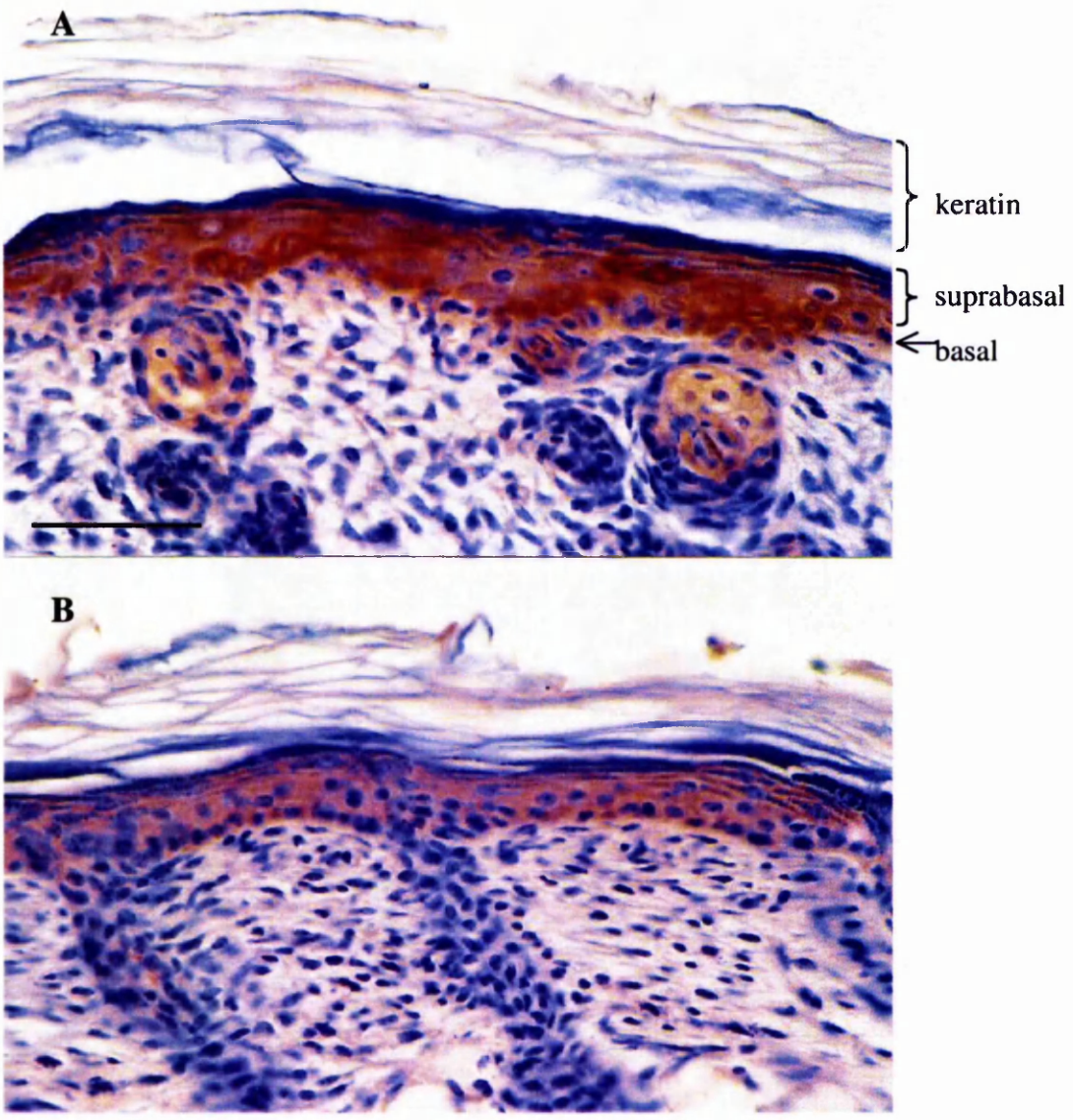
Figure 3.16
Expression of transgenic human BCL-2 in K10-*BCL*-2 mouse adult epidermis



Skins of adult C-line mice were stained with a BCL-2 antibody (BCL-2 N19, Santa Cruz) which should detect both human and mouse BCL-2.
A transgenic mice (A) and negative littermate (B) are shown.

Transgenic skins have patchy staining whoch appears suprabasal when the skin is thick enough to notice, while negative littermates have very little positive staining.
Bar: 100μm

Figure 3.17
Expression of transgenic human BCL-2 in K10-*BCL*-2 mouse neonatal epidermis



Skins of neonatal C-line mice were stained with a BCL-2 antibody (BCL-2 N19, Santa Cruz) which should detect both human and mouse BCL-2.
A transgenic pup (A) and negative littermate (B) are shown.
Transgenic skins have a patchy suprabasal staining pattern while negative littermates have a weaker, more uniform pattern.
Bar: 100μm

3.7 Functional Analysis of K10-BCL-2 Transgenic Mice

3.7.1 Introduction

Following confirmation of expression of the K10-*BCL*-2 transgene, the next stage involved an investigation of the phenotypic consequences of BCL-2 overexpression in the epidermis. K10-*BCL*-2 transgenic mice appeared grossly phenotypically normal. There was no obvious skin or hair abnormalities. Haemotoxylin and eosin staining of adult and neonatal skins also showed no histological differences between transgenic and wild-type skins. Therefore, if functional BCL-2 was being expressed in the epidermis, its presence did not produce any noticeable changes in skin morphology. A number of experiments were carried out to assess any effects that recombinant BCL-2 might have on cell proliferation or growth.

3.7.2 Epidermal cell proliferation rates are not affected in K10-*BCL*-2 transgenic mice

BCL-2 has been reported to play a role in growth control in some systems (Vairo *et al*, 1996) and to act as a suppressor of apoptosis. It may thus disrupt cell turnover. Cellular proliferation in the epidermis of K10-*BCL*-2 transgenic mice was therefore investigated. Three transgenic and three wild-type mice were injected with BrdU one hour before sacrifice. Both adult (12 weeks old) and neonatal pups were analysed in this way. Dorsal skins of these mice were stained with an anti-BrdU antibody as described in Materials and Methods and the labelled cells of interfollicular epidermis were counted.

No difference in labelling indices was found between K10-*BCL*-2 transgenic mice and their wild-type littermates (see table 3.4A). Therefore aberrant expression of human BCL-2 in mouse skin epidermis did not induce a proliferative change. It was therefore not surprising that no hyperplasia or hypoplasia was observed either in neonate or adult transgenic mice.

3.7.3 Immunohistochemical Bax expression is not disrupted in K10-*BCL*-2 mice

BCL-2 is known to interact with a number of proteins as discussed in Chapter 1. BAX is one protein with which it forms a heteromer during the regulation of

Table 3.4A

Proliferation rates in the interfollicular epidermis of K10-*BCL*-2 transgenic mice are similar to that of wild-type littermates

	K10-*BCL*-2 Transgenic	Wild-type
Adult mouse	2.21 1.77 1.92 **Mean: 1.97**	1.48 2.0 2.11 **Mean: 1.86**
Neonatal pup	2.97 3.16 3.78 **Mean: 3.30**	3.72 3.82 3.06 **Mean 3.52**

Each number is the BrdU labelling index of an individual mouse and represents the average number of BrdU+ve cells per 100 interfollicular basal epidermal cells.

Table 3.4B

Proliferation rates in the interfollicular epidermis of K10-*BCL*-2 transgenic mice are similar to that of wild-type littermates following chronic TPA treatment

K10-*BCL*-2 Transgenic	Wild-type
32.3 25.3 26.8 **Mean: 28.1**	38.4 24.4 33.1 **Mean: 31.9**

Each number is the BrdU labelling index of an individual mouse and represents the average number of BrdU+ve interfollicular cells per 500μm skin epidermis.

apoptosis. Transgenic and wild-type skins were stained with a Bax antibody as described in Materials and Methods.

Bax staining was observed throughout the epidermis as had previously been shown for wild-type mice (figure 3.8). However, no difference in staining was evident between K10-*BCL*-2 transgenics and wild-type littermates. Therefore overproduction of BCL-2 protein in the epidermis did not affect endogenous Bax expression as assessed by immunohistochemistry.

3.7.4 Response to treatment of skin with a tumour promoter is not altered in K10-*BCL*-2 transgenic mice

The tumour promoter TPA (12-O-tetradecanoylphorbol-13 acetate) perturbs the homeostasis of the skin and induces hyperplasia of the epidermis. To investigate the effects of aberrant Bcl-2 expression during such a chemical insult to the skin, K10-*BCL*-2 transgenic mice were treated with TPA. Female mice between 10 and 12 weeks old were used for the TPA treatments. Two different types of TPA treatment (chronic and acute) were carried out.

3.7.4.1 Chronic TPA treatment

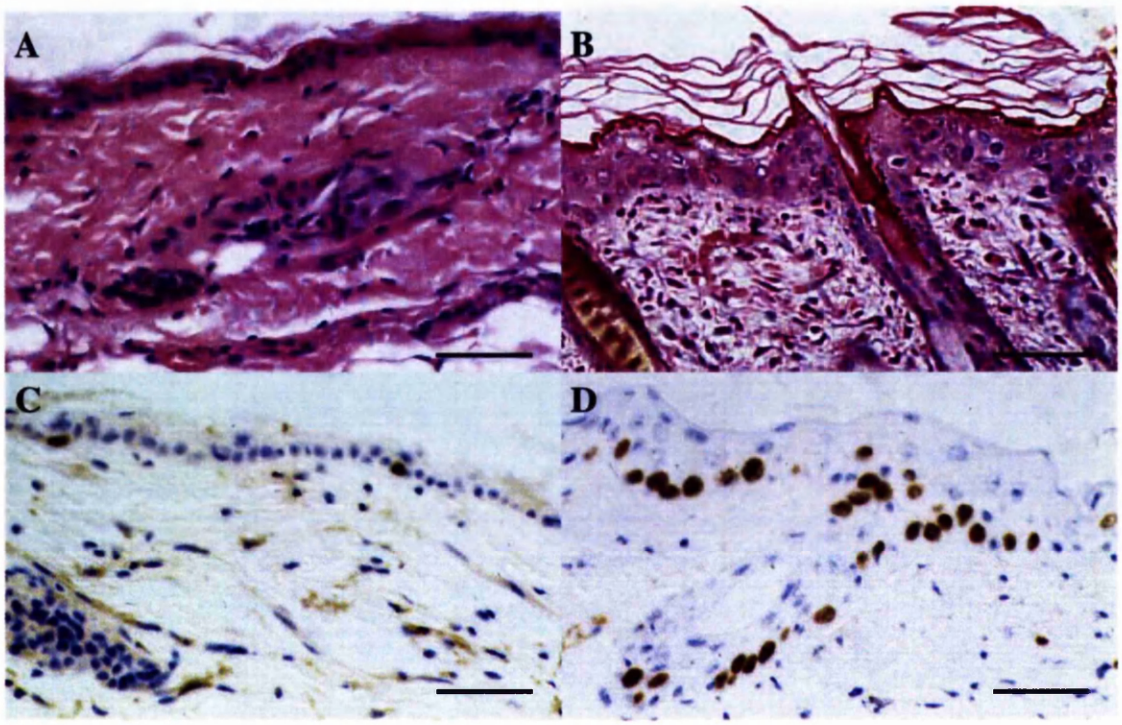
Firstly, the effects of a chronic application of TPA were examined. Three female transgenic mice and three wild-type female littermates had their dorsal skins treated with 200µl of 10^{-4}M TPA solution (0.02moles). The mice were treated a further three times at three days, six days and nine days after the initial application. Twelve days after the first treatment, mice were injected with BrdU and dorsal skin was collected.

Chronic treatment with TPA caused the skins of both transgenic and wild-type mice to undergo hyperplasia and the epidermis became markedly thickened (see figure 3.18). There was no noticeable morphological difference in skins of transgenic and wild-type littermates following the treatment.

