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Epithelial carcinogenesis in the mouse: correlating the genetics and the biology

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Tumour formation relies on a complex combination of genetic and environmental factors. In particular, the contributions from inherited predisposition genes as well as carcinogens, for example from cigarettes or in the diet, are amongst the major contributors to tumorigenesis. Since the study of such processes is particularly difficult in human cancers, the availability of a well-defined model system is of obvious benefit. The mouse skin model of multistage carcinogenesis offers an excellent tool for the study of the target cells, the target genes and the biological events associated with neoplasia. In this system, tumorigenesis occurs in a series of defined stages, each of which is characterized by specific and reproducible alterations in genes such as H-*ras*, cyclin D1, *p53* and $p16^{INK4A}$. Additional changes occur in the production of, or response to, factors such as transforming growth factor β (TGFβ). These genetic and biological alterations are mirrored in human tumours of epithelial origin. Hence, research into the general principles of tumour initiation, promotion and progression in the context of the mouse skin model is likely to prove valuable in the continual search for new methods for the diagnosis, prevention, and therapeutic treatment of human cancers.

Keywords: skin; mouse; carcinogenesis; stem cells; TGFβ; invasion

1 INTRODUCTION

Epithelial cells constitute the first line of defence against many exogenous carcinogens to which the human body is exposed. The epidermis of the skin has been exposed throughout evolution to ultraviolet (UV) light, which is both mutagenic and immunosuppressive. The epithelial cells of the gastrointestinal tract are exposed on a daily basis to mutagens or tumour promoters in the diet, either naturally occurring or produced as a consequence of protein pyrolysis during cooking at high temperatures (Wakabayashi *et al.* 1992). Similarly, the bronchial tract epithelial cells are constantly being exposed to pollutants, viruses or other damaging agents present in the atmosphere, while the bladder epithelia are continuously being washed in liquid containing the by-products of cellular metabolism excreted through the urinary tract. In view of this heavy mutational load, it is not surprising that most human tumours arise in these epithelial tissues.

Epithelial cells have not accepted these insults without evolving their own specific defence mechanisms. These may involve the production of protective proteins or chemicals, for example the melanin pigment produced by melanocytes or urocanic acid derived from breakdown of filaggrin in the upper epidermal layers, both of which afford protection from the harmful effects of UV light. Epithelia have also developed sophisticated architectural arrangements to protect their most important cells, or at least to minimize the overall detrimental effects on the organism if damage does occur. This phenomenon has been most widely studied in the epidermis and the intestinal epithelium, where it has been shown that the stem cells, most important for the long-term survival of the tissue, and consequently the organism, are few in number and are generally located in a relatively protected niche tucked as far as possible out of harm's way. For example, in the small intestine, the stem cells are located near the base of the intestinal crypts, and in the case of the epidermis, there are putative stem cell populations within the hair follicle or at the base of the deep ridges in the surface epithelium (Sun *et al.* 1991; Potten *et al.* 1997). This compartmentalization of cells has two main effects. First, the small number of stem cells and their relatively protected location should minimize the number of genetic hits leading to transformation. Second, although there are many other potential target cells within epithelial tissues, the likelihood of mutations in these cells doing any major damage is minimized by the fact that they are already approaching the end of their life-span, e.g. the keratinaceous squames at the skin surface or intestinal epithelial cells that are shed into the gut lumen (Cairns 1975). Mutations in these cells will presumably only have long-term consequences if they result in the anchoring of the cell to a position in the tissue where it can multiply and acquire additional genetic alterations.

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Other protective mechanisms involve controlling the cell cycle or the response of particular cell types to potentially damaging lesions in their DNA. The crucial stem cells are long-lived, slowly cycling cells, which only undergo division when necessary for tissue repopulation after removal of cells by wounding or during natural cell turnover (Hall & Watt 1989). This presumably allows ample time to repair any DNA damage, which occurs during the long resting phase in G0/G1. It has also been demonstrated that some stem cell populations may be particularly sensitive to certain threshold levels of DNA damage, and undergo self-destruction through apoptosis if the damage can not be adequately repaired. This is particularly evident in the small intestine, where the putative stem cells, located just above the crypt base, enter a p53-dependent apoptotic pathway in response to radiation (Clarke *et al.* 1994; Merritt *et al.* 1994). It has been proposed that this mechanism protects the organism from the potentially lethal effects of transformation of a long-lived cell population, and could explain the differences between tumour incidence in the small and large intestines, as the latter does not appear to have such a sophisticated stem cell protection mechanism (Potten *et al.* 1992).