Expression of the K10-*BCL*-2 transgene following chronic TPA treatment was examined both by *in situ* hybridisation and immunohistochemistry. Human *BCL*-2 RNA and protein were both strongly induced (see figure 3.19). This might be due to individual cells expressing higher levels of transgene mRNA and protein and/or the fact that there are more suprabasal-like cells in the epidermis of TPA-treated skin. This TPA treatment did not appear to induce endogenous *Bcl*-2 mRNA or protein

Figure 3.18

Epidermal hyperplasia and hyperproliferation following topical application of TPA to mouse dorsal skin

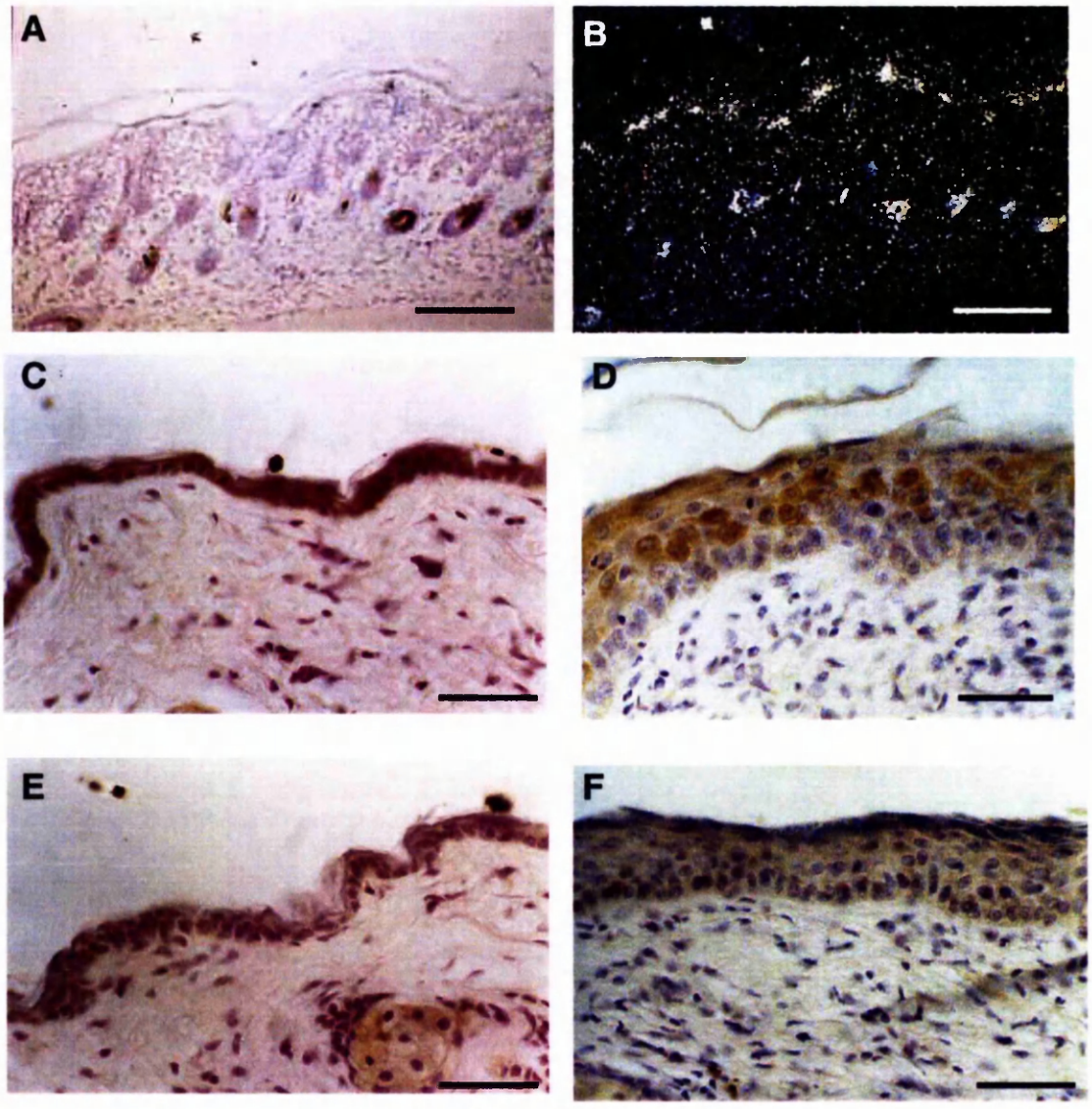


Mouse adult skin was either untreated (A, C) or treated (B, D) with the the phorbol ester TPA 4 times over a period of 10 days. 12 days after the initial treatment, mice were injected with BrdU and dorsal skin was harvested 1 hour later. Tissue was fixed in 4% paraformaldhyde and 6μm paraffin sections were made. Sections were either stained with haematoxylin and eosin (A, B) or labelled with an anti-BrdU antibody (C, D).

TPA treatment resulted in hyperproliferation and hyperplasia of the epidermis including the appearance of proliferating cells in suparabasal layers.

Bars: 100μm (A, C), 50μm (B, D)

Figure 3.19
Tumour promoter TPA causes induction of the K10-*BCL*-2 transgene



Following chronic TPA treatment, 6μm paraffin sections of K10-*BCL*-2 transgenic mice or their wild-type littermates were subjected to *in situ* hybridisation with a ^{35}S-labelled human *BCL*-2 cDNA riboprobe (A and B) or stained with a Santa Cruz N19 BCL-2 antibody (C, D, E and F).
A and B are bright-field and dark-field images respectively of the same section of a K10-*BCL*-2 transgenic skin following TPA treatment.
C and E are untreated adult skins: C is transgenic and E is non-transgenic.
D and F are TPA-treated skins: D is transgenic and F is non-transgenic.
Bars: 200μm (A,B) or 60μm (C, D, E, F).

expression. Staining with an anti-BrdU antibody showed that there was no difference in cellular proliferation between transgenic and wild-type mice. Counts of BrdU-positive cells are shown in Table 3.4B.

3.7.4.2 Acute TPA Treatment

Acute TPA treatment consisted of one application of 200µl of 10^{-4}M TPA solution (0.02moles) followed by analysis of the treated mice at various timepoints after the initial application. Animals were 8 – 14 weeks old females. At each of 7 timepoints post-TPA, three transgenic and two wild-type littermate mice were injected with BrdU one hour before sacrifice and their dorsal skins fixed in 4%PFA. Treated mice were analysed at 0 hours, 6 hours, 12 hours, 24 hours, 48 hours, 4 days, 7 days and 10 days post-TPA treatment.

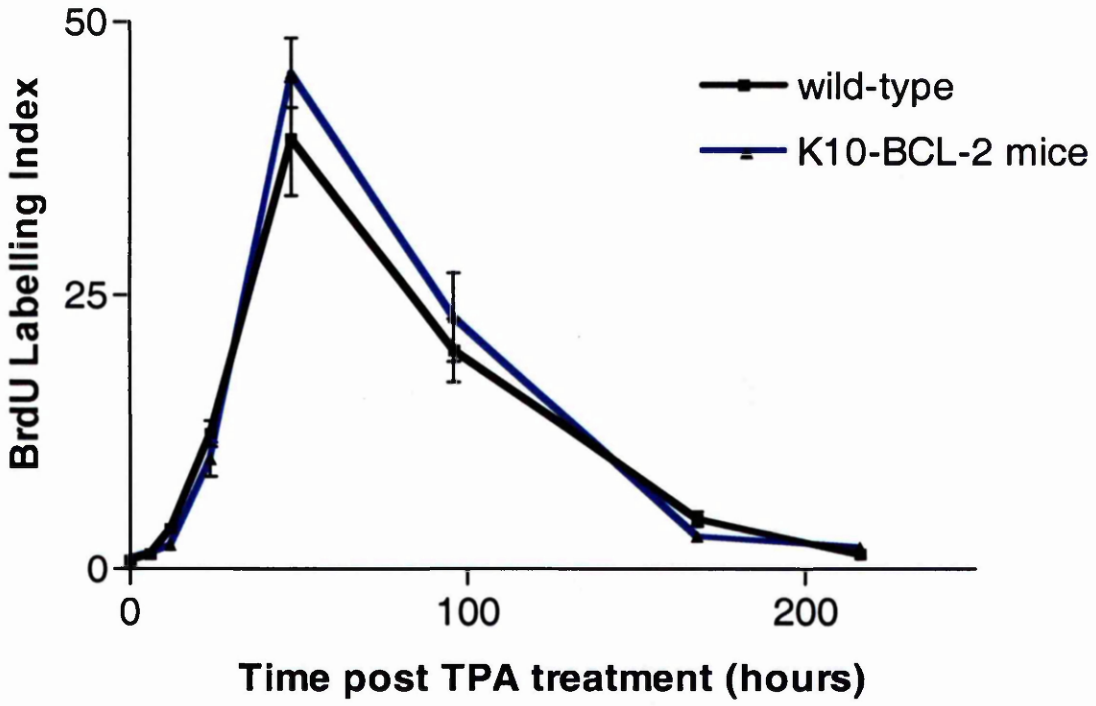
Expression of the transgene was examined by *in situ* hybridisation and immunohistochemistry at 24 hours post-TPA. As with the chronic TPA treatment, both the transgenic mRNA and protein expression were induced.

Skins were labelled with an anti-BrdU antibody and numbers of proliferating cells at all timepoints were counted (see figure 3.20). There was a peak in basal cell proliferation at 48 hours post-TPA in both transgenics and controls and there was no significant difference in the number of S-phase cells throughout the rest of the time course. The final timepoint examined was 10 days post-TPA at which point one would expect the skin to have returned to its quiescent state. This timepoint was looked at to determine whether the presence of excess Bcl-2 would delay the process of the epidermis returning to quiescence from the hyperplastic state. As can be seen from figure 3.20, and from histological appearance of the skins, this process was not affected.

It can be seen from these two TPA experiments that the presence of human BCL-2 in the epidermis did not alter the response of the skin to the phorbol ester, TPA. The increased cellular proliferation and profile of hyperplastic responses was similar in both transgenics and controls.

Figure 3.20

Profile of BrdU labelling indices in interfolicular epidermis following acute TPA treatment is similar in both K10-*BCL*-2 transgenics and wild-type mice



K10-*BCL*-2 transgenic mice and wild-type littermates were applied with 10^{-4}M TPA and skins were harvested at 0, 6, 12, 24, 48 hours and 4, 7 and 9 days after TPA treatment. One hour before sacrifice, mice were injected with BrdU. Three mice of each genotype were collected at each timepoint and the epidermal labelling indices were estimated. The BrdU labelling index is the number of interfollicular BrdU+ve cells per mm.

3.8 Transgenic Model of Skin Carcinogenesis

3.8.1 Introduction

TGFβ1 is a potent inhibitor of cell growth but various reports have shown that it can act as a stimulator or suppressor of tumour growth and progression (Cui and Akhurst, 1996 and references therein). A previous study in our lab had demonstrated this biphasic action of exogenous Tgfβ1 in skin (Cui *et al*, 1996). Transgenic mice overexpressing Tgfβ1 in the suprabasal layer of the epidermis were subjected to long-term chemical carcinogenesis. The transgenics were more resistant to induction of benign skin tumours than control mice, but the malignant conversion rate was vastly increased. The *Tgf*β transgenics utilised in the study included H-line (K10-*Tgf*β1) transgenics, which have been discussed previously, and the M2-line (K6-*Tgf*β1). Like the H-line, the M2-line drives expression of activated *Tgf*β1 in suprabasal keratinocytes, but the K6 gene promoter is inducible and is only switched on in the hyperplastic state.

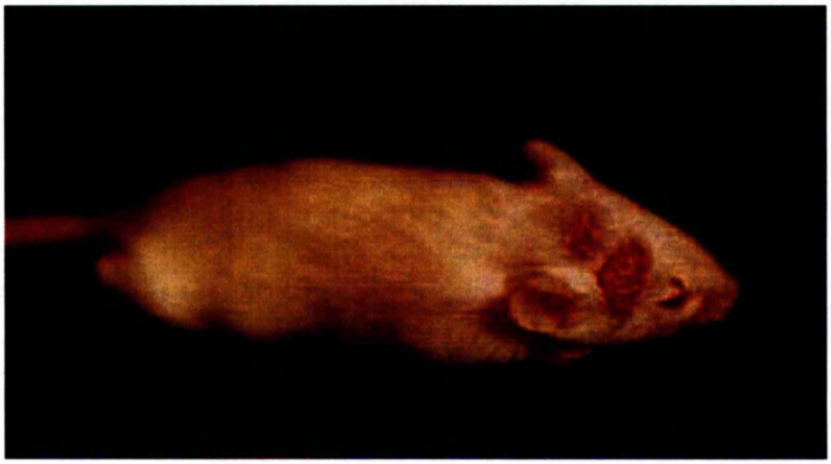
The aim of the next experiments was to assess whether Tgfβ was predominantly tumour suppressing or tumour progressing in a different model of mouse skin carcinogenesis, namely in K5-*RAS* transgenic mice (see Materials and Methods figure 2.1C). These mice have been previously characterised (Brown *et al*, 1998) and express mutant Harvey *RAS* (Ha-*RAS*) in basal keratinocytes. To assess the effect of exogenous Tgfβ1 expression on tumorigenesis in this system, the K5-*RAS* transgenic mice were bred with K10- and K6-*Tgf*β1 transgenic mice to produce mice expressing both active mutant RAS and Tgfβ1 in skin epidermis. Tumour development and progression were followed on the various crosses.