In view of this multiplicity of defence systems that guard against transformation, it is not surprising that multiple genetic events must take place to allow tumour cells to grow to the extent where they have clinical consequences. These events presumably target individual facets of cell growth and tissue architecture such as growth factor responses and production (e.g. *ras,* EGFR, TGFβ receptor), cell cycle control and the balance between proliferation and differentiation (cyclin D1, p16, Rb), cell location, adhesion and longevity within the tissue (APC, E-cadherin, telomerase) and responses to DNA damage, either intrinsic or extrinsic, which would normally induce apoptosis (p53, bcl2). Yet more changes may be necessary for the further steps of invasion, metastasis and avoidance of the immune response. The main purpose of this review is to consider these alterations in the context of a mouse-skin model system for the study of epithelial carcinogenesis, with appropriate reference to parallel studies on human epithelial cells.

2. PRECARCINOGENESIS: THE ROLE OF PREDISPOSING GENES

The probability that a certain individual develops cancer will be determined by lifestyle and luck, but also by the complement of resistance or susceptibility genes which that individual inherits through the germline. The notion that cancer predisposition can be inherited came from the early studies of Knudson, and has been amply confirmed by the isolation and identification of a large and increasing number of genes that can be passed through the germline in mutant form, causing susceptible individuals to develop tumours in particular tissues (Knudson 1993). In addition to these high penetrance genes, it has been proposed that a number of low penetrance genes may be segregating within the population, which could also play an important role (Ponder 1990). If inherited in particular combinations of alleles, these genes could confer high cancer risk to a particular individual within a family, without this being recognized as a 'family trait' passed from one generation to the next. Although the identification of these genes may be difficult based only on the human population, studies with mice have indeed demonstrated that epithelial tumour susceptibility is under polygenic control, and is amenable to mapping and isolation using recombinant congenic (Moen *et al.* 1991) or interspecific hybrid backcross mice (Nagase *et al.* 1995).

3. EARLY EVENTS IN GROWTH CONTROL

The nature of the first genetic event in carcinogenesis—initiation—must be determined by a combination of the type of mutation induced by the initiating agent, and the selective pressures applied to the target cells within the tissue. Some agents may be particularly good at inducing large deletions within genes, and are therefore likely to induce frequent inactivation of tumour suppressor genes. Others will have a preference for inducing gene amplification or point mutations, both of which can stimulate growth through activation of oncogenes. One of the main advantages of a mouse-model system for carcinogenesis is that the types of carcinogenic agents used, and the selection pressures applied, can be manipulated. Consequently, treatment of mouse skin with dimethylbenzanthracene and promotion with TPA gives rise to multiple benign tumours—papillomas—most of which show the same activating mutation in the H-*ras* proto-oncogene (Bizub *et al.* 1986; Quintanilla *et al.* 1986). The activity of this gene is also important at later stages of tumour development, as the chromosome carrying the mutant allele, or the mutant gene itself, can be duplicated or amplified during tumour progression (Bianchi *et al.* 1990; Bremner & Balmain 1990; Bremner *et al.* 1994). In human squamous tumours, the same H-*ras* gene can be mutated, but the frequency, although variable, is not as high as in this model system (Leon *et al.* 1988; Pierceall *et al.* 1991). This could be due to intrinsic differences between mouse and human cells, or to the greater heterogeneity of the carcinogens to which human squamous cells are exposed. Evidence in favour of the latter possibility comes from studies using other initiating carcinogens, which give a reduced frequency of tumours carrying the H-*ras* gene mutations (Brown *et al.* 1990; Bremner *et al.* 1994; Ruggeri *et al.* 1994). Similarly, although head and neck tumours from European or North American patients have a relatively low frequency of H-*ras* mutations, similar tumours from patients in the Indian subcontinent have a substantially higher frequency, possibly due to the different aetiology. It is possible that the lack of *ras* mutations in many human squamous tumours is compensated for by amplification of the EGF receptor, which mediates signalling through the *ras* signal transduction pathway (Medema & Bos 1993).