3.8.2 Genetic background affects the phenotype of K5-*RAS* mice

The K5-*RAS* mice were originally received from Dr K. Brown on C57Bl/6J and FVB genetic backgrounds. Both strains were observed over a period of 2-3 months. It was noticeable that K5-*RAS* mice on the FVB background had a much more severe phenotype than C57Bl/6J K5-*RAS* mice i.e. the expressivity was greater on the FVB background. FVB K5-*RAS* mice tended to develop acanthotic lesions as pups (figure 3.21) and keratoacanthomas as young adults (figure 3.22). On the other hand, C57Bl/6J

Figure 3.21
K5-*RAS* transgenic neonates and pups develop acanthotic lesions

A. A region of acanthosis of the skin of a K5-*RAS* transgenic 2-week old mouse



B. Haematoxylin and eosin staining of an acanthotic lesion

Region of acanthotic skin from a 6-day old mouse. The skin has become hyperplastic forming folds of epithelium Note the absence of hair follicles.
Bar: 200μm

Figure 3.22

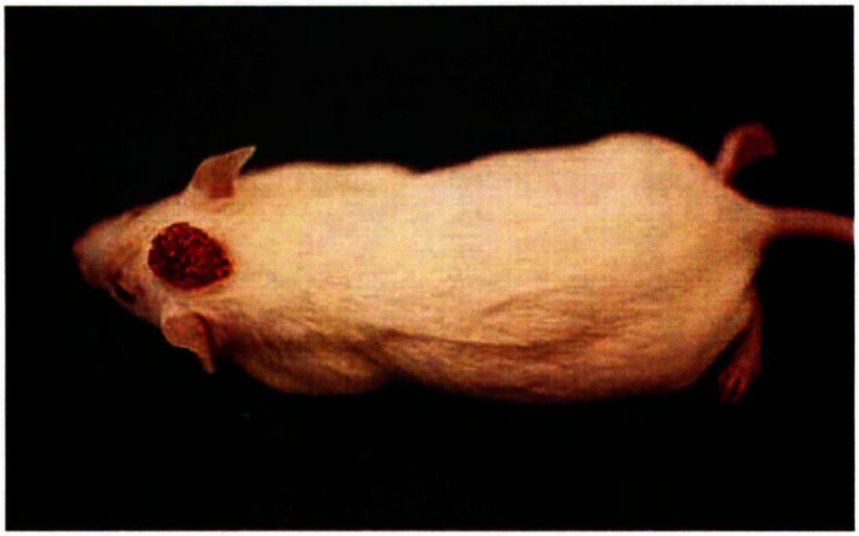
Keratoacanthomas develop spontaneously on the skins of K5-*RAS* transgenic adult mice

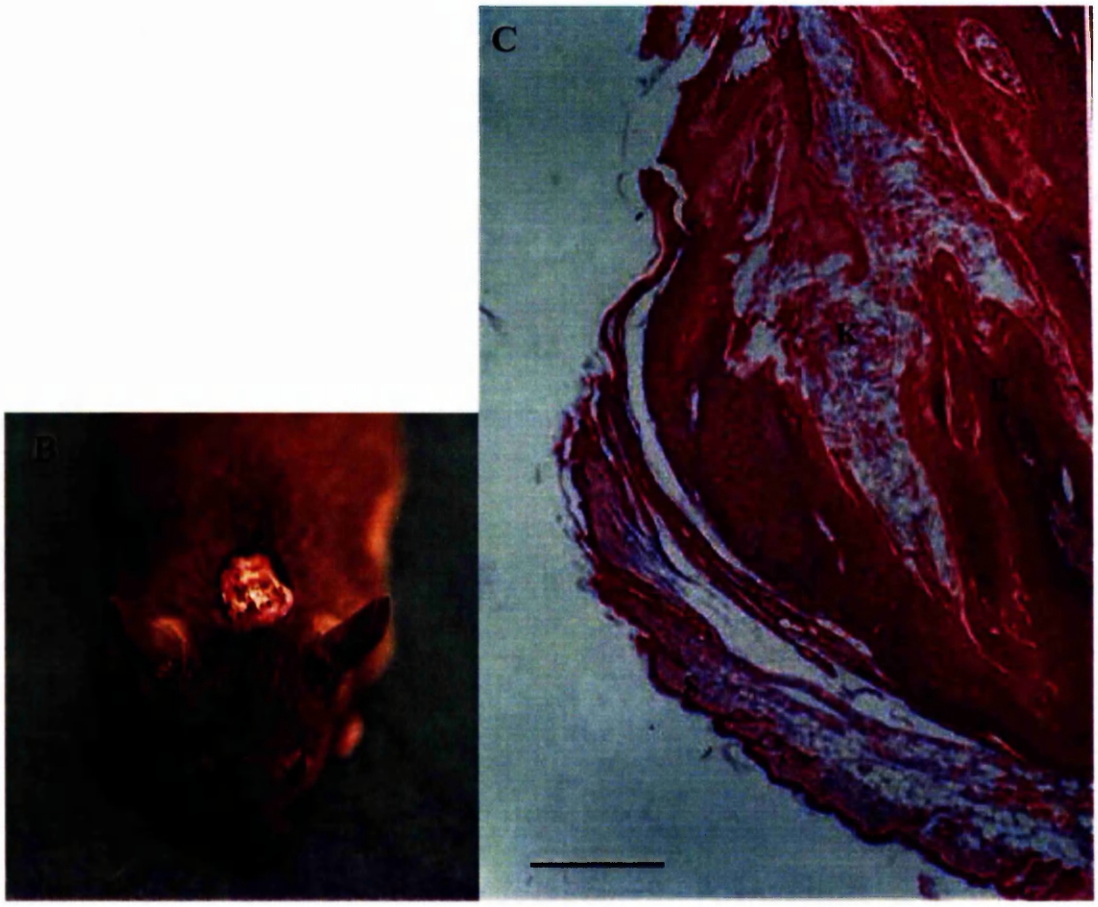
A and B. Keratoacanthomas on the heads of adult K5-*RAS* transgenic mice

C. Haematoxylin and eosin staining of a keratoacanthoma from a K5-*RAS* transgenic mouse

A 6μm section of a keratoacanthoma was stained with haematoxylin and eosin. Skin (S) enapsulates the tumour forming a cup-shaped mass which consists of epithelial cells (E) and an abundance of keratin (K).

Bar: 300μm




C
K

K5-*RAS* mice rarely developed skin lesions as pups and developed keratoacanthomas later than mice on the FVB background.

There was also a difference in penetrance of a skin phenotype between the two genetic strains. Within 8 weeks of birth, 16 out of 24 K5-*RAS* transgenic mice on a FVB background had developed a skin lesion of some sort, while 2 out of 22 C57Bl/6J K5-*RAS* mice displayed a phenotype over the same period. Within each particular genetic strain there was also incomplete penetrance of the transgene and considerable variation in expressivity, making a quantitative analysis of characteristics such as tumour formation difficult.

To ensure that the genetic background was consistent, and since the *Tgf*β1 transgenic mice were on a NIH background, the FVB K5-*RAS* mice were firstly bred on to a NIH background. The K5-*RAS* mice were backcrossed three times onto stock NIH mice. Ensuing crosses with other transgenic lines on the NIH background thus resulted in 93% NIH offspring. As with the C57/Bl6 and FVB genetic backgrounds, the NIH background displayed variable penetrance. It had been thought that on a NIH background there would be a phenotype as least as severe as that of FVB: previous chemical carcinogenesis studies had shown NIH to be one of the most susceptible to tumour formation (Balmain *et al*, unpublished observations). Surprisingly, K5-*RAS* mice on a NIH background had a less severe phenotype than those on the FVB background. The main difference was that NIH transgenics tended to get single tumours as opposed to the multiple tumours of FVB transgenics.

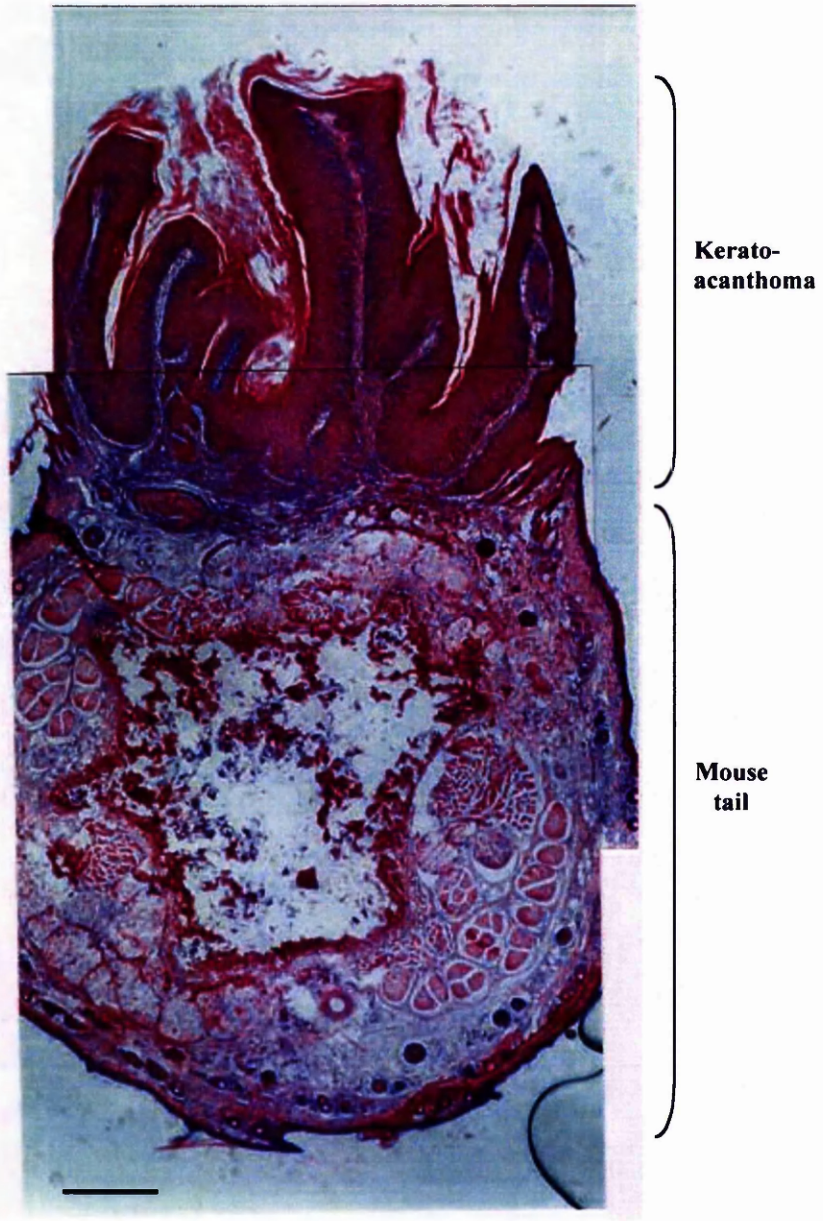
3.8.3 NIH K5-*RAS* mice develop spontaneous follicle-derived tumours

On a NIH background the main type of tumour was a keratoacanthoma - this is a benign lesion which grows quite rapidly, spreading outwards beneath the epidermis and projecting above the surface of the skin as a cup-shaped mass which undergoes keratinisation in the centre. A buttress of tissue encircles the keratin-filled centre (see figure 3.22). In terms of position of the keratoacanthomas, there was great variability: the skin of the back, abdomen, ears, face/snout, genitals, limbs and even tail (see figure 3.23) all gave rise to keratoacanthomas on various mice.

The NIH K5-*RAS* mice developed other types of tumours too. Other skin tumours include papilloma-like lesions on the ears and regions of acanthosis on snout, limbs or

Figure 3.23

Haematoxylin and eosin staining of a cross-section of a keratoacanthoma on the tail of a K5-*RAS* transgenic mouse



A 6μm paraffin section of a keratoacanthoma on a transgenic mouse tail was stained with haematoxylin and eosin.

Bar: 1mm

tail. Non-skin tumours noted include salivary gland tumours, teratomas and in one case hyperplasia of the Vas Deferens tubules.

There was tremendous variation in the age of mice when tumours/acanthosis first appeared. A small number of pups developed acanthotic lesions between 2 days and 2 weeks of age. This acanthosis tended to spread very rapidly, often covering 50% of the dorsal skin area by 48 hours after onset. The animal would then be culled complying with Home Office regulations. However, mice developing skin tumours beyond 2 weeks of age tended to develop more localised keratoacanthomas which expanded more slowly, usually reaching an endpoint (>10mm in diameter) within 2 weeks of appearance.

Injection with BrdU before sacrifice, and subsequent labelling with an anti-BrdU antibody revealed a high BrdU labelling index within the keratoacanthomas. There was also evidence of proliferating cells in suprabasal layers of the tumours (figure 3.24). This is reflected by the speed with which these tumours grew.

3.8.4 Transgene expression in tumours

In order to examine the expression pattern of the K5-*RAS* transgene in the tumours, *in situ* hybridisation was carried out on sections of normal skin and tumours from transgenic mice using a probe specific to the SV40 intron and polyadenylation sequences of the K5-*RAS* transgene construct. A strong signal was observed in basal and basal-like cells of tumours including keratoacanthomas and regions of acanthosis (see figure 3.25). Expression was not uniform throughout these cells, but was patchy. No expression of the transgene was observed in normal skin of K5-*RAS* transgenic mice showing that the transgene is induced in dysplastic tissue.

3.8.5 Exogenous suprabasal Tgfβ1 expression does not affect tumour formation in K5-*RAS* transgenic mice

K5-*RAS* transgenic mice were crossed with the *Tgf*β1 transgenic lines: line H (K10-*Tgf*β1) and line M2 (K6-*Tgf*β1). The breeding scheme and consequent lines that were produced are shown in figure 3.26. Lines RH and RHC are half-littermates, as are RHH and RHN, RM and RMC, RMM and RMN. This means that each pair of lines share one parent (the father in all these cases).

Figure 3.24

Spontaneous acanthotic lesions in K5-*RAS* transgenic mice exhibit a high BrdU labelling index and contain proliferating cells in suprabasal layers

Mice were injected with BrdU 1 hour before sacrifice and tumours were fixed in 4%PFA before being stained with an anti-BrdU antibody.

The arrow indicates a proliferating cell in a suprabasal layer of the lesion.

Bar: 100μm

Figure 3.25

***In situ* hybridisation analysis shows that tumours from K5-*RAS* mice express the K5-*RAS* transgene**

A kerataoacanthoma was removed for the skin of a K5-*RAS* transgenic mouse and probed with a ^{35}S-labelled PolyA cDNA fragment.

Low-power views of dark-field (A) and bright-field (B) images are shown. A higher magnification of a section of the tumour (C) shows localisation of transgene in basal-like epithelial cells of the tumour.

Expression of the transgene in normal skin of K5-*RAS* transgenic mice is undetectable.

Bars: A and B, 500μm; C, 100μm

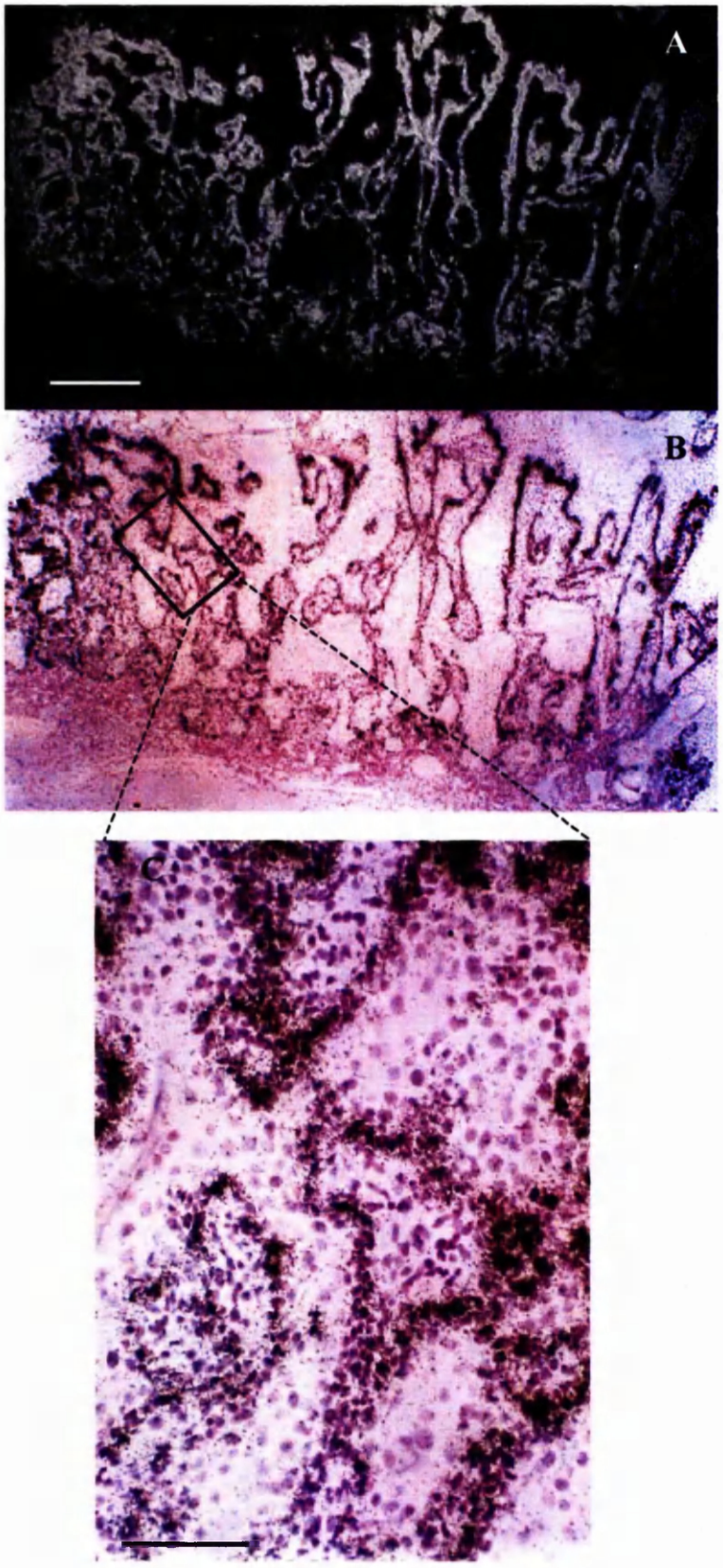
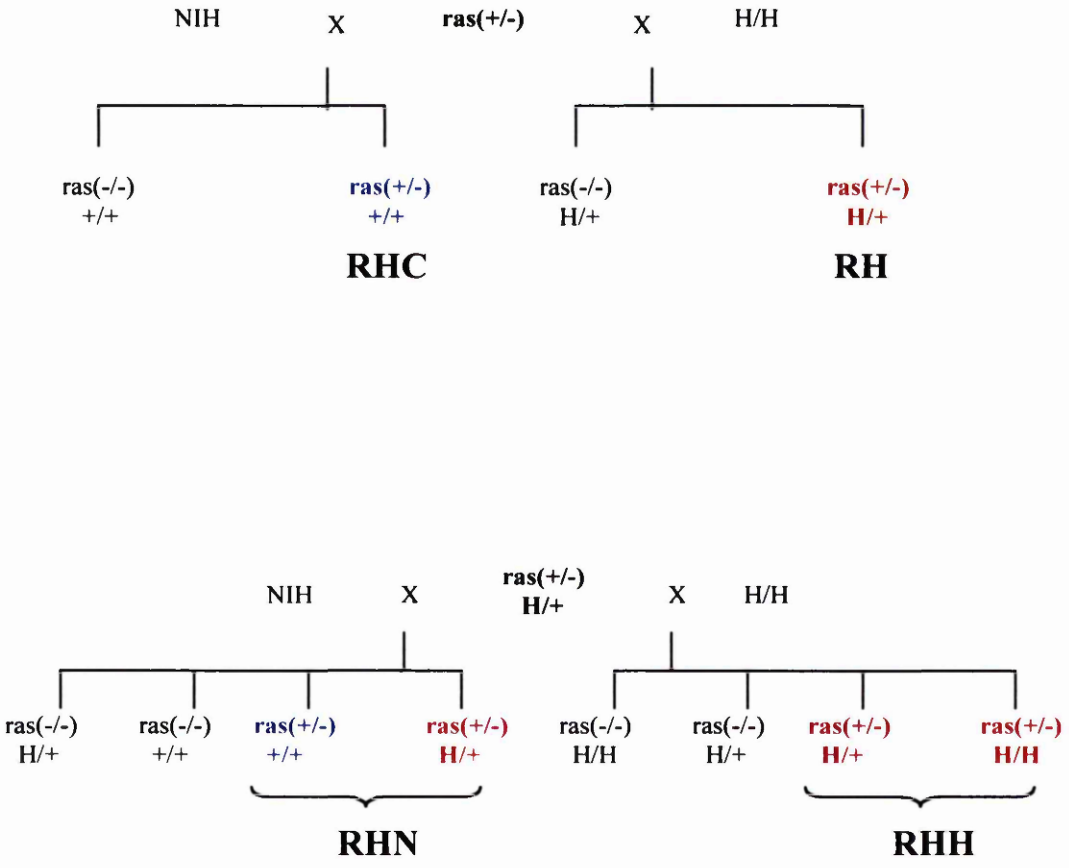

A
B
C

Figure 3.26

Schematic outlining breeding protocols for crossing K5-*RAS* transgenic line with K10-*Tgf*β1 (H line) and K6-*Tgf*β1 (M2 line)



ras(+/-) mouse negative for H-line K10-*Tgf*β1 transgene

ras(+/-) mouse positive for H-line K10-*Tgf*β1 transgene

The K5-*RAS* cross with the M2 line (K6-*Tgf*β1) was set up in the same way.

Mice were screened for the *RAS* transgene using primers specific for the human Ha-*RAS* cDNA (see figure 3.27). Previously, H-line and M2-line mice had been screened using a probe or primers specific to the SV40 polyA fragment. However, since the K5-*RAS* mice also contained the SV40 polyA fragment, a new pair of primers was designed to screen for the presence of the *Tgf*β1 transgene (S4-SR5, see Materials and Methods).

Offspring from the above crosses were observed daily and examined for evidence of skin abnormalities or tumours. Notes were made of age at appearance of tumours, types of tumours, location of tumours, how quickly the tumours progressed and when and why the mice were culled (see appendices 1 and 2). Table 3.5 shows numbers of mice generated by the crosses and the proportions of K5-*RAS* mice that consequently developed a skin phenotype.

Mice with tumours were generally culled when the tumours either reached the maximum size permissible by Home Office regulations (>10mm in diameter) or because the tumour caused the mouse to become sick.

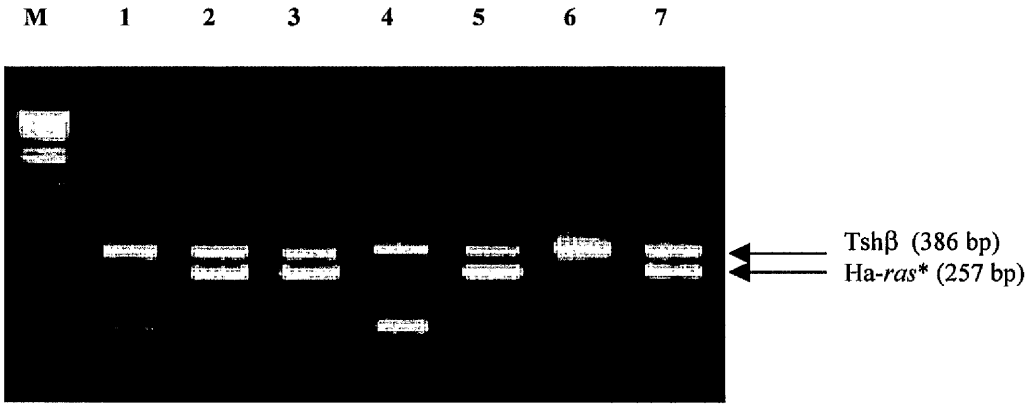
Occurrence of skin tumours in single (K5-*RAS*) and double (K5-*RAS*/*Tgf*β1) transgenic mice is portrayed in figure 3.28. 187 mice were analysed in the K5-*RAS*/H-line cross and 86 mice were analysed in the K5-*RAS*/M2-line cross. No significant differences were found between single and double transgenics in terms of latency of tumour formation. Unlike the study by Cui *et al*, 1996, where exogenous Tgfβ1 had a major effect on both tumour suppression and malignant conversion, the presence of exogenous Tgfβ1 did not inhibit tumour formation by prolonging latency.