4. CELL CYCLE CONTROL

Control of the balance between proliferation and differentiation is obviously a major determinant of the rate at which benign tumours grow and reach a critical size. An obvious link between early mutations in *ras* genes and subsequent alterations in cell cycle control comes from the observation that mutant *ras* can induce expression of cyclin

D1 (Filmus *et al.* 1994). Cyclin D1 has known oncogenic activity in epithelial cells, and is frequently amplified in human squamous tumours (Lammie *et al.* 1991). It is also known to be overexpressed in mouse epidermal papillomas which carry activated *ras* genes (Bianchi *et al.* 1993), and to undergo further changes in expression in some malignant skin tumours (Robles *et al.* 1994; R. Crombie, S. Haddow and A. Balmain, unpublished results). Cyclin D1 forms a complex with the cyclin-dependent kinases, cdk4 and cdk6, which is necessary for their activation in late G1, and phosphorylation of critical substrates such as the Rb protein, thereby allowing entry of cells into S phase (Weinberg 1995). Deregulation of cyclin D1 in tumours therefore presumably leads to a shorter G1 period, and consequently to an increase in cellular proliferation. However, this is likely to be an oversimplification. Cyclin D1 expression can be associated with inhibition of cell growth or with differentiation in certain cell types (Savatier *et al.* 1995) and its effects are therefore likely to be tissue specific. Indeed, the same can be said for the *ras* gene itself, because activated forms of this gene can induce growth arrest in primary fibroblasts and in epithelial cells (Hirakawa & Ruley 1988; Quintanilla *et al.* 1991) and differentiation of suprabasal keratinocytes *in vivo* (Bailleul *et al.* 1990). More recent data suggests that *ras* may exert its growth inhibitory effects through induction of $p16^{INK4a}$ or *p53* (Serrano *et al.* 1997).

Although *Rb* mutations have been found in some epithelial tumours arising from the bladder or breast (Lee *et al.* 1988), very few such mutations have been seen in squamous tumours arising from the skin or oral mucosa (Yoo *et al.* 1994) or in colon carcinomas. This appears to be true even when these tumours show loss of heterozygosity (LOH) on chromosome 13q (Yoo *et al.* 1994). In addition, allelotype studies indicate that mouse skin tumours do not show LOH at the *Rb* locus (Kemp *et al.* 1993*b*), and carcinogenesis studies in heterozygous *Rb* knockout mice have not shown any increase in tumour incidence with respect to normal littermates (A. Street, A. Clarke and A. Balmain, unpublished results). The reasons for this apparent specificity of *Rb* involvement in development of particular epithelial tumours are unclear, but suggest that the *Rb* gene may play an essential role in the differentiation programme or survival of certain cell types within these tissues.

Although *Rb* loss-of-function is rare in mouse skin tumours, clearly other components of the cell cycle are disrupted in this system. *p16* is a member of a family of cyclin-dependent kinase (CDKs) inhibitors which form stable complexes with CDKs, leading to displacement and turnover of cyclins and loss of kinase activity (Sherr & Roberts 1995). In agreement with a possible role in negative growth regulation, *p16* has been identified as a tumour suppressor gene that is homozygously deleted in a large number of tumour types (Kamb *et al.* 1994; Hall & Peters 1996). The biological consequences of these deletions are however still unclear. Some studies on human squamous tumours have implicated *p16* in the immortalization step of tumour development (Loughran *et al.* 1994), but in mouse skin tumours homozygous deletions occur at a later stage, in association with the acquisition of invasive properties (Linardopoulos *et al.* 1995). A similar association between *p16* deletions and the later events in progression have been seen in studies of human melanoma (Reed *et al.* 1995) and brain tumours (Jen *et al.* 1994; Nishikawa *et al.* 1995). The homozygous deletions that have been seen in both mouse and human tumours frequently lead to loss not only of *p16*, but of at least two other genes located within 20 kb on the same chromosome. One of these (*p15*) shares extensive sequence similarity with *p16* and interacts with the same CDK targets (Hannon & Beach 1994), whereas the other ($p19^{ARF}$) is translated from an alternative reading frame of the *p16* coding sequence (Quelle *et al.* 1995). Although $p19^{ARF}$ has no homology at the protein level with *p16*, it does appear to block cell proliferation, and to cooperate with *p16* in suppression of *ras* transformation (Quelle *et al.* 1995; Serrano *et al.* 1996). One possible scenario is that whereas point mutations in *p16*, or its inactivation by promoter methylation (Merlo *et al.* 1995), may lead to loss of growth control and possibly contribute to the immortalization step of transformation, simultaneous (or later) loss of its neighbouring genes by homozygous deletion may have a substantially different outcome related to acquisition of more invasive properties.

5. DNA DAMAGE RESPONSES IN EPITHELIAL CELLS

It is self evident that different mammalian tissues are programmed to respond to damage in different ways. Studies on mice that have been subjected to whole body irradiation have illustrated several types of responses. The spleen and the thymus respond to relatively low levels of DNA damage (e.g. 4 Gy γ-radiation) by strong *p53* induction, followed by massive apoptosis and subsequent regeneration (Clarke *et al.* 1993; Lowe *et al.* 1993). In contrast, neither is seen in the liver. Such a robust apoptotic response in the epithelial cells of the skin, gut or liver would have lethal consequences for the organism and perhaps this is why these tissues show much lower levels of apoptosis, which is restricted to specific cell types (Clarke *et al.* 1994; Merritt *et al.* 1994). A complicated pattern is emerging from studies aimed at understanding the relationships between DNA damage exposure, p53 protein expression and downstream events such as transcriptional activation of target genes or induction of apoptosis. Some tissue types show *p53* induction but no evidence of apoptosis, suggesting that tissue-specific modifications of the induced *p53* may determine the biological outcome. This is further illustrated in recent studies of transgenic mice carrying a *lacZ* reporter gene under the control of a consensus *p53* transcriptional control element. Irradiation of these mice shows that the ability of induced *p53* to act as a transcriptional activator through this particular recognition sequence is very cell-type specific (MacCallum *et al.* 1996). Irradiation of the skin induces relatively high levels of *p53* in the matrix region of the hair follicle, but much lower levels are detected in the interfollicular epidermis (D. Stuart and A. Balmain, unpublished results). Nevertheless, the cells within the hair follicles, which undergo apoptosis in response to radiation, fail to activate the reporter gene. This is compatible with the possibility that apoptosis can occur in the absence of transcription of *p53*-dependent genes (Caelles *et al.* 1994). Notably, extensive radiation-dependent activation of the reporter gene can be seen in

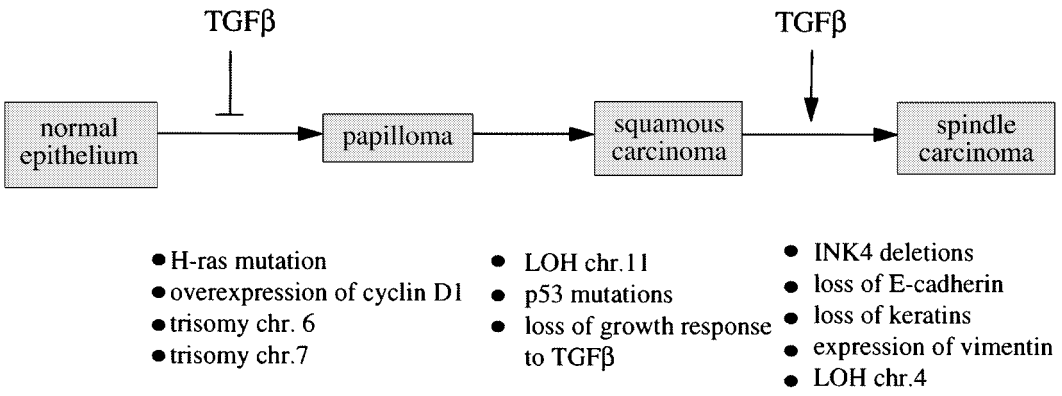

Figure 1. The mouse-skin model of tumour progression.

other tissues. All of this evidence indicates that the responses of individual cells to the same level of DNA damage are highly variable, and that the forces which select cells with mutant *p53* alleles during tumour development are also likely to vary between tissues. In some cases this may be due to loss of growth control or apoptosis, but in others the driving force may be survival of hypoxic stress (Graeber *et al.* 1996) or loss of negative regulation of angiogenesis (Dameron *et al.* 1994; Van Meir *et al.* 1994).