It was impossible to assess whether the presence of the K10-*Tgf*β1 transgene influenced the malignant conversion rate of K5-*RAS* tumours. This was because these tumours tended to grow quite large relatively quickly, and before they had a chance to progress to carcinomas. Under Home Office regulations a mouse must be culled if a tumour reaches more than 10mm in diameter and because of the nature of growth of keratoacanthomas, most mice under this regime had to be sacificed before malignant conversions.

Not all K5-*RAS* positive mice developed skin tumours. In this study, approximately 50% of mice developed a skin phenotype of some sort over a period of 55 weeks. Occurrence of other tumour types such as teratomas and salivary gland tumours had no correlation with *Tgf*β1 transgenic status.

Figure 3.27

K5-*RAS* transgenic mice were screened by PCR using primers tcr1 and tcr2



Primers tcr1 and tcr2 (see figure 2.1D) are specific for human Ha-*RAS* cDNA and produce a 257 bp fragment. Primers for thyroxine stimulating hormone β (*Tsh*β) produce a fragment of 386 bp that acts as an internal control.

M: 1kb DNA ladder (Gibco BRL)
Lanes 2, 3, 5, and 7 contain K5-*RAS* positive DNAs

Table 3.5

Genotype and Phenotype Status of Mice from the K5-*RAS*/*Tgf*β1 Transgenic Crosses

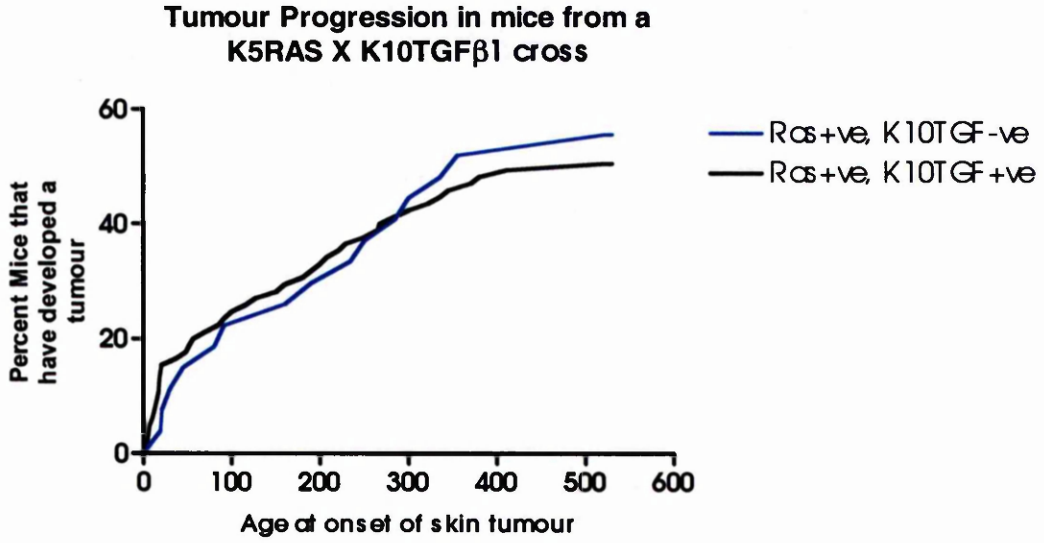
LINE	Number of mice	Number of mice with skin phenotype	Percentage of mice that developed a skin phenotype	Statisistical significance of difference between the two groups
K5-*RAS*+ve K10-Tgfβ1-ve	61	28	46%	p = 0.239
K5-*RAS*+ve K10-Tgfβ1+ve	126	53	42%	p = 0.239
K5-*RAS*+ve K6-Tgfβ1-ve	32	18	56%	p = 0.591
K5-*RAS*+ve K6-Tgfβ1+ve	54	27	50%	p = 0.591

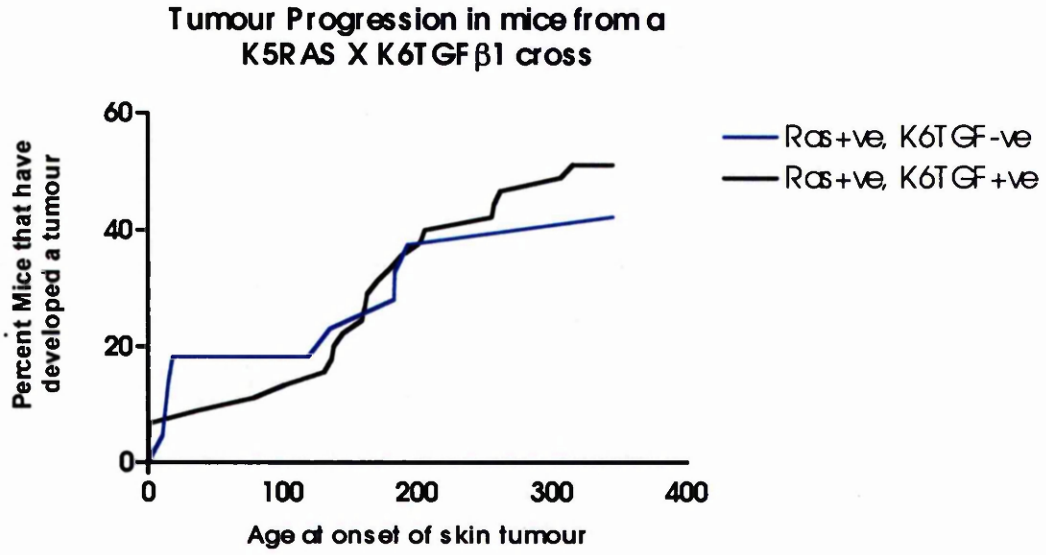
The table shows the numbers of mice from each transgenic group that developed skin tumours. Kaplan Meier survival curves were drawn for each group with the endpoint being age at onset of tumour and these are shown in Figure 3.28. The p value is gennerated from the log-rank test and represents the chance that random sampling of subjects would lead to as big a difference in tumour onset as was observed.

The p values show that there is no difference between K5-*RAS*+ve K10-Tgfβ1-ve vs K5-*RAS*+ve K10-Tgfβ1+ve mice (p = 0.239) or between K5-*RAS*+ve K6-Tgfβ1-ve vs K5-*RAS*+ve K6-Tgfβ1+ve mice (p = 0.591).

Figure 3.28

Rate of tumour appearance in K5-*RAS* transgenic mice which have been crossed with K10-*Tgf*β1 (H) or K6-*Tgf*β1 (M2) transgenic lines





Graphical portrayal (Kaplan-Meier survival curve) of tumours accumulated in mice generated from crosses of K5-RAS transgenics with either K10-Tgfβ1 or K6-Tgfβ1 lines. Numbers of mice observed are depicted in Table 3.5 and individual mice with types of tumours and transgenic status are listed in Appendices 1 and 2.

The results presented show that there is no major effect on tumour formation. Cui *et al* (1996) saw up to a 6-fold decrease in chemically-induced tumour formation due to the presence of the K10-*Tgf*β1 transgene after a period of 26 weeks. The tumours which develop in K5-*RAS* mice are derived from the hair follicle while those which arise due to chemical treatment of the skin tend to be derived from interfollicular keratinocytes. The suprabasal site of exogenous *Tgf*β1 expression may account for the difference in action on the two different tumour models.

Chapter 4
DISCUSSION

An imbalance between the rates of cell proliferation and cell death is considered to be important during the pathogenesis of many human tumours. This imbalance may be reflected in an enhanced rate of proliferation and/or a decreased susceptibility to undergo apoptotic cell death or terminal differentiation. In fact, the most common malignancies, including low grade lymphomas, skin cancer, and prostate cancer are characterised, initially, by a period where the rate of cell division does not exceed that of the normal tissue of origin, thus implicating abnormalities in the cell death pathway that lead to tumour growth. It is relevant, therefore, to determine the molecules and pathways that control the homeostasis in normal tissue and whose perturbations result in tumorigenesis.

4.1 Proliferation rate in K10-*Tgf*β1 transgenic skins is not altered by p53 deficiency

Absence of p53 failed to alter the heightened epidermal proliferative index phenotype of K10-*Tgf*β1 transgenic mice. Even though the difference between K10-Tgfβ1 transgenics and those wild-type for the transgene on a p53-null background was not significant, the p value was just outside the standard cut-off for significance, and analysis of the raw data suggests the difference is real. Therefore, whether or not Tgfβ1 induces apoptosis or terminal differentiation in K10-*Tgf*β1 transgenic mouse skin, the mechanism must be p53-independent.

Whether P53 is involved in the response to TGFβ1 has been investigated in a number of cell types. Gerwin *et al* (1992) found that when a mutant *TP53* cDNA was expressed in bronchial epithelial cells, the growth inhibitory response and induction of terminal differentiation response to TGFβ1 were decreased. This study suggested that the action of wild-type P53 could facilitate the effects of TGFβ1 on these cells. Similarly, introduction of mutant *TP53* into epidermal keratinocytes reduced sensitivity of these cells to TGFβ1 (Reiss *et al*, 1993). One possibility was that TGFβ1 might signal through P53 via MYC. TGFβ1 has been shown to induce a decrease in c-*MYC* expression that correlates with growth inhibition and this effect is blocked by expression of HPV-16 E7, or SV40 T Ag. Since the *Tp53* promoter region contains a

MYC/MYOD consensus binding site (Ronen *et al*, 1991), a shift in the various binding interactions of the MYC protein might constitute an area of intersection between P53 and TGFβ1 in control of cellular growth.

Wyllie *et al* (1991) looked at epithelial thyroid cells which had lost wild-type P53 function. As with the above studies, TGFβ responsiveness was shown to be lost in correlation with loss of wild-type p53. However, in a more comprehensive study, the same group found that P53 function was not necessary for TGFβ1 response – the result suggesting that P53 and TGFβ generate separate but interacting inhibitory signals (Blaydes *et al*, 1995). This agrees with a study on HaCaT cells which found that TGFβ could induce the CDK inhibitor $p21^{WAF1}$ in a P53-independent manner (Datto *et al*, 1995). Finally, Ewen *et al*, (1995) showed that, in mink lung epithelial cells, TGFβ1 down-regulates the translation of CDK4 in a pathway leading to G1 arrest and this pathway is dependent on the presence of wild-type p53. Therefore it appears that involvement of P53 in the growth responsiveness of cells to TGFβ1 probably depends on factors such as cell type and the specific signalling pathway activated by TGFβ1.

TGFβ1 has been shown to be able to induce apoptosis in a number of cell types and many apoptotic pathways have been shown to be P53-dependent. However, the literature suggests that the induction of apoptosis by TGFβ1 may also vary between cell or tissue types in terms of whether P53 is involved in mediating the signal. p53 was shown to be up-regulated during TGFβ1-induced apoptosis of rat liver epithelial cells (Teramoto *et al*, 1998). However, a number of reports have found that apoptosis triggered by TGFβ1 can occur in cells expressing no functional P53(Yamamoto *et al*, 1996; Hipp *et al*, 1997; Christensen *et al*, 1998).

A further consideration is the role of p53 in normal skin growth control. p53 null mice develop spontaneous sarcomas and lymphomas, but exhibit no skin abnormalities (Donehower *et al*, 1992) and it seems likely that there may be differences in susceptibility of different cell types to p53 loss. Furthermore, p53 null mouse epidermis has a surprising resistance to early-stage carcinogenesis after topical exposure to a chemical carcinogenesis protocol (Kemp *et al*, 1993b) or by breeding with transgenic mice expressing epidermally targeted oncogenes (Greenhalgh *et al*, 1996). Thus, it appears that the epidermis, which is frequently exposed to environmental carcinogens, has a strong defence system to compensate for p53 loss. One possibility is that expression of *p73*, a putative tumour suppressor gene with significant homology to *Tp53* in the DNA-binding, transactivation, and oligomerisation domains (Kaghad *et al*, 1997; Jost *et al*, 1997), may compensate for the loss of p53.

4.2 Immunohistochemical bax staining is no different between wild-type, K10-*Tgf*β1 transgenic or *Tp53*-null skins

Bax protein expression in the skins of mice from a K10-*Tgf*β1/*Tp53* knockout cross were examined by imunohistochemistry. Staining for Bax protein was similar in all skins of K10-*Tgf*β1 transgenics and their wild-type littermates whether the background was p53 wild-type or p53 null.

In the skins of mice of all genotypes, Bax staining was present in all layers of the epidermis as has been reported previously (Krajewski *et al*, 1994). However, this is the first report of Bax staining in skins of p53 null mice. Miyashita *et al* (1994) observed lower levels of Bax protein by either intensity of immunohistochemical staining or Western blot levels in several tissues of p53-deficient mice compared to wild-type littermates. Several neuronal populations as well as cells of the small intestine, thymus, lungs and liver showed reduced levels of Bax. If loss of p53 in skin affects Bax protein induction, it is not detectable by immunohistochemistry.

It is not known if BAX plays a direct role in TGFβ1-induced apoptosis. In a myeloid leukaemia cell line, Selvakumaran *et al* (1994) found that BAX expression was up-regulated during p53-mediated apoptosis but not in an apoptotic pathway induced by TGFβ1. More recently, however, TGFβ1 has been shown to induce Bax during apoptosis of rat liver epithelial cells (Teramoto *et al*, 1998). There are no reports though of an association in skin between TGFβ1 signalling and Bax induction and, if the increased cell turnover in the epidermis of K10-*Tgf*β1 is due to an increase in apoptosis, perturbation of Bax protein is not an associated feature.

4.3 Lack of altered proliferation in *Tgf*β1 null mice

In this lab, it has been reported that when Tgfβ1 is overexpressed in suprabasal cells of the epidermis, there is an increase in proliferation in epidermal basal cells (Cui *et al*, 1996). Glick *et al* (1993) had found an increased BrdU labelling index in the epidermis of Tgfβ1 null mice. The fact that a relatively small number of mice of disparate ages and undefined genetic background had been used in the latter study led us to repeat this experiment with our colony of *Tgf*β1 knockout mice. In contrast to this study no difference was found between Tgfβ1 null mice and their wild-type littermates either on a mixed or inbred genetic background.

It should be noted that there was larger variation in the BrdU counts of mixed genetic background mouse skins than in those of an inbred (NIH) background. This is perhaps not surprising based on the variation in survival rates of Tgfβ1 null mice on different genetic backgrounds observed by Bonyadi *et al* (1997). It seems reasonable to assume that one or more genetic modifiers might affect factors such as proliferation index of epidermis. Alleles of these genetic modifiers might vary in their modifying capacities depending on the genetic background of the mouse from which they originated. Consequently, a litter of mice on a mixed genetic background would harbour more versions of the modifying alleles than those of an almost pure inbred background.

This interpretation might also provide an explanation for the conflicting results of Glick *et al* (1993). The genetic background of the mice analysed in their study is not defined but appears to be mixed. They examined two wild-type, four heterozygote, and 3 homozygote knockout *Tgf*β1 mice from three different litters, two of which were 5 weeks old and one of which was 3 weeks. Even among their controls, there was a five-fold difference in BrdU labelling index between the largest and smallest indices. This large variation (probably due to genetic background) combined with the small number of mice examined and the differences in their ages means that the possibility that their findings are artefactual must be considered. Examination of *Tgf*β1 knockout epidermal labelling indices of significant numbers of mice with various inbred genetic backgrounds and a range of ages might help explain the variation.

It is perhaps not surprising that loss of Tgfβ1 in the epidermis does not cause a proliferation defect. One possible explanation is that other proteins compensate for the loss of Tgfβ1. There is evidence that there may be cross-regulation between the three Tgfβ isoforms, Tgfβ1, β2, and β3. Signalling through Tgfβ2 and Tgfβ3 is still present in Tgfβ1 null mice and may compensate for loss of Tgfβ1. Tgfβ2 is expressed in the epidermis (Glick *et al*, 1991) and Tgfβ3 is probably dermally-expressed, but may still affect epidermal proliferation as it is a secreted protein and can probably diffuse from dermis to epidermis (Escherick *et al*, 1993).

Two recent studies involving expression of a dominant-negative type II Tgfβ receptor (βRII-DN) in skin epidermis provide further insight into the role of Tgfβ in the control of epidermal homeostasis (Wang *et al*, 1997; Amendt *et al*, 1998). Expression of the βRII-DN only in basal cells of the epidermis resulted in no change in proliferation of the skin (Amendt *et al*, 1998), but when expressed in both basal and

suprabasal cells, the labelling index of the skin was doubled (Wang *et al*, 1997). There are a number of possible explanations for the difference in these two studies. One possibility is that there is a quantitative difference in expression levels of the βRII-DN. The loricrin promoter of Wang *et al* may have more efficient basal expression than the K14 promoter of Amendt *et al*. In fact, the latter study reports not only a more severe phenotype in homozygote transgenics than heterozygotes, but also a reduction in the phenotype of heterozygote mice as the adult matures. Difference in phenotype due to different expression levels of a transgene is illustrated by two studies describing the control of *Tgf*β1 by suprabasal promoters in the epidermis (Sellheyer *et al*, 1993; Fowlis *et al*, 1996).

An alternative explanation is one suggested by Amendt *et al* implying that Tgfβ1's prime function in normal homeostasis is in regulation within the suprabasal layer and this impacts on the basal layer. This would agree with reports of the localisation of Tgfβ1 in the suprabasal layer of the epidermis (Akhurst *et al*, 1988) and would also be consistent with the phenotype of K10-*Tgf*β1 transgenic mice described above and in the following section.

4.4 Proliferation rates in K10-*Tgf*β1 transgenic skin in the absence of endogenous Tgfβ1

In order to further assess the importance of Tgfβ1 signalling in the epidermis, K10-*Tgf*β1 transgenic mice were crossed with the *Tgf*β1 knockout line. Due to the small number of Tgfβ1 null mice which reach 3 weeks of age, and the fact that the proliferation phenotype of K10-*Tgf*β1 mice is more pronounced in homozygote transgenics than heterozygotes, it was not possible to generate sufficient numbers of K10-*Tgf*β1 homozygote mice on a Tgfβ1 null background in order to make a statistically significant observation. However, of the mice that were analysed, the K10-*Tgf*β1 transgene induced the increase in basal cell proliferation on a Tgfβ1 null background just as it does in the presence of endogenous Tgfβ1. Therefore, the epidermis can cope with a loss of endogenous Tgfβ1 in terms of homeostasis, yet expression of transgenic Tgfβ1 in the suprabasal layer disrupts the balance. Perhaps Tgfβ1 is only a differentiation-inducing factor in the epidermis and not a growth-inhibitory factor.

4.5 Expression of transgenic and endogenous BCL-2 in skin epidermis

Characterisation of a *BCL*-2 transgenic line under the control of a K10 promoter determined the presence of a 26 kDa human BCL-2 protein expressed in suprabasal cells of transgenic epidermis. The size of this protein agrees with previously published results (Allsopp *et al*, 1993) although it can not be ruled out that the protein is slightly truncated. The strong but relatively patchy expression of the transgene as demonstrated by immunohistochemistry agrees with reports of expression of other transgenes driven by the K10 promoter (Bailleul *et al*, 1990; Cui *et al*, 1995).

Endogenous mouse *Bcl*-2 mRNA was demonstrated by RT-PCR but was not detected by either Northern blot or the more sensitive radioactive *in situ* hybridisation. A previous study demonstrated that, in mouse, the *Bcl*-2 gene generates two mRNA species (7.5kb and 2.4kb) (Negrini *et al*, 1987). However, levels of *Bcl*-2 mRNA in skin have not been previously reported and the lack of murine *Bcl*-2 signal may indicate a relatively low endogenous *Bcl*-2 expression in the skin.

BCL-2 protein expression in skin has been demonstrated by both Western blotting and immunohistochemistry (Hockenbery *et al*, 1991; Rodriguez-Villanueva *et al*, 1998). Immunohistochemistry is less quantatative than Western blotting, but, since the antibody used detected both human and mouse BCL-2, a comparison between relative expression of endogenous and transgenic BCL-2 could be made. Transgenic BCL-2 expression was stronger and patchier than the endogenous Bcl-2 which was weaker and more uniformly spread throughout the cellular layers of the epidermis. Previous reports of BCL-2 immunohistochemical staining have been ambiguous. The staining data presented here agrees with Krajewski *et al* (1994) and Stenn *et al* (1994) who saw BCL-2 protein in both basal and suprabasal layers of the epidermis, whereas others reported staining in just the basal compartment of the epidermis (Hockenbery *et al*, 1991; Verheagh *et al*, 1995). On the basis of the Western blot analysis and immunohistochemistry it may be stated that the K10-*BCL*-2 transgene results in the deregulated expression of BCL-2 in terms of absolute amounts of BCL-2 protein and the distribution of BCL-2 protein within the epidermis.

4.6 Consequences of exogenous BCL-2 expression in transgenic epidermis

None of the experiments carried out on the K10-*BCL*-2 transgenic mice demonstrated any functional potential for transgenic BCL-2 protein targeted to

suprabasal cells of the epidermis. Indeed, several concurrent studies from other labs have now been published which come to the same conclusion. Other transgenic lines which consist of aberrant BCL-2 expression in the epidermis show a similar resilience in terms of disruption of normal homeostasis or response following chemical insult. Firstly, another K10-*BCL*-2 transgenic mouse line, similar to the lines characterised here, has been recently generated independently in another laboratory (Rodriguez *et al*, 1997b). As with the lines characterised here, they observed no histological or proliferative defects. Marthinuss *et al* (1995) generated K5-*BCL*-2 transgenic mice so that *BCL*-2 was overexpressed in the basal layer of the epidermis but, again, there was no apparent phenotype. In fact, the only report in which aberrant expression of *BCL*-2 in the epidermis has resulted in any noticeable growth alterations is from a recent study by Rodriguez-Villanueva *et al* (1998).

They generated transgenic mice expressing *BCL*-2 in the epidermis driven by a K1 promoter. Newborn pups carrying the transgene developed multifocal areas of hyperplasia. Apart from these scattered sites, the skin was normal and, in fact, in older pups and adult transgenics, this phenotype disappeared. The K1 promoter is a strong promoter and directs expression to both basal and suprabasal layers of the epidermis. The multifocal nature of the hyperplasia in these young mice is unusual as the transgene is expressed uniformly throughout the epidermis. But, as the authors suggest, it may indicate that compensatory, homeostatic mechanisms are operative which, in general, restore the normal rate of keratinocyte turnover (Rodriguez-Villanueva *et al*, 1998). It is worth noting that there is no change in BrdU labelling indices in these transgenic mice, even in the areas of hyperplasia. However, the K1-*BCL*-2 transgene did confer resistance to cell death induction by UV-B *in vitro*, and papillomas developed at a greater frequency in the K1-*BCL*-2 mice compared to control littermates following chemical carcinogenesis (Rodriguez-Villanueva *et al*, 1998).

In the K10-*BCL*-2 mice examined here, the histomorphology of the epidermis was not altered. If BCL-2 plays a role in controlling apoptosis in the epidermis it might be reasonable to anticipate that the high levels of BCL-2 protein produced in the epidermis of K10-*BCL*-2 would result in hyperplasia. The lack of dysplasia may be indicative of compensatory devices in the skin working to balance hoeostasis. It might therefore be predicted that the proliferative rate within the epidermis of the transgenics would be lower than that of control littermate mice. However, steady-state levels of proliferation, as assessed by incorporation of BrdU were comparable in K10-*BCL*-2 mice and control mice. It is conceivable that the mechanism of cell death induction in the normal epidermis proceeds via a Bcl-2-independent mechanism.

Chemical insult to the skins of K10-*BCL*-2 transgenic mice, in the form of tumour promoter treatment, revealed no role for the transgenic BCL-2 in affecting histomorphology or epidermal proliferation rate. TPA (in both chronic and acute treatments) induced hyperplasia in the skins of both transgenic and wild-type littermate mice to a similar extent. The profile of cell proliferation rate following TPA treatment was also similar in K10-*BCL*-2 transgenic skin and control skins with a peak in proliferation at 48 hours. Treatment of mouse skin with TPA induces many of the molecules involved in cell cycle and proliferation control including c-myc, cyclins D1, E and A, $p21^{Cip1}$, Tgfβ1, and Rb phosphorylation. However, exogenous BCL-2 expression (which was also induced) did not appear to interfere with the process by which these factors respond to chemical insult and restore normal homeostasis to the skin.

The lack of demonstration of functionality of the K10-*BCL*-2 transgene begs the question as to whether functional BCL-2 is actually being produced in these mice. However, it has been shown here that full-length protein is produced, expressed in the cellular compartment that it's meant to be, and is produced at a detectable level. Furthermore, the human *BCL*-2 fragment used to generate the K10-*BCL*-2 transgenic mice has been shown to possess functionality when expressed *in vitro* (Allsopp *et al*, 1993). The lack of any phenotype in either quiescent or growth-perturbed transgenic skins may represent the relevance of the involvement of BCL-2 in apoptosis or cellular proliferation in the epidermis. Bcl-2 null mice show loss of melanocytes in skin but show no defect in development or homeostasis of the epidermis (Veis *et al*, 1993). Therefore, this organ appears to be relatively refractile to alterations in levels of Bcl-2.