An unexpected result that has emerged from recent studies on epithelial carcinogenesis is that wild-type *p53* may play an essential role in the development of particular tumours. In a series of experiments on carcinogenesis in *p53*-null mice, it was observed that chemically induced, benign skin papillomas were reduced in frequency in these animals in comparison with wild-type or heterozygous littermates (Kemp *et al.* 1993*a*). More recently, Greenhalgh *et al.* (1996) have crossed transgenic mice that develop papillomas as a consequence of expression of a mutant *ras* gene in the epidermis on to a *p53*-null background. In these crosses, the benign papillomas failed to develop in the absence of wild-type *p53* (Greenhalgh *et al.* 1996). We have recently obtained a similar result by crossing an independent line of *ras* transgenic animals with *p53*-deficient mice. In this study (K. Brown and A. Balmain, unpublished results), mice that developed salivary adenomas showed evidence of increased tumour progression when crossed to *p53* heterozygotes, but failed to develop any salivary tumours on a *p53*-null background. The reasons for this are unclear, but may reflect a requirement of wild-type *p53* function at an early stage of tumorigenesis. Interestingly, it has recently been shown that low level expression of wild-type *p53* may protect cells from apoptosis induced by DNA damage (Lassus *et al.* 1996).

6. INVASION AND METASTASIS: GENETIC OR EPIGENETIC?

Epithelial tumours can progress to an invasive, metastatic phenotype through loss of cell adhesion molecules, increased expression of metalloproteinases and altered cell motility (Liotta *et al.* 1991). A cascade of events is required for the successful establishment of metastatic deposits, and a great deal of debate has centred on whether these events require additional genetic changes during tumour development or happen as a consequence of reprogramming of cell behaviour through an epigenetic mechanism. Evidence has emerged that genetic alterations can indeed influence the metastatic phenotype. Mutations have been found in genes such as E-cadherin, loss of which leads to loss of cell adhesion and increased invasive properties (Behrens *et al.* 1989; Vleminckx *et al.* 1991; Oda *et al.* 1994). Loss of E-cadherin is also seen in the most invasive forms of mouse skin tumours (Navarro *et al.* 1991). In this model, the genetic changes that lead to the invasive phenotype are recessive, as the phenotype is fully reversible in hybrids with squamous cells (Stoler *et al.* 1993).

A substantial body of evidence suggests that control of the invasive phenotype may also be epigenetic. First, behavioural changes similar to those seen during tumour progression, such as loss of E-cadherin expression and alteration of cytoskeletal components, can occur naturally and without any necessity for genetic changes during mammalian development (Hay 1990). Second, treatment of certain epithelial cells with growth factors can also induce changes reminiscent of the epithelial–mesenchymal transition (Jouanneau *et al.* 1991). Of particular interest in this context are a number of studies implicating transforming growth factor-β (TGFβ) in control of the invasive phenotype. TGFβ plays multiple roles during development, and is involved in epithelial–mesenchymal interactions and transitions, cell migration, angiogenesis and vasculogenesis. Various members of this family and their downstream signalling molecules are also involved in mesoderm induction in *Xenopus*.

Recent studies using transgenic animals that express TGFβ in the epidermis under the control of keratin gene promoters have provided evidence for these multiple functions also during tumour development (Cui *et al.* 1996; figure 1). Mice that overexpress either latent or activated forms of TGFβ1 develop fewer benign tumours than normal mice after treatment with initiators and promoters of carcinogenesis. However, the same animals showed a higher rate of malignant conversion, and had a higher than normal proportion of tumours showing invasive properties.