The resistance to disruption of homeostasis in skin, of course, does not rule out a critical role for apoptosis in the epidermis. For example, in some tissues considered to undergo extensive apoptosis in relation with cell renewal such as the small intestine, even a very high expression of transgenic BCL-2 in enterocytes does not lead to tissue changes, nor protects enterocytes against apoptotic stimuli (Rodriguez *et al*, 1997a). Apoptosis has been shown to be a response to damage incurred by sunlight in the form of UV irradiation (Kraemer, 1997) and it has also been suggested that apoptosis may contribute to the process of skin aging (Haake *et al*, 1998).

Other transgenic lines have demonstrated the resistance of skin to disruption of homeostasis. Mice overexpressing the *BCL*-2 related gene *BCL*-x in the epidermis have been created (Pena *et al*, 1997). *BCL*-x_L or *BCL*-x_S was expressed in the basal compartment of the epidermis driven by the K14 promoter. Like the K10-*BCL*-2 transgenics, there was no effect on normal proliferation or terminal differentiation. The

oncogene c-*MYC* is thought to be coupled with *BCL*-2 in the regulation of cell growth. Recently it's been shown that mice overexpressing a c-*myc* transgene in the epidermis do not display increased apoptosis *in vivo* despite undergoing hyperplasia (Pelengaris *et al*, 1999). However, when keratinocytes from these mice were cultured in appropriate serum conditions, the transgene was able to induce apoptosis. It would seem that *in vivo* survival signals act to suppress myc-induced apoptosis. Similarly, other homeostatic mechanisms may work to counteract over-expression of BCL-2.

4.7 Genetic background and penetrance effects on the K5-*RAS* transgene

The role of Tgfβ1 in tumorigenesis has been investigated in a number of tissues using both transgenic and non-transgenic mice, and conflicting and sometimes seemingly irreconcilable results have been observed. In this lab, *Tgf*β1 transgenic mice, overexpressing the growth factor in skin epidermis were subjected to long term chemical carcinogenesis. It was found that the transgenics were more resistant to the induction of benign skin tumours than control mice, and that the malignant conversion rate of papillomas to carcinomas was increased in the *Tgf*β1 transgenics. To further characterise the effects of Tgfβ1 during skin tumorigenesis, these transgenic mice were crossed with a Ha-*RAS* transgenic line that devlops spontaneous skin tumours.

It was observed that K5-*RAS* transgenic mice on a FVB background had a phenotype more severe than those on a C57Bl/6J background in terms of age of onset of acanthosis or keratoacnthomas and numbers of tumours appearing. This observation correlates with previous chemical carcinogenesis studies which demonstrated differences in susceptibility to skin tumorigenesis by mice of different genetic background (Balmain *et al*, pers.comm.; Balmain *et al*, 1998). C57Bl/6J strain tended to be more resistant to carcinogenesis than FVB mice. Differences in tumour prevalences and severities between mouse strains have also been observed in other transgenic and tumour-suppressor knockout animal models (Threadgill *et al*, 1995; Donehower *et al*, 1995b).

The chemical carcinogenesis studies mentioned above had shown the NIH genetic background to be one which was relatively susceptible to skin tumorigenesis, and in fact, at least as susceptible as the FVB background (Balmain *et al*, unpublished observations). Therefore it was surprising to find a reduced tumour load with K5-*RAS* mice on the NIH background compared to FVB. Differences in tumour susceptibility of

mice on different genetic backgrounds following chemical carcinogenesis have been shown to be due to the presence or absence of modifying loci (Balmain *et al*, 1998). The greater resistance of K5-*RAS* mice on a NIH background to formation of skin tumours than the resistance of NIH mice to chemically-induced tumours may be due to the differential effects that genetic modifying loci have on different tumour types. Skin tumours arising from mice undergoing chemical carcinogenesis arise from both interfollicular and follicular epidermal subpopulations whereas tumours due to the K5-*RAS* transgene are all follicular in origin. Different NIH modifier loci may influence susceptibility to follicularly-derived tumours and those arising from interfollicular epidermal compartments.

4.8 Effects of transgenic *Tgf*β1 on tumorigenesis in K5-*RAS* mice

It was found that neither K10-*Tgf*β1 nor K6-*Tgf*β1 transgenic mice when crossed on to the K5-*RAS* mice caused a significant change in tumour development compared to K5-*RAS* single transgenic mice. This is in contrast to the findings of Cui *et al* (1996) who showed that the presence of either of the *Tgf*β1 transgenes could reduce papilloma formation and also increase malignant progression during chemical carcinogenesis.

The lack of inhibition of tumour development might be explained by the cellular compartments in which transgenic Tgfβ1 acts. K10-*Tgf*β1 and K6-*Tgf*β1 transgenic mice express exogenous *Tgf*β1 in suprabasal layers of the epidermis. It is not known though if the overexpressed Tgfβ1 acts in a paracrine or autocrine manner. Papillomas arising through chemical carcinogenesis originate from thickened skin with a large population of suprabasal-like, K10-expressing keratinocytes. Expression of transgenic Tgfβ1 in these cells inhibits the outgrowth of these papillomas and this might be due to quite a localised autocrine effect of the exogenous protein.

In contrast, tumours arising in K5-*RAS* transgenic mice are follicular in origin. Cells expressing the K5-*RAS* transgene are restricted to the outer root sheath of the hair follicle where there is no *K10* expression and to small patches of interfollicular cells (Brown *et al*, 1998). Therefore, the initial clonal expansion preceding tumour outgrowth is from a cell or cells that are not in immediate contact with cells expressing transgenic Tgfβ1. And so, the growth inhibitory effects of the exogenous Tgfβ1 are not encountered by the developing keratoacanthomas. The differences between autocrine,

paracrine and juxtacrine effects of Tgfβ have been demonstrated before in other systems. Glick *et al* (1994) used a skin grafting model to show that autocrine Tgfβ1 could inhibit malignant progression of tumours derived from initiated keratinocytes and that autocrine and paracrine Tgfβ1 could have opposite effects on the labelling index of the tumours.

The relationship between TGFβ and RAS is still being elucidated but there is some evidence that RAS may play a role in TGFβ signalling events. It has been shown that *RAS* transformation of intestinal and other epithelial cell lines inhibits the antiproliferative effects of TGFβ (Filmus *et al*, 1993; Schwarz *et al*, 1988; Valverius *et al*, 1989; Houck *et al*, 1989). Mink lung epithelial cells are normally growth arrested by TGFβ1. When transfected or microinjected with Ha-*RAS* however, they acquire a complete resistance to growth inhibition by TGFβ1 (Longstreet *et al*, 1992; Howe *et al*, 1993). Furthermore, in these cells, TGFβ1 has been shown to decrease the levels of active GTP-bound RAS, suggesting that TGFβ controls proliferation by modulating the activity of RAS (Howe *et al*, 1993).

Further evidence of the role of RAS in TGFβ growth-inhibition came from Yan *et al* (1994). In two sublines of the same colon carcinoma cell line, which respond in opposite ways to TGFβ (proliferation or growth inhibition), RAS has been shown to play role in the response leading to growth inhibition. In U9 cells where TGFβ1 acts as a mitogen, the activity of the myelin basic proteins was increased without any detectable activation of RAS. On the other hand, when TGFβ1 inhibited the growth of HD3 cells, RAS was activated.

Elucidation of the downstream signalling pathway of TGFβ has revealed more about the interactions of RAS with this pathway. As discussed in Introduction, TGFβ signalling causes phosphorylation of SMAD2 and/or SMAD3. These SMADs associate with SMAD4, move into the nucleus and assemble transcriptional complexes. Kretzschmar *et al* (1999) have shown that oncogenically activated *RAS* inhibits TGFβ-induced nuclear accumulation of SMAD2 and SMAD3 and SMAD-dependent transcription in mammary and lung epithelial cells. This inhibitory effect is strong enough to significantly limit the activity of the TGFβ/SMAD pathway.

Hartsough *et al* (1996) showed that RAS activation was necessary and sufficient for TGFβ-mediated ERK1 activation in intestinal epithelial cells. The same group later showed that inactivation of RAS, either by expression of a dominant-negative mutant or addition of a MAP and ERK kinase inhibitor decreased the ability of TGFβ to induce

phoshorylation of SMAD1 in the same cells. Recently, Calonge & Massague (1999) were able to restore TGFβ-responsiveness to a colon cancer cell line by co-transfection of *SMAD4* and a *RAS* phosphorylation-resistant *SMAD3*. The result suggested that loss of SMAD4 function and inhibition of SMAD2/3 function by a hyperactive RAS pathway jointly prevent TGFβ antiproliferative responses in this cell line.

TGFβ1 and RAS have also been shown to collaborate in bringing about changes in the invasive properties of epithelial cells. When grown in collagen gels, both normal and *RAS*-transformed mammary epithelial cells maintain their epithelial characteristics. However, when treated with TGFβ1, the normal cells undergo growth arrest while *RAS*-transformed cells become fibroblastoid, invasive and resistant to growth inhibition by TGFβ (Oft *et al*, 1996).

In the current study, any effect on malignant progression of the tumours arising in K5-*RAS* mice was difficult to assess as there were very few carcinomas observed. This was probably because many of the K5-*RAS* transgenic mice bearing tumours had to be sacrificed after a certain period of tumour progression due to the size of the tumour. Some of these mice may have gone on to develop carcinomas. A much larger number of mice under study would be needed to assess whether Tgfβ1 could influence malignant progression in the same way as it did during chemical carcinogenesis.

4.9 Summary and future directions

A major factor in the generation of results throughout this project has been the breeding strategies employed to cross various transgenic and/or knockout mouse models. In general, as a rule, all crosses were performed so that comparisons could be made directly between either littermates or half-littermates (mice sharing one parent) to try to minimise any variation due to differences in genetic background of the mice. The workload could have been reduced by not having this as a requirement. For example, in the analysis of the K10-Tgfβ1 transgene on various p53 knockout backgrounds, the parents were heterozygous for both the K10-Tgfβ1 transgene and the p53 knockout allele. This meant that mice carrying both homozygous transgene and knockout alleles were represented by one-sixteenth of the offspring. To produce sufficient numbers of mice carrying the various relevant combinations of transgene and knockout allele

genotypes, many mice had to be generated and genotyped which were not actually used in the final analysis of proliferation rates in the epidermis. Two separate crosses, with both parents either homozygous for the K10-Tgfβ1 allele or both parents wild-type at this locus would have reduced the numbers of mice used and subsequently the workload.

There is still much to be learned about how homeostatic mechanisms work in the skin in order to preserve the balance between proliferation, cell death and differentiation. It is clear that Tgfβ does play some role in skin epidermis and mice such as K10-*Tgf*β1 transgenic mice will be extremely useful in defining that role. Perhaps Tgfβ1 is solely a differentiation-inducing factor in the epidermis. Examination of the expression of differentiation-specific markers such as K6 and loricrin in the epidermis of these mice may help answer that question. On the other hand, K1-*Tgf*β1 transgenic mice have the opposite phenotype (lack of proliferation) and why two seemingly similar transgenic lines can have such different phenotypes is interesting in itself.

The generation of K10-*Tgf*β1 transgenic keratinocytes *in vitro* may make them more amenable to experiments such as examination of their response to treatment with exogenous growth factors or chemicals, however even the action of culturing keratinocytes can switch on genes such as *K6* which are normally off *in vivo*. Therefore, a better approach may be to carry out other *in vivo* crosses with mice such as the *myc* transgenic mice described by Pelengaris *et al* (1999) where inducible expression of c-*myc* in the suprabasal epidermis triggers proliferation and disrupts differentiation.

Similarly, crosses with the K10-*BCL*-2 transgenic mice described here would be very useful tools in analysing why transgenic *Tgf*β1 or c-*myc* expression in the epidermis perturbs proliferation or differentiation. Initial crosses were carried out between K10-*Tgf*β1 mice and K10-*BCL*-2 mice during this study but unfortunately there was not enough time to complete them satisfactorily. In hindsight, it may have been advantageous to immediately set up a long-term chemical carcinogenesis experiment when mice were initially received to determine whether exogenous BCL-2 affects tumour formation or malignancy. Other obvious experiments to carry out on the K10-*BCL*-2 mice would be an analysis of the cell death response following UV irradiation both *in vivo* and on keratinocytes *in vitro*. It has been shown more recently that phenotypes of similar transgenic mice might be easier to establish in primary keratinocytes from such animals, especially following a UV insult. However, caution must be observed about the relevance of *in vitro* studies before applying them back to an *in vivo* model.