All of this evidence suggests that TGFβ family members play a positive role in increasing tumour invasion and metastasis, which is in apparent contradiction to recent studies that implicate TGFβ as a negative growth regulator for epithelial cells. Treatment of responsive epithelial cells

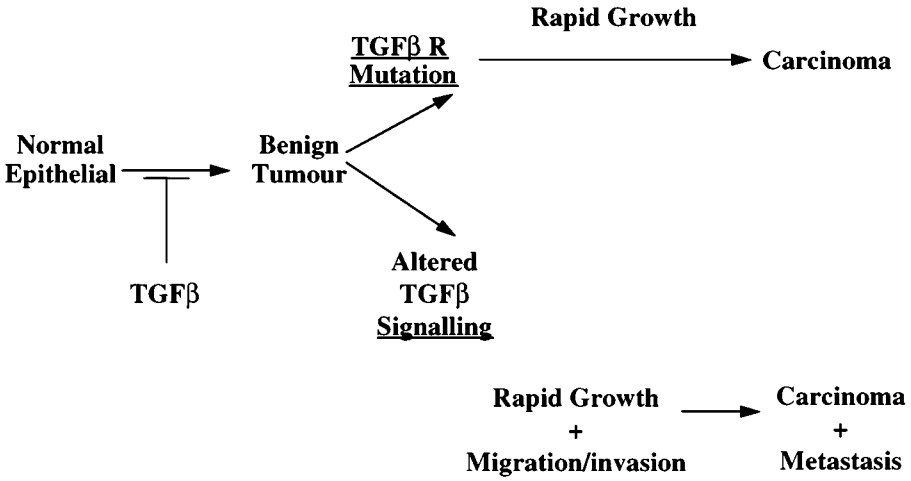

Figure 2. Role of TGFβ in tumour progression.

with TGFβ can alter cell cycle control by various mechanisms involving activation of the cdk inhibitors (Hannon & Beach 1994; Reynisdottir *et al.* 1995). In addition, in accordance with a putative role in negative control, the TGFβ type II receptor has been shown to be inactivated in colon tumours (Parsons *et al.* 1995). The genes encoding two of the downstream signalling molecules in the TGFβ pathway are both thought to be tumour suppressor genes on chromosome 18q, and they are both frequently deleted in several human tumour types.

How might these apparently disparate sets of observations be resolved? It would appear that there can be divergent routes to tumour development in epithelial tissues (figure 2). In the skin of the transgenic mice, loss of the growth inhibitory response to TGFβ is required to permit growth of benign tumours. However, the fact that these cells retain the ability to respond to other aspects of TGFβ signalling facilitates the transition to a more invasive malignant phenotype. In contrast, if a mutation in the TGFβ type II receptor or one of its downstream signalling molecules takes place at a relatively early stage of tumour development, this should cause more rapid cell growth and a greater likelihood of acquiring additional mutations leading to malignancy. However, such changes, which lead to complete loss of TGFβ signalling, might also give rise to tumours that are less able to activate the 'invasion' gene expression programme later in progression. Paradoxically, therefore, mutations in either TGFβ receptors, *DPC-4* or *Madr2* may be positive prognostic indicators for survival, as such tumours may be less likely to metastasize. Interestingly, tumours from hereditary non polyposis colorectal cancer (HNPCC) patients in which the TβRll receptor is frequently inactivated reportedly have a better prognosis than sporadic tumours (Bubb *et al.* 1996), although this could also be due to factors other than receptor loss.

Finally, an interesting connection can be seen between the previously discussed work on TGFβ and the types of genetic changes associated with the acquisition of invasiveness. Studies on mouse skin carcinogenesis have demonstrated an association between increased threshold levels of mutant *ras* (Buchmann *et al.* 1991) and development of genetic alterations leading to the invasive phenotype. A recent report showed that TGFβ induces an epithelial–mesenchymal conversion in epithelial cells only after transfection of a mutant *ras* gene (Oft *et al.* 1996), suggesting that mutant *ras* may reprogramme TGFβ signalling from growth arrest to mesenchyme induction. This agrees with the results of Caulin *et al.* (1995), who showed that TGFβ could induce PDV cells, transformed epithelial cells, which express a mutant *ras* allele, to undergo an epithelial–mesenchymal transition, but not immortalized keratinocytes expressing a normal *ras* allele. Whether TGFβ induces the mesenchymal transition via its effects on cell cycle control or by some other route is not yet known. TGFβ may ultimately prove to be a point of convergence of the genetic and epigenetic mechanisms leading to metastatic tumour cells. Further investigation of this pathway should shed additional light on the rate-limiting genetic events in tumour invasion and consequently provide additional targets for tumour therapy.

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