The reason why overexpression of Tgfβ1 leads to an increase in proliferation in K10-*Tgf*β1 epidermis has still not been determined. Newer technologies may help unravel the mechanism of action of the overexpressed growth factor. For example, a comparison of RNA expression profiles of wild-type and transgenic epidermal RNA using cDNA arrays might give some clue as to which other genes are induced or down-regulated by expression of the recombinant Tgfβ1. This may lead to some idea as to which, if any, pathway is triggered in the transgenic mice.

The actions of Tgfβ and related molecules during multistage carcinogenesis are obviously very important both scientifically and clinically. The precise mechanisms as to how Tgfβ can act as a suppressor of tumorigenesis at some stages of multistage carcinogenesis and in some tissue types, and yet can promote malignant progression at other stages, must be determined. This is especially relevant in terms of the therapeutic consequences of using TGFβ antibodies, the cytokine itself, or drugs targeted to block or enhance its activity or activity of a TGFβ pathway.

The difference of action of transgenic Tgfβ on two similar mouse cancer models (chemical carcinogenesis and K5-*RAS* transgenic) illustrates how differences such as the particular cell compartment from which a tumour develops can determine the progression of tumours arising in the same tissue. This study has also emphasised the pitfalls of transgenic breeding experiments for example when a transgene is not fully penetrant, or a change in genetic background affects the penetrance. However, crossing the K10-*Tgf*β1 mice with other transgenic models of skin tumorigenesis such as K10-*RAS* mice would help clarify exactly how TGFβ1 acts when suppressing tumour formation for example, whether the effect is due to a paracrine or autocrine action.

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Appendix 1

Table of mice generated by crossing K5-*RAS* transgenic mice with K10-*Tgfb1* transgenics showing transgenic staus of each mouse, age at tumour onset, age at death, and types and numbers of tumours borne (all mice listed are K5-*RAS* -positive)

Mouse ID	K10-*Tgfb1* Transgenic?	Age at appearance of first tumour (days)	Age at death/sacrifice	Reason for death/sacrifice	Phenotype due to transgene?	Type(s) of tumour
RHN 1	Y	15	35	Endpoint	Y	KA, PAP
RHN 8	Y		97	Endpoint	Y	TER
RHN 15	N		149	Fighting	N	
RHN 17	N		149	Fighting	N	
RHN 19	N		149	Fighting	N	
RHN 21	N	160	171	Endpoint	Y	KA
RHN 25	N		200	Endpoint	Y	SAL
RHN 2	N		97	Endpoint	Y	TER
RHN 35	Y	335	355		Y	KA
RHN 37	N		202	Sick	Poss	Mid ear
RHN 43	N	335	355		Y	KA
RHN 47	Y		335	F.D.	N	
RHN 49	N	80	150	Endpoint	Y	KA
RHN 12	N	30	59	Endpoint	Y	PAP
RHN 14	N		425		N	
RHN 22	N		287	?	?	
RHN 32	Y		405		N	
RHN 34	Y		405		N	
RHN 55	Y		?		N	
RHN 57	N	21	36	Endpoint	Y	PAP
RHN 59	N	234	264	Endpoint	Y	KA
RHN 61	N		405		N	
RHN 73	N	285	315	Endpoint	Y	KA
RHN 85	Y		405	Ear Infec	N	
RHN 89	Y		125	Fighting	N	
RHN 93	Y	85	118	Endpoint	Y	KA
RHN 95	N	355	385	Endpoint	Y	PAP

RHN 101	Y		125	Fighting	N	
RHN 103	Y		125	Fighting	N	
RHN 107	N	190	210	Endpoint	Y	SAL, PAP
RHN 109	N		123	Fighting	N	
RHN 48	N	91	116	Endpoint	Y	KA
RHN 50	N		113	Fighting	N	
RHN 54	N	522	545		Y	KA
RHN 56	Y		545	F.D.	N	
RHN 62	N		116	Endpoint	Y	SAL
RHN 115	N	19	243	F.D.	Y	PAP
RHN 117	N	45	164	Endpoint	Y	KA
RHN 119	Y		305		N	
RHN 125	Y		165	Endpoint	Y	SAL
RHN 129	Y	15	28	Endpoint	Y	PAP, KA
RHN 133	N		182	Endpoint	Y	SAL
RHH 5	Y	160	210	F.D.	Y	PAP
RHH 2	Y		23	F. D.	?	
RHH 8	Y		186	Endpoint	Y	SAL
RHH 10	Y		220	Endpoint	Y	SAL
RHH 15	Y	18	81	Endpoint	Y	PAP
RHH 17	Y		358	F.D.	N	
RHH 14	Y	18	29	Endpoint	Y	ACA
RHH 18	Y	36	281	Endpoint	Y	PAP
RHH 25	Y	20	36	Endpoint	Y	KA, PAP
RHH 29	Y	91	223	Endpoint	Y	KA
RHH 31	Y	217	259	Endpoint	Y	KA
RHH 24	Y	280	299	Endpoint	Y	KA
RHH 33	Y	48	143	Endpoint	Y	KA
RHH 35	Y		271	F.D.	N	
RHH 39	Y	229	259	Endpoint	Y	KA
RHH 45	Y		372	Sick	N	
RHH 49	Y		220	F.D.	N	
RHH 32	Y	12	36	Endpoint	Y	PAP

RHH 34	Y	70	287	Endpoint	Y	KA, PAP
RHH 53	Y		?	Fighting	N	
RHH 55	Y		175	F.D.	N	
RHH 61	Y		219	Endpoint		
RHH 63	Y		189	F.D.	N	
RHH 65	Y		254	F.D.	N	
RHH 42	Y	322	339	Endpoint	Y	KA
RHH 46	Y	370	415	Endpoint	Y	KA
RHH 48	Y		238	Endpoint	Poss	
RHH 52	Y	5	39	Endpoint	Y	KA
RHH 69	Y	52	112	Endpoint	Y	PAP
RHH 73	Y		224	Fighting	N	
RHH 77	Y		224	Fighting	N	
RHH 81	Y		224	Fighting	N	
RHH 54	Y	6	22	Endpoint	Y	KA,CAR
RHH 56	Y	10	49	Endpoint	Y	PAP
RHH 58	Y	17	32	Endpoint	Y	KA
RHH 62	Y	200	227	Endpoint	Y	KA
RHH 68	Y		375		N	
RHH 70	Y		290		Y	TER
RHH 74	Y	7	28	Endpoint	Y	ACA
RHH 97	Y	265	285	Endpoint	Y	KA
RHH 101	Y	115	129	Endpoint	Y	KA
RHH 107	Y	127	187	Endpoint	Y	KA
RHH 111	Y		178	F.D.	N	
RHH 78	Y		378	Endpoint	Y	TER
RHH 80	Y		563	F.D.	N	
RHH 88	Y	410	435	Endpoint	Y	KA
RHH 92	Y		608	F.D.	N	
RHH 94	Y	522	537	Endpoint	Y	KA
RHH 96	Y		? >563		N	
RHH 117	Y		435	F.D.	N	
RHH 125	Y		270	Endpoint	Y	TER
RHH 129	Y		108	Fighting	N	

RHH 133	Y		150	Fighting	N	
RHH 137	Y		150	Fighting	N	
RHH 100	Y	20	45	Endpoint	Y	KA,PAP
RHH 104	Y	267	317	Endpoint	Y	KA, TER
RHH 114	Y		332		N	
RHH 116	Y		332		N	
RHH 116b	Y	207	247	Endpoint	Y	KA
RHH 139	Y		182	F.D.	Y	PAP
RHH 120	Y		573		N	
RHH 126	Y		573		N	
RHH 130	Y		573		N	
RHH 132	Y	380	410	Endpoint	Y	KA
RHH 147	Y		?		N	
RHH 149	Y		310		N	
RHH 140	Y		310		N	
RHH 153	Y		225	F.D.	N	
RHH 155	Y		290		N	
RHH 144	Y		215	Sick	N	
RHH 146	Y		290		N	
RHH 157	Y		147	Fighting	N	
RHH 163	Y		147	Fighting	N	
RHH 148	Y		288		N	
RHH 175	Y	345	360	Endpoint	Y	KA
RHH 177	Y		335	F.D.	N	
RHH 156	Y		455		N	
RHH 158	Y		152	Endpoint	Y	TER
RHH 162	Y		455	Endpoint	Y	KA
RHH 164	Y		415	Endpoint	Y	KA
RHH 179	Y	5	15	Endpoint	Y	ACA

KA: keratoacnathoma
PAP: papilloma
ACA: acanthosis
Mid Ear: middle ear disease
SAL: salivary gland tumour
TER: teratoma
F.D.: Found Dead

Appendix 2

Table of mice generated by crossing K5-*RAS* transgenic mice with K6-*Tgfb1* transgenics showing transgenic staus of each mouse, age at tumour onset, age at death, and types and numbers of tumours borne (all mice listed are K5-*RAS* -positive)

Mouse ID	K10-*Tgfb1* Transgenic?	Age at appearance of first tumour (days)	Age at death/sacrifice	Reason for death/sacrifice	Phenotype due to transgene?	Type(s) of tumour
RMN 1	N		302		N	
RMN 3	Y		302		N	
RMN 5	N		229	Endpoint	Poss	Mid Ear
RMN 9	N	270	309		Y	KA
RMN 11	Y		302		N	
RMN 2	N	21	70	Endpoint	Y	PAP
RMN 6	N		402		N	
RMN 8	Y	156	171	Endpoint	Y	KA
RMN 13	Y		390		N	
RMN 15	N	365	390	Endpoint	Y	KA
RMN 19	Y	360	390		Y	V. small PAP
RMN 21	Y	75	98	Endpoint	Y	ACA/PAP
RMN 23	N		390		N	
RMN 23b	Y	276	390		Y	V. small PAP
RMN 18	Y		390		N	
RMN 26	N		390		N	
RMN 28	N		390		N	
RMN 31	N		380		N	
RMN 33	N		275	F.D.	N	
RMN 34	Y		380		N	
RMN 40	N		380		N	
RMN 42	Y	289	317	Endpoint	Y	EAR
RMN 37	N	238	305		Y	PAP
RMN 39	Y		330		N	
RMN 41	Y		330		N	
RMN 48	Y	325	342	Endpoint	Y	KA
RMN 50	Y		440		N	

RMN 52	Y	404	421	Endpoint	Y	KA
RMN 53	Y	322	409	Endpoint	Y	KA
RMN 55	Y	261	337	Endpoint	Y	TER
RMN 54	N		421	Sick	Poss	
RMN 56	N	386	404	Endpoint	Y	KA
RMN 67	N		290		N	
RMN 74	Y	514	563	Endpoint	Y	KA
RMN 73	Y	377	387	Endpoint	Y	ACA
RMN 75	N		302		N	
RMN 80	N	30	42	Endpoint	Y	KA
RMN 82	N	36	90	Endpoint	Y	PAP
RMN 84	N	30	52	Endpoint	Y	KA, PAP
RMN 86	N	690	724	Endpoint	Y	KA
RMN 88	N	367	394	Endpoint	Y	KA
RMN 90	Y		431		N	
RMM 1	Y	272	272	Endp.	Y	Vas Deferens
RMM 5	Y		123	Fighting	N	
RMM 2	Y	523	537	Endp.	Y	KA
RMM 4	Y		628	F.D.	N	
RMM 6	Y		706		N	
RMM 10	Y	612	628	Endp.	Y	SAL
RMM 15	Y		347		N	
RMM 19	Y		305	F.D.	N	
RMM 14	Y	510	540	Endp.	Y	TER/KA
RMM 25	Y	340	363	Endp.	Y	KA
RMM 27	Y		?		?	
RMM 20	Y		453	F.D.	N	
RMM 22	Y	317	325	Endp.	Y	SAL
RMM 24	Y	411	423	Endp.	Y	KA
RMM 26	Y	631	635	Endp.	Y	Lymphoma
RMM 29	Y	202	212	Endp.	Y	KA
RMM 31	Y		363	F.D.	N	
RMM 33a	Y	2	10	Endp.	Y	ACA

RMM 33b	Y		350	F.D.	N	
RMM 37	Y		430		N	
RMM 42	Y		340		N	
RMM 41	Y	3	5	Endp.	Y	ACA
RMM 43	Y		308		N	
RMM 47	Y		470		N	
RMM 49	Y	2	17	Endp.	Y	ACA

KA: keratoacnathoma
PAP: papilloma
ACA: acanthosis
Mid Ear: middle ear disease
SAL: salivary gland tumour
TER: teratoma
F.D.: Found Dead